Aberrant somatic hypermutation in transformation of follicular lymphoma and chronic lymphocytic leukemia to diffuse large B-cell lymphoma

Davide Rossi
Eva Berra
Michaela Cerri
Clara Deambrogi
Caterina Barbieri
Silvia Franceschetti
Monia Lunghi
Annarita Conconi
Marco Paulli
Andràs Matolcsy
Laura Pasqualucci
Daniela Capello
Gianluca Gaidano

The molecular mechanisms involved in histologic transformation of follicular lymphoma (FL) and B-chronic lymphocytic leukemia (B-CLL) to diffuse large B-cell lymphoma (DLBCL) are heterogeneous and largely unknown. Here we explored whether aberrant somatic hypermutation, leading to the acquisition of new mutations in *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* genes, is involved in transformation from FL or B-CLL to DLBCL. Eighteen sequential pairs of FL/DLBCL (n=9) and B-CLL/DLBCL (n=9) were investigated. Our findings demonstrate that acquisition of novel mutations due to aberrant somatic hypermutation was associated with DLBCL transformation in 5/9 (55.5%) cases of FL and 2/9 (22.2%) cases of B-CLL.

Key words: aberrant somatic hypermutation, histologic transformation, diffuse large B-cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia.

Haematologica 2006; 91:1405-1409

From the Division of Hematology, Department of Clinical and Experimental Medicine & IRCAD, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy (DR, EB, MC, CD, CB, SF, ML, AC, DC, GG); Anatomic Pathology Section, Department of Human Pathology, University of Pavia, Pavia, Italy (MP); 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary (AM); Institute for Cancer Genetics, Columbia University, New York, NY 10032, USA (LP).

Correspondence:
Davide Rossi, M.D., Division of Hematology, Department of Clinical and Experimental Medicine & IRCAD, Amedeo Avogadro University of Eastern Piedmont, Via Solaroli 17, 28100 Novara, Italy.
E-mail: rossidav@med.unipmn.it

A histological shift to diffuse large B-cell lymphoma (DLBCL) occurs in 25-35% of cases of follicular lymphoma (FL) and 3-5% of cases of B-cell chronic lymphocytic leukemia (B-CLL).[1] The molecular mechanisms associated transformation of FL and B-CLL have been elucidated only in part.[2-5] The observation that the histological shift from FL to DLBCL is associated with acquisition of new mutations in the translocated *BCL-2* gene and in the 5' non-coding region of *BCL-6* suggests that somatic hypermutation may be involved in the transformation.[6,7] In DLBCL, somatic hypermutation aberrantly targets the 5' sequences of several proto-oncogenes relevant to lymphomagenesis, including *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC*. This phenomenon, called aberrant somatic hypermutation, occurs in >50% DLBCL arising *de novo*, while it is rare in FL and virtually absent in B-CLL.[8] On these bases, we explored the association of aberrant somatic hypermutation with the transformation of FL and B-CLL.

Design and Methods

This study included: (i) 18 paired specimens from nine patients with FL collected at diagnosis and at the time of DLBCL transformation; and (ii) 18 paired specimens from nine patients with B-CLL collected at diagnosis and at the time of DLBCL transformation. All cases were classified according to the World Health Organization classification of hematopoietic tumors.[1] B-CLL specimens were from peripheral blood mononuclear cells. FL and DLBCL specimens were from lymph node biopsies. All FL cases were grade 1 or 2. Genomic DNA was isolated using a QIAamp DNA mini kit (QIAGEN, Milan, Italy). Mutational analysis of *BCL-6*, *PIM-1*, *PAX-5*, *RhoH/TTF* and *c-MYC* was performed on selected regions containing >90% of mutations found in DLBCL.[8,9] Mutations of *BCL-2* gene were also analyzed.[6] Immunoglobulin heavy chain variable region (IGHV) rearrangements were amplified with family-specific primers hybridizing to sequences in the IGHV leader, framework region 1 (FR1) or FR2 in conjunction with JH primers, in separate reactions for each VH family. Amplicons were sequenced using the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Milan, Italy). IGHV sequences were aligned to the V-BASE sequence directory (*MRC Centre for Protein Engineering, Cambridge, UK; http://vbase.mrc-cpe.cam.ac.uk/*) using the MacVector 6.0.1 software (Oxford Molecular Group PLC, Oxford, UK) and to the international ImMunoGeneTics database (IMGT, *http://imgt.cines.fr*; initiator and coordinator: Marie-Paule Lefranc, Montpellier, France), using the IMGT/V-QUEST and IMGT/Junction Analysis software. IGHV sequences were considered mutated if deviation from the corresponding germline gene was greater than 2%. The study was approved by the local Institutional Review Board and was conducted in accordance with the Helsinki Declaration. All patients provided informed consent.

Results and Discussion

Nine pairs of FL and corresponding DLBCL samples were analyzed for aberrant somatic

Table 1. Mutation analysis of *PAX-5*, *RhoH/TTF*, *PIM-1*, *c-MYC*, *BCL-6* and IGHV in transformation from FL and B-CLL to DLBCL

Case	Diagnosis	Mutation type and position[a,b]					IGHV gene[c]	IGHV mutation (%)	IGHV clonal evolution[d]
		BCL-6	PAX-5	RhoH/TTF	PIM-1	c-MYC			
1A	FL	n.a.	–	–	–	–	3-07	18.8	–
1B	DLBCL	n.a.	–	T319C, T495G, A518T, T562A, G694A, T732C, C788G, A848C, A857C, A880G, G884T, C890G, T895C, A985C	–	G4652T, C4653G:Pro45Ala	3-07	18.8	–
2A	FL	–	–	–	–	–	n.a.	n.a.	n.a.
2B	DLBCL	–	–	–	–	–	n.a.	n.a.	n.a.
3A	FL	–	–	–	–	–	4-61	3.13	–
3B	DLBCL	–	–	–	–	–	4-61	3.13	–
4A	FL	n.a.	–	G938A	–	–	3-73	13.7	+
4B	DLBCL	n.a.	–	G938A, G963A	C1458T:Val58Ala	–	3-73	17.5	+
5A	FL	n.a.	–	–	–		3-15	13.6	+
5B	DLBCL	n.a.	–	–	–		3-15	12.6	+
6A	FL	–	–	–	–		3-07	7.82	+
6B	DLBCL	T837C	–	–	–	–	3-07	7.82	+
7A	FL	C480G, C484T, G642A	G847A	–	–	–	3-11	13.51	+
7B	DLBCL	C480G, C484T, G642A	G847A, C938T	–	G1540C	–	3-11	11.82	+
8A	FL	G625A	-	–	–	–	3-23	11.26	+
8B	DLBCL	G545A, Δ T603, T707G, T902A, C1048G, T1060G, A1073C, C789T, G800A, T839C	G1025A	–	–	–	3-23	11.26	+
9A	FL	–	G656T	–	–	–	3-23	8.11	+
9B	DLBCL	T440C, G579A, T929C, T1114G	G656T, G1333A, G1325T, C1207T	T732G	–	–	3-23	8.11	+
10A	B-CLL	–	–	–	–	–	1-03	0	–
10B	DLBCL	–	–	C817T	–	–	1-03	0	–
11A	B-CLL	–	–	–	–	–	3-72	3.32	–
11B	DLBCL	–	G1134A	–	C1563T	–	3-72	3.32	–
12A	B-CLL	–	–	C390T	–	–	4-39	0	–
12B	DLBCL	–	–	C390T	–	–	4-39	0	–
13A	B-CLL	–	–	–	–	–	4-34	4.5	–
13B	DLBCL	–	–	–	–	–	4-34	4.5	–
14A	B-CLL	–	–	–	–	–	1-24	0	–
14B	DLBCL	–	–	–	–	–	1-24	0	–
15A	B-CLL	–	–	–	–	–	1-02	0	–
15B	DLBCL	–	–	–	–	–	1-02	0	–
16A	B-CLL	–	–	–	–	–	1-e	0	–
16B	DLBCL	–	–	–	–	–	1-e	0	–
17A	B-CLL	–	–	–	–	–	4-39	0	–
17B	DLBCL	–	–	–	–	–	4-39	0	–
18A	B-CLL	–	–	–	–	–	3-21	0	–
18B	DLBCL	–	–	–	–	–	3-21	0	–

[a]: wild-type sequence; [b]Numbering according to GenBank accession Nos. AY189709 (BCL-6), AF386792 (PIM-1), AF386791 (PAX-5), AF386789 (RhoH/TTF), X00364 (c-MYC); [c]n.a., not available; [d]IGHV clonal evolution was considered when IGHV mutations present in the FL or B-CLL phase were not preserved in the DLBCL phase and/or mutations appeared only in the DLBCL phase; -, absence of IGHV clonal evolution; +, presence of IGHV clonal evolution.

hypermutation of *PAX-5*, *Rho/TTF*, *PIM-1* and *c-MYC* (Table 1 and Figure 1). Acquisition of new mutations in the transformed DLBCL occurred in 5/9 (55.5%) cases (cases 1B, 4B, 7B, 8B, 9B). In three cases, a single mutation was already present in the FL phase (cases 4A, 7A, 9A) and was preserved after transformation. Mutations targeting more than one gene were found in 0/9 FL and in 4/9 (44.4%) DLBCL.

Nine pairs of B-CLL and corresponding DLBCL samples were analyzed for aberrant somatic hypermutation (Table 1 and Figure 1). Mutations targeting at least one of the four proto-oncogenes were found in 1/9 (11.1%) B-CLL and in 3/9 (22.2%) DLBCL. Acquisition of new mutations in the transformed sample occurred in two patients (cases 10B and 11B), one with IGHV mutated B-CLL and the other with unmutated B-CLL. Mutations targeting more than one gene were found in 0/9 B-CLL and in 1/9 (11.1%) DLBCL. As previously observed in B-CLL and FL in general,[8] also in the subset of patients undergoing histologic transformation, aberrant somatic hypermutation is virtually absent in the B-CLL phase and affects the FL phase at low frequency, reflecting an association of aberrant somatic hypermutation with aggressive histology.

At the time of transformation from FL, 5/9 (55.5%) DLBCL acquired a total of 25 new mutations (Table 1). Transformation was characterized by acquisition of novel mutations of *PAX-5* in 3/9 cases, *RhoH/TTF* in 3/9 cases, *PIM-1* in 2/9 cases and *c-MYC* in 1/9 cases. Two cases acquired two novel mutations in *PIM-1* and *c-MYC* coding exons, leading to amino acid substitutions (Table 1). Case 4B acquired one missense mutation in PIM-1 exon 2 in the proximity of the ATP binding pocket of the catalytic domain, leading to the substitution of Ala for Val at position 58. As reported for other *PIM-1* mutations generated

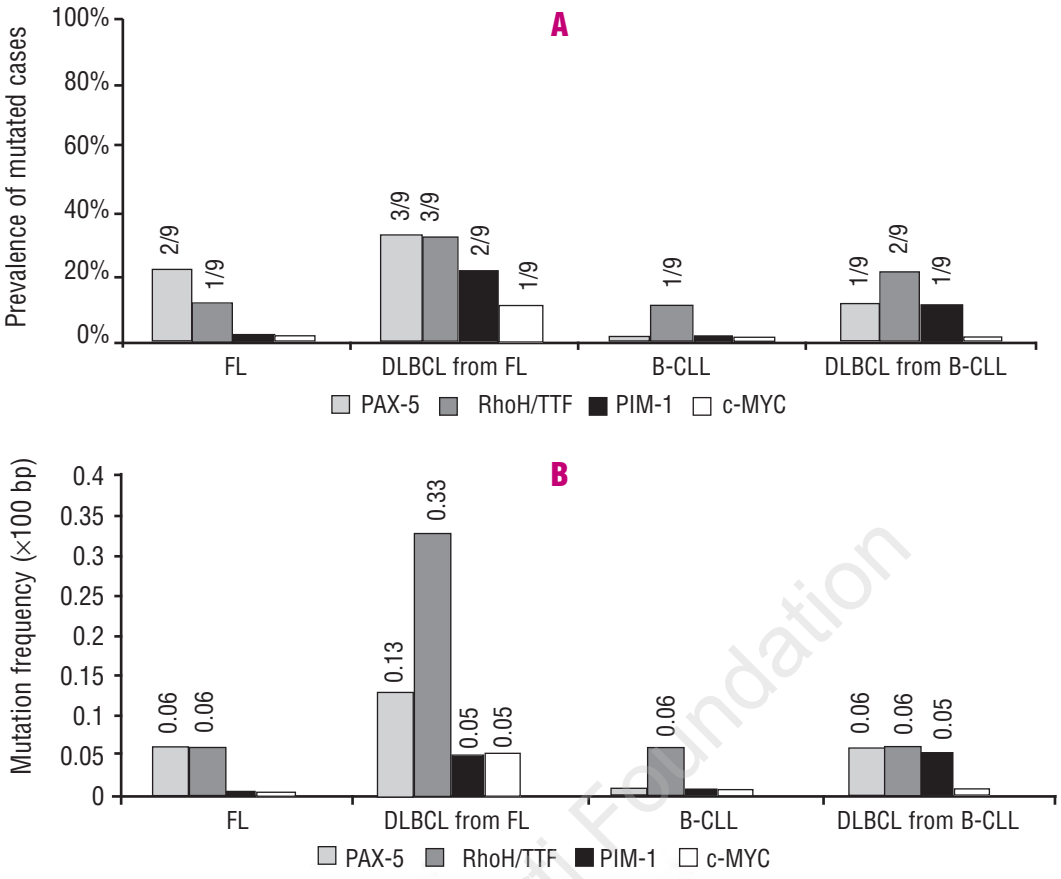

Figure 1. Prevalence (Panel A) and mean mutation frequency (Panel B) of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* mutations in follicular lymphoma (FL) at diagnosis, diffuse large B-cell lymphoma (DLBCL) transformed from FL, B-cell chronic lymphocytic leukemia (B-CLL) at diagnosis and DLBCL transformed from B-CLL.

by aberrant somatic hypermutation, the Val58Ala mutation may change the structure, and possibly the activity, of this serine-threonine kinase.[10] Alternatively, since the Val58Ala mutation is located on the surface of the PIM-1 protein, the mutation may affect protein-protein interactions.[10] Case 1B acquired one missense mutation in *c-MYC* exon 2, which encodes the gene transactivation domain, leading to the substitution of Ala for Pro at position 45. Mutations in this region may alter c-MYC function by impairing susceptibility to p107 induced suppression and possibly conferring increased transforming activity.[11] During transformation from B-CLL, 2/9 (22.2%) DLBCL acquired three new mutations (Table 1). No case displayed evidence of aberrant somatic hypermutation during the B-CLL phase. Transformation was characterized by novel mutations of *PAX-5* in 1/9 cases, *RhoH/TTF* in 1/9 cases and PIM-1 in 1/9 cases.

Overall, the molecular profile of mutations in transformed DLBCL is similar to that reported for *de novo* DLBCL and is consistent with the process of somatic hypermutation (Table 2).[8] In fact, mutations in *PAX-5*, *RhoH/TTF*, *PIM-1*, and *c-MYC* in transformed DLBCL: i) are predominantly represented by single nucleotide substitutions; ii) display a preference for transitions over transversions and an elevated ratio of G+C over A+T substitutions; and iii) display a preferential distribution within the RGYW/WRCY motifs (Table 2). Six pairs of FL and corresponding DLBCL were analysed for somatic hypermutation of BCL-6 (table 1) and BCL-2. BCL-6 mutations occured in 2/6 FL and in 4/6 corresponding DLBCL. Acquisition of new mutations in the transformed DLBCL occurred in 3/6 (50.0%) cases (cases 6B, 8B, 9B). In two cases (6A, 9A), BCL-6 was not mutated in the FL phase and acquired new mutations in the transformed sample. In one case (8A), a single mutation was present in the FL phase and was lost after transformation concomitantly with acquisition of new mutations. No case of transformed DLBCL acquired BCL-2 mutations. Nine pairs of B-CLL and corresponding DLBCL were analysed for somatic hypermutation of BCL-6 (Table 1). No BCL-6 mutations were detected in the B-CLL or in the corresponding DLBCL.

The clonal relationship between FL or B-CLL and the respective DLBCL phase was analysed by IGHV gene sequencing. A functional VDJ rearrangement was obtained in 8/9 FL/DLBCL pairs (table 1). In all pairs, the FL and the DLBCL phases displayed identical VDJ rearrangements, indicating a common clonal origin. Mutation analysis of

Table 2. Features of *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* mutations in transformation from FL and B-CLL to DLBCL.

	Mutation frequency (range)[a,b]	*Transitions/ Transversions*	*RGYW-WRCY*	*G+C/A+T*	*Single bp substitutions*	*Deletions/ insertions*
PAX-5						
FL	0.06 (0.06-0.06)×10^{-2}	1/1 (1.00)	0	2/0	2	0/0
DLBCL	0.13 (0.06-0.23)×10^{-2}	5/2 (2.50)	4	7/0	7	0/0
B-CLL	0	0	0	0	0	0/0
DLBCL	0.06×10^{-2}	1/0	0	1/0	1	0/0
RhoH/TTF						
FL	0.06×10^{-2}	1/0	0	1/0	1	0/0
DLBCL	0.33 (0.06-0.82)×10^{-2}	7/10 (0.70)	5	7/10	17	0/0
B-CLL	0.06 x 10-2	1/0	0	1/0	1	0/0
DLBCL	0.06 (0.06-0.06)×10^{-2}	2/0	1	2/10	2	0/0
PIM-1						
FL	0	0	0	0	0	0/0
DLBCL	0.05 (0.05-0.05)×10^{-2}	1/1 (1.00)	0	2/0	2	0/0
B-CLL	0	0	0	0	0	0/0
DLBCL	0.05×10^{-2}	1/0	0	1/0	1	0/0
c-MYC						
FL	0	0	0	0	0	0/0
DLBCL	0.05×10^{-2}	2/0	0	2/0	2	0/0
B-CLL	0	0	0	0	0	0/0
DLBCL	0	0	0	0	0	0/0
All genes						
FL	NA	2/1(2.00)	0	3/0	3	0/0
DLBCL	NA	15/13 (1.15)	9	18/10 (1.80)	28	0/0
B-CLL	NA	1/0	0	1/0	1	0/0
DLBCL	NA	4/0	1	4/0	4	0/0

[a]*NA indicates not applicable;* [b]*calculated on the entire region analyzed and on mutated cases only, considering 2 alleles/gene/case.*

IGHV genes revealed that all FL and DLBCL pairs were somatically mutated. In 6/8 FL/DLBCL pairs, some IGHV mutations were found in the FL phase but not in the DLBCL phase, while some mutations appeared only in the DLBCL phase. This phenomenon has been previously interpreted as clonal evolution.[7,12,13] As a result of ongoing IGHV somatic hypermutation, FL cells might become increasingly heterogeneous until a subclone different from the predominant clone of the FL phase emerges and gives rise to DLBCL.[7,12,13] At variance with somatic hypermutation in IGHV and BCL-6 genes, all proto-oncogene mutations due to aberrant somatic hypermutation in the FL phase are maintained at the time of DLBCL transformation, concomitantly with the appearance of additional mutations (Table 1). This finding suggests that aberrant somatic hypermutation might be active only in the subclone that subsequently gives rise to DLBCL. A functional VDJ rearrangement was obtained in all 9 B-CLL/DLBCL pairs (Table 1). In all pairs, the B-CLL and DLBCL phases displayed identical VDJ rearrangements, indicating a common clonal origin of the tumor samples. Mutation analysis revealed germline IGHV genes in 7/9 pairs. The remaining two pairs (11A-11B and 13A-13B) displayed mutations at a frequency of 3.3% and 4.5%, respectively. No DLBCL transformed from B-CLL displayed *IGHV* clonal evolution (Table 1).

In one DLBCL transformed from FL (case 1B) and in 2 DLBCL transformed from B-CLL (cases 10B and 11B), novel proto-oncogene mutations consistent with aberrant somatic hypermutation were acquired despite the absence of novel IGHV mutations (Table 1). Extensive sequencing analysis of subclones confirmed that the newly acquired proto-oncogene mutations were absent in the indolent phase. This observation points to a divergence between IGHV somatic hypermutation and aberrant somatic hypermutation that has not been previously described in the setting of DLBCL and immunodeficiency-related lymphomas.[8,9,14-16] However, a similar phenomenon has been documented in the case of *IGHV* unmutated B-CLL with BCL-6 mutations.[17] While it is possible that a low somatic hypermutation activity targets stochastically different loci, leading to a discordant pattern of mutations, this finding supports the hypothesis that aberrant somatic hypermutation is due to a qualitative, rather than to a quantitative defect of this process. Overall, our findings document that the accumulation of novel mutations in the *PAX-5*, *RhoH/TTF*, *PIM-1* and *c-MYC* genes is associated with transformation from FL to DLBCL in 55% of cases. Richter transformation is also associated with aberrant somatic hypermutation, although at a lesser extent, as recently reported by an independent study.[18]

DR, DC, and GG contributed to the conception and design of the study and to drafting the article; EB, MC, CD, CB, SF and ML participated in the analysis and interpretation of data; AC, MP, AM and LP contributed to the study design and interpretation of data and revised the article critically for relevant intellectual content. All authors gave their approval to the final version of the manuscript.

This study was supported by Ricerca Sanitaria Finalizzata and Ricerca Scientifica Applicata, Regione Piemonte, Torino, Italy; Novara-AIL Onlus, Novara, Italy; and Fondazione "Piera Pietro Giovanni Ferrero", Alba, Italy. The authors have no conflict of interest to declare.

Manuscript received April 12, 2006. Accepted August 3, 2006.

References

1. Jaffe ES, Harris NL, Stein H, Vardiman JW. World Health Organization Classification of Tumours, Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC Press, 2001.
2. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, et al. p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc Natl Acad Sci USA 1991;88:5413-7.
3. Yano T, Jaffe ES, Longo DL, Raffeld M. MYC rearrangements in histologically progressed follicular lymphomas. Blood 1992; 80:758-67.
4. Lo Coco F, Gaidano G, Louie DC, Offit K, Chaganti RS, Dalla-Favera R. p53 mutations are associated with histologic transformation of follicular lymphoma. Blood 1993; 82:2289-95.
5. Pinyol M, Cobo F, Bea S, Jares P, Nayach I, Fernandez PL, et al. 16(INK4a) gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. Blood 1998;91:2977-84.
6. Matolcsy A, Casali P, Warnke RA, Knowles DM. Morphologic transformation of follicular lymphoma is associated with somatic mutation of the translocated Bcl-2 gene. Blood 1996;88:3937-44.
7. Lossos IS, Levy R. Higher-grade transformation of follicle center lymphoma is associated with somatic mutation of the 5' noncoding regulatory region of the BCL-6 gene. Blood 2000;96:635-9.
8. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 2001;412:341-6.
9. Gaidano G, Pasqualucci L, Capello D, Berra E, Deambrogi C, Rossi D, et al. Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma. Blood 2003; 102:1833-41.
10. Kumar A, Mandiyan V, Suzuki Y, Zhang C, Rice J, Tsai J, et al. Crystal structures of proto-oncogene kinase Pim1: a target of aberrant somatic hypermutations in diffuse large cell lymphoma. J Mol Biol 2005;348: 183-93.
11. Hoang AT, Lutterbach B, Lewis BC, Yano T, Chou TY, Barrett JF, et al. A link between increased transforming activity of lymphoma-derived MYC mutant alleles, their defective regulation by p107, and altered phosphorylation of the c-Myc transactivation domain. Mol Cell Biol 1995;15:4031-42.
12. Matolcsy A, Schattner EJ, Knowles DM, Casali P. Clonal evolution of B cells in transformation from low- to high-grade lymphoma. Eur J Immunol 1999;29:1253-64.
13. Szereday Z, Csernus B, Nagy M, Laszlo T, Warnke RA, Matolcsy A. Somatic mutation of the 5' noncoding region of the BCL-6 gene is associated with intraclonal diversity and clonal selection in histological transformation of follicular lymphoma. Am J Pathol 2000;156: 1017-24.
14. Cerri M, Capello D, Muti G, Rambaldi A, Paulli M, Gloghini A, et al. Aberrant somatic hypermutation in post-transplant lymphoproliferative disorders. Br J Haematol 2004;127:362-4.
15. Libra M, Capello D, Gloghini A, Pasqualucci L, Berra E, Cerri M, et al. Analysis of aberrant somatic hypermutation (SHM) in non-Hodgkin lymphomas of patients with chronic HCV infection. J Pathol 2005;206:87-91.
16. Rossi D, Cerri M, Capello D, Deambrogi C, Berra E, Franceschetti S, et al. Aberrant somatic hypermutation in primary mediastinal large B-cell lymphoma. Leukemia 2005;19:2363-6.
17. Sahota SS, Davis Z, Hamblin TJ, Stevenson FK. Somatic mutation of bcl-6 genes can occur in the absence of V(H) mutations in chronic lymphocytic leukemia. Blood 2000; 95:3534-40.
18. Reiniger L, Bodor C, Bognar A, Balogh Z, Csomor J, Szepesi A, Kopper L, Matolcsy A. Richter's and prolymphocytic transformation of chronic lymphocytic leukemia are associated with high mRNA expression of activation-induced cytidine deaminase and aberrant somatic hypermutation. Leukemia 2006;20:1089-95.