Detection of *BRAF* mutations in patients with hairy cell leukemia and related lymphoproliferative disorders

Piers A. Blombery,[1] Stephen Q. Wong,[2] Chelsee A. Hewitt,[3] Alexander Dobrovic,[2,4] Ellen L. Maxwell,[5] Surender Juneja,[6] George Grigoriadis,[7] and David A. Westerman[1,4]

[1]Peter MacCallum Cancer Centre, Division of Cancer Medicine, East Melbourne, Victoria; [2]Peter MacCallum Cancer Centre, Molecular Pathology Research and Development, Department of Pathology, East Melbourne, Victoria; [3]Peter MacCallum Cancer Centre, Molecular Pathology, Department of Pathology, East Melbourne, Victoria; [4]Department of Pathology, University of Melbourne, Parkville, Victoria; [5]Melbourne Pathology, Collingwood, Victoria; [6]Melbourne Health Pathology, Royal Melbourne Hospital, Parkville, Victoria; and [7]Alfred Pathology Service and Department of Clinical Haematology, Alfred Health and Monash University, Victoria, Australia

ABSTRACT

Hairy cell leukemia has been shown to be strongly associated with the *BRAF V600E* mutation. We screened 59 unenriched archived bone marrow aspirate and peripheral blood samples from 51 patients with hairy cell leukemia using high resolution melting analysis and confirmatory Sanger sequencing. The *BRAF V600E* mutation was detected in 38 samples (from 36 patients). The *BRAF V600E* mutation was detected in all samples with disease involvement above the limit of sensitivity of the techniques used. Thirty-three of 34 samples from other hematologic malignancies were negative for *BRAF* mutations. A *BRAF K601E* mutation was detected in a patient with splenic marginal zone lymphoma. Our data support the recent finding of a disease defining point mutation in hairy cell leukemia. Furthermore, high resolution melting with confirmatory Sanger sequencing are useful methods that can be employed in routine diagnostic laboratories to detect *BRAF* mutations in patients with hairy cell leukemia and related lymphoproliferative disorders.

Key words: *BRAF V600E*, mutation, hairy cell, lymphoproliferative disorders.

Citation: Blombery PA, Wong SQ, Hewitt CA, Dobrovic A, Maxwell EL, Juneja S, Grigoriadis G and Westerman DA. Detection of BRAF *mutations in patients with hairy cell leukemia and related lymphoproliferative disorders. Haematologica 2012;97(5):780-783. doi:10.3324/haematol.2011.054874*

Introduction

Hairy cell leukemia (HCL) is a rare lymphoproliferative disorder with distinct clinicomorphological features and specific cytochemical[1] and immunohistochemical[2] characteristics. An important step in clarifying the pathogenesis of hairy cell leukemia was the description of the *BRAF* c.1799T>A p.Val600Glu *V600E* mutation in 48 patients with HCL.[3] In this cohort, the *BRAF V600E* mutation was detected by direct Sanger sequencing (SS) of PCR products from Ficoll density gradient enriched, magnetic-activated cell sorted peripheral blood from patients with known HCL. Whilst this enrichment technique results in high purity leukemia samples (>90%), in the context of a diagnostic molecular laboratory, a more cost-effective, rapid and less labor intensive method that is capable of high throughput is desirable. High resolution melting (HRM) analysis is a DNA mutation screening technique which is fast, relatively simple and has previously been shown to be a sensitive and reliable way of detecting the *BRAF V600E* mutation in tumor samples from patients with melanoma,[4] colorectal carcinoma[5] and, more recently, HCL.[6]

Our aim was to determine the feasibility of using HRM analysis and subsequent SS to detect the *BRAF V600E* in DNA extracted from unenriched bone marrow aspirate and peripheral blood samples from patients with HCL. We also aimed to confirm and extend the initial observation of Tiacci *et al.*[3] using HRM and SS to detect the *BRAF V600E* mutation in patients with HCL (including immunophenotypic variants) as well as screening morphological mimics of HCL (HCL-variant (HCL-v), and marginal zone lymphoma (MZL)) and other hematologic malignancies.

Design and Methods

Case identification

Archived bone marrow aspirate and peripheral blood samples from patients between 1998 and 2011 with HCL, HCL-v, MZL and other hematologic malignancies were retrieved from Peter MacCallum Cancer Centre (Melbourne, Australia), The Royal Melbourne Hospital (Melbourne, Australia), The Alfred Hospital (Melbourne, Australia) and Melbourne Pathology (Melbourne, Australia) after case identification from institutional databases. The assigned diagnosis was confirmed using current classification standards[7] by review of clinical,

Acknowledgments: the authors would like to thank the Victorian Cancer Cytogenetics Service for performing the cytogenetic analysis, Suneet Sandhu for assistance in data collection and Danilo Acosta, Ravikiran Vedururu and Aleksandra Rynska for performing some of the BRAF assays.
Manuscript received on September 5, 2011. Revised version arrived on November 21, 2011. Manuscript accepted on November 22, 2011.
Correspondence: David Westerman, Department of Pathology, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett Street, Victoria, Australia 8006. Phone: international +61396561518. Fax: international +61396561593. E-mail: david.westerman@petermac.org

immunophenotypic and morphological features. Specifically, HCL was identified by the constellation of characteristic morphological features (small to medium-sized lymphocytes with oval/reniform nucleus and circumferential hairy cytoplasmic projections, fried-egg appearance on trephine), monocytopenia, bone marrow fibrosis, pancytopenia, splenomegaly and durable response to purine analog therapy. HCL-v was identified by the constellation of morphological features (medium to large-sized lymphocytes, prominent nucleoli), leukocytosis at diagnosis, absence of monocytopenia and bone marrow fibrosis and lack of typical hairy cell leukemia immunophenotype on flow cytometry. MZL was identified by the presence of morphological features (small to medium-sized lymphocytes, intrasinusoidal involvement on trephine (splenic marginal zone lymphoma, SMZL), typical immunophenotype ($CD5^-$, $CD10^-$, $CD20^+$), and correlation with spleen and lymph node histology.

Only aspirate/peripheral blood samples with morphological evidence of involvement by disease (regardless of extent) were included in the HCL cohort. For HCL samples, the estimated mutant allele percentage was calculated as half (assuming heterozygosity) the percentage of typical hairy cells assessed morphologically on a 200 cell differential count. For patients with other hematologic malignancies, the morphological burden of disease was confirmed to be more than 15% of nucleated cells in order to be above the limit of detection of HRM (>7.5% estimated mutated alleles assuming heterozygosity). The study was approved by the institutional review board at the Peter MacCallum Cancer Centre.

High resolution melt analysis

For HRM analysis, DNA was extracted from bone marrow smears using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR and HRM were performed on the LightCycler 480 (Roche Diagnostics) and all reactions were performed in duplicate. Primers for the 88bp amplicon were 5′-CCTCACAGTAAAAATAGGTGATTTTGG-3′ and 5′-GGATCCAGACAACTGTTCAAACTGA-3′. The reaction mixture included 1× PCR buffer, 2.5 mM $MgCl_2$, 200 nM of each primer, 200 μM of dNTPs, 5 μM of SYTO 9 (Invitrogen, Carlsbad, CA, USA), 0.5U of HotStarTaq polymerase (Qiagen), 10ng DNA and PCR grade water in a total volume of 10 μL. PCR conditions included an activation step of 15 min at 95°C followed by 55 cycles of 95°C for 10 s, annealing for 10 s comprising 10 cycles of a touchdown from 65°C to 55°C at 1°C/cycle followed by 35 cycles at 55°C, and extension at 72°C for 30 s. Samples with aberrant melting curves were directly sequenced from a 1/10 dilution of the HRM product using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Results and Discussion

Fifty-nine samples (58 bone marrow aspirates and one peripheral blood) from 51 patients with HCL and 34 samples (33 bone marrow aspirates and one peripheral blood) from 34 patients with other hematologic malignancies were identified. In the HCL group, 32 samples were from specimens taken at initial diagnosis and 27 were taken after treatment, either at time of clinical relapse or as part of a post-treatment assessment. The results of HRM analysis and subsequent SS on HRM positive samples are summarized in Table 1.

All samples from HCL patients with an estimated mutant allele percentage of greater than 6.75% had mutations evident by HRM analysis (33 samples from 32 patients). Nine out of 26 samples from HCL patients with an estimated mutant allele percentage of less than or equal to 6.75% had mutations evident by HRM analysis. The sensitivity of HRM in both the published literature[5,6,8] and in our own experience is a mutant allele percentage of approximately 5-10% depending on the precise mutation. Therefore, all samples with an estimated mutant allele percentage that was above the accepted limits of detection (LOD) of HRM analysis showed mutations.

Of the 42 samples from patients with HCL with mutations detected by HRM, the *BRAF V600E* was detected by confirmatory sequencing in 38. Of the remaining 4 samples, 3 had equivocal sequencing results (estimated mutant allele percentages of 8.5%, 7.5% and 5.25%) and one sample had a wild-type sequence (estimated mutant allele percentage of 7%). In our experience, the sensitivity of SS in this context is a mutant allele percentage of 10-20%. Given the typical mutation curves evident on HRM analysis in these patients, it is likely that these samples contained the *BRAF V600E* mutations but were below the sensitivity of SS.

Overall, 36 out of 51 patients with HCL had the *BRAF V600E* detected by both HRM and SS. Of these 36 patients, 9 patients had immunophenotypes detected by flow cytometry that varied from the classic HCL immunophenotype ($CD5^-$, $CD10^-$, $CD11c^+$, $CD25^+$, $CD103^+$, $CD123^+$). Three patients were $CD123^-$, 2 patients were both $CD25^-$ and $CD123^-$, while 4 patients were $CD10^+$ (with an otherwise typical HCL immunophenotype). All cases with immunophenotypic variations showed otherwise typical morphological features of HCL (leukopenia, monocytopenia and bone marrow fibrosis).

One patient with SMZL had a mutation detected by HRM analysis with a *BRAF* c.1801A>G p.Lys601Glu (K601E) mutation identified on subsequent sequencing. The pathological features of the case are shown in Figure 1. The patient, a 75-year old male, presented with a

Table 1. Results of high resolution melting (HRM) analysis for BRAF mutations in patients with hairy cell leukemia (HCL) and other hematologic malignancies.

	Mutated HRM	Wild-type HRM	Equivocal HRM	Inadequate/ insufficient DNA extracted for analysis
HCL (n=59)	42*	11	2**	4
Diagnosis (n=32)	27	1	2	2
Post-treatment (n=27)	15	10	0	2
Marginal zone lymphoma (MZL) (n=15)	1***	14	0	0
Nodal MZL (n=5)	0	5	0	0
Splenic MZL (n=10)	1	9	0	0
HCL-variant (n=1)	0	1	0	0
Other (n=18)****	0	18	0	0

**BRAF V600E detected by confirmatory sequencing in 38 samples, 1 had wild-type sequence and 3 equivocal results; **1 wild-type and 1 equivocal result by confirmatory sequencing; ***BRAF K601E identified on confirmatory sequencing; ****Lymphoplasmacytic lymphoma (n=7), plasmablastic myeloma (n=4), T-cell prolymphocytic leukemia (n=2), hepatosplenic T-cell lymphoma (n=2), peripheral T-cell lymphoma not otherwise specified (n=1), hematodermic neoplasm (n=1), intravascular large B-cell lymphoma (n=1).*

marked lymphocytosis (130×10⁹/L), massive splenomegaly and constitutional symptoms. The disease course of this patient was aggressive and was refractory to both single agent chlorambucil and then subsequently to cyclophosphamide plus etoposide. There was a brief hematologic response to single agent fludarabine but the patient died of progressive lymphoma ten months after initial diagnosis. The *BRAF K601E* mutation was detected in aspirate samples taken both at diagnosis and later in the course of the disease.

The pathological features of this case strongly favor a diagnosis of SMZL rather than HCL according to current classification standards.[7] The disease course was more aggressive than is typically observed for SMZL; however, immunohistochemistry showed strong nuclear staining for p53 which suggests a *TP53* mutated status that has been associated with a poorer prognosis in SMZL.[9] Importantly, there was no morphological evidence on bone marrow biopsy of large cell transformation.

The precise molecular pathogenesis of SMZL remains unclear. The identification of the first case of SMZL harboring a *BRAF K601E* mutation has potential implications for the molecular pathogenesis of this condition. The *BRAF K601E* affects the kinase activation segment of *BRAF* and has been associated with Raf/MEK/Erk pathway activation comparable to *BRAF V600E*.[10] It has also been described in cases of papillary thyroid cancer.[11] Whilst the Raf/MEK/Erk pathway has not been a focus of research in

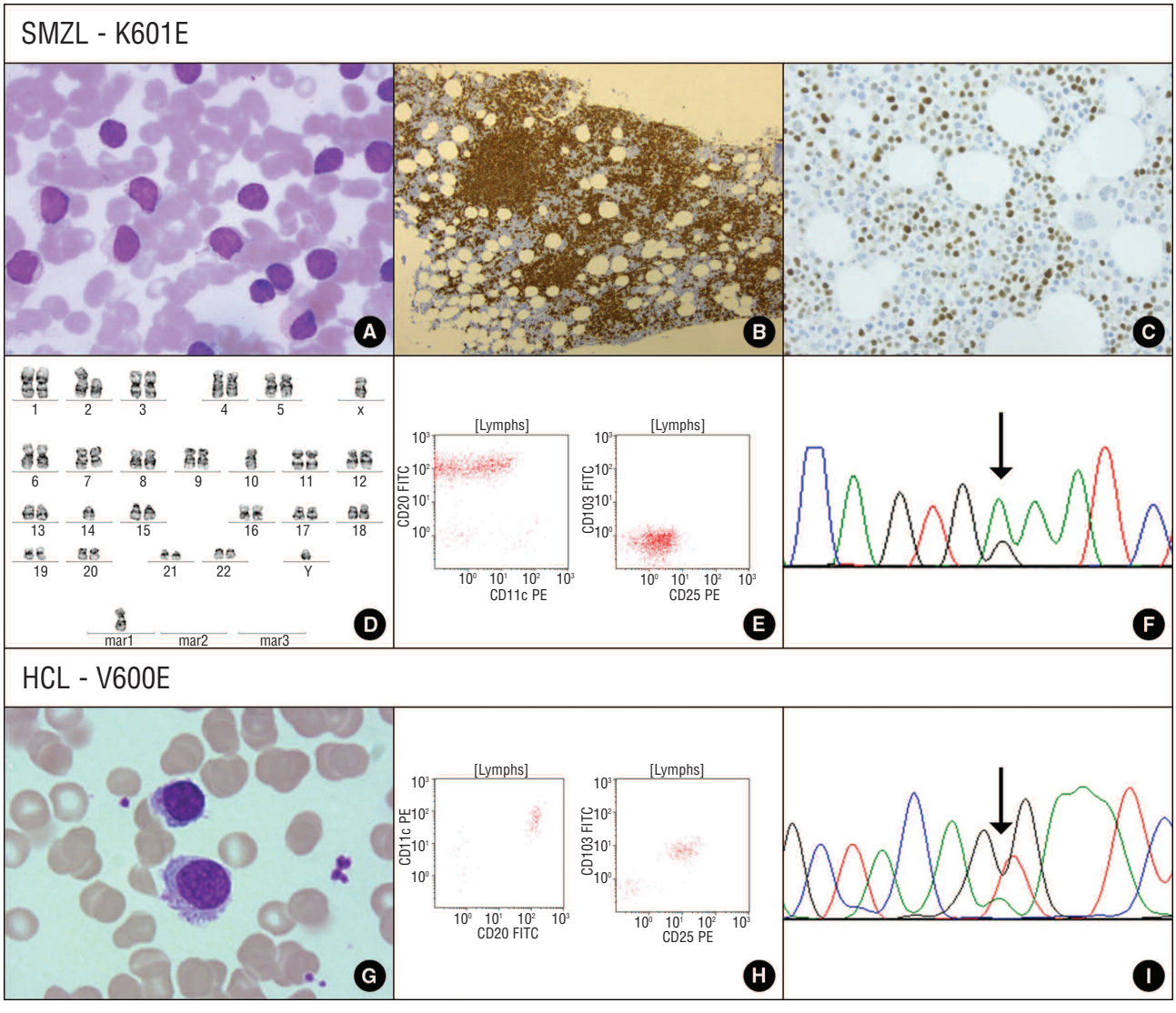

Figure 1. Pathological features of patient with BRAF K601E mutated splenic marginal zone lymphoma (SMZL) compared with hairy cell leukemia (HCL). (A) Peripheral blood showing marked lymphocytosis with small mature forms with occasional nuclear clefts and cytoplasmic projections (x200, Romanowsky). (B) Bone marrow biopsy showing both nodular and interstitial infiltrate of CD20⁺ lymphocytes (x50, CD20). (C) Strong nuclear staining for p53 in the majority of lymphocytes (x100, p53). (D) A representative metaphase from the neoplastic clone. Karyotype: 39-46,XY,del(2)(p10),-10,-14,+mar1,+mar2[cp7]/45-46,idem,del(7)(q21q31)[cp7] /46,XY[14]. (E) Flow cytometry (gated on lymphocytes) showing heterogenous CD11c expression, negative CD103 expression and weak CD25 expression. (F) Sequencing data from a section of reverse strand showing an A to G substitution at nucleotide position 1801 (arrow) in exon 15 of BRAF. (G) Typical hairy cell lymphocytes with oval nuclei and cytoplasmic projections (x400). (H) Flow cytometry (gated on lymphocytes) showing expression of CD20, CD11c, CD103 and CD25. (I) Sequencing data from a section of reverse strand showing a T to A substitution at nucleotide position 1799 (arrow) in exon 15 of BRAF.

SMZL thus far, there are reasons to suspect that dysregulation of this pathway may be involved in its pathogenesis. Deletions involving the long arm of chromosome 7 are observed in a significant number of patients with SMZL[12] with the common deleted region on chromosome 7 (7q32[13]) situated close to the BRAF locus (7q34). One possible mechanism by which this deletion may affect the Raf/MEK/Erk pathway is via altered expression of the numerous microRNAs that are located at 7q32 that may regulate multiple target oncogenes and tumor suppressor genes, including *BRAF*.

Our data confirm the initial findings[3] that *BRAF V600E* is likely to be present in 100% of patients with HCL with classic morphological and immunophenotypic features. In addition, we observed that patients with morphologically classic HCL who have immunophenotypic variations ($CD25^-$, $CD10^+$, $CD123^-$) also contain the *BRAF V600E* mutation.

From our data it appears that HRM and SS analysis are feasible strategies for the detection of the *BRAF V600E* mutation in unenriched bone marrow aspirate samples from patients with HCL; however, they also highlight potential challenges. Bone marrow fibrosis is common in patients with HCL and this is probably secondary to cytokines produced by hairy cells themselves, including FGF-2[14] and TGF-beta.[15] Fibrosis often results in suboptimal quality marrow aspirate samples containing only small numbers of hairy cells (despite extensive involvement on trephine biopsy). If HRM and SS are to be used on unenriched specimens in a diagnostic setting, it would be important to ensure adequate disease burden to avoid false-negative results. In the cases where the HRM is positive but SS is negative, it is possible to confirm the mutation by the low copy number approach.[16] An alternative technique in cases of low disease burden is allele-specific oligonucleotide PCR. This has recently been shown to be a highly sensitive method for *BRAF V600E* detection in cases of hairy cell leukemia.[17-18] In our cohort, bone marrow aspirates taken at initial diagnosis *versus* post treatment had higher rates of *BRAF V600E* detection. This phenomenon presumably reflects higher estimated mutant allele percentages in these specimens but also provides evidence that *BRAF V600E* positive clones persist in relapsed disease without evidence of further mutations within the region sequenced.

In conclusion, our results confirm and extend the recent initial observations of Tiacci *et al.*[3] and support the recent finding of a disease defining point mutation in HCL. They also demonstrate that in the presence of adequate disease burden assessed morphologically, HRM and SS are feasible techniques to detect *BRAF V600E* in patients with HCL. We have also described what is to our knowledge the first case of an activating *BRAF* mutation in a patient with SMZL. The use of these simple, rapid techniques that avoid complex sample processing will enhance their implementation in the diagnostic laboratory, and aid the diagnosis of HCL and related lymphoproliferative disorders.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Katayama I, Li CY, Yam LT. Ultrastructural cytochemical demonstration of tartrate-resistant acid phosphatase isoenzyme activity in "hairy cells" of leukemic reticuloendotheliosis. Am J Pathol. 1972;69(3): 471-82.
2. Falini B, Tiacci E, Liso A, Basso K, Sabattini E, Pacini R, et al. Simple diagnostic assay for hairy cell leukaemia by immunocytochemical detection of annexin A1 (ANXA1). Lancet. 2004;363(9424):1869-70.
3. Tiacci E, Trifonov V, Schiavoni G, Holmes A, Kern W, Martelli MP, et al. BRAF mutations in hairy-cell leukemia. N Engl J Med. 2011;364(24):2305-15.
4. Willmore-Payne C, Holden JA, Tripp S, Layfield LJ. Human malignant melanoma: detection of BRAF- and c-kit-activating mutations by high-resolution amplicon melting analysis. Hum Pathol. 2005;36(5): 486-93.
5. Pichler M, Balic M, Stadelmeyer E, Ausch C, Wild M, Guelly C, et al. Evaluation of high-resolution melting analysis as a diagnostic tool to detect the BRAF V600E mutation in colorectal tumors. J Mol Diagn. 2009;11(2): 140-7.
6. Boyd EM, Bench AJ, van 't Veer MB, Wright P, Bloxham DM, Follows GA, et al. High resolution melting analysis for detection of BRAF exon 15 mutations in hairy cell leukaemia and other lymphoid malignancies. Br J Haematol. 2011;155(5):609-12.
7. Swerdlow SH, International Agency for Research on Cancer., World Health Organization. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon, France: International Agency for Research on Cancer, 2008.
8. Simi L, Pratesi N, Vignoli M, Sestini R, Cianchi F, Valanzano R, et al. High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. Am J Clin Pathol. 2008; 130(2):247-53.
9. Gruszka-Westwood AM, Hamoudi RA, Matutes E, Tuset E, Catovsky D. p53 abnormalities in splenic lymphoma with villous lymphocytes. Blood. 2001;97(11): 3552-8.
10. Ikenoue T, Hikiba Y, Kanai F, Tanaka Y, Imamura J, Imamura T, et al. Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors. Cancer Res. 2003;63(23):8132-7.
11. Trovisco V, Soares P, Preto A, de Castro IV, Lima J, Castro P, et al. Type and prevalence of BRAF mutations are closely associated with papillary thyroid carcinoma histotype and patients' age but not with tumour aggressiveness. Virchows Arch. 2005;446 (6):589-95.
12. Mateo M, Mollejo M, Villuendas R, Algara P, Sanchez-Beato M, Martinez P, et al. 7q31-32 allelic loss is a frequent finding in splenic marginal zone lymphoma. Am J Pathol. 1999;154(5):1583-9.
13. Gruszka-Westwood AM, Hamoudi R, Osborne L, Matutes E, Catovsky D. Deletion mapping on the long arm of chromosome 7 in splenic lymphoma with villous lymphocytes. Genes Chromosomes Cancer. 2003;36(1):57-69.
14. Aziz KA, Till KJ, Chen H, Slupsky JR, Campbell F, Cawley JC, et al. The role of autocrine FGF-2 in the distinctive bone marrow fibrosis of hairy-cell leukemia (HCL). Blood. 2003;102(3):1051-6.
15. Shehata M, Schwarzmeier JD, Hilgarth M, Hubmann R, Duechler M, Gisslinger H. TGF-beta1 induces bone marrow reticulin fibrosis in hairy cell leukemia. J Clin Invest. 2004;113(5):676-85.
16. Do H, Dobrovic A. Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low level mutations in cancer biopsies. Mol Cancer. 2009;8:82.
17. Tiacci E, Schiavoni G, Forconi F, Santi A, Trentin L, Ambrosetti A, et al. Simple genetic diagnosis of hairy cell leukemia by sensitive detection of the BRAF-V600E mutation. Blood. 2012;119(1):192-5.
18. Arcaini L, Zibellini S, Boveri E, Riboni R, Rattotti S, Varettoni M, et al. The BRAF V600E mutation in hairy cell leukemia and other mature B-cell neoplasms. Blood. 2012; 119(1):188-91.