The *BRAF* V600E mutation in hairy cell leukemia and other mature B-cell neoplasms

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*Running title: BRAF V600E mutation in lymphoid B-cell neoplasms*

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Abstract

The somatically acquired V600E mutation of the \textit{BRAF} gene has been recently described as a molecular marker of hairy cell leukemia (HCL). We developed an allele-specific PCR for this mutation, and studied 62 patients with HCL, one with HCL variant, 91 with splenic marginal zone lymphoma, 29 with Waldenström macroglobulinemia, and 57 with B-cell chronic lymphoproliferative disorders. The \textit{BRAF} V600E mutation was detected in all HCL cases, and in only two of the remaining 178 patients. These two subjects had a B-cell chronic lymphoproliferative disorders that did not fulfill the diagnostic criteria for HCL. Despite the PCR positivity, the mutation could not be detected by Sanger sequencing in these two cases, suggesting that it was associated with a small subclone. We conclude that the \textit{BRAF} V600E mutation is present in all HCL patients and that, in combination with clinical and morphological features, represents a reliable molecular marker for this condition.

\textbf{Keywords}: \textit{BRAF}, hairy cell leukemia, splenic marginal zone lymphoma, mutation
Introduction

Hairy cell leukemia (HCL) is a distinct lymphoid neoplasm characterized by splenomegaly and peripheral cytopenias.1-4 Recently, Tiacci et al5 performed massively parallel sequencing of the whole exome of leukemic and matched normal cells an index HCL patient, and identified five somatically acquired mutations, including an heterozygous V600E mutation in the \textit{BRAF} gene. Targeted resequencing detected this mutation in 46 additional HCL patients but in none of 193 cases of peripheral B-cell lymphoma or leukemia.

The above observation is of paramount importance for a clear-cut diagnosis of HCL, and also for its differential diagnosis from HCL-mimicking diseases such as splenic marginal zone lymphoma (SMZL),6,7 splenic lymphoma/leukemia undifferentiated,8 and B cell chronic lymphoproliferative disorders (B-CLPD).9,10

The aim of this study was to define the incidence of the \textit{BRAF} V600E mutation in a large series of diverse lymphoid disorders. To this aim, we developed an allele-specific PCR for this mutation and studied a series of 240 mature B-cell lymphoid neoplasms, including 62 HCL cases.
Methods

Patients

These investigations were approved by the Ethics Committee of the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, and other local Institutional Review Boards. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects provided informed consent.

We studied 62 patients with HCL, one with HCL-variant (HCL-V), 91 with SMZL, 29 with WM, and 57 with B-CLPD. Diagnosis of HCL and HCL-V was made according to the WHO 2008 classification; no case analyzed in this study was included in the original report on \textit{BRAF} mutations. Diagnosis of SMZL was based on the WHO 2008 classification and on the criteria defined by Matutes et al. it was based on spleen histology in 20 patients, and on bone marrow (BM) histology combined with immunohistochemistry and flow cytometry findings in 71 cases. The diagnosis of WM was made according to the consensus recommendations from the Second International Workshop on WM. Fifty-seven patients were diagnosed with B-CLPD in absence of specific phenotypic and/or genetic alterations (24 cases were CD5-positive, 33 CD5-negative, and 13 HCV-positive); in particular, a diagnosis of CLL or a leukemic phase of mantle cell lymphoma or follicular lymphoma were ruled out in each subject.

Histology and immunohistochemistry

BM biopsies were formalin-fixed, briefly decalcified (2 h) with EDTA-HCl solution and embedded in paraffin. Immunohistochemistry was carried out by means of the streptavidin-biotin peroxidase technique, with enzymatic or microwave oven pretreatments, when needed.
DNA extraction

In patients with HCL and in the HCL-V case, genomic DNA was extracted from BM biopsies using QuickGene DNA tissue kit S (DT-S) (Life Science) on the FUJIFILM QuickGene-810 extraction platform (Fujifilm, Kyoto, Japan) according to the manufacturer's instructions.

Peripheral blood (PB) or BM mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation. Genomic DNA was extracted from BM in 90 cases (33 SMZL, 29 WM, 28 B-CLPD) and from PB in 88 cases (1 HCL, 58 SMZL, 29 B-CLPD) or using Puregene Blood DNA isolation kit (Qiagen, Milan, Italy) according to the manufacturer's recommendations. DNA concentration was quantitated by A²⁶⁰ absorbance with a BioPhotometer (Eppendorf, Hamburg, Germany). Genomic DNA (100 ng/sample) was used as template.

PCR amplification and sequencing of exon 15 of BRAF gene

BRAF gene exon 15 sequencing was performed to identify positive controls (c.T1860A) for ASO PCR. Two primers (forward, 5’- TACCTAAACTCTTCATAATGCTTGC-3’; reverse, 5’- GTAACTCAGCAGCATCTCAGGG-3’), were used to amplify a 256 bp fragment. PCR was carried out in a final volume of 50 µl containing 1X reaction buffer, 0.2 µM of each primer, 200 µM dNTPs, 2 mM MgCl₂ and 2.5 U of HotStarTaq (Qiagen, Milan, Italy). PCR consisted of an initial denaturation step of 15 minutes at 95°C, followed by 40 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 60 seconds, with a final extension step of 10 minutes at 72°C.

The amplification product was separated on 3% agarose gel and visualized by SYBR safe staining (Invitrogen, Inc., Carlsbad, CA, USA). The PCR products were purified and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI 3500 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Healthy donors were used as negative controls.
Allele Specific Oligonucleotides (ASO)-PCR assays

Two different forward primers with substitution of a single base at the end of the primer (FW 5’-TAGGTGATTGTTTCTAGCTACCGT-3’ and FM 5’-TAGGTGATTGTTTCTAGCTACCGA-3’) were designed to amplify the wild-type allele or BRAF T1860A transversion mutation respectively. To prevent the amplification of the nonmatching primer, an additional nucleotide mismatch (A > C) located 3 bases from the 3’ termini of the allele-specific primers was incorporated. The sequence of the reverse primer was the same as used before. Mutated or wild-type sequences from PB or BM samples were specifically amplified in a noncompetitive PCR as described before. BM biopsies were amplified in a final volume of 50 µl containing 1 µM of each primer, 200 µM dNTPs, 1.5 mM of MgCl2 and 1 U of AmpliTaq Gold DNA polymerase, with the reaction buffer supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). PCR consisted of an initial denaturation step of 15 minutes at 95°C, followed by 38 cycles of 95°C for 40 seconds, 58°C for 60 seconds and 72°C for 20 seconds, with a final extension step of 10 minutes at 72°C. All PCR reactions were performed in triplicate.
Results and Discussion

Clinical features of the HCL patients studied are reported in the Supplemental Table S1. The \textit{BRAF} V600E mutation was found in all the 62 HCL cases studied. In 61 cases, DNA derived from BM biopsies with a hairy cell infiltration ranging from 15% to 95%, while in the remaining one it derived from PB with 2% of hairy cells detected by flow cytometry immunophenotyping. Sanger sequencing in patients with heavily infiltrated BM showed an heterozygous pattern for the mutation. Detailed description of HCL samples is reported in the Supplemental Table S2.

The \textit{BRAF} V600E mutation was absent in the HCL-V case, and in patients with SMZL or WM. Two patients with B-CLPD showed a weak positivity in the BM (Figure 1) by ASO-PCR, but DNA Sanger sequencing did not reveal the mutation in these cases. Histological and flow cytometry features of these 2 cases are summarized in Table 1.

Case #1. In November 2008, a 41 year-old female presented with asymptomatic lymphocytosis, without any evidence of lymphadenopathy or organomegaly. Laboratory data showed hemoglobin (Hb) 12.9 g/dL, white blood cells (WBC) 16 x 10^9/L (45% circulating clonal B-cells by FC) and platelets (PLT) 283 x 10^9/L. On the BM biopsy (Figure 1), an interstitial lymphoid infiltrate (60% of the whole cellularity) composed by small, lymphocyte/centrocyte-like cells was found and. FISH for t(11;14), performed for cyclin D1 expression, was negative. Immunoglobulin rearrangement was IGHV3-48*02, IGHD7-27*01 IGHJ4*02. In this case, the \textit{BRAF} V600E mutation was confirmed also in PB. At the last follow-up, lymphocytosis was stable and the patient did not have any need of treatment.

Case #2. In 2006, a 62 year-old male presented with thrombocytopenia and splenomegaly, and a diagnosis of HCL was established in another Hospital. A cladribine treatment produced only a partial response. We saw this patient in May 2008, when his spleen was palpable 3 cm under the costal margin. A blood cell count showed Hb 15.4 g/dL, WBC count 3.9 x 10^9/L with 14% circulating clonal B-cells by flow cytometry immunophenotyping, and PLT count 95 x 10^9/L. A BM biopsy showed
20% lymphoid infiltrate with interstitial and sinusoidal pattern, composed by small to medium sized cells, with evident nucleoli, similar to pro-lymphocytes. Since this patient was asymptomatic, a watch-and-wait policy was adopted.

The \textit{BRAF} V600E mutation has been previously found in a significant proportion of solid cancers\textsuperscript{12-14} and in Langerhans cell histiocytosis,\textsuperscript{15} while \textit{BRAF} mutations other than V600E have been observed in small proportions of patients with ALL\textsuperscript{16,17} or B-cell lymphomas.\textsuperscript{18}

The findings of this study indicate that the allele-specific PCR we developed is able to detect the \textit{BRAF} V600E mutation in all patients with HCL. The same results have been recently obtained using another PCR approach,\textsuperscript{19} or high-resolution melting analysis.\textsuperscript{20} The fact that we successfully analyzed DNA extracted from BM biopsies is of crucial importance, as this most often represents the only available material containing hairy cells in HCL patients.

Within diverse mature B-cell neoplasms, the \textit{BRAF} V600E mutation was highly specific for HCL. In fact, the allele-specific PCR was positive in only two out of 57 patients with B-CLPD, who did not fulfill the diagnostic criteria for HCL. Despite the PCR positivity, the mutation could not be detected by Sanger sequencing, suggesting that it was associated with a small subclone. The two patients had different features, and no conclusion can be drawn at present concerning the prevalence of \textit{BRAF} V600E-positive clones in B-CLPD.

We conclude that the \textit{BRAF} V600E mutation is present in all HCL patients and that, in combination with clinical and morphological features, represents a reliable molecular maker for the laboratory diagnosis this mature B-cell neoplasm.
Authorship

Contribution: LA and MC designed the research; SZ developed the allele-specific PCR and did molecular investigations; EB, ML and MP reviewed histological diagnosis; RR extracted DNA from bone marrow biopsies; AT performed flow cytometry analysis; SaR, MV, MLG, MM, SiR, LM, CC and MDV collected clinical data; LA and MC wrote the paper. All authors critically revised the manuscript and approved the version to be published.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Table 1. Histological and flow cytometry features of the two patients affected with non-HCL mature B-cell lymphoproliferative disorders carrying the **BRAF** V600E mutation

<table>
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<th>Case #2</th>
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**Flow cytometry immunophenotyping**

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Legends to Figure

**Figure 1. Upper part: ASO-PCR assay.** PCR products were separated on 3% agarose gel electrophoresis, and for each sample the wild-type (WT) and the mutant (M) allele were amplified. Panel A: HCL samples presented the *BRAF* V600E mutation, while the MW cases did not present the mutant allele. Panel B: whiled B-CLPD cases #1 and #2 presented both the wild type and the mutant allele, the SMZL case did not present the *BRAF* V600E mutation. An HCL sample with an heterozygous *BRAF* V600E mutation was used as a positive control, and a healthy donor was used as a negative control.

**Lower part: bone marrow histology of case #1.** Panel C: On bone marrow biopsy, an abundant (60%) lymphoid infiltrate was observed with interstitial pattern and composed by small lymphocyte-like, centrocyte-like cells, without the typical morphology of hairy cells (Giemsa, 40x). Panel D: By immunophenotyping, either on histological sections (here) or by means of flow cytometry on BM aspirate (Table 1), the only similarity between this case and HCL was the expression of cyclin D1/bcl1 oncoprotein (streptavidin-biotic-peroxidase complex method/SABC, DAB chromogen, 40x). Panel E: While ANXA-1, the most specific marker of HCL, was negative on the lymphoid population, and only expressed by myeloid precursors, which serve as internal control (SABC, DAB chromogen, 60x).
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