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Correlation of BRAF V600E Mutation in Peripheral Blood Smears of Hairy Cell Leukemia with Morphology and Immunophenotyping

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ABSTRACT

Hairy cell leukemia being defined as a mature B-cell lymphoma shows unique clinicopathological, immunophenotype, and genetic alteration features among another mature B-cell malignancy. Although lymphocytes with hairy appearance and co-expression of CD25, CD11c and CD103 markers on B-cells are compatible with hairy cell leukemia, there are some difficulties in differential diagnosis between HCL with its variant and splenic marginal zone lymphoma. The presence of kinase-activating BRAF V600E mutation in classical HCL clone and in no other mature B-cell lymphomas/ leukemia has implication in differential diagnosis. In this study, we evaluated BRAF V600E mutation detection of H&E stained peripheral blood smears by Sanger sequencing method in patients with classical HCL and findings related to morphology and immunophenotype. Peripheral smear review indicated a subpopulation of atypical lymphocytes with villous cytoplasmic projections. By flow cytometry analysis, B-cells represented immunophenotype of CD19+ CD20(bright)+ CD11c+ CD25+ CD103+. Sanger sequencing demonstrated that presence of BRAF V600E mutation in 86.6% (13/15) of analyzed HCL DNA. Hence, gain-of-function mutation of BRAF V600E is detectable in DNA of peripheral blood smear of patients with classical HCL that were evaluated by immunophenotyping. In conclusion, DNA from peripheral blood smear can be used to identify BRAF V600E mutation using Sanger sequencing method in samples with at least 10% HCL clone as supplementary approach to aid in the diagnosis of classical HCL.

Keywords: Hairy cell leukemia, Immunophenotype, Chronic lymphoproliferative disorder, BRAF mutation

INTRODUCTION

Hairy cell leukemia (HCL) is a well-defined clinicopathological entity that is characterized by diffuse bone marrow infiltrate, pancytopenia, monocytopenia, and rare circulating tumor cells [1,2]. HCL most frequently affect elderly men with male to female ratio of 4:1 at median age of 52 years [3,4]. The presence of splenomegaly, hepatomegaly, and abdominal adenopathy are the symptoms of HCL patients. The medium-sized neoplastic cells with featured villous cytoplasmic projections, oval/bean-shaped nuclei and clumped chromatin are the characteristics of HCL. Fried-egg appearance, prominent cell border and clear cytoplasm, is a microscopic description of HCL [5]. However, HCL may be difficult to distinguish on Hematoxylin and Eosin (H&E) slide. So far, diagnosis of HCL is based on morphologic, immunophenotypic and immunohistopathology criteria [6], which can distinguish HCL from other B-cell lymphoproliferative disorder.

Hairy cells are found at low number in the blood and have a characteristic immunophenotype. Leukemic cells display slightly increased side-scatter (SSC) and placing in monocytic region on CD45 versus SSC. Flow cytometry analysis show co-expression of CD103 and CD25, bright expression of CD11c and CD20. CD5 expression is usually negative, only rare cases show expression on B-cells. CD23 is negative on leukemic cell and CD10 is often negative, but occasional cases may show aberrant expression of CD10. Markers like FMC7, CD22, CD79a, and CD123 have positive expression on neoplastic cells. Dim expression of CD11c is unusual in HCL phenotype [7-10].

Recently, more sensitive and specific test, BRAF V600E mutation, has been introduced for a genetic-based diagnosis of HCL [11]. Description of BRAF c.1799T>A p. Val600Glu helped to clarify the pathogenesis of HCL. This mutation is present in all hairy cell leukemia samples, while is not exist in other B-cell malignancies [12]. BRAF V600E mutation has been identified in different cancers, including melanoma [13] and papillary thyroid cancer [14]. In spite of high accuracy of this test, it is not a routinely diagnostic approach for HCL. For reliable detection of HCL clone with heterozygous mutation, the presence of at least 30% leukemic cells is necessary [11]. Hence, cell sorting for purification of rare HCL cells is helpful. However, detection of BRAF V600E mutation using Sanger sequencing method is sensitive test for the routine clinical diagnosis of HCL in blood samples [15].

In this study, we evaluated 20 suspicious samples for hairy cell leukemia, as well as 5 control samples by morphology, multiparameter flow cytometry, and BRAF V600E mutation analysis to find the frequency of BRAF V600E mutation and evaluate the utility of BRAF molecular test using Sanger sequencing method in parallel with morphology and immunophenotyping parameters for accurate diagnosis of HCL.

MATERIALS AND METHODS

Study design

A total of 25 peripheral blood samples including 20 patients clinically suspicious to HCL and 5 samples of previously diagnosed of B-cell chronic leukemia (B-CLL) as controls obtained from Razavi hospital Laboratory during 2015 to 2017. All sample patients were from presentation, and not from relapse of disease. Control samples were evaluated and confirmed by immunophenotyping approach; they showed co-expression of CD19/CD5 and CD5/CD23, as well as negative expression of FMC7 marker which are criteria of B-CLL diagnosis. For all samples, morphological evaluations of H&E stained blood smears were done independently by multiple pathologists. Specifically, HCL was identified by morphological features as described previously. All 20 peripheral blood samples regardless of morphological manifest of involvement of HCL were included for flow cytometry analysis. In this study, the only samples that confirmed as HCL cases by the method of immunophenotyping and also, 5 control B-CLL samples were followed by BRAF mutation analysis.

Flow cytometry

Immunophenotyping of atypical B lymphocytes was performed on peripheral blood or bone marrow aspiration mononuclear cells by multicolor flow cytometry (Attune acoustic focusing cytometer, Applied Biosystems, USA) with a panel of five-colour conjugated monoclonal mouse anti-human antibodies (BD Bioscience, USA); CD5 APC (Clone L17F12, cat no: 345783), CD11c PE (Clone S-HCL-3, cat no: 333149), CD19 PerCP (Clone 4G7, cat no: 345778), CD20 PE (Clone L27, 345793), CD25 PE-Cy7 (Clone 2A3, cat no: 335824), CD45 PerCP-Cy5.5 (Clone 2D1, cat no: 332784), and CD103 FITC (Clone Ber-ACT8, cat no: 333155) antigen expression. A tube of Fluorescence minus one (FMO) control was included to check background fluorescence and positive populations in experiment. Then, cells underwent erythrocytes lysis using BD FACS Lysing solution (BD Bioscience, USA), followed by a wash step with PBS containing 1% BSA and 0.1% sodium azide and finally samples analyzed on flow cytometer.

BRAF exon 15 amplification

To isolate DNA from HE-stained glass slide smear, slides were soaked in xylene at room temperature for 48h to remove the coverslip. The cellular material was then scraped off and placed into 1.5 ml microcentrifuge tube containing absolute ethanol for 2 wash steps. The cells were pelleted at full speed for 5 min. Then, cells were desiccated and followed by DNA extraction using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The following PCR primer pair (F: 5'-TCATGAAGACCTCACAGTAAAAATAGG-3' and R:

5'-AGCAGCATCTCAGGGCCAAA-3') was applied to amplified BRAF exon 15 sequence. The reaction mixture included 1x PCR buffer, 1.8 mM $MgCl_2$, 200 nM of each primer, 200 µM of dNTPs, 0.5 U of HotStart Taq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 25 µL. PCR condition included an activation step of 15 min at 95˚C followed by 35 cycles of 94°C for 1min, annealing 60°C for 45 sec, extension at 72°C for 1min, and final extension at 72°C for 10min. PCR products were visualized and quantified on 2% agarose gel containing 0.1% ethidium bromide.

Samples were Bi-directionally sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were analyzed using Sequencer software version 4.9 (Genes Codes Cooperation, Ann Arbor, MI).

RESULTS

By the method of immunophenotyping, 15 samples from 20 patients were identified with HCL, and flow cytometry findings of 5 samples represented as reactive lymphocytosis or other hematological malignancies. Morphological examination identified atypical lymphocytes consistent with HCL phenotype detected by flow cytometry analysis (Figure 1). In flow cytometry analysis of HCL patients, none of them had CD25 negative expression and all showed classic HCL immunophenotype on B-cells (CD5-, CD11c+, CD25+, and CD103+) except two sample patients in which absence of CD103 marker expression was seen on B-cells. Hence, hairy cell variants were not identified immunophenotypically among patient samples studied. The median age of samples with HCL was 56.2 years (Range 36-73 years) with a male/female ratio of 1.5. Clinical and phenotypical characteristics of HCL patients presented in Table 1.

Table 1 Clinical and phenotypical features of 15 patients with hairy-cell leukemia (HCL)

Patient NO	BRAF V600E mutation	Sex	Age at diagnosis	WBC count at diagnosis	Immunophenotype of Leukemic B Cells			HCL Clone (%)	Previous therapy
			years	per mm^3	CD11c	CD25	CD103		
1	Yes	F	48	5,270	+	+	+	24	No
2	Yes	M	56	7,700	+	+	+	29	No
3	Yes	F	61	8,970	+	+	+	65	No
4	Yes	M	49	6,680	+	+	+	30	No
5	Yes	M	37	3,120	+	+	+	18	No
6	Yes	F	73	2,800	+	+	+	22	No
7	Yes	M	63	11,460	+	+	+	53	No
8	Yes	M	67	2,620	+	+	+	25	No
9	Yes	M	50	6,050	+	+	+	60	No
10	Yes	F	36	9,260	+	+	-	63	No
11	Yes	M	58	2,670	+	+	+	51	No
12	No	F	66	6,980	+	+	+	9	No
13	Yes	F	53	9,980	+	+	+	38	No
14	No	M	63	97,510*	+	+	+	8	No
15	Yes	M	63	2,510	+	+	+	60	No

*Bone Marrow Aspiration sample, Overall 3/15 (86.6%) patients with classical HCL phenotype had the BRAF V600E mutation which was detected in peripheral blood sample by Sanger sequencing method (Fig.1). The percentage of HCL clone varies between 8 and 65. The BRAF V600E was not detected in control samples, B-CLL, were diagnosed by the method of flow cytometry, Control samples showed CD5+/CD23+/FMC7- expression on B-cells.

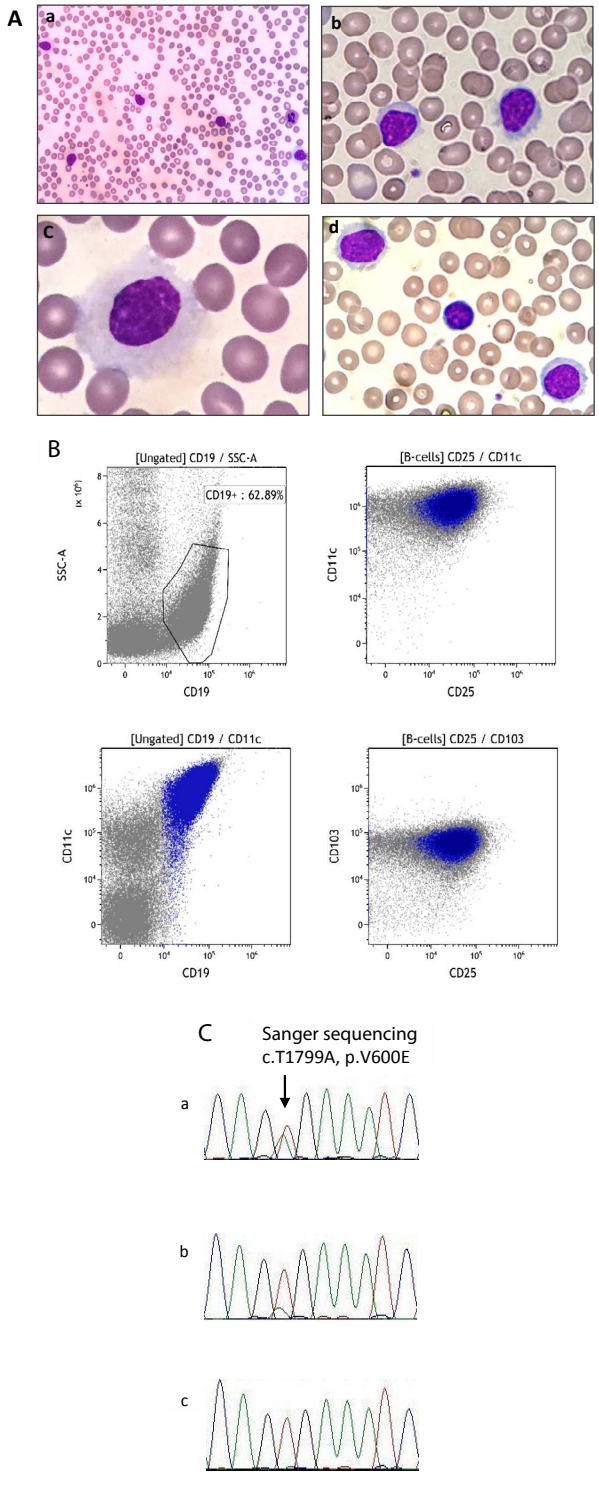

Figure 1. Cytomorphology, Imunophenotyping and Sanger sequencing assays in HCL. Panel A represent cytological features of HCL in the peripheral blood. Images shows leukemiac cells with cytoplasmic projections in compared to atypical lymphocyte (H&E stained, subpanel a: magnification 40x; subpanel b, c and d, magnification 100x). In Panel B, flow cytometry dot-plot of a sample from a patient with classical HCL shows peripheral-blood mononuclear cells (PBMCs) expressing CD19 together with CD11c, CD25, and CD103. Panel C, Sanger sequencing on DNA extracted from scraped cellular materials off the slides in HCL cases reveal a heterozygous T→A mutation (subpanel a and b, respectively). The leukemic cells do not carry the T→A mutation in B-CLL patient (subpanel c)

DISCUSSION

Diagnosis of hairy cell leukemia, a low-grade B-cell lymphoproliferative disorder, relies on morphological and immunophenotyping criteria being discussed in detail previously. In this study, patients were identified with HCL by combination of morphology characteristics, immunophenotype and molecular. None of these samples had not request for cytochemistry. It seems that immunophenotyping as a more powerful and reproducible diagnostic approach for identification of atypical B-cell population has been inhibited cytochemistry [16]. Detection of atypical lymphocytes by cytochemical staining techniques may lack of specificity and are often not well-controlled. For accurate diagnostic and classification of B-cell lymphoma, confirmatory laboratory test including immunophenotyping assay is needed [17,18]. However, there are some diagnostic barriers in immunophenotyping due to low number of circulating hairy cells. Moreover, CD25 expression is often negative in hairy cell variants which make diagnostic difficulties in differential diagnosis [19]. The differential diagnosis of among classical HCL and its variants is crucial for appropriate treatment selection [7]. Hence, sensitive and specific diagnostic test is essential for differential diagnosis of hairy cell from other peripheral B-cell lymphomas including splenic marginal zone lymphoma. The recent identification of BRAF V600E mutation which is observed and limited to classical HCL not variant cases, allowed molecular differential diagnosis of HCL [20,21]. Furthermore, molecular testing BRAF V600E mutation helps to improve diagnostic accuracy of HCL in peripheral blood sample. And also as a high sensitive test, it allows early detection of HCL relapse and determination of treatment response in the long-term follow-up [12,22]. In 2011, the study using whole exome sequencing detected that BRAF V600E mutation is present in all 48 hairy cell samples and none of 114 other peripheral B-cell disorders and, these results were confirmed by high-resolution melting (HRM) analysis [11]. Other molecular approaches have been developed for BRAF V600E mutation detection in order to Minimal Residue Disease (MRD) monitoring in peripheral blood of HCL patients [22], including allele-specific PCR [23], pyrosequencing [24] and allele-specific quantitative PCR [25]. Tiacci, et al. showed that allele-specific oligonucleotide PCR is a highly sensitive method for BRAF V600E mutation in cases of low HCL burden [15]. Although it has been claimed that Sanger sequencing method can miss a significant proportion of low-level heterozygous mutations [26], we identified BRAF c.1799T>A mutation in HCL diagnosed samples having more than 10% HCL clone. The absence of BRAF V600E mutation in non-HCL samples and control samples was detected by DNA sequencing approach. These finding are in consistence with study that BRAF V600E mutation is not present in other B-cell Lymphoproliferative disorder [27]. On the other hand, Langabeer, et al. reported BRAF V600E mutation in B-cell chronic lymphocytic leukemia and prolymphocytic leukemia [28]. However, it has been established that HCL relies strongly on BRAF V600E mutation other than solid tumors [29]. Interestingly, it has been claimed that this point mutation is detectable in relapsed disease which reflects that BRAF V600E positive clone remain in post treatment [30,31]. HCL patients had a good response to purine analogs but relapse more frequently. Hence, identification of BRAF V600E mutation in HCL has significance for introducing minimal residue disease monitoring and as potential targeted therapy which has been established in melanoma treatment [13,32]. In 2015, it was established that oral BRAF inhibitor vemurafenib was highly effective in HCL patients who had refractory to purine analogues, or relapsed following purine analogue treatment [12,33]. Therefore, this key driver mutation that distinguishes HCL from other B-cell lymphomas could be act as an ideal therapeutic target in HCL [29].

From our data, it appears that detection of BRAF V600E mutation in H&E-stained glass slide using Sanger sequencing analysis is achievable strategy. In this study, the minimum percentage of HCL clone obtained by flow cytometry analysis was 8% in total cells. In addition, all samples were at initial diagnosis, not post treatment. We could not identify this key driver mutation in two samples which presented 8 and 9% HCL clone in total cells. One explanation about false-negative result of Sanger sequencing in these two low disease burden samples is that we assessed BRAF mutation detection on peripheral blood smear, which is unenriched sample. Since, fresh whole blood sample of HCL patients were not available. Then, we could not extract DNA from purified lymphocytes using Ficoll-paque density that make increase in specificity and sensitivity of the BRAF mutation detection. Furthermore, evaluation of BRAF V600E mutation on leukemic cells that were purified using Ficol-hypaque gradient following cell sorting to select CD19+ cells is the most sensitive approach for MRD monitoring [29]. The same false-negative result in BRAF V600E mutation detection by Sanger analysis has been reported on bone marrow smear samples of HCL patients with lower that 15% HCL clone in total cells [30]. In those cases, the BRAF mutation was detected positive by HRM analysis, but failed using Sanger method regarding to this point that limitation detection of HRM assumed >7.5% (heterozygosity of mutated alleles). Hence, assurance of adequate disease burden is crucial to avoid false-negative result in Sanger sequencing analysis for BRAF mutation detection in HCL peripheral blood smear.

CONCLUSION

This study confirms the high specificity of BRAF V600E mutation detection by the method of Sanger sequencing on peripheral blood smear of HCL patients having at least 10% of HCL clone. BRAF V600E mutation detection on peripheral blood smear could be considered as a further layer of diagnostic power especially in those cases that further material is restricted.

DECLARATIONS

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Conflict of Interest

The authors declare no financial or commercial conflict of interest regarding this manuscript.

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