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Original Article

Immunohistochemical analysis using a BRAF V600E mutation specific antibody is highly sensitive and specific for the diagnosis of hairy cell leukemia

Xuan J Wang, Annette Kim, Shaoying Li

Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, USA

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Abstract: Hairy cell leukemia (HCL) is usually diagnosed by morphology and flow cytometry studies. However, it is challenging sometimes to distinguish HCL from its mimics. Recently, the *BRAF* V600E mutation has been described as a disease-defining molecular marker for HCL which is present in nearly all cases of HCL but virtually absent in mimics of HCL. In this study, we investigated the possibility of using immunohistochemical detection of the BRAF V600E mutant protein to differentiate HCL from its mimics. A total of twenty-eight FFPE tissue specimens were studied, including HCL (n=12), HCL variant (HCL-v, n=3), splenic marginal zone lymphoma (SMZL, n=6), and other marginal zone lymphomas (MZL, n=7). Immunohistochemical studies were performed using a mouse monoclonal antibody (clone VE1, Spring Bioscience, CA) specific for *BRAF V600E* mutation. Molecularly confirmed *BRAF* V600E mutation positive and negative cases were used as the positive and negative controls respectively. All 12 cases of HCL showed cytoplasmic BRAF V600E protein expression in leukemia cells by immunohistochemical study regardless of tumor burden, whereas all cases of HCL mimics including HCL-v, SMZL, and MZL were negative for BRAF V600E protein. Using this *BRAF* V600E mutation specific antibody, this immunohistochemical study has 100% sensitivity and 100% specificity for the diagnosis of HCL in our cohort. In conclusion, immunohistochemical detection of the BRAF V600E mutant protein is highly sensitive and specific for the diagnosis of HCL. Compared to PCR or sequencing-based methodologies, immunohistochemistry is a relatively rapid and inexpensive alternative for the differential diagnosis between HCL and its mimics.

Keywords: *BRAF* V600E, hairy cell leukemia, immunohistochemistry

Introduction

Hairy cell leukemia (HCL) is a mature B-cell malignancy characterized by splenomegaly, pancytopenia, and circulating lymphoid cells with circumferential "hairy" cytoplasmic projections. The hairy cell leukemia cells typically have a distinctive immunophenotype: coexpression of CD25, CD11c, CD103, CD123 and the pan B-cell markers CD19, CD20, and CD22 [1]. Thus, the diagnosis of HCL can usually be established on the basis of tumor cell morphology and flow cytometry immunophenotypic studies alone. However, rare cases of HCL may show some variation in morphologic or immunophenotypic features. In addition, some HCL mimics, which include HCL variant (HCL-v), splenic marginal zone lymphoma (SMZL), and rarely other marginal zone lymphomas (MZL) can display variable degrees of morphologic and immunophenotypic features similar to those of HCL. These variations make it very difficult to make a definitive diagnosis in some cases.

The differential diagnosis between HCL and its mimics is crucial because HCL, but not its mimics, is uniquely sensitive to alpha interferon or nucleoside analogs such as cladribine and pentostatin [2]. Although immunohistochemical stains such as Annexin A1, tartrate-resistant acid phosphatase, DBA.44, and T-bet, may aid in the diagnosis of HCL, these markers lack sufficient sensitivity and specificity for the differential diagnosis between HCL and its mimics [3]. Unlike other B cell neoplasms, HCL has a very stable genome and lacks any recurrent translocations [1, 4, 5]. In 2011, Tiacci et al showed

Table 1. Immunohistochemical analysis of HCL and its mimics

Diagnosis	No of Cases	Tissue/Organ	BRAF V600E (% positive)
HCL	12	Bone marrow	100%
HCL-v	3	Bone marrow (n=2); Spleen (n=1)	0%
SMZL	6	Spleen	0%
MALT	4	Parotid (n=3); Stomach (n=1)	0%
MZL	3	Lymph node (n=1); Bone marrow (n=2)	0%

HCL: Hairy cell leukemia; HCL-v: HCL variant; SMZL: Splenic marginal zone lymphoma; MZL: nodal Marginal zone lymphoma; MALT: extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue.

that *BRAF* V600E mutation was present in 100% of 48 patients with HCL but in none of 195 patients with other B-cell malignancies, which included 22 SMZL and 16 unclassifiable splenic B-cell lymphoma/leukemia, including HCL-v and splenic red pulp small B-cell lymphoma [6]. *BRAF* V600E mutation was independently confirmed as a disease defining molecular marker for HCL in subsequent studies [7-10]. All of these previous studies used molecular techniques such as Sanger sequencing, high resolution melting analysis, or pyrosequencing. These methods are highly specific and analytically sensitive. However, they are usually more expensive with a relatively longer turn-around-times, and may not be available in all pathology practice settings.

Recently, a mouse monoclonal antibody (clone VE1) specifically recognizing the BRAF V600E mutant protein was developed and shown to exhibit a high sensitivity and specificity for the detection of BRAF V600E in a variety of tumors [11-16]. Here we performed an independent study to further confirm the sensitivity and specificity of this antibody in the diagnosis of HCL and to evaluate if immunohistochemistry using this mutation specific antibody can serve as an alternative for molecular methods for the detect of *BRAF* V600E mutation in the differentiation of HCL from its mimics.

Materials and methods

Tissue selection

All tissue material was obtained from the Department of Pathology, Microbiology, and Immunology at Vanderbilt University Medical Center with appropriate approval from the Institutional Review Board. A total of 28 cases were studied (bone marrow, n=15; spleen, n=6; lymph node and other, n=7) which including 12 cases of HCL, 3 cases of HCL-v, 6 cases of SMZL, and 7 cases of nodal and extranodal MZL (**Table 1**). Slides and flow cytometry were reviewed for all cases to confirm the diagnoses according to the 2008 World Health Organization criteria [1]. All 12 HCL demonstrated typical morphology and immunophenotype.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded (FFPE) tissue specimens from the above 28 cases. The BRAF V600E immunohistochemical stain was performed on an automated immunostainer (Leica Bond-Max IHC stainer, San Diego, CA). The 4-μm-thick tissue sections were deparaffinized and underwent a heat induced antigen retrieval using the Bond Max Epitope Retrieval 2 solution for 20 minutes. The sections were incubated with a mouse anti-human BRAF V600E specific monoclonal antibody (Clone VE1, Spring Bioscience, Inc., Pleasanton, CA) diluted at 1:100 for one hour. The Bond Refine Polymer detection system was used for visualization. A HCL-v case with molecularly confirmed negative *BRAF* V600E mutation was used as the negative control. A melanoma case with molecularly confirmed positive *BRAF* V600E mutation was used as the positive control.

Data analysis

All immunohistochemical slides were evaluated by three pathologists blinded to the diagnoses and were scored as either positive or negative. Cases were scored as positive if tumor cell cytoplasmic staining was evident with staining intensity significantly higher than background non-specific staining. The negative and positive control samples stained appropriately. Additional immunohistochemical studies for CD20 and/or CD79a originally performed at diagnosis were reviewed and compared with the BRAF V600E staining in all cases.

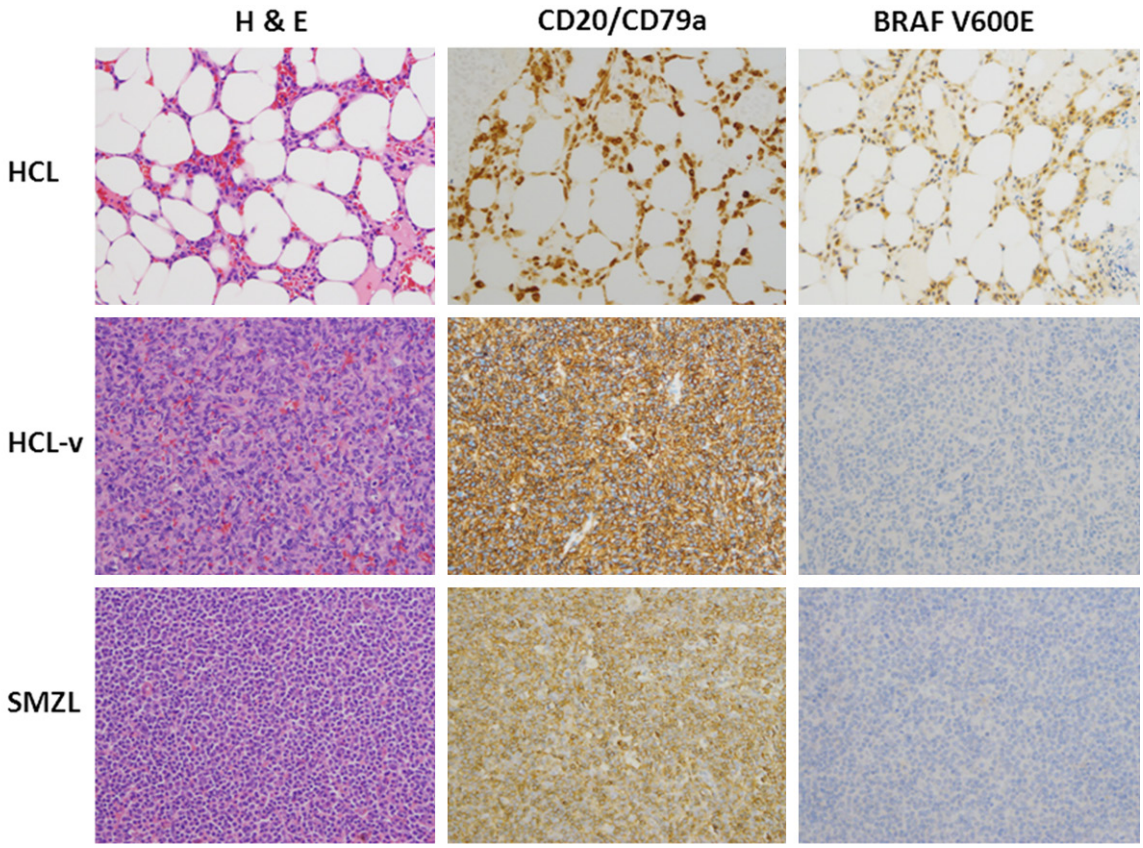

Figure 1. Immunohistochemical analysis using VE1 clone specific to BRAF V600E mutation protein. A representative case of hairy cell leukemia (HCL, upper panel) showed cytoplasmic BRAF V600E protein expression in all CD79a positive cells; a representative case of HCL variant (HCL-v, middle panel) and splenic marginal zone lymphoma (SMZL, lower panel) demonstrated lack of BRAF V600E expression in CD20+ tumor cells.

Results

All 28 (100%) cases were evaluable by immunohistochemistry for the *BRAF* V600E mutant protein. All 12 cases of HCL were bone marrow biopsies and all showed cytoplasmic *BRAF* V600E protein expression in leukemia cells by immunohistochemistry regardless of the tumor burden (**Table 1** and **Figure 1**). When compared to CD20 or CD79a immunostains, cells with positive BRAF V600E staining corresponded to the CD20/CD79a positive cells. In contrast, BRAF V600E staining was not identified in any of the 3 cases of HCL-v, 6 cases of SMZL, and 7 cases of nodal and extranodal MZL (**Table 1** and **Figure 1**). Non-specific staining in rare plasma cells was seen in some cases of HCL [11]. Immunohistochemistry using the BRAF V600E mutation specific antibody (VE1) demonstrated 100% sensitivity and 100% specificity for the diagnosis of HCL in our study.

Discussion

BRAF, a serine-threonine protein kinase, is a member of the RAF kinase family and plays an important role in the RAS-RAF-MAPK signaling pathway, which regulates cell survival, proliferation and differentiation [17]. Somatic *BRAF* mutations, with c.1799T>A (V600E) being the most common, have been previously reported in a variety of cancers including melanoma, thyroid, colonic and ovarian carcinomas [18, 19]. In recent years, the *BRAF* V600E mutation has been shown to be a disease-defining mutation for HCL, while it is virtually absent in other hematopoietic tumors [20, 21], with the exception of Langerhans cell histiocytosis [22]. The identification of this mutation is not only important for the diagnosis of HCL, but also potentially for targeted therapy. Although traditional purine analogues have induced high response rate in HCL, the relapse rate is high. A *BRAF* inhibitor, Vemurafenib, has demonstrated

effective response in standard chemotherapy-resistant HCL patients [23-25].

The availability of these targeted *BRAF* inhibitors as well as the exquisite sensitivity of HCL to purine analogues makes it critical to differentiate HCL from its mimics. Although detection of the *BRAF* V600E mutation using various molecular techniques can be a direct and very effective method to aid in the diagnosis of HCL in some morphologically and immunophenotypically atypical cases, molecular testing can also be relatively expensive and time consuming. By contrast, immunohistochemical detection of the BRAF V600E mutant protein is a rapid and inexpensive method which may be quickly implemented in the majority of the diagnostic pathology practices. Another benefit of immunohistochemistry is that it requires less tissue than molecular methods.

In our study of 12 HCLs and 16 HCL mimics, we have shown that the immunohistochemical stain using a BRAF V600E mutation specific antibody demonstrates 100% sensitivity and 100% specificity for the diagnosis of HCL and can be used as a useful tool to differentiate it from its mimics. However, there have been rare reports of HCL cases that were notable for an absence of the mutation. A study from the antibody development group found that two cases of HCL that were positive for BRAF V600E by immunohistochemistry (VE1 clone) lacked the mutation by DNA Sanger sequencing [11]. However, both cases had a low tumor burden that was below the detection limit of Sanger sequencing. This suggests that in cases with a low tumor burden, immunohistochemical analysis is likely to be even more sensitive than certain molecular techniques. Other studies that have also identified HCL cases with no *BRAF* V600E mutation include Xi et al, who studied 53 cases of HCL and found 11 cases lacking the BRAF V600E mutation [26]. Langabeer et al. recently reported a case of HCL with a classic clinical, morphological, immunophenotypic, and cytochemical profile but no *BRAF* V600E mutation [27]. There are 3 postulations regarding why BRAF V600E mutation were not detected in some HCL cases: 1) Some are truly wild type; 2) Some lack the specific *BRAF* V600E and instead harbor a non-exon 15 mutation, as demonstrated by Tschernitz et al [28]; 3) The *BRAF* V600E mutation status may be falsely negative due to low tumor cell burden and the use of assays with limited analytical sensitivity. Under the first two circumstances then, similar to molecular tests, immunohistochemical studies will be negative for the BRAF V600E protein. In the third circumstance, immunohistochemistry may detect the BRAF V600E protein [11]. None of the HCL cases in our small cohort lacked the BRAF V600E expression.

It is well known that most cases of HCL and its mimics can be diagnosed based on the typical morphology and immunophenotype. For diagnostically challenging cases with atypical features, we suggest the following strategy to diagnose HCL and differentiate it from its mimics: 1) Immunohistochemical study for BRAF V600E protein expression using the VE1 clone; a positive stain supports the diagnosis of HCL; 2) If the immunostain is negative or equivocal, appropriate PCR-based molecular analysis, rather than Sanger sequencing, should be performed to confirm the absence of *BRAF* V600E mutation. The utilization of this strategy will ensure an accurate, rapid and cost effective diagnosis of HCL or its mimics.

In summary, the results of our study demonstrate that immunohistochemical detection of BRAF V600E protein is highly sensitive and specific for the diagnosis of HCL and can aid in the differential diagnosis from its mimics. Compared to molecular techniques, immunohistochemistry is an accurate, rapid, and cost-effective alternative for the diagnosis and differential diagnosis of HCL.

Disclosure of conflict of interest

No authors have any conflict of interest.

Address correspondence to: Dr. Shaoying Li, Division of Hematopathology, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, 4519 TVC, 1301 Medical Center Drive, Nashville, TN 37232-5310, USA. Tel: 615-343-9149; Fax: 615-3437961; E-mail: shaoying.li@Vanderbilt.edu

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