

Original Research Article

Real-time PCR-based analysis of BRAF V600E mutation in low and intermediate grade lymphomas confirms frequent occurrence in hairy cell leukaemia

Mark Ewalt^{1†}, Subhadra Nandula^{1†}, Adrienne Phillips², Bachir Alobeid¹, Vundavalli V. Murty¹, Mahesh M. Mansukhani^{1*} and Govind Bhagat^{1*}

¹Department of Pathology and Cell Biology, Columbia University Medical Center and New York Presbyterian Hospital, New York, NY, USA

²Department of Medicine, Division of Hematology and Oncology, Columbia University Medical Center and New York Presbyterian Hospital, New York, NY, USA

*Correspondence to:

Govind Bhagat and Mahesh M. Mansukhani, Department of Pathology and Cell Biology, Columbia University Medical Center and New York Presbyterian Hospital, 630 West 168th Street, VC 14–228, New York, NY, USA.
E-mail: gb96@columbia.edu;
E-mail: mm322@columbia.edu
[†]These authors contributed equally to this work.

Abstract

Hairy cell leukaemia (HCL) is a rare type of B-cell non-Hodgkin lymphoma (B-NHL), which is not known to be associated with any characteristic recurrent karyotypic abnormality. A recent study that used massively parallel whole exome sequencing identified an activating V600E mutation in BRAF, which appeared specific for HCL. Here, we confirm the specificity of BRAF V600E for HCL among low and intermediate grade B-NHL and describe a real-time polymerase chain reaction method for detecting this mutation in cases with low tumour burden. The V600E mutation does not appear to be associated with microsatellite instability, unlike the case in colorectal cancer. Thus, in conjunction with prior data, our results suggest incorporation of BRAF V600E mutation analysis in the diagnostic workup of HCL cases. Additionally, targeting the Ras-Raf-Mek-Erk-Map kinase pathway should be investigated as a potential therapeutic strategy for patients with this disease. Copyright © 2011 John Wiley & Sons, Ltd.

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Introduction

Recent advances in technology, especially high resolution whole genome analyses, have yielded clues to the molecular pathogenesis of a variety of neoplasms that lacked recurrent cytogenetic alterations, as ascertained by conventional methods [1]. In an early study analysing cancer genomes for recurrent mutations, Davies *et al.* discovered activating mutations of BRAF in exons 11 and 15, most frequently in melanoma (up to 66% of cases harboured such a mutation), with the V600E (c.1799 T>A) mutation being the most common [2]. Until recently, data regarding the presence and frequency of BRAF mutations in lymphomas were limited. Lee *et al.* identified somatic BRAF mutations in 4 of 164 (2.4%) non-Hodgkin lymphomas (NHL), all representing diffuse large B-cell lymphomas. The V600E mutation was not identified in this series [3]. BRAF mutations were recently detected in 4% of multiple myeloma by a multi-institutional consortium on performing whole genome and exome analyses of 38 samples and screening an additional 161 cases, with four (2%) cases harbouring the V600E mutation [4]. More recently, Tiacci *et al.* also identified a V600E BRAF mutation in an index case of hairy cell leukaemia (HCL) with the use of massively parallel whole exome sequencing, and the authors further showed that this mutation was present in all 47 additional HCL analysed with Sanger sequencing [1]. These authors suggested that the BRAF V600E mutation

may account for the activated phenotype of HCL, as a BRAF inhibitor could inhibit downstream ERK and MEK phosphorylation *in vitro*, and that targeting this pathway may be of therapeutic value [1]. Recently, Boyd *et al.* verified the presence of the V600E mutation in HCL patients with the use of a high resolution melting analysis assay [5].

In this study, we sought to investigate the presence of BRAF V600E mutations in a large panel of low and intermediate grade B-cell non-Hodgkin lymphoma (B-NHL) comprising different subtypes, and to assess any association of this mutation with microsatellite instability (MSI). We used a real-time polymerase chain reaction (PCR)-based assay to detect the mutation in unpurified peripheral blood, bone marrow and tissue samples, obtained fresh and also after fixative treatment. Limiting dilution analysis demonstrated utility of this assay in detecting V600E mutation in samples with a low tumour burden.

Materials and methods

Case selection

Our departmental archive was searched for cases of low and intermediate grade B-NHL diagnosed at our institution between 1 January 2000 and 15 August 2011. The selected B-NHL ($n = 102$) were classified according to the 2008 World Health Organization Classification [6], as HCL ($n = 12$ from eight patients), chronic lymphocytic leukaemia/

small lymphocytic lymphoma ($n = 20$), mantle cell lymphoma ($n = 19$), marginal zone lymphoma (MZL, $n = 24$); representing 18 extranodal MZL, stomach ($n = 6$), lung ($n = 4$), eye/ocular adnexa ($n = 4$), and thymus, kidney, ileum and rectum ($n = 1$ each); four nodal MZL; and two splenic MZL, follicular lymphoma ($n = 20$) and lymphoplasmacytic lymphoma ($n = 7$). DNA was extracted with the use of DNAzol[®] (Invitrogen, Carlsbad, CA) for samples fixed with Carnoy's solution as previously described [7], or with the use of the QIAamp[®] tissue and blood kits (Qiagen, Valencia, CA) for fresh and formalin-fixed samples, as per manufacturer's instructions. Tumour percentage was evaluated through flow cytometry in nine HCL samples, and immunohistochemistry for CD20, TRAP and DBA-44 in three HCL samples. This study was approved by our institutional review board.

Detection of BRAF V600E mutation

Presence of BRAF V600E mutation was determined with the use of a real-time PCR assay and allele-specific hydrolysis ('Taqman') probes. Taqman[®] Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used to amplify a 136 bp DNA fragment. Wild type and mutant alleles were distinguished with the use of competing Taqman[®] probes. The primers and probes used for PCR amplification (B-raf-51 F and B-raf-176R) have been previously described [8]. Briefly, the PCR assay was performed on an ABI 7500 (Applied Biosystems, Carlsbad, CA) standard real-time ddCT relative quantification plate with the use of the BRAF-mutant—FAM-TAGCTACAGAGAAATC and BRAF-wild type—VIC-TAGCTACAGTGAAATC probes. A sample was considered to harbour the BRAF V600E mutation if the difference in the threshold cycle (dCt) between amplification of mutant and wild type sequence was less than or equal to eight cycles. (Figure 1). The detection limit (percent tumour cells) was determined by diluting DNA, which was extracted from a fresh HCL sample with known neoplastic cell percentage (Patient 6, Table 1), with normal high molecular weight DNA extracted using the same method. The mutant allele was also detected through the dilution of the A2058 cell line DNA in normal DNA.

Analysis for microsatellite instability

Microsatellite instability analysis was performed using the MSI Analysis System, version 1.2 (Promega Corp, Madison, WI). This assay entails a multiplex reaction of five quasi-monomorphic mononucleotide repeat markers (BAT-25, BAT-26, MONO-27, NR-21 and NR-24) and also entails two highly polymorphic pentanucleotide markers (Penta C and Penta D) as sample identifiers. After PCR amplification, samples were analyzed with ABI 3100 Avant[™] (Applied Biosystems, Carlsbad, CA) for altered alleles. In the absence of normal DNA for comparison, a sample was considered microsatellite-high (MSI-H) if greater than two markers showed an altered pattern, indeterminate if only two showed an alteration and microsatellite stable (MSS) if no marker showed an altered allele (or MSS with an allele polymorphism if only one marker showed an altered allele).

Results

The clinical characteristics of hairy cell leukaemia cases are presented in Table 1. Of note, one case (Patient 8) had an atypical immunophenotype, as detected by flow cytometry (CD103 negative), but it expressed CD25 and TRAP by immunohistochemistry. Another case (Patient 7) presented with axillary lymphadenopathy without significant splenomegaly. We purified DNA from tumour samples and used the real-time PCR assay to assess for the presence of BRAF V600E mutation (Table 2). The BRAF V600E mutation was detected in all cases where the tumour percentage in the analysed tissue exceeded 5%. In two patients (Patients 2 and 5), no mutation was detected in the samples with low tumour burden ($\leq 5\%$ tumour cells) but was detected in additional samples containing a higher frequency of neoplastic cells. We, thus, confirm the results of Tiacci *et al.* indicating a high prevalence of BRAF V600E mutation in HCL. The detection of the V600E mutation in an atypical CD103 negative HCL case and a case with an atypical clinical presentation highlights the utility of this analysis to diagnose challenging cases of HCL.

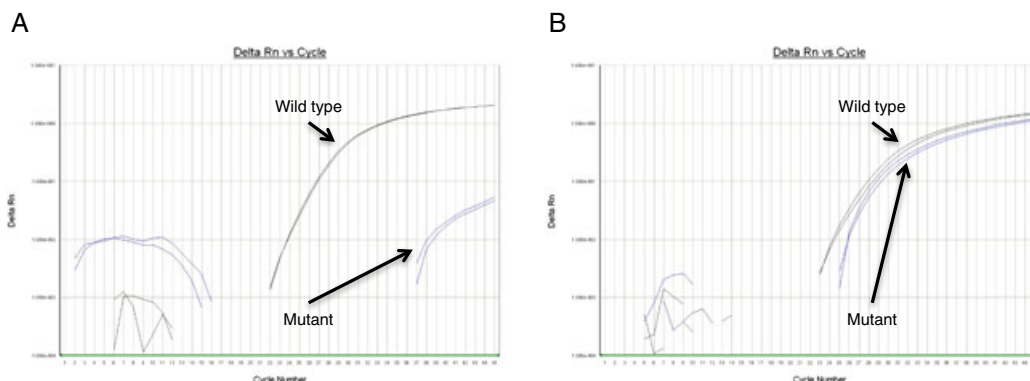


Figure 1. Representative results from the real-time PCR assay for detection of BRAF V600E mutation. (A) Negative case (extranodal marginal zone lymphoma, stomach). (B) Positive case (hairy cell leukaemia, Patient 6, spleen). Black curves represent wild type probes (VIC), and blue curves represent mutant (c1799T>A pV600E) probes (FAM)

Table 1. Clinical characteristics of HCL patients

Patient	Age at diagnosis	Sex	Presentation	Treatment [†]
1	58	M	Pancytopenia	Rituximab
2	69	M	Pancytopenia	Cladribine
3	46	M	Pancytopenia	Unknown
4	41	M	Pancytopenia	Unknown
5	53	M	Pancytopenia	Cladribine, Rituximab/Cladribine, Cladribine
6	43	F	Pancytopenia, splenomegaly	Cladribine, Pentostatin (2 rounds), Rituximab/Pentostatin, Splenectomy
7	61	M	Pancytopenia, lymphadenopathy, weakness	None
8*	30	M	Pancytopenia	None

[†]Sequential treatment regimens.

*This case represents an HCL with an atypical phenotype.

Table 2. BRAF V600E mutation detection in HCL samples

Patient	Tissue type	Tumour%	WT Ct	Mutant Ct	dCt	BRAF V600E
1	Bone marrow	3.7%*	32.817	35.478	2.661	Mutant
2	lung	5%**	29.932	39.83	9.898	Wild type
	Bone marrow	13.4%*	29.273	31.6	2.327	Mutant
	Lymph node	80%**	30.045	32.145	2.1	Mutant
3	Bone marrow	35.3%*	31.135	33.78	2.645	Mutant
4	Bone marrow	0.6%*	34.142	45	10.858	Wild type
5	Peripheral blood	3.1%*	29.757	37.78	8.023	Wild type
	Bone marrow	23.0%*	31.089	33.531	2.442	Mutant
6	Peripheral blood	42.9%*	28.341	30.298	1.957	Mutant
	Spleen	78.4%*	24.81	24.968	0.158	Mutant
7	Lymph node	70%**	27.819	31.116	3.297	Mutant
8	Bone marrow	5.7%*	30.337	36.597	6.26	Mutant

dCt: difference in the threshold cycle; WT: wildtype; Ct: threshold cycle.

*Tumour percentage based on flow cytometry.

**Tumour percentage assessed by immunohistochemical staining for CD20, DBA-44 and TRAP.

To further evaluate the sensitivity of our real-time assay, we performed limiting dilution analysis with the use of the spleen sample from Patient 6 (Table 2), diluting the extracted DNA with wild type genomic DNA. We were able to detect the BRAF V600E mutation at a dilution ratio of 1:8 (9.8% tumour cells) (Table 3). We were similarly able to detect the mutant allele in a 10% dilution of the A2058 cell line DNA in normal DNA, or approximately 5% mutant allele (data not shown). The absolute limit of detection may be improved with further refinement of this assay. At present, it provides sensitivity within the range of 5% and 10% tumour cells, demonstrating the value

Table 3. Limiting dilution analysis to determine mutation detection sensitivity of the real-time PCR assay

Dilution	Tumour%	dCt	BRAF V600E
1:1	78.4%	0.3	Detected
1:2	39.2%	0.6	Detected
1:4	19.6%	3.1	Detected
1:8	9.8%	5.2	Detected
1:16	4.9%	11.4	Not Detected
1:32	2.5%	13.1	Not Detected

dCt: difference in the threshold cycle.

Tumour% based on flow cytometry.

DNA was extracted from fresh splenic tissue (Patient 6).

of real-time PCR in screening for the BRAF V600E mutation in HCL samples where the neoplastic cellular yield is often limited because of underlying myelofibrosis.

We next sought to evaluate the specificity of the BRAF V600E mutation for HCL among low and intermediate B-NHL by evaluating for the presence of this mutation in 90 additional cases of B-NHL with a >20% tumour burden. We found that none of these additional cases harboured the BRAF V600E mutation, supporting the notion that this alteration is specific to HCL (Table 4).

Previous studies of mutations in the Ras-Raf-Mek-Erk-Map kinase pathway have found an association between BRAF activating mutations and MSI in colorectal neoplasms [9].

Table 4. BRAF V600E mutation detection in low-intermediate grade B-NHL

Neoplasm type	Frequency of V600E mutation
Hairy cell leukaemia	9/12
Chronic lymphocytic leukaemia/small lymphocytic lymphoma	0/20
Mantle cell lymphoma	0/19
Marginal zone lymphoma	0/24
Follicular lymphoma	0/20
Lymphoplasmacytic lymphoma	0/7

The assay failed for one lymphoplasmacytic lymphoma case.

BRAF V600E mutations were also described in three of nine (33%) immunodeficiency-related NHL displaying MSI, which consisted of EBV + diffuse large B-cell lymphomas, including a primary CNS lymphoma, and an EBV-T-cell post-transplant lymphoproliferative disorder [10]. We, thus, investigated whether HCL with BRAF mutations showed evidence of MSI. Interestingly, all cases exhibited an MSS phenotype with using a sensitive assay.

Discussion

Hairy cell leukaemia is a rare and enigmatic B-NHL that predominantly involves the bone marrow and spleen. It lacks characteristic chromosome abnormalities, and its aetiology has remained elusive ever since its recognition as a unique subtype of B-NHL [6,11–13]. The neoplastic small B-cells exhibit 'hairy' cytoplasmic projections and have an activated phenotype, which may explain their response to purine analogues and interferon therapy.

The ubiquitous occurrence and high specificity of the BRAF V600E mutation for HCL among B-NHL, as recently described [1,5], was confirmed in our series of HCL with the use of a real-time PCR assay. We were able to identify this mutation in seven of eight (87.5%) patients. We also have shown that this mutation was not present in 90 additional samples comprising a variety of low and intermediate grade B-NHL, further supporting the notion that the BRAF V600E mutation is a specific genetic alteration in HCL.

Tiacci *et al.* reported a sensitivity of approximately 30% tumour cells for the detection of BRAF V600E using Sanger sequencing [1]. Here, we describe a method that detects this mutation in cases with a lower tumour burden by using either fresh or fixed archival tissue, within range of the method by Boyd *et al.* [5].

Taken together, our data and other published data suggest that the BRAF V600E mutation is specific for HCL among low to intermediate grade B-NHL. Prior studies have shown that the BRAF V600E mutation leads to activation of the MAP kinase pathway via sequential phosphorylation of downstream signalling mediators. BRAF inhibition abrogates this activation, suggesting a potential role for BRAF inhibition in the treatment of refractory/resistant HCL [1,2]. Further studies will help elucidate the exact pathogenic effects of this mutation. Given the high specificity of this mutation, it might also become an integral part of disease classification in the future. Our study did not include cases of HCL variants and only two cases of splenic MZL were evaluated, which were both negative. Analysis of additional such cases needs to be undertaken in order to confirm specificity of this mutation for HCL among low and intermediate grade B-NHL.

Conclusion

In summary, we describe a sensitive method to specifically detect the BRAF V600E mutation in patient samples down

to a concentration of 5% to 10% tumour cells. The current assay demonstrates an improved detection limit compared to direct Sanger sequencing and could thus be useful for diagnosis and therapy selection. Disease monitoring following treatment, however, may require an allele-specific PCR approach, or other sensitive methods that can detect low level mutant alleles.

Authorship contributions

Govind Bhagat and Mahesh Mansukhani designed the study and analysed data. Adrienne Phillips, Govind Bhagat, Bachir Alobeidi and Mark Ewalt provided and characterized patient samples. Subhadra Nandula performed the experiments and analysed data. Mark Ewalt and Govind Bhagat wrote the manuscript. All authors reviewed the manuscript and provided critical comments.

Conflicts of interest

The authors declare no conflict of interest.

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