

TITLE: Comparison of methodologies for the detection of *BRAF* mutations in bone marrow trephine specimens.

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ABSTRACT

AIMS: *BRAF* V600E detection assists in the diagnosis of hairy cell leukaemia (HCL), however testing practices vary. We evaluated the clinical utility of 5 *BRAF* mutation testing strategies for use on bone marrow trephines (BMT).

METHODS: 11 HCL, 5 HCL 'mimic', 2 treated HCL and 10 normal BMT specimens were tested for mutant *BRAF*, comparing Sanger sequencing, pyrosequencing, amplicon-based next generation sequencing (NGS), automated (Idylla) polymerase chain reaction (PCR) and immunohistochemistry (IHC).

RESULTS: PCR and IHC were cheaper and identified V600E in 100% of HCL cases. Pyrosequencing detected the mutation in 91%, NGS in 55% of cases and Sanger sequencing in 27%. All assays gave wild-type *BRAF* results in HCL mimics and normal BMT samples.

CONCLUSIONS: PCR and IHC were most sensitive and cost-effective, but these have limited scope for multiplexing and are likely to be replaced by NGS gene panels or whole genome sequencing in the medium to long term.

INTRODUCTION

Hairy cell leukaemia (HCL), a rare low-grade, generally indolent, B cell neoplasm, accounts for around 2% of lymphoid leukaemias, typically presenting in middle-age, with splenomegaly and bone marrow infiltration leading to pancytopenia. Treatment with nucleoside analogues (cladribine and pentostatin) usually leads to remission.¹ Around 50% of patients relapse and require second-line therapies such as rituximab, in combination with nucleoside analogues.² More recently low dose BRAF inhibitors, such as vemurafenib, have been used successfully in patients with recurrent or progressive HCL, either alone or in combination with rituximab.³⁻⁶

Histology classically shows a characteristic 'fried egg' cytomorphology and immunohistochemistry (IHC) and/ or flow cytometry confirm expression of CD20, CD11c, CD25, CD103, CD72 and annexin-A1.¹ Differentiation from other lymphoproliferative malignancies (e.g. splenic marginal zone lymphoma) on bone marrow trephine (BMT) biopsy, critical for informing management, is often straightforward, but occasionally more challenging. In recent years, the *BRAF* V600E mutation has been demonstrated in almost 100% of HCL infiltrates but is usually absent in other lymphoproliferative disorders.^{1,7} The V600E codon change renders the B-Raf serine/threonine protein kinase (on the mitogen-activated protein kinase (MAPK) pathway) constitutively active, promoting cell proliferation and neoplasia.⁸ V600E is a disease-defining driver mutation in HCL and detection of the mutation is both diagnostically useful in cases with non-classical features and important in guiding targeted therapy.^{1,4-11}

Commercially available methodologies for detecting *BRAF* mutations in formalin-fixed paraffin embedded (FFPE) tissue include polymerase chain reaction (PCR) assays (e.g. Cobas, Roche Diagnostics) and traditional sequencing techniques (Sanger, pyrosequencing). Recent developments include next-generation sequencing (NGS) panels, novel automated PCR (e.g. Idylla, Biocartis) systems and monoclonal antibody immunohistochemistry (e.g. VE1 IHC, Ventana Medical Systems).^{12,13} There are no guidelines for *BRAF* testing methodologies in HCL. In this study we evaluate the clinical utility of a range of commercially available assays as applied to BMT, comparing concordance and cost.

METHODS

TISSUE SAMPLES: 27 fully anonymised archival FFPE, EDTA-decalcified bone marrow trephine biopsies (BMT), with diagnoses and percentage involvement by haematological neoplasms (where appropriate) as shown in Table 1, were obtained with full ethical approval (IRAS reference 162057) from the Oxford Radcliffe Biobank, John Radcliffe Hospital, Oxford, UK.

IMMUNOHISTOCHEMISTRY: BRAF V600E mutation-specific immunostaining on 4µm thick FFPE sections was carried out on all samples using the VE1 mouse monoclonal antibody clone (Ventana Medical Systems, Tucson, AZ, USA) using a Ventana Benchmark Ultra immunostainer according to the manufacturer's instructions (Ventana Medical Systems). All results were analysed by a specialist haematopathologist (EJS).

AUTOMATED PCR: Two 5µm-thick FFPE tissue sections of samples H1-17 and D6 were loaded separately into a disposable cartridge for analysis using the Idylla (Biocartis, Mechelen, Belgium) fully-automated real-time PCR (qPCR) system, following the manufacturer's instructions.

DNA EXTRACTION: Ten 5µm sections of each FFPE BMT specimen were mounted on glass slides. Bone marrow tissue was visually identified, manually macrodissected using a scalpel, and deparaffinised using the M220 focused ultrasonicator (Covaris, Woburn, MA, USA). DNA was extracted using the Covaris truXTRAC FFPE DNA kit according to the manufacturer's instructions.

DNA ANALYSIS: DNA concentration was measured using both UV spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Bishop's Stortford, UK) and fluorometric quantification (Qubit dsDNA BR Assay, Thermo Fisher Scientific). DNA quality (in terms of amplifiability) was assessed by qPCR using a QuantStudio Real-Time PCR System, following the manufacturer's instructions (Thermo Fisher Scientific). The same primers (primer 204; sequence subject to commercial confidentiality) and fluorescent dye (SYBR green in SYBR KAPA 2x mastermix) were used for all reactions and ROX low was used as a reference dye. Samples were cycled as follows: 50 degrees for 2 minutes, 95 degrees for 10 minutes, followed by 40 cycles of 95 degrees for 30 seconds, 60 degrees for 30 seconds, and 72 degrees for 31 seconds. Because D1-D10 were acting only as negative control samples, not all techniques were applied to all samples. Due to its excellent DNA yield, sample D6 was selected for automated PCR and Sanger

sequencing, while samples D1 - D5 and D7 - D10 underwent Sanger sequencing, NGS and pyrosequencing.

SANGER SEQUENCING: *BRAF* exon 15 (a 255-base pair (bp) fragment containing codon 600) was amplified from the extracted DNA by PCR using a TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany) and primers containing V600 M13 tails for sequencing (M13F: GTAAAACGACGGCCAGTCCTAACTCTTCATAATGCTTGC & M13R: CAGGAAACAGCTATGACAGTAACTCAGCAGCATCTCAGG). Cycling conditions: 97°C for 15 minutes, 30 cycles of 97°C for 30 seconds, 60°C for 90 seconds, and 72°C for 1 minute; final extension at 72°C for 10 minutes. 5µl of each PCR product was visualised on a 3% ethidium bromide-containing TBE agarose gel. PCR product was sent to the Oxford Regional Genetics Laboratory (Churchill Hospital, Oxford, UK) for Sanger sequencing (using M13 tail primers) on an ABI 3130 Genetic Analyser (Thermo Fisher Scientific). Sequence analysis was performed using Mutation Surveyor (SoftGenetics LLC, State College, PA, USA). The resulting electropherograms were visually assessed for the c.1799T>A nucleotide substitution (V600E). Results with only a very small peak (as shown in figure 1 panel F) were classified as 'equivocal'.

PYROSEQUENCING: A 151 bp DNA region encompassing *BRAF* exon 15 was amplified by PCR (*BRAF* pyro forward: CTTTACTTACTACACCTCAGA, *BRAF* pyro reverse: CTCAATTCTTACCATCCACAA). Cycling conditions: 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute; final extension at 72°C for 10 minutes. 5µl of each PCR product was visualised on a 3% TBE agarose gel. Pyrosequencing was carried out using 20µl of PCR product and primer *BRAF* V600 pyro: TGATTTTGGTCTAGCTACA on a Pyromark Q24 according to manufacturer's instructions (Qiagen, Manchester, UK). A variant allele frequency of 5% was used as a cut-off for calling base changes.

NEXT GENERATION SEQUENCING: DNA extracted from all samples, excluding D6, was subject to NGS using a TruSeq Custom Amplicon (Illumina, Cambridge, UK) 'lymphoid panel' (Illumina) designed in-house using DesignStudio (Illumina): This consisted of 1146 175 bp amplicons which comprise most of the coding regions of 23 genes: *ATM*, *B2M*, *BRAF*, *CARD11*, *CD79B*, *CDKN2A*, *CREBBP*, *EZH2*, *KMT2D*, *KRAS*, *MEF2B*, *MYD88*, *NOTCH1*, *NOTCH2*, *NRAS*, *PIM1*, *PRDM1*, *PTEN*, *SOCS1*, *TET2*,

TNFAIP3, *TP53*. Up to 250 ng DNA (Table 1) was used for library preparation with a TSCA v1.5 kit as per the manufacturer's instructions. The low amount of input DNA for some samples (Table 1) meant that the number of PCR amplification cycles during library preparation was increased from the recommended 24 to 27 in some cases. Eight indexed samples were multiplexed and subjected to 2 x 150 bp paired end sequencing on a MiSeq (Illumina) using v2 chemistry. Alignment and variant calling were performed in Basespace (utilising a Custom banded Smith-Waterman aligner and the Somatic Variant Caller respectively) while filtering and annotation were performed using Variant Studio (both Illumina). Results were filtered on consequence (i.e. predicted either to change the amino acid sequence within the expressed protein, introduce a termination codon or alter splice sites) and quality with variants with a Q score of <100 being discarded. *BRAF* exon 15 sequence results were visualised using IGV (The Broad Institute, Cambridge, MA, USA). A variant allele frequency of 5% was used as a cut off for calling base changes.

RESULTS

IMMUNOHISTOCHEMISTRY: All (11/11; 100%) HCL cases samples showed diffuse, moderate to strong cytoplasmic staining with VE1 IHC (Table 1, Figure 1). None of the normal, HCL mimics or treated HCL in complete remission (HCL-CR) specimens exhibited any staining.

AUTOMATED PCR: All (11/11; 100%) HCL cases were found to be positive for a *BRAF* V600E/E2/D mutation, denoted collectively as *BRAF* V600 mutation. This assay cannot distinguish between these three different codon changes. All HCL mimic, HCL-CR and normal samples were identified as *BRAF* wild type (WT).

DNA EXTRACTION: 22/27 (81%) samples passed the 1.8 threshold for an acceptable 260/280 ratio on the Nanodrop, suggesting DNA was sufficiently free of RNA or protein contamination for downstream analyses. However, Qubit-determined DNA concentrations were consistently 30-50% of those measured using the Nanodrop (Qubit mean [DNA] = 33.4 ng/μl (SD = 28.7 ng/μl); Nanodrop mean [DNA] = 70.5 ng/μl (SD = 51.2 ng/μl)). As Qubit is less susceptible to overestimating the DNA concentration by including contaminants in the quantification, the Qubit DNA concentration was used for all subsequent calculations.

Poorer DNA yields were obtained from pathological samples H1-17 (mean [DNA] = 29.9 ng/μl) compared with normal samples D1-10 (mean [DNA] = 39.3 ng/μl), reflecting the fact that the greater numbers of 'normal' BMT specimens in the biobank, compared with those containing HCL or HCL-mimics, allowed selection of larger sized 'normal' samples.

A qPCR assay (Table 1) indicated that DNA of poorer quality was extracted from pathological specimens (H1-17) compared with normal samples (D1-10), (D1-10 mean = 3.19 ΔCt, H1-17 mean = 4.92 ΔCt), with 12/17 (71%) pathological samples compared with 2/10 (20%) normal samples having a ΔCt over 3, indicating greater DNA fragmentation in specimens H1-17.

SANGER SEQUENCING: Sanger sequencing failed to give a definitive result for 7/11 (64%) HCL cases, while it identified the *BRAF* c.1799T>A (i.e. V600E) mutation in 3/11 (27%) HCL cases, but misidentified 1 case (9%) as WT. 4/11 (36%) were equivocal and 3/11 (27%) failed due to poor quality DNA. These samples had the highest ΔCt values in the HCL-containing group as determined by qPCR, with a mean ΔCt of 7.78. 9/10 (90%) of the normal samples gave *BRAF* WT results and 1/10 (10%) failed due to poor quality DNA (ΔCt = 9.87). The 4 HCL-mimics and 2 HCL-CR cases were *BRAF* WT.

PYROSEQUENCING: 10/11 (91%) of HCL samples demonstrated a *BRAF* c.1799T>A base substitution. The remaining HCL sample (H10) had a *BRAF* c.1799T>A base substitution recorded at a level of 3% and was therefore determined to be negative, assuming an assay sensitivity of 5%. The HCL tumour burden in this sample was very low (~5%) and assuming the *BRAF* mutation is heterozygous, the VAF would only be expected to be ~2-3%. 100% normal samples, 100% of HCL mimics and both HCL-CR cases were identified as *BRAF* WT.

NEXT GENERATION SEQUENCING: The *BRAF* c.1799T>A base substitution was detected in 6/11 (55%) HCL cases. 4/11 (36%) HCL cases were identified as WT and 1/11 (9%) HCL case was not sequenced due to library preparation failure. 8/9 (89%) normal samples were found to be *BRAF* WT. In the remaining normal sample, the region on exon 15 containing the potential mutation site was not amplified and therefore was not sequenced. 100% of HCL mimics and HCL-CR cases were identified as *BRAF* V600 WT.

DISCUSSION

BRAF testing offers an additional diagnostic modality in challenging cases of HCL and is useful for directing targeted therapy, e.g., with vemurafenib. *BRAF* V600E analysis can help identify HCL-variant (HCL-v) cases, which resemble classical HCL, but lack the *BRAF* V600E mutation.^{7,11,12,14} HCL-v patients respond very poorly to treatment with purine analogues alone,¹⁵ but are much more amenable to combined therapy with rituximab,¹⁶ and are unsuitable for vemurafenib. While we recognise that in many cases of suspected HCL, a bone marrow aspirate sample will be available to allow extraction of high quality DNA, this study was undertaken to consider the most clinically effective approach to determining *BRAF* mutation status when only a BMT is available.

We investigated concordance between differing methodologies in the detection of the *BRAF* V600E mutation and considered their clinical utility in the diagnosis of HCL. Automated PCR and IHC showed complete (100%) concordance and were able to detect *BRAF* mutations in all untreated HCL cases. Given that Idylla and IHC identified *BRAF* V600 mutation in all cases in which it was expected and did not identify the mutation in any cases in which it was not expected and there was 100% concordance between the methodologies, results with these methodologies were taken as the “gold standard” against which the sensitivity of other methodologies was assessed. No method identified the mutation in cases in which it was not expected or in cases in which it was not identified by Idylla or IHC and thus all methods were 100% specific. Considering only cases in which *BRAF* V600 mutation was definitively identified by Idylla and IHC, sensitivities for pyrosequencing, NGS and Sanger sequencing were 91%, 55% and 27%, respectively. If equivocal cases were also included for pyrosequencing and Sanger sequencing, sensitivities rose to 100% and 64%, respectively. No cases gave equivocal results by NGS, with this method giving 45% false negatives.

IHC and the Idylla system currently appear most attractive for detection of *BRAF* mutations, based on diagnostic accuracy. IHC is the cheapest (around £30 per slide), fastest (within 4 - 6 hours) and most accessible for histopathology laboratories and thus may overall be the preferred option for most centres. Furthermore, IHC detects only the V600E and this is probably sufficient to detect most common mutations in HCL – other rare variants may be more common in other tumour types such as melanoma (the tumour which the Idylla test was originally designed for). As a DNA-based test, Idylla is more

expensive than IHC, however it is relatively competitive against other molecular techniques at around £100 per sample (cf. around £150 for conventional PCR or £350 for NGS). N.B. Costs quoted are average assay prices for the referrer in the UK for the different technologies at the time of writing. Hands-on time and experience needed for Idylla are minimal and the test is extremely rapid, with results in 90 minutes. The potential for multiplexing is currently limited with no more than a few genes in any one cartridge.¹⁷⁻²⁰ The system is currently impractical for high throughput work flows. Obviously, there are other PCR-based assays that are commercially available (e.g. cobas) and in-house, non-commercial assays are also popular in some laboratories. While this study did not specifically look to address such established methodologies in general, the price comparison here may help laboratories compare with their own current pipelines. Finally, newer methodologies with very low limits of detection, such as digital PCR, are also used by some centres. These techniques may be of particular use in monitoring minimal residual disease and would not be as hampered by DNA degradation as more traditional PCR.

Pyrosequencing demonstrates high sensitivity, making it an attractive option for *BRAF* testing, but it is not widely available to routine laboratories. Sanger sequencing demonstrated low sensitivity, either failing or giving equivocal results for the majority of HCL cases, likely due to low quality and/or low quantities of DNA, a common problem with FFPE samples, especially BMT samples, which have been decalcified. However, many of the equivocal results were likely to be low-level mutations (the variant allele frequency [VAF] detection threshold being 10-20%). Sanger sequencing thus remains useful for validating novel mutations with a sufficient VAF, but is less attractive for everyday clinical use. Sanger sequencing and pyrosequencing are relatively inexpensive (around £130 and £200 respectively) and produce fairly rapid results. Depending on the quality of sequencing, pyrosequencing can provide a more unambiguous read-out than Sanger sequencing electropherograms which require more subjective interpretation. Both methods are often performed using in-house assays meaning significant validation (as opposed to verification) is required to achieve ISO 15189 accreditation.

NGS has the advantage of massively-parallel sequencing of multiple regions within multiple gene targets, potentially addressing a wider range of clinical questions. However, in this study NGS demonstrated relatively low sensitivity compared with pyrosequencing,

Idylla and IHC. This was likely due to poor DNA quality, low DNA yield and/ or low variant allele frequency (VAF) due to low percentage involvement by HCL in some cases. Disadvantages of NGS include lengthy and complex bench work (although this is increasingly being automated), bioinformatic support requirements, a higher cost (£200-£300 per case) and longer turn-around times (if batched, sometimes up to a week). The high cost and long turn-around time are offset, however, by the ability to sequence multiple targets at once and, in the longer term, this will bring down costs per target and turnaround times.

NGS requires good quality DNA, making FFPE BMT samples, as currently processed, a challenging substrate for sequencing. We envisage better results in future with changes to fixatives, improved decalcification methods and laboratory processing, coupled with DNA capture technologies. In this context, it makes logistical and financial sense to move to a supraregional or national level service offering standardised NGS panels. There will remain a need, however, for rapid testing of urgent cases and/ or screening, prior to NGS testing. These tests may need to be done in a near-patient context and our data suggest the most rapid, reliable (and cost-effective) methods are automated PCR and IHC. Given its lower cost, we suggest that IHC currently has the greatest clinical utility for screening, with automated PCR or NGS for confirmation if targeted treatment is being considered. We anticipate that if the current plan for a national genomic service with central molecular testing is realised, NGS will eventually become the preferred methodology in the longer term for most testing. This, of course, will not obviate the diagnostic usefulness of IHC when assessing a first presentation bone marrow trephine.

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CONTRIBUTIONS:

All authors devised the project, carried out aspects of the practical work and contributed to the writing and editing of the manuscript.

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TABLE 1

Sample	Diagnosis	% Involvement	Nanodrop (260/280) (Acceptable >1.8)				qPCR (ΔCt) (pass<2.5)	NGS DNA input (ng)	Sanger Sequencing (stated sensitivity 20%)	Pyro-sequencing (stated sensitivity 5%)	MiSeq Variant Studio	MiSeq IGV	Idylla (stated sensitivity 1%)	IHC
D1	Normal: no haematological diagnosis	n/a	76.5	1.9	42	1.515	250	250	WT	-	-	0/361	n/a	n/a
D2		n/a	61.7	1.89	31	1.452	250	250	WT	-	-	6/2954	n/a	n/a
D3		n/a	100.8	1.89	66.2	1.978	250	250	WT	-	-	6/3314	n/a	n/a
D4		n/a	32.6	1.96	17.2	2.0725	150	150	WT	-	-	29/2973	n/a	n/a
D5		n/a	42.5	1.91	24.8	1.947	150	150	WT	-	-	4/4279	n/a	n/a
D6		n/a	145.8	1.87	90.2	2.066			WT	n/a	n/a	n/a	-	n/a
D7		n/a	50.1	1.94	30	2.284	250	250	WT	-	-	4/4725	n/a	n/a
D8		n/a	39.4	1.88	19	2.488	150	150	WT	-	-	0/284	n/a	n/a
D9		n/a	84.3	1.84	49	6.183	250	250	WT	-	-	1/1009	n/a	n/a
D10		n/a	58.4	1.79	23.2	9.869	150	150	Fail	-	Fail	Fail	n/a	n/a
H1	SMZL	>90	185.8	1.92	76.6	2.9965	250	250	WT	A:1% T:99%	-	14/5113	-	-
H2	HCL	70	51	1.93	19.9	2.8425	150	150	+	A:30% T:70%	+	1175/3961	V600E/E2/D	+
H3	HCL	30	52	1.59	11.7	3.8775	150	150	Equivocal	A:9% T:91%	+	418/4863	V600E/E2/D	+
H4	Treated HCL (HCL-CR)	0	61.3	1.86	23	5.7605	250	250	WT	A:2% T:98%	-	0/509	-	-
H5	HCL	70	25.2	2	7.3	5.9985	109	109	+	A:15% T:85%	+	200/2570	V600E/E2/D	+
H6	LPL	30	97	1.86	54	5.7835	250	250	WT	A:1% T:99%	-	4/3023	-	-
H7	LPL; treated HCL (HCL- CR)	>90	65.2	1.91	27	2.3745	250	250	WT	A:1% T:99%	-	13/4659	-	-
H8	HCL	50	53.6	1.81	21.8	6.6195	250	250	Equivocal	A:12% T:88%	+	231/825	V600E/E2/D	+
H9	HCL	>90	31	1.82	12.1	8.3213	150	150	Fail	A:26% T:74%	Fail	Fail	V600E/E2/D	+

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H10	HCL	5	58.2	1.9	22.6	3.3685	250	WT	A:3% T:97%	-	0/38	V600E/E2/D	+
H11	CLL	40	214.2	1.89	117	2.6117	250	WT	A:2% T:98%	-	0/1400	-	-
H12	HCL	>90	62.2	1.87	22.8	7.6385	250	Fail	A:42% T:58%	+	201/203	V600E/E2/D	+
H13	HCL	80	46.6	1.63	7.62	5.4352	120	+	A:35% T:65%	+	681/1751	V600E/E2/D	+
H14	HCL	50	16	1.78	5	7.3826	72	Fail	A:18% T:82%	-	6/2481	V600E/E2/D	+
H15	HCL	70	13.3	1.76	3.86	5.2944	63	Equivocal	A:23% T:77%	-	2/1132	V600E/E2/D	+
H16	MCL	15	160.8	1.88	71.2	2.3457	250	WT	A:2% T:98%	-	0/41	-	-
H17	HCL	50	16.7	1.8	5.52	5.0566	89	Equivocal	A:18% T:82%	-	0/74	V600E/E2/D	+

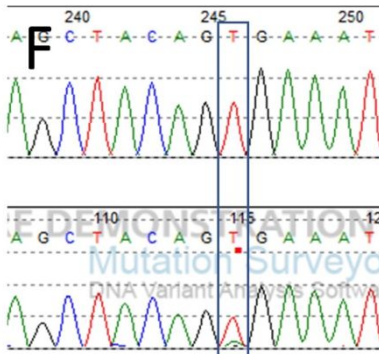
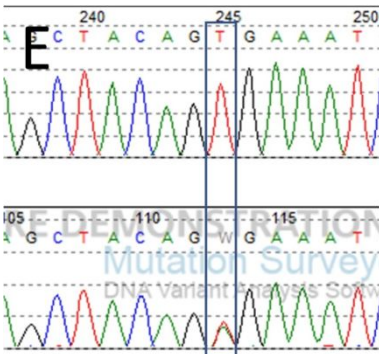
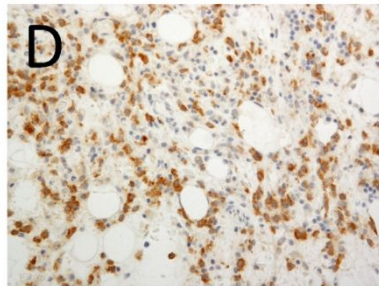
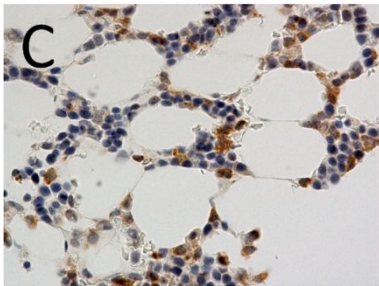
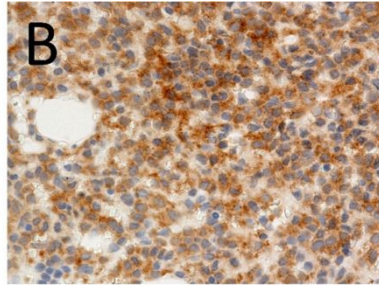
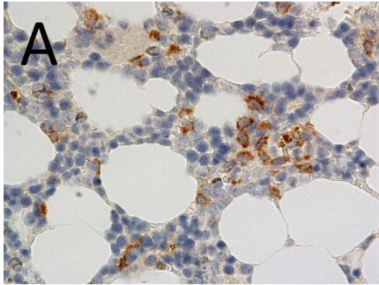


FIGURE AND TABLE LEGENDS:

Table 1: Sample details, extracted DNA parameters and results of *BRAF* V600E mutation analysis for all cases in the study. 17 cases (H1-H17) of histologically confirmed lymphoproliferative neoplasm included 11 classical HCL cases with tumour burdens between 5% and >90%; 4 other lower grade B-cell lymphomas (potential HCL ‘mimics’ including chronic lymphocytic leukaemia (CLL), lymphoplasmacytic lymphoma (LPL), splenic marginal zone lymphoma (SMZL) and mantle cell lymphoma (MCL)); BMT samples from 2 patients with treated HCL in complete remission (HCL-CR), one of which had persisting coexistent lymphoplasmacytic lymphoma (LPL). Ten normal BMTs (denoted D1-D10) were used as negative controls: These were taken to stage B-cell lymphomas other than HCL and showed no BMT involvement by lymphoma on standard morphological and immunohistochemical examination. Sample diagnosis and percentage involvement by a given neoplasm (assessed by a haematopathologist estimating percentage of cells showing immunohistochemical CD20 positivity), where present, are included, together with data on extracted DNA concentration and quality. With regards to qPCR analysis, DNA preparations from samples highlighted green were deemed to have passed this quality control assay ($\Delta C_t < 2.5$), those highlighted yellow were close to a threshold of adequacy ($\Delta C_t < 3.0$), those highlighted red were deemed to be of poor quality ($\Delta C_t > 3.0$). For NGS DNA input, green cells indicates the recommended amount of DNA input for NGS (250 ng), orange cells denote the lower limit for adequate DNA input (150 ng) and red cells denote sub-optimal DNA input (<150 ng). For *BRAF* V600E assay results, red cells indicate a WT result, and green cells indicate the presence of a *BRAF* V600E mutation. Orange cells indicate equivocal results for Sanger sequencing, meaning that a small peak was present on the electropherograms suggestive of the c.1799T>A nucleotide substitution (encodes the *BRAF* V600E mutation). In the absence of confirmatory tests such an equivocal result for Sanger sequencing would be insufficient to call the mutation. Grey cells containing ‘Fail’ indicate failure to produce a result in the given assay. The MiSeq Variant Caller column shows filtered *BRAF* V600E mutations detected by NGS (+), while the MiSeq IGV column shows V600E reads/total reads at that locus. “MiSeq” denotes targeted resequencing NGS, “Idylla” denotes a *BRAF* mutation assay carried out on an Idylla machine; “IHC” denotes immunostaining with an anti-*BRAF* V600E antibody (as per Methods section).

Figure 1: Representative examples of *BRAF* V600E detection by immunostaining and Sanger sequencing. Brown staining indicates the expression of V600E mutant protein. Panel A: Immunostaining of sample H10 (5% involvement) with mutation-specific monoclonal antibody (VE1, Ventana, Tuscon, AZ), demonstrates strong staining on a small number of cells. Panel B:

sample H13 (80% involvement) shows a high percentage of mutant cells. Panel C: sample H14 (50% involvement) was a fragmented specimen, meaning accurate assessment of the percentage of B cells was difficult. Panel D: sample H5, a sample taken from an individual with a concurrent mycobacterial infection. Panel E: Sanger sequencing of sample H2. Electropherogram demonstrates clear dual peaks for the WT T base (red) and the mutated A base (green). Panel F: Electropherogram (sample H3) demonstrates the presence of the WT T base with a suggestion of the mutated A base; this result was classified as equivocal.