Short report

Development and Validation of a Real Time Quantification Assay to Detect and Monitor BRAFV600E Mutations in Hairy Cell Leukemia

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Abstract
The \textit{BRAFV600E} mutation was recently detected in hairy cell leukemia (HCL) by whole exome sequencing. To make use of this new marker for diagnosis and follow up of HCL we developed a \textit{BRAFV600E}mut-specific quantitative real-time PCR assay and validated it in 117 HCL patients and 102 non-HCL/\textit{BRAFwt} patients. The cut-off level to discriminate \textit{BRAFV600E}-positive/-negative cases was set at 0.023 \%\textit{BRAFV600E}/\textit{BRAFwt}. 115/117 HCL (98.3\%) demonstrated \%\textit{BRAFV600E}/\textit{BRAFwt} above the cut-off (mean, 29.6±41.1). The remaining 2/117 HCL with lower \%\textit{BRAFV600E}/\textit{BRAFwt} ratios were also \textit{BRAFwt} by deep-sequencing technology. 16 HCL-variant patients showed \%\textit{BRAFV600E}/\textit{BRAFwt} values corresponding to “non-HCL”. Follow-up studies in 19 HCL cases demonstrated a decrease of \%\textit{BRAFV600E}/\textit{BRAFwt} during therapy. The log-reductions as determined by RQ-PCR and immunophenotyping correlated significantly (p<0.001). In conclusion, we confirmed \textit{BRAFmut} as a useful marker in HCL, its absence in HCL-variant, and developed a RQ-PCR based assay to monitor MRD in HCL.

**Key words:** Hairy cell leukemia (HCL), \textit{BRAFV600E}, quantitative real-time PCR, minimal residual disease (MRD), multiparameter flow cytometry (MFC).
Introduction

Hairy cell leukemia (HCL) is a clearly characterized disease which differs from other mature B-cell neoplasms by its clinical presentation, unique immunophenotype and morphology, as well as the occurrence of bone marrow (BM) fibrosis.\textsuperscript{1,2} So far, diagnosis of HCL was based on cytomorphology, immunophenotyping, and immunohistopathology. Recently, a V600E mutation of  \textit{BRAF} (\textit{v-raf murine sarcoma viral oncogene homolog B1}) was detected in all 48 patients with HCL analyzed by whole-exome and Sanger sequencing.\textsuperscript{5} The \textit{BRAFV600E} was restricted to typical HCL and was not observed in hairy cell leukemia-variant (HCL-v) or splenic marginal-zone lymphoma.\textsuperscript{5} \textit{BRAF} mutations have been described before in different cancers, including melanoma,\textsuperscript{6} papillary thyroid cancer, or lymphoblastic leukemia.\textsuperscript{7} Here, we developed a new quantitative real-time PCR assay to detect the \textit{BRAFV600E} mutations. We evaluated this assay in 117 patients at diagnosis of HCL as confirmed by multiparameter flow cytometry (MFC) and performed follow-up studies in comparison to MFC in a proportion of these patients.

Patients

We investigated 117 patients at diagnosis of HCL as confirmed by MFC (28 females; 89 males; median age: 57.9 years; 24.3-88.4 years), 9 of whom had been part of the recently published sequencing study.\textsuperscript{5} In addition, two independent cohorts, i) 16 patients with HCL-v, and ii), 102 patients with different acute/chronic myeloid/lymphatic leukemias or non-malignant diseases were included for comparison studies. BM or peripheral blood (PB) samples were sent to the Munich Leukemia Laboratory between 08/2005-06/2011. All patients gave written informed consent with genetic analysis and research studies in accordance with the Declaration of Helsinki. The study was approved by the Munich Leukemia Laboratory Institutional Review Board.

Methods

Following erythrocyte lysis of the BM/PB samples, all HCL and all HCL-variant cases were investigated by 5-color flow cytometry.\textsuperscript{8} Classification as HCL was based on bright monotypic surface immunoglobulin, bright coexpression of CD20, CD22, and CD11c, and expression of CD103 and CD25.\textsuperscript{1} HCL-v was diagnosed in the absence of CD25.\textsuperscript{1}

As the \textit{BRAFV600E} is characterized by an invariant T>A nucleotide substitution we decided to develop an mRNA-based reverse transcription allele-specific real-time quantification (RQ-PCR) assay. Quantification of the \textit{BRAFV600E} was performed by measuring the expression of \textit{BRAFV600E} mRNA in comparison to \textit{BRAF} wild-type (\textit{BRAFwt}) mRNA. Values were given in %\textit{BRAFV600E}/\textit{BRAFwt}. RQ-PCR was performed by LightCycler\textsuperscript{®} 1.5 System.


(Roche Diagnostics, Mannheim, Germany) with the application of hybridization probes as the detection format as detailed in Figure 1A. Standard curves for both assays were produced by 10-fold dilution series of 6 different plasmid concentrations. In all following runs a reference dilution was analyzed and the external standard curve was loaded by comparison to a reference sample. To analyze the efficiencies and sensitivities we performed serial dilution experiments of the plasmids and \textit{BRAF}\textsuperscript{mut} patient samples in \textit{BRAF}\textsuperscript{wt} cDNA. Efficiencies were calculated from the slope of the standard curves and ranged from 1.93 to 1.96, and were similar in the \textit{BRAF}\textsuperscript{wt} and \textit{BRAF}\textsuperscript{mut} assays (Figure 1B-C). Limiting dilution series of cDNA of 2 cases with highly infiltrated HCL in cDNA of a normal control revealed a sensitivity of \(10^{-4}\) to \(10^{-5}\) (Figure 1D).

For confirmation of the RQ-PCR results 15 cases with and 15 without \textit{BRAF}\textsuperscript{V600E} were verified by Sanger Sequencing. In addition, two cases with \textit{BRAF}\textsuperscript{V600wt} as assessed by RQ-PCR were investigated by deep-sequencing (454 Life Sciences, Branford, CT).\textsuperscript{9} Correlation between PCR and MFC results was performed according to Spearman, expression levels of \textit{BRAF} were compared between groups using Student’s t-test.

Results and Discussion

First, we defined the non-specific background of the assay that can be caused by the normal wild-type allele in the 102 “non-HCL” controls. This cohort was comprised of: AML n=9, MDS n=7, MPN n=13, CML n=4, CML in MMR n=10, CMML n=4, CEL n=2, T-ALL n=1, B-ALL n=2, follicular lymphoma: n=3, mantle cell lymphoma: n=8, SMZL: n=1, CLL n=11, other mature B-NHL: 20, non-malignant diseases n=7. Unspecific %\textit{BRAF}\textsuperscript{V600E}/\textit{BRAF}\textsuperscript{wt} values of “non-HCL” controls were very low (median: 0.003, range: 0.000-0.030; mean±SD, 0.005±0.006). The cut-off level for discrimination of \textit{BRAF}\textsuperscript{V600E}-positive and -negative cases was therefore defined as 0.023 %\textit{BRAF}\textsuperscript{V600E}/\textit{BRAF}\textsuperscript{wt} (3xSD above the mean). Of note, also the 16 patients with HCL-variant showed %\textit{BRAF}\textsuperscript{V600E}/\textit{BRAF}\textsuperscript{wt} values in the range of the “non-HCL” controls (median: 0.006, range: 0.000-0.012; mean, 0.006±0.004).

Subsequently, the 117 patients with a proven diagnosis of HCL were analyzed. In 115 of 117 HCL (98.3%), the %\textit{BRAF}\textsuperscript{V600E}/\textit{BRAF}\textsuperscript{wt} expression was above the cut-off (median, 17.2, range, 0.077-280.3; mean, 29.6±41.1). Expression levels measured in PB samples (n=37) were slightly higher (median %\textit{BRAF}\textsuperscript{V600E}/\textit{BRAF}\textsuperscript{wt}: 13.89, range 0.138-280.3) than those in BM (median: 25.09, range: 0.077-131.6) (p=0.042). This higher expression correlates with a higher infiltration of pathologic cells in PB (23.9%) compared to BM (12.4%) (p=0.002). MFC detected a median of 10.0% leukemic cells (range, 0.2-74.0%; mean, 16.1±17.2%). \textit{BRAF}\textsuperscript{V600E} expression and the percentage of HCL cells by MFC at diagnosis showed a significant correlation (r=0.741, p=0.001) (Figure 2A). Two of the 117 (1.7%) HCL patients
had \%BRAFV600E/BRAFwt values in the range of normal controls (0.000 and 0.006, respectively) and thus are considered as having BRAFwt. The percentage of HCL cells by MFC amounted to 4.0% and 8.0%, respectively, in these cases. We confirmed absence of the V600E or a variant mutation by 454 deep sequencing of exon 15 with 198- and 398-fold coverage, respectively, which was corresponding to a detection limit of ~0.5-1% of cells. Both patients were females (aged 52 and 61 years) and showed good response to cladribine (patient #1) and splenectomy (#2). Besides BM assessment by cytomorphology and MFC the diagnosis of HCL was confirmed in patient #1 by BM immunohistochemistry and in patient #2 by immunohistochemistry following splenectomy. In a recently published paper\textsuperscript{15} not only variant but also IGHV4-34-expressing hairy cell leukemia were reported to lack the BRAFV600E mutation (11 out of 53 examined HCL cases, all with IGHV4-34 usage). However, both our cases did not reveal IGHV4-34 usage. Thus, it remains to be studied which HCL are BRAFwt and whether this rare subset has a common still unknown genetic defect.

Follow-up studies including 1-4 samples per patient were performed in 19 HCL cases in parallel by MFC and RQ-PCR. At diagnosis, the mean \%BRAFV600E/BRAFwt was 38.0±60.8, and was decreasing to a mean of 7.3±19.8 by a mean log reduction of 2.0±1.6 during therapy. MFC revealed a mean of 17.4±22.0% leukemic cells at diagnosis, decreasing by a mean log reduction of 1.8±1.2 to a mean of 3.0±7.7% during follow-up. The log reduction as observed by RQ-PCR and immunophenotyping again showed a significant correlation (r=0.896; p<0.001; Figure 2B). Only one of seven patients who achieved immunologic CR during follow-up maintained a slightly increased \%BRAFV600E/BRAFwt of 0.05, while six obtained also a molecular CR with \%BRAFV600E/BRAFwt values in the range of negative controls. One patient showed an increasing percentage of HCL-cells by MFC and in parallel an increasing \%BRAFV600E/BRAFwt (from 0.988 to 1.9) 36 days before the cytomorphologic relapse appeared.

In conclusion, our study confirms the high specificity of the BRAFV600E for HCL\textsuperscript{10,11}. In HCL patients, RQ-PCR for the detection of BRAFV600E provides a new valid, rapid, and highly sensitive molecular MRD parameter which may be clinically applied similarly to the settings in CLL and mantle cell lymphoma\textsuperscript{12,13} and provides a high sensitivity level which is even higher as compared to an allele-specific PCR applied to an independent cohort as published recently\textsuperscript{14}. Furthermore, this new RQ-PCR assay facilitates the diagnosis of HCL by detection of the BRAFV600E in all mutation carriers and improves the discrimination of HCL from HCL variant cases. However, as shown here for the first time, single cases with classical HCL do not carry BRAF gene mutations. Furthermore, this RQ-PCR assay may be used to monitor minimal residual disease with sensitivity comparable to and potentially higher than that achieved by flow cytometry. Additionally RQ-PCR has the advantage to be
applicable also retrospectively without the need for viable cells. In general, we would recommend use of RQ-PCR and immunophenotyping in combination to further improve and strengthen the diagnosis and MRD monitoring in patients with HCL.
Authorship and Contributions

SS, TH, CH, and WK performed design of study. UB and WK analyzed data and wrote the first draft of the manuscript. SS, FD, NW, MU, and VG performed molecular studies. TH performed cytomorphology, WK was responsible for immunophenotyping. All authors contributed to write the manuscript and approved the final version.

Disclosure of Conflicts

SS, TH, CH, and WK declare part ownership of the Munich Leukemia Laboratory GmbH. FD, NW, MU and VG are employed by the Munich Leukemia Laboratory GmbH. UB has nothing to declare.
References


Figure Legends

**Figure 1A)** BRAFV600E real-time quantification assay. i) Assays for **BRAF**wt and **BRAF**mut were almost identical with the exception of the **BRAF**V600E mutation-specific primer which includes the nucleotide substitution of the T>A at the 3´end and an additional mismatch nucleotide (A>G at the third position from the 3´end) that further enhances the specific detection of mutated transcripts. Forward and reverse primer are underlined. Mutated nucleotide and mismatch nucleotide in the forward primer are given in red. Fluorescein labelled detection probe is given in green and LC640 labelled probe is given in red. In detail, the reaction was performed in a final volume of 20 µl by use of 2 µl mastermixin (LightCycler Fast Start DNA Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany), 4 mmol/L MgCl₂, 0.5 µmol/L of each forward (**BRAF**wt-F: TGGTGAATTTTGTCTAGCTACAGT or **BRAF**mut-F: TGGTGAATTTTGTCTAGCTACGA) and reverse primer (**BRAF**-R: TCTGACTGAAAGCTGTATGGATT) (Metabion, Martinsried, Germany), 0.25 µmol/L of each of the two fluorescent hybridization probes (**BRAF**-Fl: GTGGGTCCCATCAGTTTGAACAG-Fluorescein and **BRAF**-LCred640: LC640-GTCTGGATCCATTTTGTGGATGGCACCA-Phosphate), and 2 µl cDNA (accounting for an equivalent of mRNA of 200,000 cells). Amplification was performed after initial incubation at 95°C for 10 minutes in a 3-step cycle procedure (denaturation 95°C, 1 second, ramp rate 20°C/s, annealing temperature 64°C, 10 seconds, ramp rate 20°C/s, and extension 72°C, 26 seconds, ramp rate 2°C/s) for 45 cycles.

ii) Position of primers (**BRAF**-F and **BRAF**-R), V600E mutation and hybridization probes at the mRNA are indicated. iii) For plasmid standards exons 14-16 of **BRAF**wt and **BRAF**mut, each, were cloned into a pCR2.1TOPO® vector. B) 10-fold dilution series of the plasmid carrying the **BRAF**V600E: 200,000 – 2 plasmid copies. C) Standard curve showing efficiency of the **BRAF**V600E specific PCR of 1.93. D) 10-fold dilution of a diagnostic **BRAF**V600E mutated patient sample in cDNA of an unmutated patient showing a sensitivity of 1/100,000. **BRAF**wt signal is indicated as “non-specific background”.

**Figure 2:** Correlation of A) **BRAF**V600E expression level and the percentage of HCL cells by MFC at first diagnosis; B) the logarithmic reduction between diagnosis and follow-up assessment as determined for **BRAF**V600E expression level by RQ-PCR and for HCL cells by MFC.
Figure 1A

**BRAFV600E Real Time Quantification Assay**

i) BRAF LC Assay

G A

TAATATTTCTTCTATGAAGACCTCACAGTTAATAGGTGATTTTGTCATCTCACTACAGTGAAATCTC
GATGGAGTGGGCATCATCATTTGTTAACAAGTTGCTTGAGACATCTTTGTGGATGCGGACCAGAAGCTCA
TCAGATAGCAAGATATAATACCTACAGGCTTTCACTGAGATGATATGATATGATATGATATGATATGATAT
GAATTGATGACTGACAGTTACCTATTCAAACATCAACAGGACAGATAATTATGTGATGGG

ii) BRAF mRNA

V600E hybridisation probes

BRAF-F  
BRAF-R

| e 13 | e 14 | e 15 | e 16 | e 17 | e 18 |

iii) BRAF Plasmid Standard

pCR 2.1-TOPO® Vector ~3900 bp

BRAF Exon 14-16 298 bp

Figure 1B

**Plasmid Dilution Series**

Fluorescence (F2/F1) vs Cycle Number
Figure 2

A

B
Development and validation of a real-time quantification assay to detect and monitor \textit{BRAFV600E} mutations in hairy cell leukemia

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