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Detection of *BRAF* V600E Mutation in Langerhans Cell Histiocytosis Using High-resolution Melting Analysis in Decalcified, Paraffin-embedded Tissue

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Abstract

The oncogenic *BRAF* V600E mutation has recently been found in a substantial number of Langerhans cell histiocytosis (LCH) cases. We developed and validated a rapid and sensitive high-resolution melting (HRM) analysis–based assay to detect *BRAF* V600E mutation in formalin-fixed, paraffin-embedded (FFPE), decalcified bone tissues, including 32 LCH cases and four primary bone lymphoma cases. *BRAF* V600E mutation was found in 18 (56%) of the LCH cases. HRM results were confirmed by Sanger sequencing. Our assay is thus a rapid, sensitive, and low-cost way to detect the *BRAF* V600E mutation in LCH FFPE samples for clinical diagnostic use.

Keywords: *BRAF* mutation; Langerhans cell histiocytosis; High-resolution melting analysis

Introduction

Langerhans cell histiocytosis (LCH) is a rare clonal proliferation disease in which affected cells have phenotypic characteristics similar to those of normal Langerhans cells. Clinically, LCH manifestations range from isolated bone lesions to multisystemic disease, with outcomes ranging from spontaneous remission to fatal progression. Recent identification of oncogenic *BRAF* V600E mutation in over half of LCH cases provided molecular evidence of the neoplastic nature of LCH [1-4].

The ability to accurately detect this mutation in LCH would be not only helpful in establishing a correct diagnosis but also important for potential target therapy, such as vemurafenib [5]. However, DNA sequencing of LCH samples is technically difficult because LCH cells usually infiltrate bone and brain tissues and the clinical samples submitted for molecular testing are often mixed with large quantities of non-tumor cells. Developing a highly sensitive and specific assay is therefore essential for mutation detection in these cases.

One assay with potential in this setting is high-resolution melting (HRM) analysis. Although used to detect the *BRAF* mutation in various types of cancers, [6] HRM analysis has not been applied to detect mutations in bone. Bone tissues require decalcification prior to paraffin embedding, which can be technically challenging for many molecular assays. In this study, we report a rapid assay using polymerase chain reaction (PCR) and HRM analysis to detect *BRAF* V600E mutation in formalin-fixed, paraffin-embedded (FFPE), decalcified bone tissues and soft tissues that involved by LCH.

Materials and Methods

Patients and samples

FFPE tissue blocks from 32 LCH patients were retrieved from the Department of Pathology at Xiangya Hospital, Changsha, China. Among these samples, 25 were from bone and seven were from soft tissue. Blocks from four primary bone *lymphoma* cases *were also used as controls. Bone samples were* decalcified in 10% formic acid for 3 hours at 50°C prior to paraffin embedding. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Mainz, Germany). For each bone sample, four to six 10-μm sections of decalcified tissue were used. Institutional review board approved this study.

PCR amplification and high-resolution melting analysis

We designed a set of primers to flank the *BRAF* exon 15 codon 600 sequence with an amplicon size of 161 bp. The primer sequences were forward, 5′-TTTTCCTTTACTTACTACACCTCA-3′, and reverse, 5′-ATAGCCTCAATTCTTACCATCCA-3′. PCR was performed in a 20-μL reaction with 30 ng of DNA, 5 μmol/L of each primer, 2.5 mmol/L of $MgCl_2$, 1.5 μmol/L of Eva Green dye (Biotium, Hayward, USA), and 1 U of Taq Polymerase (Roche, Rotkreuz, Switzerland). PCR and HRM were performed consecutively on a Light Cycler 480 system (Roche) using the following profile: an initial denaturation at 95°C for 1 minute followed by 50 cycles of 10 seconds at 95°C, 15 seconds at 60°C, and 25 seconds at 72°C. The PCR product was then denatured at 95°C for 1 minute and annealed at 40°C for 1 minute. The final HRM step was performed from 65°C to 95°C with a ramping temperature of 0.04°C/second. Melting curve analysis was performed using Gene Scanning Software (Roche).

Serial dilution study

To assess the sensitivity of the assay, we performed a serial dilution study using a pair of plasmid constructs containing either the wild-type or mutated sequence from the HT29 cell line, which harbors a heterozygous GTG to GAG mutation at codon 600 (V600E) of the *BRAF* gene [7]. The dilution series were prepared by mixing the

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mutated plasmid DNA into the wild-type plasmid DNA to yield 0%, 1%, 5%, 10%, 20%, 50%, and 100% of the mutated plasmid DNA.

Results

We first performed a serial dilution study to determine the reliability and sensitivity of our HRM assay. DNA extracted from the plasmid containing the *BRAF* V600E mutant sequence was serially diluted into plasmid DNA of the wild-type sequence. The results confirmed that our assay could successfully distinguish heterozygote of the *BRAF* V600E mutant sequence with a detection sensitivity of at least 1% of mutated alleles (Figure 1A).

Using our HRM assay, we were able to screen all FFPE samples for the *BRAF* V600E mutation. Of the 32 LCH samples, 18 (56%) were found to show the *BRAF* V600E mutation profile. No difference in amplification was found between bone tissue and soft tissue. Figure 1B shows representative HRM profiles of five samples, three of which

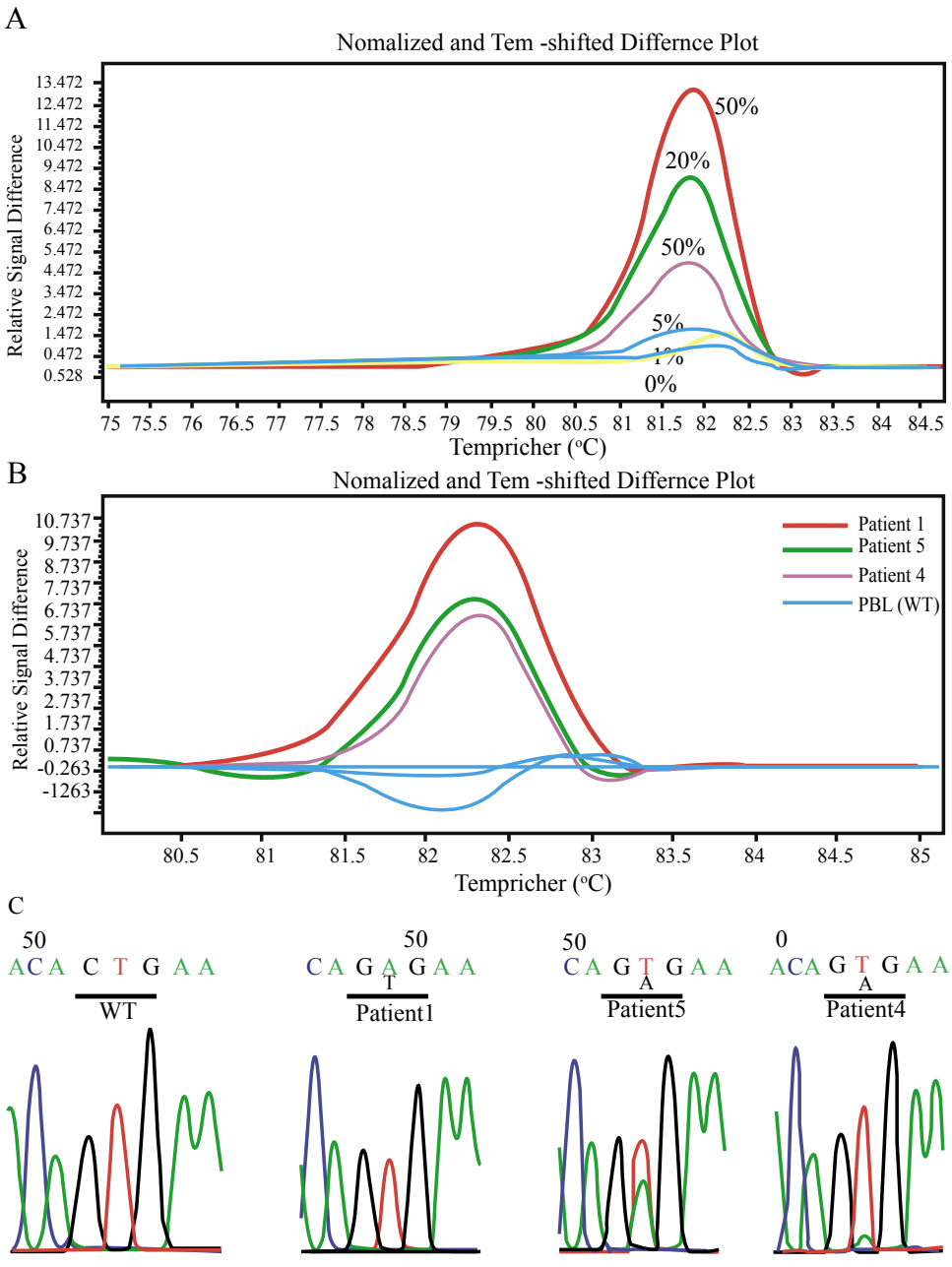

Figure 1A: Detection of the *BRAF* V600E mutation using high-resolution melting (HRM) analysis. **A, A** HRM difference plot of the dilution series of the *BRAF* V600E mutation plasmid DNA mixed into wild-type DNA. The detection sensitivity of this assay is at least 1% of mutated alleles. **B:** Representative HRM difference plots for Langerhans cell histiocytosis (LCH) samples (patients 1, 4, and 5) and primary bone lymphoma (PBL, indicated in the figure by blue lines) samples. All of the three LCH patients show a GTG to GAG (V600E) mutation, whereas the two PBL patients show wild-type (WT) DNA profiles. **C:** Corresponding Sanger sequencing results of the *BRAF* exon 15. The black line below the sequence indicates codon 600.

were positive for the *BRAF* V600E mutation and two of which were negative. Parallel testing by Sanger sequencing confirmed that all of the cases determined to be positive by the HRM assay carried the GTG to GAG (V600E) mutation. Figure 1C shows the corresponding Sanger sequencing results of these example cases. In three cases, however, the initial Sanger sequencing results were equivocal while HRM profiles were clearly positive for the mutation. An example of these equivocal results is shown for Patient 4 in figures 1B and 1C. As controls, we also tested four cases of primary lymphoma of the bone using our HRM assay. No *BRAF* V600E mutation was detected in these cases. In addition, no mutation was detected by Sanger sequencing in any of the HRM negative cases, suggesting a specificity of close to 100% for this HRM assay.

Since majority of these cases were referrals from outside hospitals, their clinical information available for analysis were scarce. We compared 3 recent LCH cases with *BRAF* V600E mutation with 3 cases with wild-type *BRAF*. The affected sites in both groups were 1 in bone tissue and 2 in soft tissues. The cases with *BRAF* V600E mutation presented at apparently younger ages, 1, 29 and 30 years versus 16, 56 and 60 years (p=0.468).

Discussion

We have developed a new HRM analysis assay to detect *BRAF* V600E mutation in LCH samples. To our knowledge, this is the first report to apply HRM analysis to decalcified bone tissue. This assay is rapid and shows a sensitivity of 1%, which is far superior to that of Sanger sequencing (about 20%). Given the infiltrative nature of LCH-which is often associated with large quantities of reactive/normal tissue in the lesions biopsied-samples with low tumor percentage can pose a challenge for direct sequencing. Therefore, the superior sensitivity of HRM is more suitable for mutation detection in LCH tissue samples with no requirement for laborious microdissection procedure.

As controls, we also tested four cases of primary lymphoma of the bone and detected no *BRAF* mutations, suggesting that our assay is specific. Since over half of the LCH patient population carries this mutation, our rapid assay can serve as a screening test to identify these patients in a timely manner, with higher sensitivity and lower cost than with sequencing. If clinically necessary, patients with negative screening test results may be further tested by different assays for other *BRAF* mutations that are relatively rare in LCH [4,8].

Our study detected the *BRAF* V600E mutation in 18 (56%) of 32 LCH cases, a proportion comparable to the observation from a larger study cohort [1]. These findings indicate that LCH belongs to a growing list of neoplasms in which transformed cells harbor the *BRAF* V600E mutation. This *BRAF* mutation constitutively activates the RAS-RAF-MEK-ERK signaling pathway, a key role in tumorigenesis [9]. As the first molecular marker identified in LCH, the *BRAF* V600E mutation may be a valuable diagnostic marker for LCH. The current diagnostic immunohistochemistry markers, including CD1a, S100, and CD207, do not differentiate between LCH and reactive Langerhans cells [10]. It is well known that reactive Langerhans cells can be abundant in both benign conditions (e.g. dermatopathic lymphadenopathy) and neoplastic conditions (e.g. lymphoma). Detection of the *BRAF* V600E mutation will help to establish a diagnosis of LCH. Moreover, identifying patients with this mutation may enable patients with refractory disease to receive targeted therapy.

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