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Rapid and sensitive detection of *CALR* exon 9 mutations using high-resolution melting analysis

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ABSTRACT

Background: Somatic *CALR* exon 9 mutations have recently been identified in patients with *JAK2*/*MPL*-unmutated myeloproliferative neoplasm, and have become an important clonal marker for the diagnosis of essential thrombocythemia (ET) and primary myelofibrosis. In the present study, we sought to use high-resolution melting analysis (HRMA) as a screening method for the detection of *CALR* mutations.
Methods: 32 *JAK2*/*MPL*-unmutated ET patients were retrospectively enrolled and 8 healthy adults were used as wild-type control. *CALR* exon 9 mutation was independently screened by HRMA with the CFX Connect real-time system and Sanger sequencing. TA-cloning was used to detect *CALR* exon 9 mutations in patients suspected to have low mutant allele burden.
Results: The maximal sensitivity of HRMA in identifying both *CALR* type 1 and type 2 mutants from patients' genomic DNA was 2.5%. Twenty-two samples were found to have distinct melting curves from wild-type. The presence of *CALR* mutations in 16 of these 22 samples was confirmed by Sanger sequencing, while the other 6 samples were wild-type by sequencing. After TA-cloning, *CALR* mutations were detected in 5 of 6 patients from 1 (6%) of 16 clones to 1 (2%) of 50 clones. Therefore, HRMA identified *CALR* mutations in 21 (65.6%) of 32 ET patients compared to 16 (50%) patients by Sanger sequencing, with a false positive rate of 3% and no false negative.
Conclusion: The HRMA developed in our system is a rapid and sensitive technique for the detection of *CALR* exon 9 mutations.

1. Introduction

The classic *BCL-ABL1*-negative chronic myeloproliferative neoplasm (MPN) is a clonal hematopoietic stem cell disorder and includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) [1]. ET is characterized by increased number of mature megakaryocytes in the bone marrow and sustained thrombocytosis in the peripheral blood. Although most ET patients have a normal life expectancy, some may encounter serious events such as thrombotic and hemorrhagic complications and leukemic transformation during their clinical course [2]. The *JAK2* V617F mutation was discovered in 2005, and has provided important diagnostic, therapeutic, and prognostic implications in MPNs. The frequency of *JAK2* V617F mutation is over 90% in polycythemia vera (PV), and about 60% in ET and PMF [3–5]. Besides, *MPL* mutations are identified in about 4–5% of *JAK2*-unmutated ET and PMF patients [6]. Following these seminal reports, other somatic mutations such as *LNK*, *TET2* and *DNMT3A* have also been detected in patients with MPN [7]. However, they are not mutually exclusive with *JAK2* and *MPL* mutations and also not specific to patients with MPN [7,8]. Despite many somatic mutations have been identified in patients with ET, clonal molecular marker is still not identified in ~40% of ET patients.

Recently, a high frequency (around 49–88%) of somatic calreticulin (*CALR*) mutations was identified in patients with *JAK2*/*MPL*-unmutated

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patients with ET and PMF [9–13]. Most *CALR* mutations in MPNs are heterozygous indels in exon 9 causing one base pair reading frameshift and resulted in the generation of a novel CALR protein C-terminus. The majority of the *CALR* exon 9 mutants were a 52 bp deletion of nt1172 to nt1223 (L367fs*46, type 1 mutation) and a 5 bp insertion of TTGTC (K385fs*47, type 2 mutation). Rarely, *CALR* exon 9 point mutations have been reported in follicular lymphoma (E403X and E405Q) [14], PMF (E379D) [9] and chronic neutrophilic leukemia (E398D) [15]. Importantly, *CALR* mutations are not only mutually exclusive with *JAK2* and *MPL* mutations, but they are also infrequently detected in other myeloid neoplasms such as myelodysplastic syndrome, chronic myelomonocytic leukemia and atypical chronic myeloid leukemia [9,10]. These findings indicate that *CALR* mutations are quite specific for ET and PMF. Based on these discoveries, *CALR* mutations have been proposed to be included in the World Health Organization classification system for the molecular diagnosis of ET and PMF [16]. Therefore, the detection of *CALR* mutations with reliable and cost-effective methods in patients suspected to have ET or PMF is very important.

Several methods have been used to detect *CALR* exon 9 mutations including direct DNA sequencing, PCR followed by fragment analysis and immunostaining [9,10,17,18]. Although fragment analysis has a relatively high sensitivity for *CALR* mutation detection, it cannot discriminate point mutation from wild-type sequence. High-resolution melting analysis (HRMA) is a closed-tube and PCR-based technique for the detection of gene polymorphism and mutations by measuring changes in the melting of a DNA duplex [19]. HRMA is a well-established method for the detection of or prescreening for mutations both in a routine molecular laboratory and in a research setting. For example, HRMA has shown high sensitivity and specificity for the detection of *JAK2* V617F and *JAK2* exon 12 mutations in patients with MPN [20–22]. Recently, the feasibility of using HRMA for the detection of *CALR* mutations in ET and persistent thrombocytosis has been reported using the LightCycler 480 platform (Roche Diagnostics) [23]. In this study, we sought to assess HRMA for rapid and sensitive detection of *CALR* exon 9 mutations in ET using the CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA, USA).

2. Materials and methods

2.1. Patient samples and DNA extraction

The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of Mackay Memorial Hospital. 32 adult patients with *JAK2*/*MPL*-unmutated ET were retrospectively enrolled based on the 2008 World Health Organization classification and 8 healthy adults were used as wild-type control. Written informed consent was obtained from all patients. Patient genomic DNA was derived from bone marrow or peripheral blood by using EasyPure Genomic DNA Spin Kit (Bioman, Taipei, Taiwan).

2.2. Assay design and the HRMA technique

Oligonucleotide primers were designed by Primer3 software to flank all *CALR* exon 9 variants reported in MPN. The primers were used to amplify a 134 bp amplicon [GenBank: NM_004343]: forward 5′-GAAA CAAATGAAGGACAAACAGG-3′, and reverse 5′-CCTCATCCTCCTCATCCT CA-3′. PCR was performed in a 20 μl reaction volume containing precision melt supermix (Bio-Rad Laboratories, Hercules, CA, USA), 100 nM of each primer, and 25 ng genomic DNA. The 134 bp amplicon was run according to the following conditions: an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. After completion of amplification, DNA was heated at 95 °C for 30 s, kept at 60 °C for 1 min, and then melted from 70 to 95 °C (increment 0.2 °C, dwell time 10 s). The results were analyzed using the Bio-Rad Precision Melt Analysis software. Melting profiles were normalized, grouped and displayed as fluorescence-versus-temperature plots or subtractive difference plots (−df/dt vs T). All samples with distinguished melting curves from wild-type were confirmed by duplicate study. Both type 1 and type 2 *CALR* exon 9 mutant cDNA were obtained by direct DNA synthesis, and *CALR* wild-type cDNA was cloned from patient sample.

2.3. Sanger sequencing

All patients were also independently screened for *CALR* exon 9 mutations spanning codons 352–417 by Sanger sequencing on an ABI 3730 sequencer based on previously described method [10]. All identified sequence variants were subjected to repeated bi-directional sequencing for confirmation. Mutations were identified using DNA Dynamo sequence analysis software (Blue Tractor Software Ltd, Conwy, UK). All patients had been screened for *JAK2* V617F and *MPL* exon 10 mutations as previously described [5,7].

2.4. Sensitivity of HRMA in detecting CALR type 1 and type 2 mutations

To study the sensitivity of the methodology, we serially diluted two plasmids carrying *CALR* type 1 and type 2 mutations with wild-type plasmid DNA in different concentrations (100% mutant, 50% mutant, 25% mutant, 10% mutant, 7.5% mutant, 5% mutant, 2.5% mutant, 1.25% mutant, and 0% mutant). The sensitivity tests were carried out in triplicate samples. The sensitivity of HRMA was validated by serially diluting two patient samples carrying *CALR* type 1 and type 2 mutations with control DNA. Based on the relative peak areas of the mutant and wild-type PCR products, the mutant allele burden of these 2 patient samples was estimated to be ~50%. *CALR* type 1 and type 2 patients' DNA were also serially diluted by wild-type DNA in different concentrations (50% mutant, 25% mutant, 12.5% mutant, 5% mutant, 3.75% mutant, 2.5% mutant, 1.25% mutant, 0.625% mutant, and 0% mutant). We did not evaluate the sensitivity of HRMA for other types of *CALR* mutations because they are less frequently detected.

2.5. TA-cloning

The PCR products of *CALR* exon 9 of 6 ET patients suspected to have a low allele burden mutant were purified using a EasyPure High Pure PCR clean-up Kit (Bioman, Taipei, Taiwan) and cloned into a pGEMT-easy vector (Promega, Madison, CA, USA). We obtained at least 16 clones in each individual. The PCR product of each clone was checked on a 2% agarose gel by electrophoresis for the presence of mutant band. All selected clones were then sent for Sanger sequencing regardless the presence or absence of mutant band.

3. Results

3.1. Sensitivity of HRMA in identifying the CALR type 1 and type 2 mutants

We first evaluated the sensitivity of HRMA in detecting the *CALR* type 1 and type 2 mutant plasmid DNA with different concentrations of mutant DNA serially diluted by wild-type plasmid DNA. HRMA could distinguish *CALR* type 1 and type 2 mutants with the maximal sensitivity of 2.5% and 1.25%, respectively (Fig. 1A and C). Whereas, the maximal sensitivity of Sanger sequencing for the detection of both *CALR* type 1 and type 2 mutants was at least 10% or higher (Fig. 1B and D). Besides, the maximal sensitivity of HRMA was validated with 2 patient samples and was found to be 2.5% for both *CALR* type 1 and type 2 mutants (Fig. 2A and B).

3.2. Detection of CALR exon 9 mutations in JAK2/MPL-unmutated ET patients

In this cohort of 32 ET patients, the normalized melting curves of 22 (68.8%) patient samples clearly showed a distinctive difference from