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Pyrosequencing-based quantitative measurement of *CALR* mutation allele burdens and their clinical implications in patients with myeloproliferative neoplasms

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ABSTRACT

Background: We developed a pyrosequencing-based method for the quantification of *CALR* mutations and compared the results using Sanger sequencing, fragment length analysis (FLA), digital-droplet PCR (ddPCR), and next-generation sequencing (NGS).
Methods: Method validation studies were performed using cloned plasmid controls. Samples from 24 patients with myeloproliferative neoplasms were evaluated.
Results: Among the 24 patients, 15 had *CALR* mutations (7 type 1, 2 type 2, and 6 other mutations). The type 1 or type 2 mutation-positive results from pyrosequencing exhibited 100% concordance with the Sanger sequencing results. One novel *CALR* mutation was not detected by pyrosequencing. The *CALR* mutation allele burdens measured by pyrosequencing were slightly lower than those measured by FLA but slightly higher than the results obtained using ddPCR. Pyrosequencing exhibited high correlations with both methods. The mutation allele burdens estimated by NGS were significantly lower than those measured by pyrosequencing. An increased *CALR* mutation allele burden was associated with overt primary myelofibrosis. Patients with > 70% mutation allele burdens in myeloid cells had a significantly longer time from diagnosis ($P = 0.007$), more bone marrow fibrosis ($P = 0.010$), and lower hemoglobin ($P = 0.007$).
Conclusions: Pyrosequencing was a useful rapid sequencing method to determine the burden of *CALR* mutations.

1. Introduction

Myeloproliferative neoplasms (MPNs) are hematopoietic disorders characterized by abnormal proliferation of the myeloid lineage. Three classic subtypes are polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) [1]. These disorders are well known for their association with the *JAK2* V617F mutation, in addition to mutations on *MPL* exon 10 and *JAK2* exon 12 [2–4]. In 2013, frameshift mutations in exon 9 of the calreticulin (*CALR*) gene were identified as key pathogenic mutations for ET and PMF [5,6]. Numerous kinds of indels on exon 9 of the *CALR* gene have been described, and among these mutations, the two most frequent are type 1, a 52-base pair (bp) deletion mutation, and type 2, a 5-bp insertion mutation [5,6].

Several methods have been developed to detect *CALR* mutations, including Sanger sequencing, fragment length analysis (FLA), real-time quantitative PCR with a hydrolysis probe or melting curve assay, digital-droplet PCR (ddPCR), and next-generation sequencing (NGS) [5–11]. Because the types of *CALR* mutations are variable [5,6], detection by direct sequencing can provide more specific information than indirect detection methods based on PCR product sizes. Sanger sequencing is considered to be the primary method of testing; however, it has a modest limit of detection (LOD) with an allele frequency of approximately 20% [12]. In addition, it is generally impossible to obtain quantitative information via Sanger sequencing. Pyrosequencing is a relatively recent bioluminescence technique that employs sequencing-

Abbreviations: BM, bone marrow; ddPCR, digital-droplet PCR; ET, essential thrombocythemia; MPN, myeloproliferative neoplasm; NGS, next-generation sequencing; PB, peripheral blood; PMF, primary myelofibrosis; VAF, variant allele frequency

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by-synthesis technology [12,13]. Compared with Sanger sequencing, pyrosequencing has a superior LOD at an allele frequency of approximately 5% and provides quantitative data. However, the read length that can be achieved by pyrosequencing is shorter (generally up to 100 bp) than that of Sanger sequencing, and pyrograms of complex mutations can yield confusing patterns that are often difficult to interpret [12]. Nevertheless, two important types of *CALR* mutations, the type 1 and type 2 mutations, are already known, and other types of mutations are located within the important calreticulin protein motif that is coded by exon 9. We hypothesized that *CALR* mutations would be good candidates for a pyrosequencing assay.

In this study, we developed and validated a novel method of quantitating the *CALR* mutation burden using a pyrosequencing technique. We explored whether the pyrograms could be easily interpreted for large deletions and insertions of the *CALR* gene. In addition, we investigated the clinical implications of the *CALR* mutant allele burden measured by pyrosequencing.

2. Materials and methods

2.1. Preparation of positive controls by DNA synthesis and cloning

To prepare the positive controls, two mutant DNA fragments were synthesized: a 485-bp DNA fragment containing the 52-bp deletion from the reference sequence (chr19:13054337-chr19:1305873, GRCh37/hg19) for the type 1 *CALR* mutation-positive control and a 542-bp DNA fragment containing the 5-bp TTGTC insertion for the type 2 *CALR* mutation-positive control (Cosmo Genetech, Seoul, Korea). Each of the DNA fragments was cloned into the *Bam*HI and *Sal*I restriction enzyme sites of a pUC57 vector and then transformed into One Shot Top 10 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Transformants harboring the recombinant plasmids were selected using brain heart infusion (BHI) broth containing ampicillin (10^{-4} g/mL).

2.2. Patient samples

This study included 24 patients who had been diagnosed with MPN from 1997 to 2016 without *JAK2* or *MPL* mutations. Two pathologists (S.Y.K. and Y.O.) reevaluated the patients' bone marrow (BM) slides and reclassified the patients' MPN classifications according to the revised WHO classification criteria [1]. Eleven patients were classified as having ET, 5 patients were classified as having prefibrotic/early PMF (pre-PMF), and 8 patients were classified as having overt PMF. A total of 31 samples of peripheral blood (PB, n = 24) and BM (n = 7) were collected. One patient was tested at diagnosis, and the remaining patients were tested during follow-up at varying times after diagnosis (median, 5.5 years; range 1.1–18.4 years). Clinical and laboratory information was collected from the patients' electronic medical records. The characteristics of the patients are presented in Supplemental Table S1. The median age of the patients was 63 years (range, 20–76 years). Eight patients were male (33.3%). For 23 patients, karyotyping results were available, and 4 of these patients presented cytogenetic abnormalities. This study complied with the Declaration of Helsinki and was approved by the Institutional Review Board at the Chungnam National University Hospital.

2.3. Plasmid and genomic DNA extraction

Plasmid DNA was collected using an AccuPrep Nano-Plus Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. Genomic DNA (gDNA) from the patients' BM and PB was extracted using the ExiPrep Dx Blood Genomic DNA Kit (Bioneer) on an ExiPrep 16 Plus instrument (Bioneer) according to the manufacturer's instructions. The quality and quantity of the gDNA were assessed using the 260 nm/280 nm absorbance ratio determined with a NanoPhotometer N50 (Implen, Munchen, Germany).

2.4. Pyrosequencing

The DNA fragment that includes the *CALR* mutations was amplified using 2 different primer sets so that *CALR* could be sequenced in both directions. The primer sequences are presented in Supplemental Table S2. In the forward direction pyrosequencing (Pyro-Fwd set), the 314-bp PCR product was sequenced from 3-bp upstream of the type-1 deletion. In the reverse direction pyrosequencing (Pyro-Rev set), the 209-bp PCR product was sequenced from 2-bp downstream of the type-2 insertion site. PCR analysis was performed using the PyroMark PCR kit according to the manufacturer's specifications (Qiagen, Hilden, Germany). Each reaction contained 12.5 μL of PCR master mix, 2.5 μL of Coral red, 2 μL of $MgCl_2$, 5 μL of Q-solution, 0.5 μL of each of the forward and reverse primers (10 pmol) and 2 μL of gDNA for a final volume of 25 μL. The PCR protocol was as follows: initial denaturation at 94 °C for 5 min; 45 cycles at 94 °C of 40 s, 62 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 7 min. Electrophoresis was performed on a Lab901 Screen Tape System (Agilent Technologies Inc., Santa Clara, USA) to confirm the target size and quantity.

The biotinylated *CALR* PCR products were pyrosequenced using PyroMark Gold reagents (Qiagen, Hilden, Germany) with a PyroMark Q24 instrument (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The nucleotide dispensing order used to detect the *CALR* type 1 and type 2 sequences are presented in Supplemental Table S2. The resulting pyrograms were then converted into peak heights for manual data processing. The synthesized type 1 and type 2 positive controls were always analyzed within each run to correct for any background signals and to standardize the percentage of *CALR* gene mutations by adjusting the peak height of the synthesized mutations to 100%.

2.5. Sanger sequencing

We performed Sanger sequencing for all the samples and confirmed the CALR mutation types. The detailed procedures of Sanger sequencing were described in a previous paper [14].

2.6. FLA

To compare the allele burden results of patient samples by pyrosequencing, we performed FLA according to a previously described procedure (Supplemental Table S2) [7]. Each fragment was analyzed using an ABI 3730xl genetic analyzer (PE Applied Biosystems, Foster City, CA).

2.7. NGS

NGS of *CALR* was performed as a part of a targeted gene panel test including 138 genes. This gene panel also included *JAK2*, *MPL*, *ABL1*, and *BCR*. The gDNA shearing, standard library production and hybridization were performed by Celemics (Celemics Inc., Seoul, Korea), followed by paired-end 150 bp rapid-run sequencing on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The raw data were mapped to the reference genome (hg19); PCR duplicate reads were removed, and variants flagged as "LowQual" and low depth (< 10) were excluded. Variants were annotated with ANNOVAR. Mapping errors were confirmed by visual inspection with the IGV browser.

2.8. ddPCR

ddPCR reactions were performed for the *CALR*-mutated samples according to a procedure described in a previously published paper (Supplemental Table S2) [11].

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