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Quantification of the Mutant *CALR* Allelic Burden by Digital PCR

Application to Minimal Residual Disease Evaluation after Bone Marrow Transplantation

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With the recent discovery of *CALR* mutations, >80% of patients with myeloproliferative neoplasms carry a phenotype-driving mutation. For *JAK2 V617F*, the most frequent mutation in myeloproliferative neoplasms, accurate determination of mutational loads is of interest at diagnosis, for phenotypic and prognostic purposes, and during follow-up for minimal residual disease assessment. We developed a digital PCR technique that allowed the accurate determination of *CALR* allelic burdens for the main mutations (types 1 and 2). Compared with the commonly used fluorescent PCR product analysis, digital PCR is more precise, reproducible, and accurate. Furthermore, this method reached a very high sensitivity. We detected at least 0.025% *CALR* mutants. It can thus be used for patient characterization at diagnosis and for minimal residual disease monitoring. When applied to patients with primary myelofibrosis who underwent hematopoietic stem cell transplant, the digital PCR detected low levels of minimal residual disease. After negativation of the mutational load in all patients, the disease reappeared at a low level in one patient, preceding hematologic relapse. In conclusion, digital PCR adapted to type 1 and 2 *CALR* mutations is an inexpensive, highly precise, and sensitive technique suitable for evaluation of myeloproliferative neoplasm patients during follow-up. *(J Mol Diagn 2016, 18: 68—74; http://dx.doi.org/10.1016/j.jmoldx.2015.07.007)*

Philadelphia chromosome—negative myeloproliferative neoplasms (MPNs) are characterized by an excessive proliferation of hematopoietic cells consecutive to the clonal acquisition of a phenotype-driving mutation. More than 95% of patients with polycythemia vera (PV), 60% to 70% with essential thrombocytemia (ET), and 50% to 60% with primary myelofibrosis (PMF) harbor a G1849T (V617F) mutation of the *JAK2* gene.[1—3] Many *JAK2 V617F*—negative PVs carry a mutation in the exon 12 of *JAK2*,[4] whereas the gene encoding for the JAK2-coupled thrombopoietin receptor *MPL* is found mutated in 5% to 10% of ET or PMF patients.[5] Recently, recurrent mutations in the *CALR* gene were discovered in most *JAK2* and *MPL* nonmutated ET and PMF patients.[6,7] In addition to better understanding the pathophysiology of MPN, the discovery of these mutations has provided very useful clonal markers for the diagnosis of MPN.

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Further characterization of the diseases has been permitted by the accurate assessment of the mutational burden, especially for the *JAK2 V617F* mutation. For instance, it has been rapidly recognized that the *JAK2 V617F* allelic burden is much higher in PV than in ET patients,[2] most of the latter having mainly heterozygously mutated cells.[8] In addition, different levels of mutant allele burden have been associated with various outcomes,[3,9,10] emphasizing the need for accurate and reproducible measurements of mutant allelic burdens. Last, these clonal markers can be used for disease burden assessments and monitoring the effect of some treatments. Interferon alpha has thus been found to efficiently decrease *JAK2 V617F* allelic burden in PV and ET patients, some of whom eventually reach a level undetectable by current techniques.[11] Hematopoietic stem cell transplant (HSCT) is equally able to strongly decrease *JAK2 V617F* levels, which can be used as a minimal residual disease (MRD) marker, the persistence of which predicts a higher risk of relapse.[12] In addition, the persistence or reappearance of the mutation can guide the modulation of the immunotherapy after HSCT to complete the molecular response.[13] These elements make an accurate sensitive and reproducible technique for evaluation of mutant allelic burden extremely useful for characterization and monitoring of MPN patients.

Whereas the *JAK2 V617F* mutation affects a single nucleotide, mutations in the exon 9 of the *CALR* gene are more diverse. Most mutations are type 1 (45% to 53%), corresponding to a 52-bp deletion, or type 2 (32% to 41%), consisting of a 5-bp insertion.[6,7] The primer-based allele-specific PCRs that were the most reliable for *JAK2 V617F* mutations[14,15] cannot be easily adapted for the quantification of the larger *CALR* mutations. This approach has been successfully used to quantify type 1 and type 2 mutations with a limit of detection of approximately 1%, a level that is suboptimal for MRD monitoring.[16] However, this approach of allelic discrimination can reach a higher sensitivity in the context of digital PCR (dPCR). This technique relies on the fragmentation of the PCR reactions into very small droplets that are clonally analyzed.[17] Thus, the quantification of the PCR results is based on the number of droplets positive for amplification of the wild-type or the mutant studied allele. This results in an absolute reliable and sensitive assessment of the mutational load.

Because all *CALR* mutations imply size variations (deletions and/or insertions), their detection and relative quantification are frequently performed using PCR fragment length analysis. These are typically achieved by capillary electrophoresis of end point PCR products and integration of the area under the peaks of the electropherograms. We compared the performances of fragment analysis to those of allelic discrimination using dPCR. We found that, even though both techniques are appropriate for routine analyses, the latter has a higher accuracy and lower detection threshold, making it a better tool for both characterization of patients and MRD monitoring.

Table 1 Primers and Probes

Application	Name	Sequence
CALR cloning, sequencing	CCF	5′-ATGCTAGCCTGCCGGAGGGTCG-TTTTA-3′
	CCR	5′-ATACGCGTAGAGACATTATTT-GGCGCGG-3′
Evagreen plasmid quantification	TopoF	5′-ACACGTAGAAAGCCAGTCCG-3′
	TopoR	5′-TAAGCCCACTGCAAGCTACC-3′
Fragment analysis	CFF	5′-GAGGTGTGTGCTCTGCCT-3′
	CFR	5′-AGAGACATTATTTGGCGCGG-3′
dPCR type 1	CD1F	5′-CAGAGAAACAAATGAAGGAC-AAAC-3′
	CD1R	5′-GGGACATCTTCCTCCTCATC-3′
	CD1PW*	5′-GGACGAGGAGCAGAGGCTTAAG GAGGA-3′
	CD1PM*	5′-GGACGAGGAGCAGAGGACAAG-GAGGA-3′
dPCR type 2	CD2F	5′-TGAAGGACAAACAGGACGAG-3′
	CD2R	5′-CCGGGGACATCTTCCTCC-3′
	CD2PW*	5′-AGGCAGAGGACAATTGTCGGA-GGATGATGA-3′
	CD2PM*	5′-GAGGAGGCAGAGGACAAGGAG-GATGATGA-3′

*Probes.

F, forward; M, mutant; dPCR, digital PCR; R, reverse; W, wild type.

Materials and Methods

Sample Preparation

DNA was obtained from granulocytes of patients with myeloproliferative disorders explored for the *CALR* mutation at the hematology laboratory of the CHU de Bordeaux or patients included in the JAKALLO clinical trial (*http://www.clinicaltrials.gov*; accession number NCT01795677). All patients had provided informed written consent in accordance with the Declaration of Helsinki for the use of the remaining DNA for investigational purposes. DNA was extracted using the QIAamp Blood DNA minikit according to the manufacturer's instructions (Qiagen, Hilden, Germany). As a means of developing standards for assessing the precision of the method, a sequence corresponding to exon 9 of *CALR* was amplified from patients with wild-type, type 1, or type 2 *CALR* mutations with primers CCF and CCR (Table 1) at 500 nmol/L and a mix that contained 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 1.25U GoTaq, and the provider's specific buffer (Promega France, Charbonnieres, France), then cloned into the TopoTA vector (Life Technologies, Carlsbad, CA). The plasmid was quantified by an Evagreen-based ddPCR using primers specific for the plasmid backbone (TOPOR and TOPOF) (Table 1) at 130 nmol/L and the ddPCR QX200 Evagreen supermix. The PCR program was as follows: denaturation for 5 minutes at 95°C, followed by 40 cycles of 30-second denaturation (95°C) and 1-minute annealing/extension (60°C), then 5 minutes at 4°C and 5 minutes at 90°C. Total *CALR* gene copy numbers were also measured in