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Comparison of Real Time PCR versus PCR with Fragment Length Analysis for the Detection of *CALR* Mutations in Suspected Myeloproliferative Neoplasms

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Running title: Real-Time PCR for *CALR* mutations in MPN

Mutations in *CALR*, the gene which encodes calreticulin, are highly recurrent in essential thrombocytosis (ET) and primary myelofibrosis (PMF), being found in the majority of cases lacking the *JAK2* V617F point mutation.[1–4] More than 50 different *CALR* mutations have been described to date, with the two most common being a 52 base pair deletion (type 1 mutation) and a 5 base pair insertion (type 2 mutation). Additional mutations include variably sized deletions or insertions, which have been divided into type-1-like, type-2-like, or other mutations based on the precise mutated amino acid sequence.[3–5] Each of these various insertion and deletion mutations lead to a +1 frame shift, and a common, novel C-terminal amino acid sequence that lacks the KDEL endoplasmic reticulum localization signal and at least one stretch of negative amino acids that function in calcium binding in the normal protein.

The diversity of *CALR* mutations presents methodological challenges for routine diagnostic laboratories. A variety of techniques have been employed including Sanger sequencing, PCR with fragment length analysis, melting curve analysis, and next generation sequencing studies.[3,4,6–8] While sequencing methods offer the potential for the most comprehensive analysis, these studies are time consuming and not available at all institutions. There therefore remains a need in many laboratories for a rapid, inexpensive method to determine *CALR* mutation status. We have previously described a fragment length PCR assay (FL-PCR) employed at our institution.[9] Real time PCR assays, however, are also rapid, widely available, and offer the potential for increased specificity compared to fragment length analysis. In this report, we compare the results of the FL-PCR assay with a commercially available real-time PCR assay.

146 consecutive samples submitted to the Cleveland Clinic Molecular Diagnostics laboratory for *CALR* mutation analysis between March 2016 and February 2017 with sufficient residual DNA for analysis were retrospectively selected for inclusion in this study. *CALR* exon 9 was amplified by FL-PCR as previously described in detail.[9] Real time PCR was performed using the CALR RGQ PCR Kit (Qiagen, Germantown, MD) on the Rotor-Gene Q 5Plex HRM platform (Qiagen), following the manufacturer's instructions. The CALR RGQ PCR Kit employs 7 separate reactions in a single run. Type 1 and type 2 CALR mutations are detected using allele-specific amplification achieved by allele refractory mutation system (ARMS) technology. Less common mutations are detected via 5 reactions utilizing a series of 3'-block (clamped) oligonucleotide probes to suppress amplification of wild type sequence. The presence of amplification in any of the 5 clamped reactions identifies the presence of a non-type 1, non-type 2 *CALR* mutation. Wild type and mutant *CALR* controls were analyzed in each run. Results of prior FL-PCR were not available to individuals performing real time PCR during analysis.

Table 1

Results of 146 samples (144 peripheral blood, 2 bone marrow) are detailed in Table 1. Using FL-PCR analysis, size altering *CALR* mutations were identified in 22 (15%) cases, including 14 type 1 mutations, 4 type 2 mutations, and 4 cases with atypically sized alterations. The 4 atypically sized deletions including one case with a 2 bp deletion, two cases with a 4 bp deletion, and one case with a 34 bp deletion. Using real time PCR, *CALR* mutations were identified in 20 (14%) cases, including 14/14 (100%) type 1 mutations, 4/4 (100%) type 2 mutations, and 2/4 (50%) cases with atypically sized alterations. All 124 cases negative by the FL-PCR assay were also negative by the real time PCR assay. Using FL-PCR as a gold standard, the real time PCR assay showed a sensitivity of 91%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 98%.

Table 2

Each of the 4 samples with atypically sized (non-type1/type2) mutations were further analyzed by Sanger or next generation sequencing (Table 2). Two of these samples were positive by both FL-PCR and real time PCR: one case with a 34 bp deletion (Type 4 mutation) and one case with 4 bp deletion (Type 36 mutation). The two samples positive by FL-PCR but negative by real time PCR included one case with a 4 bp deletion (Type 22 mutation), and one case with a novel, complex insertion/deletion mutation. Interestingly, this novel mutation did not produce the expected altered C-terminal amino acid sequence that is common to other *CALR* mutations. Rather, the predicted amino acid sequence shared only a stretch of only 18 amino acids with the usual mutant calreticulin sequence, followed by a novel 36 amino acid C-terminus. The C-terminal KDEL sequence of wild type calreticulin was notably absent in the mutant sequence.

The limit of detection of each method was compared using dilution series prepared from patient samples. As previously reported, by FL-PCR analysis, type 1 and type 2 mutations were detectable in samples containing approximately 10% and 5% mutant alleles, but not in samples containing approximately 1% mutant alleles, yielding a limit of detection of approximately 5% mutant alleles. Using the real time PCR assay, type 1 mutations were detectable at 5% mutant alleles, but not 1% or lower, for a limit of detection of approximately 5% mutant alleles. For type 2 mutations, real time PCR results were positive at 5%, 1%, and 0.5%, but negative at 0.1% and below, yielding a limit of detection of 0.5% mutant alleles.

Identifying *CALR* mutations and distinguishing between the varying types of mutations is important both for diagnosis and for prognostic assessment. The presence of a *CALR* mutation, or other driver mutation in *JAK2* or *MPL*, is one criterion for the diagnosis of ET and PMF in the updated 2016 WHO classification of myeloproliferative neoplasms, and detection of such mutations assist in the differential diagnosis with reactive conditions.[1,2,10] Furthermore, cases of ET and PMF with various *CALR* mutations are reported to show differing clinicopathologic characteristics compared to cases with *JAK2* or *MPL* mutations. In ET, cases with a *CALR* mutation are associated with less risk of thrombosis than cases with a *JAK2* V617F mutation. In PMF, cases with *CALR* mutations show improved survival compared to cases with a *JAK2* mutation or "triple negative" cases that lack *JAK2*, *MPL* and *CALR* mutations. When examined by mutation types, PMF with *CALR* type 1 mutations show better outcomes compared to type 2 *CALR* or *JAK2* V617F mutation.

This analysis showed that each of the 18 type 1 and type 2 *CALR* mutations were detected by the allele specific assay, which employs mutation-specific primers for each of these variants. The diverse

non-type 1, non-type 2 mutations are detected through the use of multiple locking probes that inhibit amplification of the wild type sequence and allow for detection of uncommon mutations, although they are not specifically distinguished. Of four non-type 1, non-type 2 mutations, 2 were positive with the real time PCR assay, while two were not detected. The two undetected cases included a 4 bp deletion (type 22 mutation) and a novel insertion/deletion mutation. The type 22 mutation is known to be recurrent,[5] but this represents a rare mutation (0.1% of *CALR* mutations, COSMIC database accessioned 10/14/2018). This real time PCR assay therefore has detected the vast majority of *CALR* mutations.

The novel insertion/deletion detected in this study shows unique features. While multiple complex insertion/deletion mutations have been described in *CALR* (COSMIC database), this case did not produce the typical altered C-terminal sequence reported in other *CALR* mutations. Rather, this mutation produced only an 18 amino acid stretch in common with the usual mutated C-terminus. This result suggests that this 18 amino acid region may contain a critical region for pathogenesis. Notably, this variant did lack the wild type KDEL sequence which mediates ER localization.[3,4] Unfortunately, additional follow-up information was not available on this patient to better characterize this presumed myeloproliferative neoplasm. Additional studies will be required to determine the clinical significance of this variant compared to more common *CALR* mutations.

This study has shown that both FL-PCR and a commercially available real time PCR kit offer sensitive detection of *CALR* mutations. Real time PCR allows for specific detection of type 1 and type 2 mutations, as well as identification of other less common variants. This assay may be particularly advantageous for laboratories performing similar allele specific assays for *JAK2* V617F and *MPL* mutations. The real time PCR assay may also be advantageous for follow up of patients under therapy, as this technique displayed a lower limit of detection than FL-PCR, at least for type 2 *CALR* mutations. Conversely, a drawback of the real time assay is that some rare variants may go undetected. The results of this study will assist laboratories in selecting the most appropriate assay for their workflow, facilitating diagnosis and prognostic evaluation of myeloproliferative neoplasms.

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Author contribution statement: Laura Doyle, Gary Procop and James Cook designed the study; Laura Doyle, Bryan Betz and Helmut Weigelin performed the research; Laura Doyle and James Cook analyzed the data; Laura Doyle, Bryan Beta, Helmut Weigelin, Gary Procop and James Cook wrote and approved the final manuscript.

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		Fragment Length Assay					
		ND	Type 1	Type 2	2 bp del	4 bp del	34 bp del
Real Time PCR Assay	ND	124			1	1	
	Type 1		14				
	Type 2			4			
	Non-Type 1, Type 2 mutant					1	1

Table 1. Results of 146 samples analyzed for *CALR* mutations by real time PCR assay and fragment length assay. ND=not detected.

Fragment Length Result	Real time PCR Result	Sequence Result (Reference transcript NM_004343.3)	Amino Acid Sequence (codon 352 to end)	Mutation type
2 bp del	ND	c.1142_1215delins72, p.E381fs	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEGQRTKRMMRTKMRMRRMRRTRRKNRGRCPRPGQGRAVERPASRAGLRPERSCRRAGRAKX	Novel
4 bp del	ND	c.1122_1125delGAAA, p.K374fs	AAEKQMKDKQDEEQRLKEEEEDNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEAX	Type 22
4bp del	Positive, not type 1 or 2	c.1147_1154delinsTGTC, p.E388fs	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEACRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEAX	Type 36
34 bp del	Positive, not type 1 or 2	c.1103_1136del, p.K368fs	AAEKQMKDKQDEEQRLRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEAX	Type 4

Table 2. Details of non-type 1/type 2 *CALR* mutations identified. Mutated amino acid sequence is indicated in red font. Regions of the common mutant C-terminal calreticulin sequence are underscored. Mutation types are classified according to the system of Klampfl et al.[3]

Abbreviations: del – deletion; ND- not detected.