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Original Article

Detection of *CALR* Mutations Using High Resolution Melting Curve Analysis (HRM-A); Application on a Large Cohort of Greek ET and MF Patients

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Abstract. ***Background and Objectives:*** **Somatic mutations in the calreticulin gene (*CALR*) are detected in approximately 70% of patients with essential thrombocythemia (ET) and primary or secondary myelofibrosis (MF), lacking the *JAK2* and *MPL* mutations. To determine the prevalence of *CALR* frameshift mutations in a population of MPN patients of Greek origin, we developed a rapid low-budget PCR-based assay and screened samples from 5 tertiary Haematology units. This is a first of its kind report of the Greek patient population that also disclosed novel *CALR* mutants.**

Methods: **MPN patient samples were collected from different clinical units and screened for *JAK2* and *MPL* mutations after informed consent was obtained. Negative samples were analyzed for the presence of *CALR* mutations. To this end, we developed a modified post Real Time PCR High-Resolution Melting Curve analysis (HRM-A) protocol. Samples were subsequently confirmed by Sanger sequencing.**

Results: **Using this protocol we screened 173 MPN, *JAK2* and *MPL* mutation negative, patients of Greek origin, of whom 117 (67.63%) displayed a *CALR* exon nine mutation. More specifically, mutations were detected in 90 out of 130 (69.23%) essential thrombocythaemia cases (ET), in 18 out of 33 (54.55%) primary myelofibrosis patients (pMF) and in 9 out of 10 (90%) cases of myelofibrosis secondary to ET (post-ET sMF). False positive results were not detected. The limit of detection (LoD) of our protocol was 2%. Furthermore, our study revealed six rare novel mutations which are to be added in the COSMIC database.**

Conclusions: **Overall, our method could rapidly and cost-effectively detect the mutation status in a representative cohort of Greek patients; the mutation make-up in our group was not different from what has been published for other national groups.**

Keywords: Myeloproliferative Diseases, Thrombocythemia, Myelofibrosis, Calreticulin, Jak2, MPL, Mutation.

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Introduction. Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (pMF).[1] The molecular basis of these disorders was partly elucidated in 2005 with the identification of the *JAK2*V617F mutation in the majority of PV patients and in about 60% of those with ET and MF.[2-6] It was later reported that somatic mutations of *JAK2* exon 12 are present in the remaining PV patients,[7] while mutations of *MPL* exon 10 were present in about 5% of the ET and MF cases.[8] 2008 WHO definition of MPNs includes *JAK2*V617F or *MPL* exon ten mutations as a major criterion for disease diagnosis.[9] Beyond these two culprits, somatic mutations in the *CALR* gene that encodes for calreticulin were identified in 2013 in about 20-25% of patients with ET and MF[10,11] and were subsequently incorporated into the 2016 WHO diagnostic criteria.[12] Determination of *CALR* mutations is relevant not only for their diagnostic contribution[12] but also for their prognostic significance as well.[13-16]

CALR mutations are deletion or insertion events or a combination of both within the DNA sequence of the last exon (exon 9) of the gene. The two most common mutations are either a 52-bp deletion (Type-1; c.1099_1150del;L367fs*46; 45-55% of the cases) or a 5-bp insertion (Type-2; c.1154_1155insTTGTC; K385fs*47; 32-42% of the cases). The remaining 15% of the cases comprise other deletions or insertions or a combination of both that are either unique or found in a small number of patients.[10,11,17] All *CALR* mutations result in a +1bp frameshift, leading to the coding of a novel amino acid peptide sequence distal to the site of the mutation that consequently generates a novel C-terminus at the protein level.[10,11]

The high incidence and specificity of *CALR* mutations in ET and MF make inevitable the need of incorporating rapid and sensitive methodologies in the diagnostic work-up of MPNs. In the relatively similar case of *JAK2*, improved PCR methods, have pushed the *JAK2*V617F mutation limit of detection (LoD) down to a burden of only 1-3%, which has been considered sufficient for the clinical correlation to PV. Such LoD levels should also be attained with the available technologies in the case of *CALR*.[18]

Detection of *CALR* exon nine mutations can be achieved by several methods such as Sanger sequencing, fragment analysis, high resolution melting curve analysis (HRM-A), TaqMan-based Real-Time PCR and targeted next-generation sequencing (NGS).[10,11,19-22] Targeted NGS provides the best LoD and is the most robust technique;[21] however, it is still a costly approach with a long turn-around time, which makes it impractical for routine diagnostic services. HRM-A, is a well-established method for the detection of gene polymorphisms and mutations, by measuring changes in the melting point of DNA duplex.[23]

In this report, we describe a rapid and sensitive HRM-A protocol, which we developed for the detection of *CALR* exon nine mutations using the LightCycler cobas 4800 platforms (ROCHE Diagnostics, Indianapolis, IN, US). Our assay was first applied in a small cohort of MF patients; subsequently, when thoroughly validated, it was used for the study of ET and MF samples from several tertiary health care centres in Greece, thus providing reliable data concerning the frequency of *CALR* mutations within the Greek population and even disclosing a few novel mutations.

Material and Methods. Patient diagnosis was based on the WHO 2008 criteria.[1,9] *CALR* mutational analysis was performed in 130 patients with ET and 43 patients with MF who were negative for the *JAK2*V617F and *MPL*W515L/K mutations, as well as in 19 patients with secondary thrombocytosis.

The study protocol was approved by the Internal Review Boards of all participating Institutes; written informed consent was obtained from all patients, and the study was conducted in accordance with the current version of the Helsinki Declaration.

DNA was extracted by standard procedures after isolation of total leukocytes from peripheral blood or bone marrow following red cell lysis. All samples were investigated for the presence of the *JAK2*V617F and the *MPL*W515L/K mutations. The *JAK2V617F* mutation was detected using a tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) assay with a sensitivity of 1% as previously described.[4] The *MPL*W515L/K mutations were detected using an allele-specific PCR (AS-PCR) assay with a sensitivity of 1% as previously described.[24]

HRM analysis assay design. The HRM analysis assay was performed using the LightCycler cobas 4800 platform (ROCHE Diagnostics, Indianapolis, IN, US). Oligonucleotide primers were designed using the Oligo7 Primer Analysis Software v.7.0.5.7 (Molecular Biology Insights Inc, Colorado Springs, CO, US) to flank all *CALR* exon 9 variants reported in MPNs. Primer sequences were CALRe9HF: 5'-AGGCAGCAGAGAAACAAATGAA-3' and CALRe9HR: 5'-TCTACAGCTCGTCCTTGGC-3',

and the amplicon size was 204 bp (GenBank: NG_029662.1). Ten nanograms of DNA was amplified in a final volume of 10μL containing 1x LightCycler 480 High-Resolution Melting Master Mix (ROCHE Diagnostics, Indianapolis, IN, US), 1.0μM of the CALRe9HF, 0.2μM of the CALRe9HR and 2.5 mM $MgCl_2$.

The cycling conditions were: initial denaturation at 95ºC for 10 min, followed by 45 cycles of 95ºC for 15s, 67ºC for 30s and 72ºC for 15s. The high resolution melting program included denaturation at 95ºC for 1 min, re-naturation at 40ºC for 1 min and melting from 65ºC to 95ºC, with a ramp of 0.03ºC per second and 17 fluorescent acquisitions per degree centigrade.

All samples were analysed in duplicate. Two samples of normal individuals (*CALR* wild-type), one positive control for *CALR* Type-1 and one positive control for *CALR* Type-2 were included in each experiment. The analysis was done with the Light Cycler 480 Gene Scanning Software v.1.5.1.74 (ROCHE Diagnostics, Indianapolis, IN, US). Melting profiles were normalized, grouped and displayed as fluorescence versus temperature plots. Normalization bars were set between 81.5 and 82.0ºC for the leading range and 88.0-88.5ºC for the tailing range. The threshold for the melting temperature (Tm) shift was set at 10. The settings were optimized to a sensitivity value of 0.4 on the analysis software.

Wild-type and mutated samples were defined as negative and positive controls respectively in the analysis. Melting curve analysis is based on the differences in melting curve shape of each sample, hence clustering the samples into groups based on the internal software calculation.

Sanger Sequencing. All patients were screened for *CALR* exon 9 mutations spanning codons 1054-1254 by Sanger sequencing at an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, US). Sanger sequencing was performed on a broader genomic area than the one used for the HRM-A. The PCR primer sequences were CALRe9SF: 5'-CCAACGATGAGGCATACGCT-3' and CALRe9SR: 5'-ATCCACCCCAAATCCGAACC-3' and the amplicon size was 469 bp (GenBank: NG_029662.1). PCR products were cleaned using the QiaQuick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. Bidirectional sequencing was performed using the BigDye Terminator, v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The analysis was performed using the Chromas v.2.4.3 (Technelysium Pty Ltd, South Brisbane, QLD, AU) software.

Results.

HRM analysis for CALR exon 9 mutations. **Table 1** summarizes our results. Of the 173 ET and MF patients analysed using our HRM-A protocol, 117 (67.63%) displayed a *CALR* exon 9 mutation; the incidence of this finding in the ET and MF cohorts was quite similar, comprising of 69.23% in the ET (90 out of 130) and 62.79% in the MF (27 out of 43) group of patients. More specifically, the latter group includes 33 pMF and 10 secondary post-ET MF, of which 54.55% (18 out of 33) and 90% (9 out of 10) carried *CALR* exon 9 mutations, respectively. In contrast, all 19 patients with secondary thrombocytosis were *CALR* negative.

Of the 90 ET patients with HRM positive curves, 46 were Type-1 (L367fs*46), 6 were Type-1-like (E364fs*55, L367fs*50, L367fs*52, D373fs*47, K375fs*49 and K377fs*47), 34 were Type-2 (K385fs*47), 1 was Type-2-like (E386fs*46) and 3 showed complex mutations consisting of D373fs*56, D373fs*51 and K377fs*55. Of the 27 MF patients with HRM positive curves, 16 were Type-1 (L367fs*46), 3 were Type-1-like (L367fs*52 and K377fs*47), 7 were Type-2 (K385fs*47) and 1 showed a complex mutation (E379fs*47).

Table 1. Distribution of *CALR* exon 9 mutations detected in the cohorts of Greek ET and MF patients.

Essential thrombocythaemia							
	Type-1	**Type-1 like**	**Type-2**	**Type-2 like**	**Complex type**	**Mutations detected**	**Samples**
n	46	6	34	1	3	90	130
%	*51.11 **	*6.67 **	*37.78 **	*1.11 **	*3.33 **	*69.23 ***	
Primary myelofibrosis							
	Type-1	**Type-1 like**	**Type-2**	**Type-2 like**	**Complex type**	**Mutations detected**	**Samples**
n	13	2	3	0	0	18	33
%	*72.22 **	*11.11 **	*16.67 **	*0.00 **	*0.00 **	*54.55 ***	
Myelofibrosis secondary to ET							
	Type-1	**Type-1 like**	**Type-2**	**Type-2 like**	**Complex type**	**Mutations detected**	**Samples**
n	3	1	4	0	1	9	10
%						*90.00 ***	

** Percentage of mutation subtypes versus overall mutations detected. ** Percentage of mutations detected versus samples under investigation.*

Table 2. Common and rare *CALR* exon 9 mutations detected in the cohorts of Greek ET and MF patients. Registration order according to increasing start site of mutation within the coding sequence. Mutations # 3, 6, 7, 9, 10 and 11 are novel and (as of May 8, 2017) are not included in the COSMIC v85 database.

	Coding sequence alteration (cDNA level)	Aminoacid sequence alteration	Recurrence	Previously reported
1	c.1091_1124del34	p.E364fs*55	1	YES
2	c.1092_1143del52	p.L367fs*46	62	YES
3	c.1094_1130del37	p.L367fs*50	1	NO
4	c.1098_1131del34	p.L367fs*52	3	YES
5	c.1116_1146del31	p.D373fs*47	1	YES
6	c.1118_1125>CTTG	p.D373fs*56	1	NO
7	c.1118_1140>CGTT	p.D373fs*51	1	NO
8	c.1122_1140del19	p.K375fs*49	1	YES
9	c.1129_1132>TTTTGCTTA	p.K377fs*55	1	NO
10	c.1129_1147del19	p.K377fs*47	2	NO
11	c.1131-1151>GGAGTGTC	p.E379fs*47	1	NO
12	c.1154_1155insTTGTC	p.E385fs*47	41	YES
13	c.1154_1155insATGTC	p.K386fs*46	1	YES

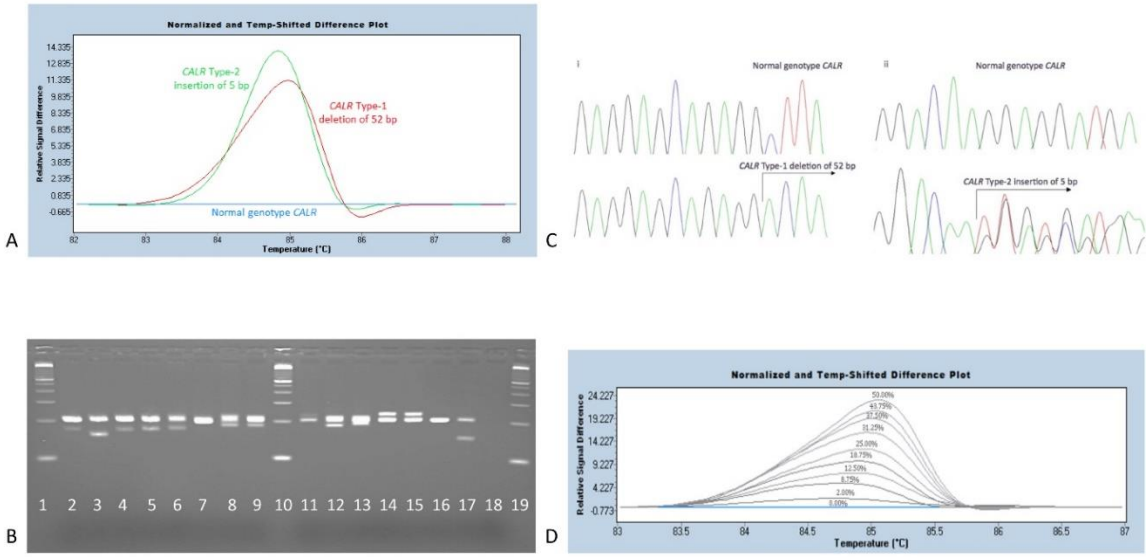

Figure 1. **A.** HRM-A curves after normalization of temperature and fluorescence data, using the LightCycler 480 SW 1.5.1 software (ROCHE Diagnostics, Indianapolis, IN, US). The two most common *CALR* exon 9 mutations detected, are Type-1 deletion (L367fs*46) and Type-2 insertion (K385fs*47). These are grouped separately, due to variations in the pattern of their melting curves. Both melting curves differ significantly compared to the normal genotype sample. Each Type-1-like, Type-2-like and complex mutation identified provided a unique melting curve pattern, that diverges from a normal control. **B.** Agarose gel 3% dense electrophoresis of PCR products. Left to right: line 1, MW marker; line 2, a 34 bp deletion (E364fs*55); line 3, a Type-1 deletion of 52 bp (L367fs*46); line 4, a 37 bp deletion (L367fs*50); line 5, a 34 bp deletion (L367fs*52); line 6, a 31 bp deletion (D373fs*47); line 7, a complex mutation (D373fs*56); line 8, a complex mutation (D373fs*51); line 9, a 19 bp deletion (K375fs*49); line 10, the MW marker; line 11, a complex mutation (K377fs*55); line 12, a 19 bp deletion (K377fs*47); line 13, a complex mutation (E379fs*47); line 14, a 5 bp insertion (E386fs*46); line 15, a Type-2 insertion of 5 bp (K385fs*47); line 16, a normal *CALR* genotype sample; line 17, a MARIMO cell line DNA sample carrying a 61 bp deletion (L367fs*43) which was initially used as a study reference; line 18, a Non Template Control sample (NTC); line 19 the MW marker. A 100 bp molecular-weight size marker (MW) was used as a DNA ladder (Thermo-Fischer Scientific). **C.** Sanger sequencing chromatograms for sequence identification of the HRM-A previously detected *CALR* mutations. i. Top to bottom: a *CALR* normal genotype sample and a common Type-1 deletion (L367fs*46). ii. Top to bottom: a *CALR* normal genotype sample and a Type-2 insertion (K385fs*47). **D.** LoD analysis of the designed HRM-A protocol. A sample harboring a Type-1 deletion mutation (L367fs*46) was used as a reference. Starting at 50% of mutation allele burden, as determined after Sanger sequencing analysis, we ended up detecting as low as 2% of mutant alleles, through serial dilutions.

Table 2 shows the coding sequence alterations (at the cDNA level) of the detected *CALR* exon 9 mutations. Most of them are already known and included in the most recent database of the Catalogue of Somatic Mutations in Cancer (COSMIC v85, as of May 8th, 2017),[25] while six are reported for the first time. As expected, most commonly found mutations were those of Type-1 and Type-2 (**Figure 1A**).

Following HRM-A, PCR products were analysed in 3% agarose gel to confirm that the detected melting curve pattern, diverging from normal genotype control samples, actually belongs to a *CALR* exon 9 mutation (**Figure 1B**). The 204 bp band corresponds to the wild-type *CALR* gene, while additional bands are evidence of *CALR* mutations.

Sanger sequencing analysis of CALR exon 9 mutations. Sanger sequencing analysis was performed in all cases; results were fully concordant with those obtained by the HRM-A technique (**Figure 1C**).

Limit of Detection of HRM analysis for CALR exon 9 mutations. In order to assess the LoD of the assay, we performed serial dilutions of a patient's sample carrying a *CALR* Type-1 mutation (52-bp deletion) and displaying a mutant allele burden of approximately 50% according to sequencing analysis. Serial dilutions were made up to 1% of the mutant in wild-type DNA. The *CALR* mutant could be detected in up to 2% dilution (**Figure 1D**).

Discussion. Recent methodological advances have shown that somatic mutations in the *CALR* gene occur in 20% to 25% of patients with ET and MF[10,11] and are now incorporated into the most recent revision of the WHO diagnostic criteria.[12] Determination of *CALR* mutations is essential for the diagnostic workup[12] and has an impact on the assessment of prognosis.[13-15]

CALR mutation analysis is carried out with several methods including Sanger sequencing,[26] fragment analysis,[27] HRM-A,[19] Real Time PCR using TaqMan probes[28] and NGS[21] with each one of these methods having their specific advantages and drawbacks. Sanger sequencing and fragment analysis have limited sensitivity (in the range of 10-25% and 5-10% respectively), while the NGS technology presents the lowest LoD (1-1.5%) but is costly and time-consuming for routine use, at present.[21,29]

More specifically, compared to NGS, our HRM-A protocol can be completed within a few hours of sample collection, providing fast turn-around times. On the other hand, NGS library preparation and sequencing require a minimum of 2 days for completion, without taking into account the downstream time-consuming analytical steps and the prerequisite of multiplexing more than one samples for cost-effectiveness. The latter is a major obstacle for labs with small sample load. In addition, the NGS output is such that aiming solely at the *CALR* mutation, would be a waste of resources. To fully exploit the capabilities of NGS, clinical laboratories either need a significant load of samples on a routine basis or the simultaneous analysis of more than one genomic region. For all other occasions, HRM-A offers a relatively simple but equally reliable technique that can support the daily routine of individual samples with fast turn-around times.

Other platforms that offer equally satisfactory sensitivity levels to HRM-A are TaqMan-based assays. The classical method uses fluorescently labeled probes, that bind specific DNA sequences. In this way, potentially unwanted PCR by-products can be ignored during analysis, thus allowing for less stringent PCR conditions and primer design. However, the use of nucleotide probes increases expenses and limits the detection to previously determined mutations only. On the contrary, the HRM-A approach is less costly and also permits the detection of novel mutations. A potential drawback of the HRM-A method is the use of fluorescent molecules that bind double-stranded nucleic acids in a non-specific manner. This feature may lead to confusing detection signals, due to potential secondary amplicons, unless experimental settings are optimized in a way that allows the amplification of specific products. This prerequisite has been fulfilled in our protocol design.

In this communication, we propose an optimized HRM-A protocol, which can identify *CALR* exon 9 mutations on the basis of the differential plots that clearly discriminate mutant from wild-type samples. Moreover, this version can efficiently classify Type-1 and Type-2 common mutations and appears to prevent the detection of false-positive results, in contrast to earlier reports.[21] These characteristics are attributed to the carefully designed highly specific primers, along with finely tunned primer annealing temperature (Ta) and PCR cycling conditions. Our modification also allowed prompt detection of novel mutations through the appearance of variable changes in the melting curve profile of undetermined genotype samples, which were then further investigated using Sanger sequencing. In our experiments, the LoD of mutated DNA in a wild-type background was 2%, which is considered fully acceptable (≥2%), marginally lower than that previously reported when using the same technique (3%) and not substantially different than the respective TaqMan assays, where LoD varies between 1-3%.[19,28]

In order to validate our standardized technique, we applied it in a study of a large cohort of Greek ET and MF patients. We observed that neither the overall frequency of the *CALR* exon 9 mutations nor their distribution in the Greek ET and MF patients was substantially different from those reported in similar surveys from other European institutes.[10,11,30,31] In addition, we identified six novel mutations, which are to be added in the COSMIC database.

Conclusions. The proposed modification of the HRM-A technique is considered reliable and has proven useful for the large-scale survey of the *CALR* exon 9 mutations across the Greek ET and MF patients.

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