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SHORT COMMUNICATION

Calreticulin Mutations in Bulgarian MPN Patients

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Abstract Somatic mutations in *JAK2*, *MPL* and *CALR* are recurrently identified in most of the cases with Philadelphia chromosome negative myeloproliferative neoplasms (MPNs). We applied four molecular genetic methods for identification of CALR exon 9 mutations, including high resolution melt (HRM) analysis, Sanger sequencing, semiconductor target genes sequencing and whole exome sequencing. A total of 78 patients with myeloid malignancies were included in the study. We identified 14 CALR exon 9 mutated cases out of 78 studied patients with myeloid malignancies. All mutated patients were diagnosed with MPN being either PMF (n = 7) or ET (n = 7). Nine cases had type 1 mutations and 5 cases had type 2 mutations. *CALR* exon 9, *MPL* exon 10 and *JAK2* p.V617F were mutually exclusive. There were no statistically significant differences in the hematological parameters between the cases with *CALR* and *JAK2* or *MPL* mutations. Notably, all four techniques were fully concordant in the detection of *CALR* mutations. This is one of the few reports on the *CALR* mutations frequency in South-eastern populations. Our study shows that the frequency and patterns of these mutations is identical to those in the patients' cohorts from Western countries. Besides we demonstrated the utility of four different methods for their detection.

Keywords MPNs · CALR · JAK2 · MPL · Mutations

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Introduction

In less than a decade the postulated by William Dameshek clinical similarity between the tree myeloproliferative diseases: primary myelofibrosis, essential thrombocythemia and polycythemia vera found it molecular genetic explanation. Somatic mutations in just three genes *JAK2*, *MPL* and *CALR* appeared to be recurrently identified in the vast majority of those Philadelphia chromosome negative myeloproliferative neoplasms (MPNs) [1]. While *JAK2* and *MPL* mutations were initially shown to affect up to half of the MPN cases and were readily shown to trigger myeloproliferation through constitutive activation of the JAK-STAT signaling pathway, the *CALR* identification as a recurrently mutated gene in PMF and ET took several years longer because its mutations were small insertion and deletions and its function in myeloid cell signaling had been less well understood [2, 3]. A number of reports in the last 3 years showed that CALR mutations are found in about 30% of the PMF and ET cases and are almost always mutually exclusive with JAK2 and MPL mutations. These mutations affect exon 9 of the gene and are either small deletions (type 1) or small insertions (type 2) or combinations of the previous two types causing frame shifts leading to the translation of an invariable neomorphic C terminus of the protein. This striking feature is obviously responsible for the pathogenetic mechanism of the mutant proteins and is most likely affecting signaling through the TPO receptor or calcium

signaling at the level of the endoplasmic reticulum in megakaryocytes. From a merely clinical point of view *CALR* mutations have obvious diagnostic value in the MPN work-up as well as prognostic power. Here we provide the first report on the prevalence of CALR mutations in Bulgarian MPN patients and discuss the implementation of several molecular methods for their detection in the routine diagnostic practice.

Materials and Methods

Study Population

Genomic DNA samples from 78 patients (36 males and 42 females) with myeloid malignanices were used in this study. The samples were collected either at Alexandrovska Hospital between 2010 and 2012 as part of a previous project or at Sofiamed University Hospital in 2015 [4, 5]. The distribution per diagnoses was as follows: PMF ($n = 25$), ET ($n = 33$), PV ($n = 3$), MPN-NOS ($n = 4$), post-ET PMF ($n = 1$), AML ($n = 3$), MDS ($n = 3$) and MPN/MDS ($n = 6$). Diagnoses were established according to the 2008 WHO classification. Molecular genetic testing of all patients was part of the routine diagnostic work-up for the patients and all of them provided written informed consent. The principles of the Helsinki Declaration were strictly followed for all patients and their DNA samples processing.

Molecular Genetic Testing

JAK2 p.V617F and *MPL* mutational status of each patient was established using either custom Luminex-based assay or Sanger sequencing as described previously [4, 5]. All samples were screened for *CALR* exon 9 mutations using high-resolution melting HRM analysis as described previously [6]. The presence of mutations was confirmed by Sanger sequencing as described previously [6]. Sixteen samples were also tested for CALR mutations using targeted amplicon resecquencing on Ion Torrent platform (ThermoFisher) [7]. These samples were included in our previous report but reanalyzed as described below. The typical results of the four assays are presented in Fig. 1.

Bioinformatic and Statistical Analysis

Raw data extraction and normalization of HRM curves was performed using EcoStudy software version 5.0 (Illumina). Sanger sequencing analysis was performed using Sequencher 5.1 (Gene Codes Corp.). Ion Torrent data analysis was performed on Galaxy platform (version 1.0.4) using the

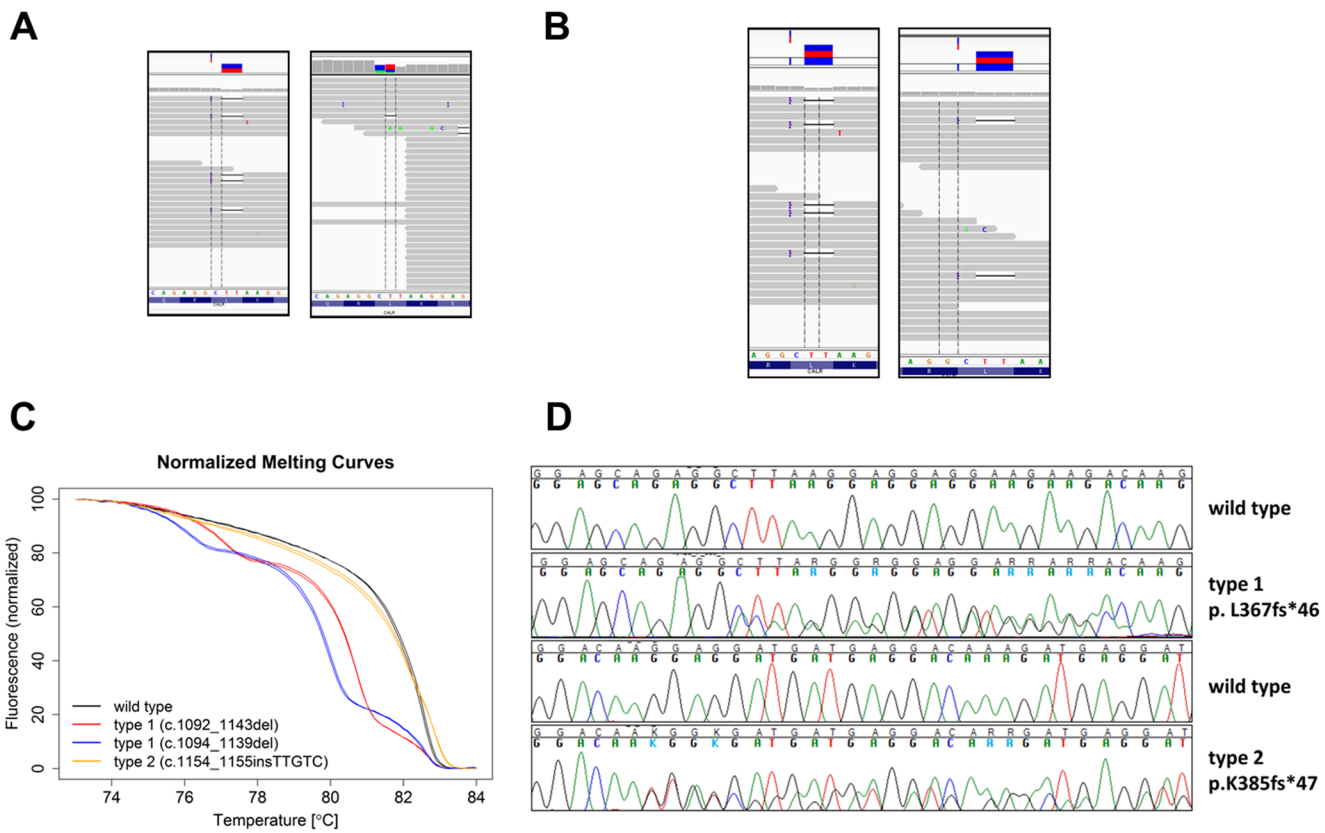

Fig. 1 Demonstartion of molecular genetic testing for CALR exon 9 mutations. **a** Deep sequencing with Ion Torrent; **b** whole exome sequencing; **c** HRM analysis; and **d** Sanger sequencing

following tools: FASTQ Groomer, BWA, FreeBayes and ANNOVAR. Whole exome sequencing analysis was performed by OGT (Oxford, UK) using proprietary software. Read alignments were visualized using Integrative Genomics Viewer (IGV) version 2.3. Continuous variables were compared using two groups comparison with two-sided t-test. *P*-values below 0.05 were considered significant.

Results

We studied a total of 78 patients for the presence of CALR exon 9 mutations using several methods such as high resolution melting (HRM) analysis (n = 43), target genes exome sequencing (n = 16), whole exome sequencing (n = 3) and Sanger sequencing (n = 78). We identified a total of 14 CALR exon 9 mutated cases. All mutated patients were diagnosed with MPN being either PMF (n = 7) or ET (n = 7). Nine cases had type 1 mutations (c.1092_1143del (p.L367 fs*46) (n = 7), c.1102_1135del (p.K368 fs*51) (n = 1) and c.1094_1139del (p. Q365fs*50) (n = 1) and 5 cases had type 2 mutations (c.1154_1155insTTGTC, p.K385 fs*47 (n = 5)). The clinical characteristics of the PMF and ET cases with CALR mutations are presented in Table 1. CALR exon 9, MPL exon 10 and JAK2 p. V617F were mutually exclusive. There was only female patient identified as carrying both CALR type 2 and JAK2 p. V617F mutation. We did not observe statistically significant differences in the hematological parameters between the cases with CALR and JAK2 or MPL mutations except for the slightly lower platelets count in ET patients with CALR mutations.

Notably, we were able to identify CALR mutations with all of the used techniques. Furthermore, all identified CALR mutations using HRM or deep sequencing were afterwards confirmed by Sanger sequencing. We did not observe any false positive cases based on HRM and deep sequencing.

Discussion

The significantly improved understanding regarding the molecular genetic background of Philadelphia chromosome negative MPNs has recently changed dramatically the diagnostic approach in these diseases as recognized by the latest WHO classification. However, the implementation of the molecular testing for *JAK2*, *CALR* and *MPL* mutations is still lagging back in many countries including some of the less developed European committee member states. For instance, a nationwide registry of Bulgarian PMF patients showed that more than 1/3 of the patients were not tested even for the JAK2 mutational pattern [8]. Therefore, continuous efforts are to be made for the implementation of novel methods for detection of these mutations in the routine diagnostic procedures worldwide.

Table 1 Patients' demographic and hematological characteristics

	PMF (sMF)				ET			
	All	JAK2 mutated	MPL mutated	CALR mutated	All	JAK2 mutated	MPL mutated	CALR mutated
n	26	12	4	7	33	18	0	7
M/F	17/9	9/3	3/1	4/3	23/10	3/15	0/0	5/2
Age(mean + SD)	67.64 ± 8.28	68.83 ± 8.18	64.75 ± 8.14	64.71 ± 11.09$^{\#,\$}$	58.85 ± 17.06	57.39 ± 18.68	-	63.43 ± 16.31*
Splenomegaly (% cases)	100	100	100	100	30.3	33.33	-	28.57
WBC	13.48 ± 10.93	17.48 ± 12.7	15.43 ± 14.16	8.79 ± 7.52$^{\#,\$}$	9.98 ± 4.05	11.21 ± 4.13	-	7.96 ± 3.72*
Hgb	87.4 ± 27.88	90.92 ± 36.25	111.0 ± 27.4	81.86 ± 16.82$^{\#,\$}$	131.72 ± 31.0	145.35 ± 30.47	-	125.86 ± 15.13**
Plt	380.36 ± 452.54	431.25 ± 578.67	545.75 ± 458.8	182.14 ± 132.32$^{\#,\$}$	856.7 ± 353.25	864.06 ± 363.91	-	914.43 ± 482.02

$^{\#}$ Not significant at $p < 0.05$ vs. PMF with JAK2 mutations

$^{\$}$ Not significant at $p < 0.05$ vs. PMF with MPL mutations

*Not significant at $p < 0.05$ vs. ET with JAK2 mutations

**$p = 0.049$ vs. ET with JAK2 mutations

Here, we performed the first study in Bulgaria on the molecular epidemiology of JAK2, MPL and CALR mutations. We identified a mutational frequency of the CALR gene (27% for PMF patients and 21% for ET patients) consistent with the previous reports from other populations [9, 10]. Type 1 mutations were more frequent than type 2, which is also in consistency with the previous reports [9, 10]. We did not detect significant clinical or laboratory features discriminating between the CALR mutated and unmutated cases of PMF and ET.

A number of assays have already been adapted for detection of CALR mutations. The optimal method for a given diagnostic laboratory obviously depends on the equipment availability and professional expertise. A recent study by Jones et al. compared four of those methods including Sanger sequencing, fragment analysis PCR, high resolution melt (HRM) and targeted next generation sequencing (NGS) [6]. They conclusively demonstrated the superiority of HRM and fragment analysis PCR and deep sequencing to detect low allele burdens of mutations in comparison to Sanger sequencing.

Although *CALR* exon 9, *JAK2* p.V617F and *MPL* exon 10 is relatively accessible for diagnostic purposes using Sanger sequencing our study and many others showed that still a number of triple negative cases exists. Those cases would require extensive sequencing of the *JAK2* or *MPL* gene or as proposed by the WHO 2016 classification testing for the most frequent clonal markers – mutations in the genes *ASXL1*, *EZH2*, *TET2*, *IDH1*, *IDH2*, *SRSF2* and *SF3B1* [11]. Most of the latter genes showed a pattern of scattered missense and small indel mutations which makes impractical their routine testing by Sanger sequencing. We and others [7, 12] developed a number of custom panels for targeted genes sequencing using the two most widely used NGS platforms. Inevitably, they will gain greater recognition also because of the opportunity to detect very low levels of mutant allele burdens providing also information regarding the subclonal architecture of the malignant population and eventually allowing for the earlier identification of novel genetic events suggestive of clonal and clinical evolution.

Compliance with Ethical Standards

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