ORIGINAL ARTICLE

Calreticulin Mutations in Myeloproliferative Neoplasms Patients Diagnosed in UKM Medical Centre

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ABSTRACT

Introduction: Calreticulin (CALR) mutations are one of the molecular markers that has been incorporated for the diagnosis of myeloproliferative neoplasms (MPN) in the revised 2017 WHO Classification of Haematopoietic and Lymphoid Tumors. This study was performed to determine the prevalence of CALR mutations in patients with MPN diagnosed in UKMMC and to compare their demographics plus laboratory features with other MPN patients. **Methods:** A total of 59 MPN patients who tested negative for JAK2V617Fmutation were selected and 21 MPN patients positive for JAK2V617F were included as controls. Screening for CALR exon 9 was done by multiplex polymerase chain reaction (PCR) followed by Sanger sequencing. **Results:** A total of six JAK2 V617F negative MPN samples were found to be positive for CALR mutations. Out of these six, three patients with CALR mutations were of type I mutation, two were type II while one was a mutation in the stretch III region. None of the twenty one JAK2 V617F positive MPN samples were positive for CALR mutation. Clinical phenotypes for those positive for CALR were restricted to Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF) and one case of atypical Chronic Myeloid Leukaemia (CML). **Conclusion:** CALR mutations constituted 10.16% from the MPN patients who were negative for JAK2V617F mutation with no significant differences in platelet counts, hemoglobin (Hb), hematocrit and white cell counts as compared to MPN patients with JAK2 V617F mutations. Testing for CALR mutations among those who are negative for JAK2V617F within Malaysian population maybe worthwhile and require larger scale studies.
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INTRODUCTION

Current diagnostic criteria for Philadelphia-negative myeloproliferative neoplasms (MPN) have been redefined with the discovery of Janus Kinase 2 (JAK2), myeloproliferative leukaemia (MPL) and calreticulin (CALR) genetic alterations. However detection for CALR and MPL mutations are not routine tests that are readily available for molecular diagnosis of MPNs in Malaysia offered by most molecular laboratories. The revised 2017 WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues states that when BCR-ABL1 or JAK-2 mutation are not detected, the search for 2 other mutations of MPL and CALR (NCBI Ref. seq: NC_000019.10) genes should be made to confirm or exclude the diagnosis since these molecular markers are major findings in the diagnostic criteria for MPNs (1). Calreticulin is a multifunctional protein that acts as a major Ca(2+)-binding (storage) protein in the lumen of the endoplasmic reticulum. It acts as an important modulator of the regulation of gene transcription by nuclear hormone receptors. As of 2018, University Malaya Medical Center (UMMC) and Ampang Hospital offer testing for both JAK-2 exon 14 and 12 mutations while MPL gene mutation testing is also offered by UMMC (2,3). Ampang Hospital offers CALR mutation testing for JAK-2 negative samples since 2016 (4) however, currently no data exists for the prevalence of CALR mutations within Malaysian population (PubMed keywords: calreticulin AND Malaysia; 9 unrelated results on 6th Sept 2018, Cochrane database keywords: calreticulin AND Malaysia; 3 unrelated results on 6th Sept 2018). Only one case report in Malaysia from 2016 by Wong et. al described a patient positive for TET-2 and CALR mutation who presented with a very high platelet and Hb (5). The author in this report outsourced the detection of both genes to an external laboratory using Next Generation Sequencing (NGS) method. Almost all previous studies for CALR mutations involved whole genome sequencing by Sanger's method or NGS which are expensive and not widely available within our setting.

In 2016, Jeong et. al from South Korea described a new method of PCR with custom made primers to detect type 1 and type 2 mutations of CALR which account for 80 to 90% of mutations within this gene (6). Type 1 mutation is a 52-bp deletion (c.1092_1143del; p.L367fs*46) while type 2 is a 5-bp insertion (c.1154_1155insTTGTC, pK385fs*47). They then compared it to Sanger sequencing and fragment analysis for accuracy. They reported a sensitivity of 94.1% and specificity of 100% when compared to Sanger's sequencing (6). Mohamed et. al used the same primers and method to study CALR mutations among JAK2 negative patients in Sudan during early 2018, reporting a 40.5% percentage of patients turning out positive (7). This study used these same primers to detect CALR mutations in 80 samples from patients who were suspected or diagnosed with myeloproliferative neoplasms from January 2017 until February 2019.

The diagnosis of Essential Thrombocytosis (one subtype of MPNs) according to the WHO 2016 can be met if all major criteria or three major criteria and one minor criteria are met. The major criteria include a platelet count > 450 x 10^9/L, bone marrow morphology suggestive of MPNs changes specific for Essential Thrombocytosis, other criteria for BCR-ABL positive MPNs are not met and a positive mutation marker for MPL, JAK2 or CALR mutation. The minor criteria include the presence of another clonal marker or the absence of evidence for reactive thrombocytosis. These major and minor criteria are also applied with the same changes for morphology and clinical findings for other MPNs.

The aim of this study was to determine the prevalence of CALR mutations in MPN patients diagnosed in UKMMC. It was hypothesized that the prevalence is around 15-20% within the samples in this study while other studies from overseas reported variable prevalence from 40-80% (7,8) in their studies for CALR with regards to JAK2 negative MPNs. The other aim was to correlate the clinical phenotypes of the MPN patients with their demographics and laboratory parameters.

MATERIALS AND METHODS

Patient selection

This was a retrospective study on 80 retrievable DNA samples of MPN patients who were referred to UKMMC, Kuala Lumpur between January 2017 until February 2019. The study was carried out in the Molecular Genetics Unit, Department of Laboratory Diagnostics Services, UKMMC, Kuala Lumpur. Out of 80 MPN samples, 59 samples were JAK-2 negative and the remaining 21 samples were JAK-2 positive samples used as controls. All the patients were selected based on the WHO diagnostic criteria 2016 for haematology parameters and/or bone marrow examination which yielded significant findings. One sample included a patient who was diagnosed with Chronic Myelomonocytic Leukaemia (CMML), an entity classed under MPN/MDS category under the 2016 WHO classification. Ethics approval for this study was obtained from the Medical Research Ethics Committees of Universiti Kebangsaan Malaysia (Code Project: FF-2019-094) and all the patients tested have consented for their DNA to be used in research prior to previous testing for JAK-2^{V617} mutation done using PCR.

DNA extraction

DNA was previously extracted from 2 ml of Ethylene diamine tetra acetic (EDTA) peripheral blood and bone marrow aspirates using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) employing the standard protocol described by the manufacturer and all extracted DNA samples had been kept at -30°C.

Detection of CALR mutations by PCR amplification

Initial PCR optimization was done on one sample at four different annealing temperatures using SelectCycler™ II thermal cycler (Select-BioProducts, USA). PCR amplification was performed in a total volume of 25µl reaction mixture containing 12.5µL Tiangen 2x Master Mix including 0.1 U/µlTaqPlus Polymerase, 500 µMdNTP, 20 mMTris-HCl (pH8.3), 100 mMKCl, 3 mM MgCl2 and Stabilizer and enhancer (Tiangen Biotech (Beijing) CO.,LTD), 1.5µl (10 pmol) of each primer F1 (forward primer 1) 5´-GCA GCA GAG AAA CAA ATG AAG G-3´and R (reverse primer) 5´-AGA GTG GAG GAG GGG AAC AA-3´ and 2.5µl (10 pmol) of primer F2 (forward primer 2) 5´-GCA GAG GAC AAT TGT CGG A-3′ (Apical Scientific Sdn. Bhd., Malaysia), and 10ng of DNA template. The reaction mixture was amplified at an initial denaturation temperature of 94°C for 10 minutes, followed up by 40 cycles with DNA denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds and DNA extension at 72°C for 30 seconds. The final extension was set up at 72°C for 7 minutes (SelectCycler™ II thermal cycler, Select-BioProducts, USA). After PCR amplification, gel electrophoresis was performed in a 2% agarose gel with Fluorosafe DNA Stain (Axil Scientific Pte Ltd, Singapore) at 110 Volts for 45 minutes to detect the amplified regions of DNA. The agarose gel was exposed under UV light using Omega Lum™ G Gel Imaging System (Applegen, Inc., Pleasanton, CA, USA). Results interpretation was done based on the expected amplicon size for CALR wild-type at 357 bp, CALR type-1 mutation at 302 bp and CALR type-2 mutation at 272 bp (6).

DNA sequencing

All positive samples were sequenced externally through bi-directional Sanger Sequencing at First Base Technologies Lab based in Seri Kembangan, Selangor. The sequences were then outsourced to Prof Dr. Zulqarnain Mohamed from the Institute of Biological Sciences, University of Malaya for interpretation and confirmation.

RESULTS

Out of 59 JAK2^{V617F} mutations negative MPN samples, only six were positive for CALR mutations. None of the 21 JAK2^{V617F} positive MPN samples, were positive for CALR mutations. Type I mutation was identified by the presence of a 302 base pair (bp)band on lane 2,4 and 5 while type II mutation was identified by the presence of a higher extra band on agarose gel electrophoresis close to the wild type band as shown on lane 6 and 7 in Figure 1. A 335 bp band was also detected on patient 11 as illustrated in lane 9 at Figure 1 which was later confirmed by Sanger sequencing to be a stretch III mutation (c.1129_1154delinsTGTC, p.K377fs*46). These mutations were all confirmed by Sanger sequencing. Prevalence of CALR mutations among JAK2^{V617F} negative MPNs were 10.16% while prevalence among total MPNs were 7.41%. Five out of six (83%) patients were Chinese females while one was a Malay male. Mean age at diagnosis was 61.5 years old among all positive samples. All samples with available bone marrow examinations showed increased megakaryocytes with some degree of dysplasia while two had evidence of fibrosis. One patient with myelofibrosis had a clinical phenotype with cytogenetic studies showing monosomy 5 and monosomy 2. For this patient, fluorescence in-situ hybridization (FISH) study showed CSF1R mutation in 43% of cells analyzed and deletion of EGR1 in 59% of cells analyzed. One patient had an atypical phenotype on the peripheral blood film with a chronic myeloid leukaemia – like picture. The patient was negative for BCR-ABL mutation by PCR study and had a low neutrophil alkaline phosphatase (NAP) score of 2. These findings are summarized in the Tables I, II and III according to their FBC/peripheral blood film, mutation types and bone marrow findings.

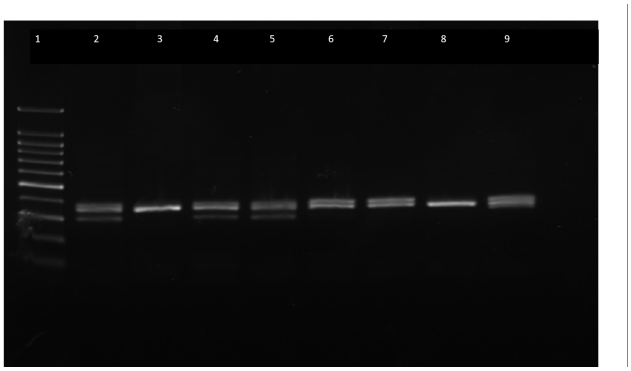

Figure 1: Gel electrophoresis showing type I mutations in lane 2, 4 and 5. Type II mutations are shown in lane 6 and 7. Lane 9 shows a stretch III mutations while Lane 3 and 8 were wild type. Lane 1 is the base pair ladder for reference.

DISCUSSION

Since first discovered in 2013 by Nangalia et al (8) and Klampfl et al (9), after performing whole exome

Table I: Demographics, bone marrow, erythropoietin and cytogenetic findings of patients with positive CALR mutations

Demographics	Patient 40	Patient 21	Patient 20	Patient 17	Patient 5	Patient 11	Mean
CALR mutations	Type I	Type I	Type I	Type II	Type II	Stretch III	NA
Age	54	70	37	65	79	64	61.5
Gender	Female	Male	Female	Female	Female	Female	NA
Race	Chinese	Malay	Chinese	Chinese	Chinese	Chinese	NA
Cellularity	Normal	Hypercellular	NA	Hypocellular	NA	Hypercellular	NA
Dysplasia	Present	Present	NA	Present	NA	Present	NA
WHO Reticulin Grade	Grade I	Grade I-II	NA	Grade II	NA	Grade II	NA
Cytogenetics	Normal	Normal	NA	Monosomy 2 & 5	Normal	Normal	NA
Serum eytrhopoietin (mu/ml)	7	NA	NA	NA	NA	NA	NA
Clinical MPN subtype	ET	ET	ET	MF	Atypical CML	MF	NA

*NA: Not Available/Applicable, ET: essential thrombocytosis, MF: myelofibrosis. CML: chronic myeloid leukaemia, NA: not available.

Table 2: Peripheral blood findings based on FBC and blood films for all positive samples taken at diagnosis.

Parameters	Patient 40	Patient 21	Patient 20	Patient 17	Patient 5	Patient 11	Mean value
Hb (g/dL)	15.9	10.1	12.0	9.5	10.1	11.8	11.5
Haematocrit (%)	49.5	30.1	35.9	30.5	35.1	38.5	36.6
White cells (10^9)	15.8	9.7	16.2	7.2	50.3	9.5	18.1
Platelets (10^9)	1350	1330	651	287	183	1336	856.2
Neutrophils count (10^9)	11.2	3.4	13.1	5.3	42.9	5.5	13.6
Eosinophils counts (10^9)	0.2	0.3	0.1	0.1	0.6	0.2	0.25
Basophils counts (10^9)	0.1	0.3	0.1	0.1	0.6	0.5	0.28
Lymphocytes	3.3	4.1	2.0	1.4	5.3	3.8	3.3
MPV (fl)	8.9	NA	9.5	11.4	10.5	9.9	41.9
LE picture on peripheral blood	No	Yes	No	Yes	Yes	No	NA

Hb: Hemoglobin, MPV: Mean Platelets Volume, LE: Leucoerythroblastic, NA: Not Available/Applicable

Table III: Comparison between the demographics, laboratory parameters and clinical phenotypes of JAK2^{V617F}, CALR and patients negative for both JAK2^{V617F} and CALR mutations in this study.

Parameters	JAK2 (n=21)	Mutations CALR (n=6)	JAK2/CALR negative (n =53)
Mean Hb (g/dL)	13.2	11.5	15.4
Mean Haematocrit (%)	41.6	36.6	47.0
Mean WCC (10^9/uL)	18.1	18.1	14.5
Mean Platelets (10^9/uL)	655	856	374
Gender	7 Females 14 Males	5 Females 1 Male	11 Females 42 Males
Mean Age	59	61	54
LE picture on FBP	5 (23.8%)	3 (50%)	5 (9.4%)
Phenotype	12 ET 4 MF 5 PV	3 ET 2 MF 1 atypical CML	8 ET 1 MF 7 PV 1 MDS/MPN 36 MPN-U
Ethnicity	15 Malays (71%) 6 Chinese (29%)	1 Malay (20%) 5 Chinese (80%)	31 Malays (58.5%) 18 Chinese (34%) 2 Burmese (3.5%) 1 Bangladeshi (2%) 1 Indian (2%)

LE: leucoerythroblastic, FBP: Full blood picture, ET: essential thrombocytosis, PV: Polycythemia Vera, MF: myelofibrosis MDS: myelodysplastic syndrome, MPN-U: MPN-Unclassifiable.

sequencing on myeloproliferative neoplasms patients who were JAK2 negative, more than 50 different types of mutations were documented in the CALR gene (10). Rapid advances in molecular techniques through PCR have since allowed these mutations to be detected through various methods such as high resolution melting (HRM) curve analysis (11), amplicon length based analysis (ALDA) (6,12) and recently an antibody based immunohistochemical method has been evaluated for use which showed good correlation with PCR based method (13). The method by Jeong JH et al was chosen primarily due to its simplicity of using only 3 primers with affordable, widely available agarose gel for detecting the mutations.

The results obtained in this study differ significantly from published evidence regarding CALR mutations in that most patients in these published evidence were young, male, have lower white blood cells counts, higher platelets and better prognosis (10,15). Five out of 6 patients that were positive in this study were females with mean age of 61.5 years and mean white cell count of 18.1 x 10^9/uL. Mean platelet count was 856 x 10^9/uL which is less than 1000 x 10^9/uL as published in previous reviews (8,10,15). At the time of this writing, 2 patients have passed away (patient 5 and patient 17), 2 are still on follow-ups (patient 11 and patient 21) and another 2 patients were lost on follow-ups (patient 20 and patient 40). Both the patients who passed away had Type II mutations which are in-line with previous reviews (10,15) that this particular mutation has a worse prognosis when compared to type I. The youngest (patient 20) is a female at 37 years old who had no symptoms but was found to have persistent thrombocytosis during her pregnancy. She had a type I mutation for CALR. The phenotypes reported was in agreement with other studies as none of these patients had polycythemia vera with 5 others being either myelofibrosis or essential thrombocytosis. Only one had an atypical phenotype with a CML-like clinical picture.

Comparison of laboratory parameters and demographics between JAK2^{V617F} and CALR positive samples were then made. A one tailed Mann-Whitney test using SPSS version 27 was then initiated to compare between the two mutations for differences in hemoglobin, haematocrit, white cell count and platelet level which yielded p-values of 0.106, 0.115, 0.330 and 0.164 respectively. Taking p value at <0.05, all these yielded no statistically significant results. Compared to other studies (22,23,24) which highlighted that patients with JAK2^{V617F} mutations were older, had a higher hemoglobin and white cell count with lower platelets, the sample population in this study with CALR mutations were also older with higher platelet counts and lower hemoglobin levels. These variations in findings highlight the need of mutational testing for prognostication as clinical phenotypes and laboratory parameters cannot reliably distinguish between the types of mutations that JAK2^{V617F} and CALR carry. Comparison with the Mann-Whitney test were also made between the patients who were CALR positive and the patients who were negative for both JAK2 ^{V617F} and CALR mutations which yielded significant differences in Hb (p=0.006), haematocrit (p=0.008) and platelets (p= 0.02) with no significant difference in white cell count (p=0.112). These results were replicated when the same comparison was made between patients who were positive for JAK2^{V617F} mutations and the patients who were negative for both JAK2^{V617F} and CALR mutations with Hb (p=0.004), haematocrit (p=0.03), platelets (p=0.002) showing significant differences and white cell count (p=0.159) showing no significant difference. The differences in the results between these three groups can be attributed to the clinical phenotypes that are associated with each of them as highlighted in Table IV.

The results in this study are mostly in agreement with a previous larger registry-based report from Yap, Law and Sathar et. al (14) who reported myeloproliferative neoplasms in Malaysia with a focus on JAK2 positivity. An overwhelming 5 out of 6 (83.3%) patients who tested positive for CALR were females and of Chinese ethnicity as compared to the national average of 43.2% after adjustment for the weightage of ethnic composition in Malaysia. Mean age was 61.5 years old as compared to 54.2 years old from the national registry. 3 (50%) of our patients who were CALR positive manifested clinically as ET while 2 manifested as primary myelofibrosis. On the other hand, the national registry recorded that most Malaysian patients with MPNs had ET (40.4%) and PV (38.1%). Despite the similar prevalence in clinical manifestations, our mean Hb at presentation was significantly lower at 11.5 g/dL as compared to 14.8

Table IV: CALR mutational profile among several studies in South East Asian Populations

Authors (year)	n	Prevalence	Mean Age	Phenotype	Mean Platelet	Mean WBC	Gender predominance
Current study	59	10.16%	61.5	MF, ET, atypical CML	856 x 10^9	18.1 x 10^9	Female
Singdong et al (2016) (16)	100	6%	80	MF, ET	NS	NS	NS
Rattarimtarong et al (2018) (17)	28	35.7%	57	ET	1449 x 10^9	10.1 x 10^9	Female
Limsuwanachot et al (2017) (18)	58	14%	75.5	ET	577 x 10^9	7.2 x 10^9	Female
Vu et al (2019) (19)	395	27.6%	51	ET	1207 x 10^9	9.7 x 10^9	ND
C.H Ng et al (2018) (20)	331	12.1%	56	ET	1065 x 10^9	10.2 x 10^9	Male

MF: myelofibrosis, ET: essential thrombocytosis, NS: Not stated, ND: No difference.

g/dL of the national average while platelet was higher at 856.2 x 10^9/uL compared to 745.9 x 10^9/uL of the national average. This maybe attributed to the difference in genotype focusing on JAK2 in the registry vs CALR in our study. White cell count was similar at 18.1 x 10^3/uL compared to the national average of 16.9 x 10^3/uL.

Among South East Asian population, Singdong et al in 2016 (16) used PCR with pyrosequencing to test 100 samples of Thai patients with MPNs for JAK2, CALR and MPL. They found out that the prevalence of JAK2-negative, CALR-positive MPNs were 6% as compared 10.16% from our study. In their study, 5 patients had the phenotype of essential thrombocytosis while one patient had myelofibrosis. Mean age reported for CALR positive patients with essential thrombocytosis was also significantly higher which was close to 80 years old as compared to this study at 61.5 years old as a whole. Another study by Rattarittamrong et al in 2018 (17) combined PCR with HRM and sequencing to test 28 Thai patients with essential thrombocytosis who were JAK2-negative for CALR mutations. The prevalence of CALR mutations in this study was 35.7% with a mean age of 57 years old as and mean platelet count of 1449 x 10^9/uL while mean white cell count was 10.1 x 10^9/uL. A female predominance was noticed among those who were positive. Another study among 58 Thai patients by Limsuwanachot et al in 2017 (18), noted the prevalence of CALR mutations was 14% among patients with essential thrombocytosis who were negative for BCR-ABL mutations. Mean age reported was 75.5 years old while mean platelet count was 577 x 10^9/uL with a female predominance. Mean white cell count was 7.2 x 10^9/uL.

A similar study in Vietnam conducted by Vu et al in 2019 (19) among 395 patients with essential thrombocytosis noted a prevalence of 27.6% with no difference in sex ratio and a mean platelet count of 1207 x 10^9/uL. Mean white cell count was 9.7 x 10^9/uL while mean age was 51 years old. Another large multicenter study done in 2018 among 331 Singaporean patients (20) with essential thrombocytosis noted almost the same findings as ours except for a male predominance. The mean age was 56 years old, mean white cell count was 10.2 x 10^9 and mean platelet count was 1065 x 10^9. Interestingly, this study also noted a Chinese predominance in a multi-ethnic population similar to our center in Malaysia while also having a male predominance. The differences between ours and these studies for CALR mutations are summarized in Table IV.

As more data emerge regarding the prevalence of these mutations, treatment is also evolving. It is now well known that JAK2-inhibitors particularly Ruxolitinib has a role to play in the management of CALR positive MPNs. New agents like fedratinib and pacritinib are currently undergoing phase III trials for this disease entity (21). This makes it more crucial for these mutations to be detected on a larger scale for these drugs to be made accessible to patients.

CONCLUSION

CALR mutations are most prevalent among Chinese patients and constituted 10.16% from the patients who are negative for $JAK2^{V617F}$ mutation. Testing for this mutation among those who are negative for $JAK2^{V617F}$mutation within Malaysian population maybe worthwhile and require more large scale studies.

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