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CASE REPORT

Atypical Presentation of Primary Myelofibrosis with Calreticulin (CALR) Mutation: A Case Report from a Single Referral Centre in Malaysia

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Abstract

Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm (MPN) characterized by a proliferation of predominantly megakaryocytes and granulocytes in the bone marrow, which in fully developed disease is associated with reactive deposition of fibrous connective tissue and extramedullary hematopoiesis. Approximately 60% of PMF cases carry JAK2 V617F, about 30% of cases have CALR mutation, 8% positive for MPL and 12% of cases are triple negative for these mutations. The laboratory findings in PMF are quite diverse and ranges from leukocytosis and thrombocytosis in early stage of the disease to marked pancytopenia, leukoerythroblastic blood film, tear drop poikilocytosis and severe fibrosis with diffuse osteosclerosis in trephine bone marrow biopsy in later stage of the disease. Here we described unusual laboratory presentation of PMF with CALR gene mutation with hematological and morphological features of chronic myeloid leukemia but molecular analysis for BCR-ABL1 transcript was negative.

Keywords: Primary Myelofibrosis, Calreticulin mutation, BCR-ABL1 transcript

Introduction

Myeloproliferative neoplasm (MPN) with BCR-ABL1-negative is a clonal haematopoietic stem cell disorder characterized by the proliferation of cells of predominantly abnormal megakaryocytes and granulocytes in the bone marrow, which in its fully developed disease is associated with reactive deposition of fibrous tissue and with extramedullary haematopoiesis (Steven et al., 2017). Myelofibrosis can present as a de novo disorder or evolve secondary to previous polycythaemia vera or essential thrombocythemia (post PV MF or post ET MF). Primary myelofibrosis (PMF) is the least frequent among the chronic myeloproliferative diseases. (Gunawardena and Bavanthan, 2015)

The estimated annual incidence of overt PMF is 0.5 to 1.5 cases per 100,000 populations. The prevalence of PMF is increasing due to earlier diagnosis and prolonged survival. PMF affect men and women nearly equally. It occurs most commonly in sixth to seventh decade of life. (Steven et al., 2017)

The disease is clinically characterized by progressive anaemia, constitutional symptoms and splenomegaly. The laboratory findings show leucocytosis, leucoerythroblastic blood film, tear drop poikilocytosis, reticulin fibrosis in bone marrow and elevated levels of various inflammatory and pro-angiogenic cytokines. (Gunawardena and Bavanthan, 2015; Steven et al., 2017).

The majority (approximately 50 to 60%) of the patients harbour JAK2 V617F mutation. MPL mutations (approximately 8%) were described in JAK2 V617F mutation negative PMF. Calreticulin (CALR) mutations are found in about 30% of PMF cases. 8% in MPL and 12% of cases are triple negative for this mutation (Steven et al., 2017). Recent discovery of the CALR mutation has improved the genetic detection of myelofibrosis to about 80% (Rumi and Cazzola, 2017).

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Overall prognosis depend on stage at which neoplasm is initially diagnosed and corresponding risk status, which can be determined using prognostic scoring system. Pre PMF is associated with 10 to 15 years relative survival rate of 72% and 59%. Major causes of morbidity and mortality are bone marrow failure (infections, haemorrhage), thromboembolic events, portal hypertension, cardiac failure or progression to leukaemia (Steven et al., 2017).

The only treatment modality that is currently capable of prolonging survival or potential cure for MF is allogeneic hematopoietic stem cell transplant (AHSCT). The treatment algorithm is based on risk stratification. In non-transplant candidates, conventional treatment for anemia includes androgens, prednisone, thalidomide, and danazol; for symptomatic splenomegaly, hydroxyurea and ruxolitinib. Fedratinib is another JAK2 inhibitor which has now been approved by FDA for use after ruxolitinib fails. Splenectomy is considered for drug-refractory splenomegaly and involved-field radiotherapy for non-hepatosplenic of extramedullary hematopoiesis and extremity bone pain (Tefferi, 2020).

Here we describe a case of primary myelofibrosis CALR positive presented with laboratory features of CML, however DNA analysis was negative for BCR-ABL1 transcript.

Case presentation

An 81-year-old ex-smoker was presented with a month history of cough and shortness of breath. She had massive hepatosplenomegaly. Full blood count (FBC) showed a haemoglobin (Hb) level of 7.2 g/dL, platelet count of 45 x 10^9 and hyperleucocytosis with white blood cells (WBC) count was 166.2 x 10^9. Absolute neutrophil count was 4.3 x 10^9.

Peripheral blood film showed presence of teardrop red blood cell and all spectrum of myeloid lineage was seen with peak in neutrophils and myelocytes. There was also eosinophilia, basophilia with 8% blasts. Neutrophils alkaline phosphatase (NAP) score was very low, 2/100 WBC (reference range 35-100/100 WBC). She refused for bone marrow aspirate and trephine biopsy.

Cytogenetic analysis of the G-banded chromosomes from cultured peripheral blood showed a normal female chromosome complement with no evidence of clonal abnormality. Fluorescence in situ hybridization (FISH) done on 200 nuclei using Vysis LSI BCR/ABL1 Dual Colour Translocation Probe. Two green and two orange signal patterns were observed in all nuclei analysed. This analysis showed no evidence of BCR-ABL1 rearrangement in all of the cells analysed. DNA analysis for BCR-ABL1 transcript by Reverse Transcriptase PCR method which has been previously described in (Wong et al., 2011) and JAK2 V617F mutation amplification-refractory mutation system PCR (ARMS-PCR) described in (Hamidah et al., 2011) using peripheral blood were negative.

Further molecular analysis for CALR mutation by conventional PCR and followed by bidirectional Sanger sequencing was done. DNA was previously extracted from 2 ml of Ethylenediaminetetraacetic (EDTA) peripheral blood using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) employing the standard protocol described by the manufacturer and this extracted DNA sample had been kept at -30°C. Initial PCR optimization for CALR mutation was done on one sample at four different annealing temperatures using SelectCycler™ II thermal cycler (Select-BioProducts, USA). PCR amplification was performed using forward primer 1 (F1) 5´-GCA GCA GAG AAA CAA ATG AAG G-3', reverse primer (R) 5´-AGA GTG GAG GAG GGG AAC AA-3´ and forward primer 2 (F2) 5´-GCA GAG GAC AAT TGT CGG A-3' (Apical Scientific Sdn. Bhd., Malaysia).

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. Agarose gel were 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 (Figure II) after which needs to be confirmed by sequencing as described by (Jeong et al., 2016). This case was found to have abnormal band 272bp.

The mutation was then confirmed by Sanger sequencing. The PCR product was initially purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and then sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems, Foster City, CA). The final sequences were then analysed using BioEdit Sequence Alignment Editor Version 7.2.6. For this patient, Sanger sequencing for CALR exon 9 mutation showed mutation (c.1154_1155insTTGTC, p.K385fs*47). Sequencing analysis confirmed to have similar finding with PCR product 272bp was CALR type 2 mutation (5 bp insertion of TTGTC) (Figure III).

Diagnosis of MPN with CALR mutation was made. The patient was treated with tablet hydroxyurea 500mg daily. Comfort measures were instituted and the patient died 10 days after admission due to septic shock secondary to hospital acquired pneumonia.

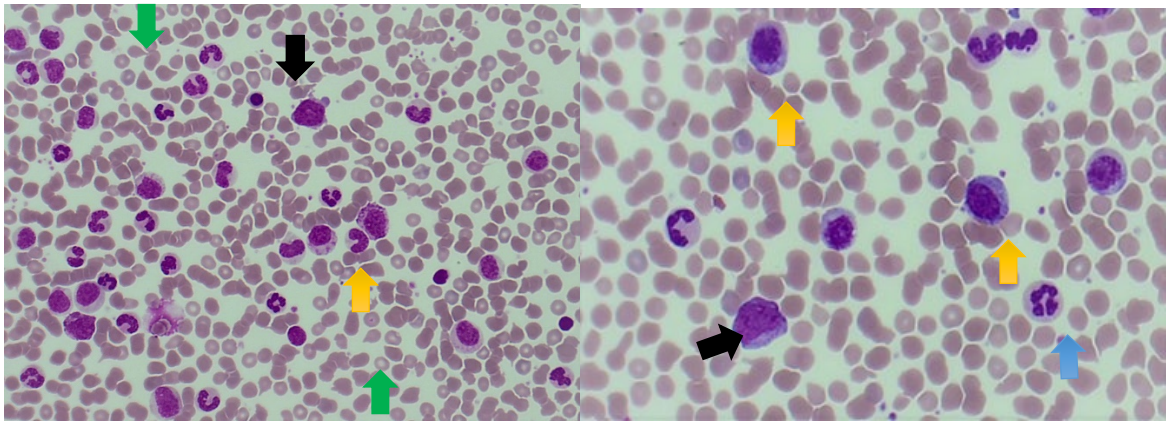

Figure I. Peripheral blood film showed presence of teardrop red blood cell (green arrow), hyperleukocytosis and all spectrum of myeloid lineage seen with peak in neutrophils (blue arrow) and myelocytes (yellow arrow); eosinophilia, basophilia and 8% blasts (black arrow).

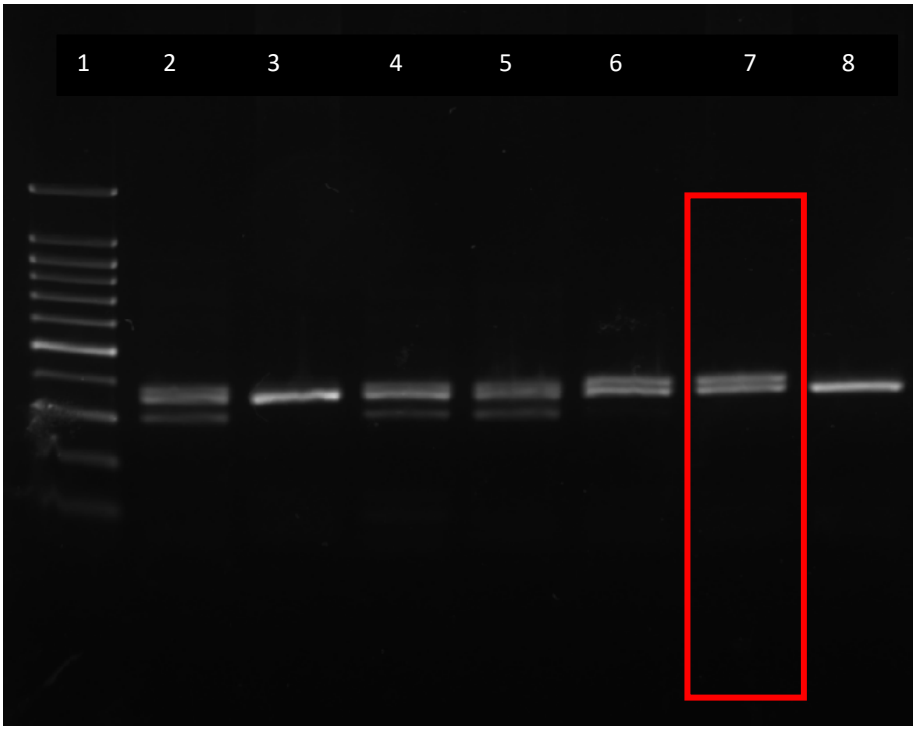

Figure II. ARMS PCR for CALR mutation showed presence of abnormal band. Lane 1: Ladder, Lane 2: Type I, Lane 3: Normal, Lane 4: Type I, Lane 5: Type I, Lane 6: Type II, Lane 7: Patient and Lane 8: Normal

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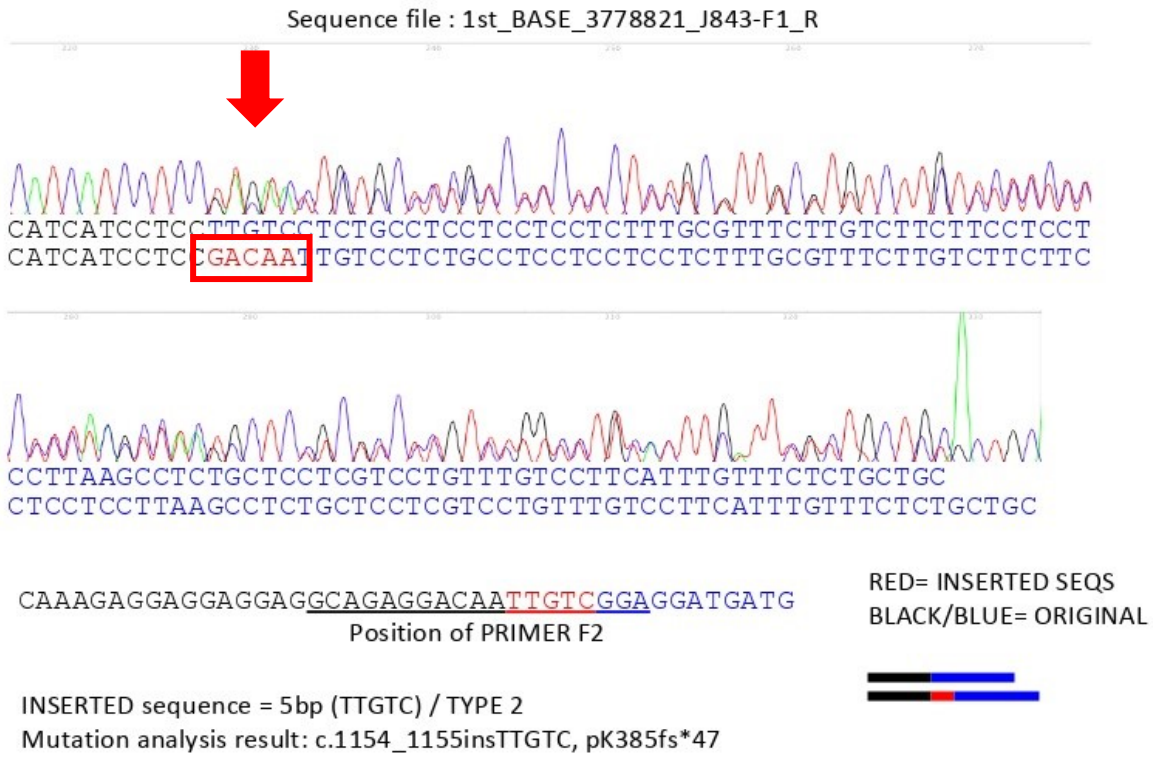

Figure III. Electropherogram showing CALR exon 9 mutation 5-bp insertion [ins]) **(TTGTC)** mutation site highlighted.

Discussion

Myeloproliferative neoplasms are a collection of diseases that have overlapping clinico-pathological features and this is a challenge to the haematologist distinguishing one from the other. The diagnosis of primary myelofibrosis is based on the 2017 World Health Organization (WHO) criteria, which include histopathological, morphological, clinical, and molecular-cytogenetic variables.

To confirm a diagnosis of PMF, patients must meet all three major criteria and at least one minor criteria. The first major criteria are largely based on histopathology. For example, megakaryocytic proliferation and atypia, without reticulin fibrosis (grade 1) for pre fibrotic PMF or with fibrosis (grade 2 or 3) for overt stage accompanied by increased age-adjusted bone marrow cellularity, granulocytic proliferation, and often decreased erythropoiesis. The second major criterion is excluding the other MPN such as BCR-ABL1 positive Chronic Myeloid Leukaemia, Essential Thrombocytosis or Polycythaemia Vera and Myelodysplastic syndrome. The third criterion is demonstration of JAK2, Calreticulin or MPL or the absence of reactive bone marrow fibrosis. Minor criteria include the presence of anaemia not attributed to a comorbid condition, leucocytosis (WBC count > 11x109/L), palpable splenomegaly and LDH level increased to above upper normal limit (Steven et al., 2017).

Clinical manifestations in PMF include severe anaemia, marked hepatosplenomegaly, constitutional symptoms (eg, fatigue, night sweats, fever), cachexia, bone pain, splenic infarct, pruritus, thrombosis, and bleeding. Ineffective erythropoiesis and hepatosplenic extramedullary haematopoiesis are the main causes of anaemia and organomegaly, respectively. It is currently assumed that aberrant cytokine production by clonal cells and host immune reaction contribute to PMF-associated bone marrow stromal changes, ineffective erythropoiesis, extramedullary haematopoiesis, cachexia, and constitutional symptoms. Causes of death include leukemic progression that occurs in approximately 20% of

patients, but many patients also die of comorbid conditions including cardiovascular events and consequences of cytopenias, including infection or bleeding (Tefferi, 2020).

Clinical features of chronic myeloid leukaemia (CML) are generally nonspecific: splenomegaly is present in 46–76% and may cause left upper quadrant pain or early satiety; fatigue, night sweats, symptoms of anaemia and bleeding due to platelet dysfunction may occur, the latter most commonly in patients with marked thrombocytosis; <5% of patients present with symptoms of hyperviscosity, including priapism; these are generally seen when the presenting white cell count (WCC) exceeds 250,000/μL (Thomson et al., 2015).

Peripheral blood film of PMF show leukoerythroblastosis (ie, presence of nucleated red cells, immature granulocytes) and teardrop cells is a typical but not invariable feature of PMF; prefibrotic PMF might not display overt leukoerythroblastosis. (Tefferi, 2020) Characteristic of CML features are as follows: absolute leukocytosis (median of 100,000/μL) with a left shift and classic "myelocyte bulge" (more myelocytes than the more mature metamyelocytes seen on the blood smear); blasts usually number <2%; absolute basophilia is nearly universal, with absolute eosinophilia. Platelet count is usually normal or elevated; thrombocytopenia suggests an alternative diagnosis or the presence of advanced stage, rather than chronic phase disease (Thomson et al., 2015).

Overall prognosis depends on the stage at which the neoplasm is initially diagnosed and prognosis scoring system. The widely used refined Dynamic International Prognostic Scoring System (DIPSS) plus include eight predictors of inferior survival at the time of diagnosis: age > 65 years, haemoglobin level<10 g/dL, leukocyte count >25 × 10^9/L, circulating blasts ≥ 1%, and the presence of constitutional symptoms, red blood cell transfusion dependency, platelet count <100 x 109/L and unfavourable karyotype includes sole complex karyotype or 2 abnormalities that include +8, −7/7q−, I (17q), inv(3), −5/5q−, 12p−, or 11q23 rearrangement. The eight DIPSS plus risk factors are used to define low (no risk factors), intermediate 1 (1 risk factor), intermediate 2 (2 or 3 risk factors), and high (≥ 4 risk factors) risk groups with respective median survivals of 15.4, 6.5, 2.9, and 1.3 years (Steven et al., 2017). DIPSS plus score for this patient is 7 and categorized as high-risk group.

The advent of mutation in CALR gene changed the landscape of MPNs. It was first recognized as a somatic mutation in patients with MPNs who had no mutations in either JAK2 or MPL by Klampfl et al., (2013).

CALR was originally identified as a calcium binding protein in the endoplasmic reticulum lumen of most cells of human origin. Its main function is to play a critical role in quality control processes during protein synthesis and folding, through binding to misfolded proteins. CALR is found at multiple subcellular localizations outside of the endoplasmic reticulum, where it mediates a variety of cellular processes, including apoptotic cell clearance, cell adhesion, and cell migration (Gold et al.,2010).

Patients with a CALR-mutated MPN appear to harbour CALR mutations only in cells of the myeloid lineage. Furthermore, samples from patients without an MPN did not detect CALR mutations, suggesting that CALR mutations are likely pathogenic in MPNs. (Klampfl et al., 2013) (Nangalia et al., 2013). All CALR mutations are insertions or deletions resulting in a frameshift, and cluster in the last exon (exon 9) of the gene. Thus far, more than 50 different types of mutations in CALR have been detected. Two specific mutations are most prominent, a 52-bp deletion (type 1) and a 5-bp insertion (type 2) (Zhiyuan et al., 2013) accounting for around 50% and 30% CALR mutations, respectively (Klampfl et al., 2013).

CALR mutations differ in their phenotype and prognostic impact. In type 1 CALR-mutated patients were younger and displayed higher platelet count, lower leukocyte counts and lower DIPSS-plus scores; they were also less likely to be spliceosome mutated, anaemic or require transfusions. Comparison of type 1 vs type 2 CALR mutations showed that type 2 associated with higher-risk DIPSS-plus scores (P=0.008), EZH2 mutations (P=0.0001), marked leucocytosis (P=0.002) and increased peripheral blast percentage (P=0.04) (Tefferi et al., 2014).

A study was done by Chunshui (2020) in a cohort of 13 patient with BCR-ABL1 and CALR mutation. In this cohort, 8 out of 13 patients were diagnosed

with Ph negative MPN (such as PMF, Essential Thrombocythemia, or post-essential thrombocythemia myelofibrosis) before CML diagnosis. There was a shift to the clinical BCR-ABL1 positive CML phenotype, with manifestations such as splenomegaly and leukocytosis, between the initial and second diagnoses. Moreover, there was always a relatively long duration for Ph negative MPN transformation to CML with median duration of 4 years (range: 2–23 years). Occurrence of CALR driver mutation and BCR-ABL1 fusion in the same patient could indicate the presence of different subclones and clonal evolution during disease progression and remission, mandating further investigation of CALR mutation and BCR-ABL1 fusion in the same or different clone.

Our index case was found to be in high-risk category according to DIPSS plus. Her molecular mutation profile was negative for JAK2, MPL, and BCR-ABL1 translocation. Interestingly, we detect type 2 CALR gene mutation in this patient. In contrast to the clinical findings associated with type 1, i.e., lesser disease severity and overall good prognosis, our patient with type 2 mutation presented in the advanced phase of disease with one-month cough, shortness of breath, severe anaemia and massive splenomegaly that required treatment. Her platelet count was low at presentation. No thrombotic or haemorrhagic event were noted. She was treated with tablet hydroxyurea 500mg daily and died 10 days after admission due to septic shock secondary to hospital acquired pneumonia. The severe disease manifestations at presentation shows that type 2 CALR gene mutation might be accountable for an aggressive disease phenotype, marked leukocytosis with increase blast count in PMF.

This case showed that the favourable prognostic impact of CALR mutations on PMF might be restricted to only those patients with type 1 CALR mutations. This is because of the segregation of adverse features (for example, increased circulating blast count and higher-risk DIPSS-plus risk scores) in patient with type 2 CALR mutation. Therefore, it is important to further explore the potential differences among different types of CALR mutations in both phenotype and prognostic impact, especially between the two most frequent ones: type 1 and type 2.

Conclusion

This case highlights the importance of detecting the type of CARL mutations in MPN. It significantly improves the diagnostic approach for MPN when complete laboratory diagnostic tests were possible, useful in the therapeutic management and also prognostic stratification of this patient.

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