

Screening for calreticulin mutations in a cohort of patients suspected of having a myeloproliferative neoplasm

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Background. The discovery of calreticulin (*CALR*) has shown it to be the second most frequent mutation after the Janus Kinase 2 (*JAK2*) mutation in myeloproliferative neoplasms (MPNs). Its structure indicates various functions, of which two are to ensure calcium homeostasis and proper folding of other target proteins. Over 36 types of *CALR* mutations have been identified, all causing a recurrent frameshift in the C-terminal domain affecting *CALR*'s localisation and calcium-binding function.

Objective. To screen a cohort of 89 patients suspected of having an MPN for the *CALR* mutations.

Methods. Capillary and gel electrophoresis were used in conjunction as confirmatory tests to screen the cohort of patients.

Results. Of three samples containing a type 1 *CALR* mutation, two were heterozygous and one homozygous for a 52-base pair deletion in *CALR*.

Conclusions. Most studies report *CALR* mutations to be present only in patients with primary myelofibrosis or essential thrombocythaemia, with mutual exclusivity to *JAK2* mutations. The findings of this study indicate that *JAK2* and *CALR* mutations are no longer considered mutually exclusive. Similarly, patients with a polycythaemia vera phenotype could also carry a *CALR* mutation.

S Afr Med J 2016;106(12):1260-1262. DOI:10.7196/SAMJ.2016.v106i12.10769

Myeloproliferative neoplasms (MPNs) can be classified according to the 2008 World Health Organization classification^[1] into Philadelphia (Ph) chromosome-negative and positive neoplasms. Ph chromosome-negative neoplasms include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF), whereas chronic myeloid leukaemia (CML) comprises Ph chromosome-positive neoplasms.

The Janus Kinase 2 (*JAK2*) mutations found in 50 - 60% of patients encode a cytoplasmic tyrosine kinase protein active in the JAK/signal transducer and activator of transcription proteins (STAT) signalling pathway. A gain-of-function mutation replaces a guanidine base with a thymine base, resulting in a valine-to-phenylalanine alteration,^[2] and consequently constitutive tyrosine kinase activity along with enhanced proliferation of myeloid cells. In 5 - 10% of patients the thrombopoietin receptor (*MPL*) gene is impaired, while in 30 - 45% of patients no specific molecular marker had been described until the discovery of calreticulin (*CALR*). In these 30 - 45% of patients *CALR* was shown to be the second most frequent mutation after *JAK2*, offering mutual exclusivity to *JAK2* and *MPL* mutations, and being absent in PV patients.^[2]

The *CALR* gene, mapped to chromosome 19p,^[3] has three structural and functional domains, referred to as the N, P and C domains.^[4] The N domain is important for proper folding of *CALR* and enables its interaction with numerous other protein molecules such as α -integrin. The P domain comprises specific amino acid sequence repeats permitting binding of high-affinity calcium ions (Ca^{2+}) and lectin-like chaperoning.^[5] The C domain contains a KDEL signal comprising four acidic amino acids (K = Lys, D = Asp, E = Glu, L = Leu) that allow *CALR* to have a high capacity for binding Ca^{2+} , thus regulating *CALR*'s interaction with other protein molecules.^[6]

Of the 36 types of insertions and deletions identified, type 1 (a 52-base pair deletion) and type 2 (a 5-base pair insertion) mutations account for >80% of *CALR* mutations.^[7] Phenotypic differences between type 1 and type 2 carriers have been implicated.^[3] All

recurrent mutations cause a frameshift in the region encoding the C domain, leading to altered subcellular localisation^[2] and impaired function. Two main functions have been indicated, a chaperone function essential in protein processing and transport in the endoplasmic reticulum (ER), ultimately eliminating incorrectly folded proteins, and a regulatory function over intracellular Ca^{2+} levels whereby *CALR* acts as a sensor and buffer to Ca^{2+} in the ER lumen. Despite inconclusive evidence regarding the mechanism by which *CALR* assists protein folding and Ca^{2+} homeostasis,^[5] its depletion has profound effects.

Methods

Samples

Eighty-nine archived samples from patient and control groups, including all samples of patients suspected of having an MPN, were screened. There were *JAK2*-positive and negative and ET, PV and PMF samples. The patient group consisted of *JAK2*-negative samples, whereas the control group consisted of *JAK2*-positive samples, as *CALR* and *JAK2* are thought to be mutually exclusive. DNA was extracted and purified using the Promega Purification Kit (Promega, USA) (www.promega.com/protocols/).

Polymerase chain reaction (PCR) and amplicon analysis

Published primers^[2] targeting exon 9 of *CALR* were used (Applied Biosystems, UK). A primer-based sizing assay was used to detect *CALR* mutations, the forward primer (GGCAAGGCCCTGAGGTGT) being labelled with 6-carboxyfluorescein (6-FAM). The reverse primer sequence was GGCCTCAGTCCAGCCCTG.

Hot-start PCR was used. The amplicons labelled with 6-FAM were electrophoresed in a 3130 Genetic Analyser (Applied Biosystems, UK), as well as on agarose gel.

A mixture of 10 μL Hi-Di Formamide and 0.5 μL GeneScan LIZ 500 (size standard), both from Life Technologies, USA, was prepared for capillary electrophoresis, of which 10 μL was added

to 1.5 µL PCR product. Nested PCR was performed for the samples subjected to gel electrophoresis, whereby the PCR products were amplified for a second time in an ABI 2700 thermocycler (Applied Biosystems, USA). PCR products were separated on a 2.5% agarose gel and analysed under ultraviolet light. Samples positive for a mutation were band-stabbed and placed in TE⁺ buffer (EDTA 0.5M, Tris 1M) overnight at 4°C. Four µL of the supernatant was used for sequencing.

The wild-type allele has 267 base pairs.^[8] Any fragments that differed in size to the wild-type allele carried a mutation.

DNA sequence analysis

Sequencing of samples was performed using the BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing products were purified using the ethanol/sodium acetate precipitation method. A volume of 25 µL Hi-Di Formamide was added to the pellet and the samples were run on an ABI Prism 3130 Genetic Analyser (Applied Biosystems, UK).

The sequencing reactions were analysed using Sequencing Analysis Software (version 5.3.1) (Applied Biosystems, UK). Two free online programs were used, Chromas version 2.4.3 (Technelysium, Australia) (www.technelysium.com.au/chromas_lite.html), which visualised the sequencing data, and Lalign (www.ch.embnet.org/software/LALIGN_form.html), which compared the sequencing data with a reference sequence obtained from Ensembl^[8] using the algorithm of Huang and Miller.^[9]

Ethical considerations

The University of the Free State Faculty of Health Sciences Ethics Committee granted ethical approval for this study (ref. no. ETOVS 15/8).

Results

The melting temperatures for all reactions were optimised at 69°C. Of the 89 samples, only three showed a 52-base pair deletion. With two samples, two distinct peaks were observed, one at 263 base pairs and the other at 210 base pairs. With the other sample, one distinct peak was observed at 210 base pairs, making it homozygous for the type 1 deletion. Fig. 1 depicts electropherograms of (A) a patient with no *CALR* mutation present, and (B) a patient with a mutated *CALR*.

DNA fragments separated on a 2.5% agarose gel confirmed three samples positive

for a 52-base pair deletion. Again, of the three samples, two were heterozygous and one was homozygous for a 52-base pair deletion in *CALR*. Fig. 2 shows a gel electropherogram of all samples positive for a *CALR* type 1 mutation. Included is a molecular weight marker sizing the fragments between 200 and 300 base pairs. The online program Lalign allowed for comparison of a reference sequence of *CALR* with the sequencing product. All three samples showed a 52-base pair deletion.

Discussion

CALR mutations were identified in three patients, all of which accounted for a type 1 mutation. Two were heterozygous for a 52-base pair deletion, while the other was homozygous. The *JAK2* and *CALR* mutations were thought to be mutually exclusive,

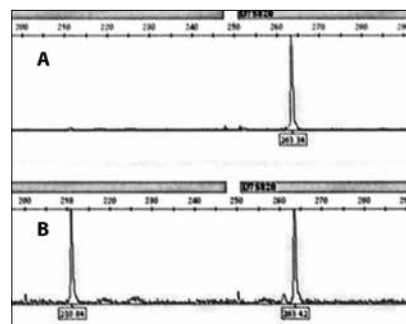


Fig. 1. Capillary electropherograms depicting samples with (A) wild-type *CALR*, and (B) 52-base pair *CALR* deletion.

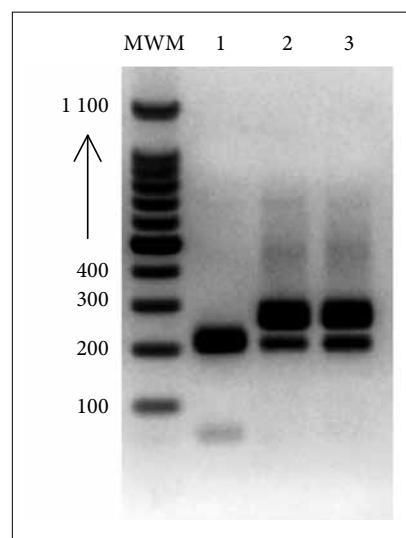


Fig. 2. Agarose gel picture samples positive for the *CALR* deletion 52 mutation. Sample 1 is homozygous and samples 2 and 3 are heterozygous. The x-axis indicates the molecular weight marker (MWM) and patient samples. The y-axis indicates the fragment size of the MWM measured in base pairs.

but the homozygous sample was double-positive for both *JAK2* and *CALR*. Studies have described similar cases in patients with ET, but not in those with PV, and the implications relating to clinical presentation and prognosis of these patients are unclear. The heterozygous samples were from patients with a typical ET phenotype, and the homozygous sample had a PV phenotype.

The loss of *CALR* function can be observed by the onset of disease due to a frameshift to an alternative reading frame occurring in the C-terminus, obscuring *CALR*'s pleiotropic role. The C-terminus is coded by exon 9 of *CALR*, which includes an ER retention signal. With the generation of a new C-terminus, through replacement with a positively charged polypeptide chain rich in arginine and methionine, the ER KDEL retention signal is lost. *CALR*'s roles are related to its distribution in the ER, in cytosol and on the cell surface. It resides mainly in the ER, where it performs its pleiotropic functions ranging from Ca²⁺ regulation to ensuring proper protein folding. If the ER KDEL retention signal is lost, *CALR* therefore cannot be localised, or perform its critical functions in many biological processes including proliferation, apoptosis and immunogenic cell death.^[10] When *CALR* is overexpressed, it suppresses cell proliferation and enhances apoptosis, which is triggered by Ca²⁺ release from the ER. Disabled cellular responses are therefore seen when there is a loss of *CALR*. In addition, it promotes the phagocytic function of macrophages to engulf hazardous cancerous cells. When *CALR* is mutated, there is therefore decreased phagocytosis of cancer cells.^[3]

The unanticipated functional involvement of the JAK/STAT proteins signalling pathway alongside *CALR* demonstrates a transcriptional *JAK2* signalling signature consistent in all MPN patients regardless of clinical phenotypes or mutational status.^[3] This signature, present in patients with a mutated *CALR*, could elucidate the double-positive cases. Cells expressing type 1 *CALR* mutations proliferate independently of interleukin 3 (IL-3). This hypersensitivity to IL-3 hypothesis was confirmed by the phosphorylation of STAT5 in the presence and absence of IL-3. Increased phosphorylation of STAT5 was observed in the absence of IL-3, supporting the hypothesis that the JAK/STAT signalling pathway is activated in type 1 *CALR* mutations.^[10]

Some data also suggest preferential expansion of the megakaryocyte cell lineage as a

result of *CALR*'s inability to export Ca^{2+} from the ER, keeping the calcineurin-nuclear factor of activated T cells (NFAT) signalling pathway notably less active. This in turn favours the megakaryocyte cell lineage instead of the erythroid lineage, providing an explanation as to why two heterozygous samples were observed in patients with high platelet counts. *CALR* expression in erythroblasts and granulocytes is also downregulated from CD34^+ cells, suggesting *CALR*'s crucial role in platelet functions.^[5] In contrast to mutant *CALR* only being observed in ET and PMF, the homozygous sample was found in a patient suspected of having PV.

Conclusions

The clinical outcomes of *CALR* differ from those of *JAK2* and *MPL*. *CALR* mutations are associated with prognostic advantages such as a lower thrombotic risk, longer median survival, and a more benign course of disease.^[3] Since *CALR* mutations are associated with better outcomes, these clinical outcomes are useful in future prognostic and therapeutic indications of Ph chromosome-negative disorders. Knowledge of *CALR* mutations can therefore allow for a more definitive classification of myeloproliferative neoplasms.

Funding. This study was funded by the Department of Haematology and Cell Biology in the Faculty of Health Sciences at the University of the Free State.

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Accepted 16 March 2016.