DETECTION OF CALR EXON 9MUTATIONS IN EGYPTIAN PATIENTS WITH PERSISTENT THROMBOCYTOSIS

Dalia Ahmed El-Sewefy, Gehan Mostafa Hamed, Yasmin Nabil Elsakhawy and Doha Osama Abdulrahman

Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Corresponding author
Doha Osama Abdulrahman
Mobile: (+20) 1099623343

E.mail:
Dohaosama42@gmail.com

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ABSTRACT:

Background: *Thrombocytosis is a commonly encountered clinical scenario, with a large proportion of cases discovered incidentally when complete blood count is obtained for some unrelated reason, creating an important diagnostic challenge.*

Aim of the Work: *To evaluate the diagnostic and prognostic value of CALR mutations testing in patients with persistent thrombocytosis, and study the relation of thismutations with clinical and hematological parameters.*

Patients and Methods: *The present study included fifty patients with persistent thrombocytosis (platelet count > 450×10^9/L) for at least 3 months. All subjects were genotyped for the CALR gene using high resolution melting PCR (HRM-PCR) technique. The study was approved by the Research Ethics Committee of Ain Shams University.*

Results: *The present study revealed that CALR gene mutations are positive in one-third of cases with 1ry thrombocytosis. The mutations are associated with younger age males with higher platelet count, lower hemoglobin level and lower total leucocytic count (TLC) than CALR wild type counterparts.*

Conclusion: *The present study revealed that CALRexon 9 mutations can influence the clinical and hematological phenotype of the patient and hence the disease diagnosis and prognosis.*

Keywords: *Thrombocytosis- Primary- Reactive- CALR*

INTRODUCTION:

Thrombocytosis is defined as elevated platelet count above 450 × 10^9/L; this threshold was recommended by WHO and by the British Committee for Standards in Hematology (BCSH) (2010). In most literature, for thrombocytosis to be persistent, it should be sustained for at least 3 months[1, 2].

The major causes of thrombocytosis can be divided into reactive and clonal thrombocytosis. Reactive causes include transient processes such as acute blood loss, acute infection or inflammation, extreme physical exertion, or other stress. Sustained forms of reactive thrombocytosis include iron deficiency, hemolytic anemia, asplenia, cancer, chronic inflammatory or infectious diseases, and rare drug reactions[3].

Clonal thrombocytosis is typically due to a chronic myeloproliferative neoplasms (MPN) particularly essential thrombocytosis (ET) and pre- primary myelofibrosis (pre-PMF). These clonal disorders are associated with adverse events related to the thrombocytosis, including thrombotic, vascular and bleeding complications[3].

According to the WHO classification of myeloid neoplasms (2016), CALR mutation is considered one of the major criteria for

diagnosis of ET, pre-PMF and overt PMF. Therefore, many studies were directed to detect the frequency of this mutation in different disorders and ethnic groups as well as its impact on the patient's clinical phenotype and hence the prognosis[4].

AIM OF THE WORK:

To evaluate the diagnostic and prognostic value of CALR mutations testing in patients with persistent thrombocytosis, and study the relation of this mutations with clinical and hematological parameters.

PATIENTSAND METHODS:

I) Patients:

The present study was conducted on 50patientswho attended Hematology and Oncology Unit of Ain Shams University Hospitals and referred to Main Hematology Lab in Clinical Pathology Department for investigation of persistent thrombocytosis (platelet count> 450×10^9/L) during the period from July 2017 till July 2018.An informed oral consent was obtained from each participant before enrolment in the study. Moreover, the study was approved by the Research Ethics Committee of Ain Shams University.

Inclusion Criteria:

a) Adult Egyptian patients.

b) Persistent thrombocytosis (Platelet count > 450 $\times10^9$/L for more than 3 months).

Exclusion criteria:

A) Start of chemotherapy.

B) Diagnosis of BCR-ABL1 positive CML.

All individuals included in this study were subjected to the following:

- Full history taking.
- Thorough clinical examination, laying stress on organomegaly.
- Complete blood count (CBC) using LH 750 cell counter (Beckman Coulter, USA), with examination of Leishman stained peripheral blood smears.
- Bone marrow aspiration, with examination of Leishman stained bone marrow smears.
- Bone marrow trephine biopsy.
- Testing for exon 9 mutation of CALR by high resolution melting PCR (HRM-PCR) using Rotor- Gene Q 5pex HRM (Qiagen, USA).

All patients were evaluated according to the criteria of ET and pre PMF in WHO classification of myeloid neoplasms (2016).

II) Sampling

A. A peripheral blood sample (2mL) was collected on EDTA coated tubes from all enrolled patients and sent for CBC.

B. Bone marrow aspirate sample (0.5mL) was withdrawn for creating bone marrow smears. Further 2 mL was withdrawn and collected on EDTA coated tube for DNA extraction, and detection of mutation. The extracted DNA was stored at -20 °C.

III) Methods:

A) Analytical Methods: *CALR* mutations detection:

1) Genomic DNA extraction:

The DNA was extracted from fresh bone marrow or peripheral blood samples using *QIAamp ® DNA Blood Mini kit (QIAGEN, USA).*

2) <u>Detection of CALR Mutation by HRM PCR</u>:

The High-resolution melting PCR (HRM PCR) was done using the *Type-it HRM PCR Kit (Qiagen, USA)* on the Rotor-Gene Q 5pex HRM instrument (Qiagen, USA). The test primers were prepared by Applied Biosystems (USA).

The reaction protocol started with initial cycle of pre incubation at 95 °C for 10 min, followed by 45 cycles of amplification composed of: heating at 95 °C for 10 s (denaturation), heating at 64 °C for 10 s (annealing/extension) and finally heating at 74 °C (elongation). Then 1 cycle of high resolution melting composed of: heating at 95 °C for 60 s (denaturation), heating at 45 °C for 60 s (annealing), and finally heating 65-95 °C for 5 s (melting).

Data analysis was done using Rotor-Gene Q Series Software 2.3.1 (Build 49). Data were presented in two formats: the normalized plot (Figure 1) and the difference plot (Figure 2).

The melting curves were normalized and temperature shifted creating the normalized plot which allows samples to be directly compared. The normalized plot is the graph in which the amount of fluorescence (due to the intercalating dye remaining at any temperature point) is expressed as a fraction of the amount prior to data acquisition**(5).**

Difference plots were generated by selecting a negative control as the baseline and the fluorescence of all other samples was plotted relative to this sample. Significant differences in fluorescence were indicative of mutations**(5).** Each mutant allele had its own distinctive melting curve when compared to the wild-type allele. The distinct melting curves of the mutant became more apparent when data were represented in a difference plot format than in a normalized plot**(6).**

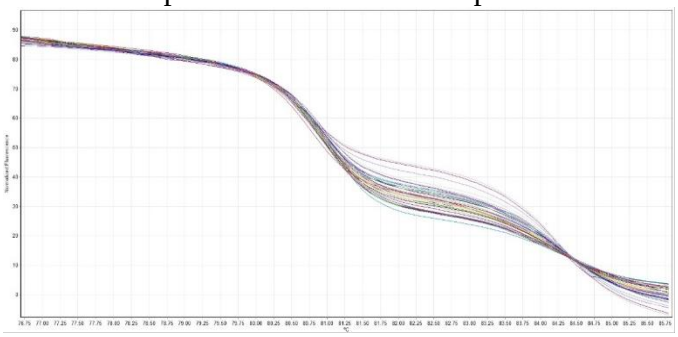

Figure (1): The normalized HRM PCR curves of the study patients

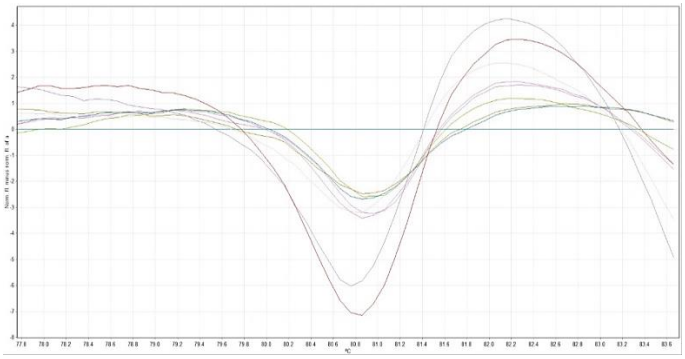

Figure (2): The difference curves showing CALR mutated samples, the baseline is the wild type control.

B) Statistical Methods:

- **<u>Sample size determination</u>:** the required sample size was calculated at the Community Department of Faculty of Medicine, Ain shams University using the G* power software version 3.1 (Universität Düsseldorf, Germany).

The collected data were revised, coded, tabulated and introduced to Statistical Package for the Social Sciences soft-ware program (SPSS, version 25.0, IBM Corp., USA, 2017-2018). Data were presented and suitable statistical analysis was done according to the type of data obtained for each parameter. Qualitative data were

expressed as number and percent (n; %); parametric quantitative data were expressed as mean and standard deviation (SD). Comparative statistics for qualitative data was done by the Chi squared test (X^2) between two independent groups and the ANOVA test between three independent groups for qualitative data. As for quantitative parametric data, and Student's t test was performed. Probability or p value of <0.05 was considered statistically significant in all analyses.

I- Overview of Study Group:

The study group included 50 patients with sustained elevation in platelet count ≥ 450×10^3/uL for at least 3 months and was divided according to the WHO classification of myeloid neoplasms (2016) into: group A which involved 30 patients with 1ry thrombocytosis and group B that included 20 patients with reactive thrombocytosis**.**

A- Epidemiological and clinical features:

Group A comprised 16 (53.3%) females and 14 (46.7%) males. Their ages ranged from 19 to 72 with a mean of 49.03 ± 15.05 yrs. At presentation, 17 (56.7%) had organomegaly. As regard **group B**, it comprised 9 females (45%) and 11 males (55%). Their ages ranged from 17-78 with a mean of 45.05± 16.78 yrs. At presentation, 2 (10%) had organomegaly.

B- Routine laboratory Data:

In group A, the total leucocytic count (TLC) ranged from 4.7 to 35.9 × 10^3 /uL, hemoglobin (Hb) showed a mean of 12.25 ± 2.63 g/dL, platelet count ranged from 455 to 2617 × 10^3 /uL and mean platelet volume (MPV) showed a mean of 9.8 ± 1.5 and ranged from 8 to 13.2.

In group B, TLC ranged from 6.8 to 33.3 × 10^3/uL, Hb showed a mean of 9.9 ± 3.6 g/dL, platelet count ranged from 536 to 1315 × 10^3 /uL and MPV showed a mean of 9.6 ± 1 and ranged from 8.5 to 12.

Regarding group A, 9 patients out of 30 (30%) were positive for CALR mutation. In group B, all the studied patients were negative for CALRmutation.

II. Comparison betweengroup A and group B

Epidemiological and clinical features (Table1)

A higher incidence of organomegaly and thrombotic events was detected in group A reaching a highly significant (HS) difference (p=0.001). In contrast, no significant difference was revealed as regard age and gender (p<0.05).

Table (1): Epidemiological and clinical features of both groups (A & B)

Variable	Group A	Group B	Test value	P-value	Sig.
	n= 30	n=20			
Age (years), Mean±SD	49.03 ± 15.05	45.05 ± 16.78	0.876•	0.385	NS
(Range)	19 – 72	17 – 78			
Gender, n (%)					
Female	16 (53.3)	9 (45)	0.333*	0.564	NS
Male	14 (46.7)	11 (55)			
Organomegally. n (%)					
No	13 (43.3)	18 (90)	11.092*	0.001	HS
Yes	17 (56.7)	2 (10)			
Thrombosis, n (%)					
No	18 (60)	20 (100)	10.526*	0.001	HS
Yes	12 (40)	0 (0)			

P-value >0.05: Non significant (NS); P-value <0.05: Significant (S); P-value< 0.01: highly significant (HS) *:Chi-square test; •: Independent t- test.

Routine Laboratory Data (Table 2):

Hemoglobin level showed significant difference (p=0.01), being lower in group B. No statistically significant difference regarding other different laboratory data.

Table (2): Comparison between group A and group B regarding routine laboratory data

		Group A n = 30	Group B n = 20	Test value	P-value	Sig.
TLCs	Range	4.7 – 35.9	6.8 – 33.3	-1.011•	0.317	NS
Granulocytes	Range	0.9 – 33.5	4 – 26.5	-0.756•	0.453	NS
RBCs	Range	2.5 – 7.9	2.3 – 7.13	1.226•	0.226	NS
Hb	Range	7.6 – 17.8	4.1 – 17.9	2.684•	0.010	S
Hct	Range	22.8 – 55.3	14.3 – 52.5	1.934•	0.059	NS
MCV	Range	48.4 – 108	55.7 – 87.9	1.232•	0.224	NS
MCH	Range	14.3 – 32.9	14.5 – 28.5	1.292•	0.203	NS
MCHC	Range	28.9 – 38	24.5 – 38.7	0.876•	0.385	NS
RDW	Range	13.4 – 26	12.4 – 40.1	-0.442•	0.661	NS
PLTs	Range	455 – 2617	536 – 1315	0.871•	0.388	NS
MPV	Range	8 – 13.2	8.5 – 12	0.584•	0.562	NS
LDH	Range	100 – 832	100 – 756	-0.951‡	0.342	NS

TLC: total leucocytic count, RBCs: red blood cells, Hb: hemoglobin, Hct: hematocrit, MCV:mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, PLTs: platelets, MPV: mean platelet volume, LDH: lactate dehydrogenase, P-value >0.05: Non significant (NS); P-value <0.05: Significant (S); P-value< 0.01: highly significant (HS): *:Chi-square test;
•: Independent t-test; ‡: Mann Whitney test

CALR mutation status (figure 3):

As for the mutation status there was a significant difference regarding *CALR* mutation (p=0.018) being positive in 1ry thrombocytosis (group A).

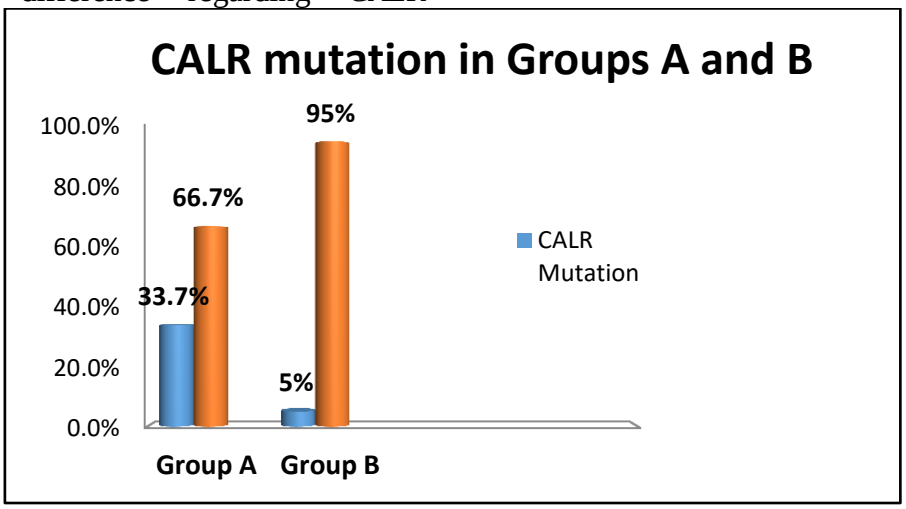

Figure (3): Frequency of CALR mutation in groups A & B.

III. Comparison between patients with 1ry thrombocytosis regarding CALR mutation

As shown in Table 3, CALR mutation were significantly higher among younger males (p<0.05).

Laboratory data showed, lower TLC, lower Hb and higherplatelet count ($p<0.05$) among 1ry thrombocytosis patients with CALR mutation. No significant difference was found regarding Hct, MCV or MCH ($p>0.5$).

Table (3): Comparison between patients with mutated and unmutated CALR

		Negative CALR mutation	Positive CALR mutation	Test value	P-value	Sig.
		n = 20	n = 10			
Age	Mean±SD	53.60 ± 14.02	39.90 ± 13.24	2.568•	0.016	S
	Range	26 – 72	19.0 – 60.0			
Sex	Female	14 (70.0%)	2 (20.0%)	6.696*	0.010	S
	Male	6 (30.0%)	8 (80.0%)			
TLCs	Mean±SD	15.51 ± 7.84	8.89 ± 2.73	2.572•	0.016	S
	Range	9 – 35.9	4.7 – 12.7			
RBCs	Mean±SD	5.10 ± 1.12	3.79 ± 1.59	2.616•	0.014	S
	Range	3.9 – 7.4	2.5 – 7.9			
Hb	Mean±SD	12.96 ± 2.15	10.84 ± 3.04	2.208•	0.036	S
	Range	10.2 – 17.8	7.6 – 17.4			
Hct	Mean±SD	38.92 ± 8.21	32.55 ± 9.20	1.926•	0.064	NS
	Range	23.7 – 55.3	22.8 – 52.5			
MCV	Mean±SD	76.14 ± 12.48	81.38 ± 10.32	-1.144•	0.262	NS
	Range	48.4 – 108	65.1 – 93.3			
MCH	Mean±SD	24.19 ± 4.30	26.55 ± 3.91	-1.461•	0.155	NS
	Range	14.3 – 32.9	19.4 – 30.4			
MCHC	Mean±SD	31.73 ± 1.95	32.55 ± 2.36	-1.012•	0.320	NS
	Range	28.9 – 34.9	29.8 – 38.0			
RDW	Mean±SD	19.24 ± 3.58	18.81 ± 3.66	0.308•	0.761	NS
	Range	13.4 – 26	13.7 – 25.9			
PLTs	Mean±SD	763.30 ± 199.90	1330.90 ± 604.73	-3.853•	0.001	HS
	Range	455 – 1085	788.0 – 2617.0			
	Yes	11 (55.0%)	1 (10.0%)			

TLC: total leucocytic count, RBCs: red blood cells, Hb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, PLTs: platelets, MPV: mean platelet volume,

P-value >0.05: Non significant (NS); P-value <0.05: Significant (S); P-value< 0.01: highly significant (HS)

*:Chi-square test; •: Independent t-test; ‡: Mann Whitney test

DISCUSSION:

Thrombocytosis is a commonly encountered clinical scenario, with a large proportion of cases discovered incidentally. It is defined as elevated platelet count above 450×10^9/L; this threshold was recommended by WHO (2016) and by the British Committee for Standards in Hematology (BCSH) (2010). In most literature, for thrombocytosis to be persistent, it should be sustained for at least 3 months[1,2,7].

The major causes of thrombocytosis can be divided into reactive and clonal thrombocytosis. Reactive causes include transient processes such as acute blood loss, acute infection or inflammation, extreme physical exertion, or other stress. Sustained forms of reactive thrombocytosis include iron deficiency, hemolytic anemia, asplenia,

cancer, chronic inflammatory or infectious diseases, and rare drug reactions[3].

Clonal thrombocytosis is typically due to a chronic MPN particularly ET and pre-PMF. According to the WHO classification of myeloid neoplasms (2016), CALR mutation is considered one of the major criteria for diagnosis of ET, pre-PMF and overt PMF[4].

The aim of the present study was to detect CALR gene mutations in patients with persistent thrombocytosis and to discover the relation of these mutations with clinical and hematological parameters.

The results of the present study showed that CALR mutations were observed in 30% of ET cases. Overall, these mutational frequencies accord well with the findings of other studies[8,9].

Wealso noticed that 1ry thrombocytosis cases having CALR mutation were younger males, with lower hemoglobin level and higher platelet count compared with cases lacking CALR mutation. These findings were described also by ***Bilbao-Sieyro et al. (2014)***[10]and by ***Marty et al. (2016)*** [11].

Of note, almost all the studied patients with reactive thrombocytosis didn't reveal to harbor the CALR mutation which accords with the findings reported by ***Bilbao-Sieyro et al. (2014)***[10].

In conclusion, detection of CALR mutations in cases of persistent thrombocytosis could help distinguishing 1ry and 2ry thrombocytosis and might uncover the presence of ongoing clonal disorder. It also could help in predicting the clinical phenotype and disease outcome of patients with persistent thrombocytosis. Eventually, detection of CALR mutation by HRM- PCR could be used as a screening test saving time and money, and then the positive samples are to be validated and further identified by sequencing techniques.

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الكشف عن الطفرات الجينية للكالريتكيولين اكسون٩ في المرضى المصريين المصابين بالزيادة المستمرة في عدد الصفائح الدموية

داليهاحمد السويفي , جيهان مصطفى حامد , ياسمين نبيل السخاوي

قسم الباثولوجيا الاكلنيكيه - كلية الطب - جامعة عين شمس

المقدمة : يعد عرض كثرة الصفائح الدموية سيناريو شائع ، حيث يتم اكتشاف نسبة كبيرة من الحالات بمحض الصدفة عندما يتم فحص صورة كاملة للدم لسبب اخر غير ذي صلة ، مما يخلق تحديًا تشخيصيًا مهمًا

الهدف :اكتشاف الطفرة الجينية للكالريتكولين CALR لدى المرضى المصريين الذين يعانون من الزيادة المستمرة في عدد الصفائح الدموية, بالاضافة الى تقييم العلاقة بين هذه الطفرة والصفات الاكلينيكية وبيانات الدم المعملية للمرضى.

المرضى و الطرق : قد اشتملت الدراسة الحالة على خمسين مريضا يعانون من كثرة الصفيحات لمدة لا تقل عن ثلاثة اشهر(عدد الصفائح الدموية اكثر من ٤٥٠×$١٠^{٩}$ / لتر).و قد تم الكشف عن الطفرة الجينية للكاريتكولين CALR باستخدام تقنية الذوبان عالية الدقة (HRM-PCR). تمت الموافقة على الدراسة من قبل لجنة أخلاقيات البحث العلمي بجامعة عين شمس .

النتائج : تم اكتشاف وجود الطفرة الجينية للكارليتكولينCALR في ثلث عددالمرضى المصابين بزيادة الصفائح الاولي.وقد كانت الحالات الايجابية للطفرةاصغر سنامع ارتفاع عدد الصفائح الدموية,وانخفاض مستوى الهموجلوبين وكرات الدم البيضاءمقارنة بنظرائهم ممن لا يعانون من وجود هذه الطفرة .

الخاتمة : يمكن للطفرات الجينيةللكالريتكولين CALR أن تؤثر على النمط الاكلينيكي للمريض وبيانات الدم المعملية، وبالتالي تؤثر على نتائج المرض.