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Presence of Polyclonal Hematopoiesis in Females with Ph-negative Myeloproliferative Neoplasms

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Classical Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-neg MPN) are considered to originate from somatic mutation(s) of a hematopoietic stem cell leading to clonal hematopoiesis, as shown by X-chromosome allelic usage in females. Somatic mutations in *JAK2, cMPL* and *calreticulin* (*CALR*) genes are the three main disease-defining mutations. Mutations in the *JAK2* gene are found in almost all polycythemia vera (PV) patients and in 45–50% of essential thrombocythemia (ET) and primary myelofibrosis (PMF) patients, while mutations in *cMPL* are found in about 2–5% of ET and PMF. *CALR* mutations ($CALR^{MUT}$) have been identified in the majority of ET and PMF patients who lack *JAK2* and *cMPL* mutations, but are generally not found in PV patients. Compared with *JAK2*- and c*MPL*-mutated patients, $CALR^{MUT}$ patients have higher platelet counts, lower hemoglobin and leukocyte counts, a lower risk of thrombosis(1) and a more favorable prognosis, but no difference in the rate of transformation to post-ET PMF.(2)

Determination of clonality based on X-chromosome inactivation has helped to differentiate reactive processes from MPNs. However, prior to the discovery of MPN disease-defining mutations, it was reported that some patients with a clinical phenotype of ET had polyclonal hematopoiesis.(3)

There are two widely used clonality assays based on X-chromosome inactivation (XCI), the HUMARA assay and the transcriptional-based clonality assay (TCA). The HUMARA clonality assay is based on assessing the DNA methylation status of adjacent CpG sites close to trinucleotide repeats in exon 1 of the human androgen receptor gene (*AR*).(4) Because some investigators observed unexpected results using the HUMARA assay, the second

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method was developed by our group based on analysis of transcripts of X-chromosome polymorphic genes subjected to X-chromosome inactivation, i.e. transcriptional-based clonality assays (TCA).(5, 6) Using both assays, discrepant data have been obtained in some females; however TCA revealed only a single transcript in individual burst forming units-erythroid (BFU-E), while heterogeneous methylation at the AR locus was obtained by the HUMARA assay.(6)

It has been reported recently that *JAK2*^{V617F} ET is more likely to have polyclonal hematopoiesis than *CALR*^{MUT} ET; this study was conducted using the HUMARA assay.(7) We evaluated 96 ET, 33 PMF and 65 PV females for *JAK2*, *cMPL,* and *CALR* mutation status, and using the TCA clonality assay, we examined the existence of polyclonal hematopoiesis in our cohort of MPN females seen over the last three decades. We also report two previously undescribed exon 9 *CALR* mutations and our analyses of *CALR*^{MUT} burden in granulocytes (GNC), CD3^{+} T-cells, and some CD34^{+} progenitors.

The 194 female patients with ET, PMF or PV were diagnosed according to the revised WHO criteria and had been treated with hydroxyurea or JAK2 inhibitors, but not pegylated interferon-α. Informed consent was obtained from all patients and 10mL of peripheral blood was used for the study. Platelets and granulocytes were isolated according to previously published protocols.(5)

Genomic DNA was isolated from GNC using the Puregene DNA purification kit (Gentra, MN, USA). *JAK2*^{V617F} and *MPL*$^{W515L/K}$ were detected by quantitative allele-specific PCR (qRT-PCR)(8) and *CALR*^{MUT} by semi-quantitative fragment analysis.(9) *CALR*^{MUT} was categorized as *CALR*^{MUT} type 1 (52-bp deletion; c.1092_1143del), *CALR*^{MUT} type 2 (5-bp insertion; c.1154_1155insTTGTC) and other previously described variants. Samples with an unreported *CALR* profile were sequenced.

The frequency of *JAK2*^{V617F} was 57% (N=55) in ET and 42% (N=14) in PMF; all PV females were *JAK2*^{V617F} mutated. *CALR*^{MUT} was present in 18% (N=17) of ET and 39% (N=13) of PMF. *MPL*^{W515L} was found in only one PMF patient. Triple-negatives accounted for 25% (N=24) of ET and 15% (N=5) of PMF (Figure 1a). This distribution of mutations is consistent with previous studies.(9–11)

To assess clonality, we first genotyped single nucleotide exonic polymorphisms from five X-chromosome genes subjected to X-chromosome inactivation [*BTK; FHL1; IDS; G6PD*; MPP1] using *TaqMan* allele-discrimination assays. Females heterozygous for any X-chromosome loci were considered informative for quantitative transcriptional clonality assay (qTCA). Total RNA was isolated from GNC using *Tri-Reagent* (Molecular Research Center, OH) and allelic usage ratio of informative X-chromosome polymorphic genes determined. Aliquots of 200 ng of DNA-free RNA were reverse-transcribed using SuperScript VILO for qRT-PCR (Invitrogen, CA, USA). Quantitative allele-specific PCR (qRT-PCR) was performed as described previously(12). A ratio of ≥80:20 was used to define clonality, accepting that ~5% of normal females will have skewing of X-inactivation sufficient to be classified as clonal hematopoiesis.(6)

qTCA evaluations of ET. Of 96 ET females, 80 were informative for TCA. Forty-seven had *JAK2*^{V617F} (30% mean allele burden) and 45 (96%) of these were clonal, whereas 12 (75%) of 16 *CALR*^{MUT} ET females had clonal hematopoiesis (p=0.032) (Figure 1b). Of the 17 triple-negatives, 14 (82%) were clonal. In contrast to the study using the HUMARA assay, we found a higher frequency of clonal hematopoiesis in *JAK2*^{V617F} (96% versus 26%, p<0.001) and triple-negative ET (82% versus 9%, p<0.001), but a similar frequency of clonal *CALR*^{MUT} ET.(7)

qTCA evaluations of PMF. Thirty of 33 PMF females were informative for TCA; 12 of 13 (94%) with *JAK2*^{V617F} (39% mean allele burden) and 11 of 12 (92%) with *CALR*^{MUT} were clonal (Figure 1c). In four triple negative PMF, three were clonal and one polyclonal. The single *MPL*^{W515L} PMF female was clonal.

qTCA evaluations of PV. All 57 informative *JAK2*^{V617F} (44% mean allele burden) PV females were clonal by qTCA (Figure 1d).

Distribution of CALR^{MUT} *variants.* In the 16 *CALR*^{MUT} ET females, seven (44%) had type 1 *CALR*^{MUT}, eight (50%) had type 2 *CALR*^{MUT} and one had another *CALR*^{MUT} mutation. Among 13 PMF females, six (46%) had type 1 *CALR*^{MUT}, five had type 2 *CALR*^{MUT} (50%) and two had other *CALR*^{MUT} mutations. Both of these "other" *CALR*^{MUT} mutations are previously undescribed 46bp and 37bp deletions [chr19:13,054,570-13,054,615 and chr19:13,054,570-13,054,606, respectively]. These sequences were deposited using the human reference genome (http://genome.ucsc.edu/cgi-bin/hgGateway) (hg19). Clonality status was not associated with the type of *CALR*^{MUT}.

Relative allelic burden of CALR^{MUT}. We also examined the relative allelic burden of *CALR*^{MUT} in GNC, T-cells and CD34^{+} progenitors using fragment analysis, a method which has a 3–5% allelic burden detection limit.(13) CD34^{+} cells and T-lymphocytes were sorted by FACS using antibodies against CD34 or CD3 (FACS Vantage SE, BD Bioscience, CA). Among 29 *CALR*^{MUT} ET and PMF female patients, the mean *CALR*^{MUT} allelic burden was ~50% in GNC and was undetectable in CD3^{+} T-cells. This is consistent with previously published data, which suggested that *CALR*^{MUT} is a somatic mutation favoring differentiation to myeloid progenitors.(14) Among six ET females patients from whom CD34^{+} cells were available, the *CALR*^{MUT} allelic burden was lower in CD34^{+} cells than in GNC (p=0.005, Figure 2), similar to previously reported in *JAK2*^{V617F} PV patients.(15)

In conclusion, we report analyses of 194 Ph- MPN females and confirm that polyclonal hematopoiesis exists in a minority of ET females. However, we found a marked difference between a >95% clonal prevalence in our *JAK2*^{V617F} ET cohort versus 26% reported by another group, and a similar discrepancy in triple negative ET.(7) We submit this difference is very likely due to different XCI clonality assays, as methylation of the AR locus in the HUMARA assay does not always reflect the X-chromosome inactivation state.(6) The prevalence of clonal hematopoiesis in *CALR*^{MUT} was lower than *JAK2*^{V617F} ET in our cohort (75% versus 96%,) suggesting that *CALR*^{MUT} clones have a weaker suppressive effect on residual normal hematopoietic stem cells than *JAK2* mutated clones, which may contribute to the more benign course of *CALR*^{MUT} ET.

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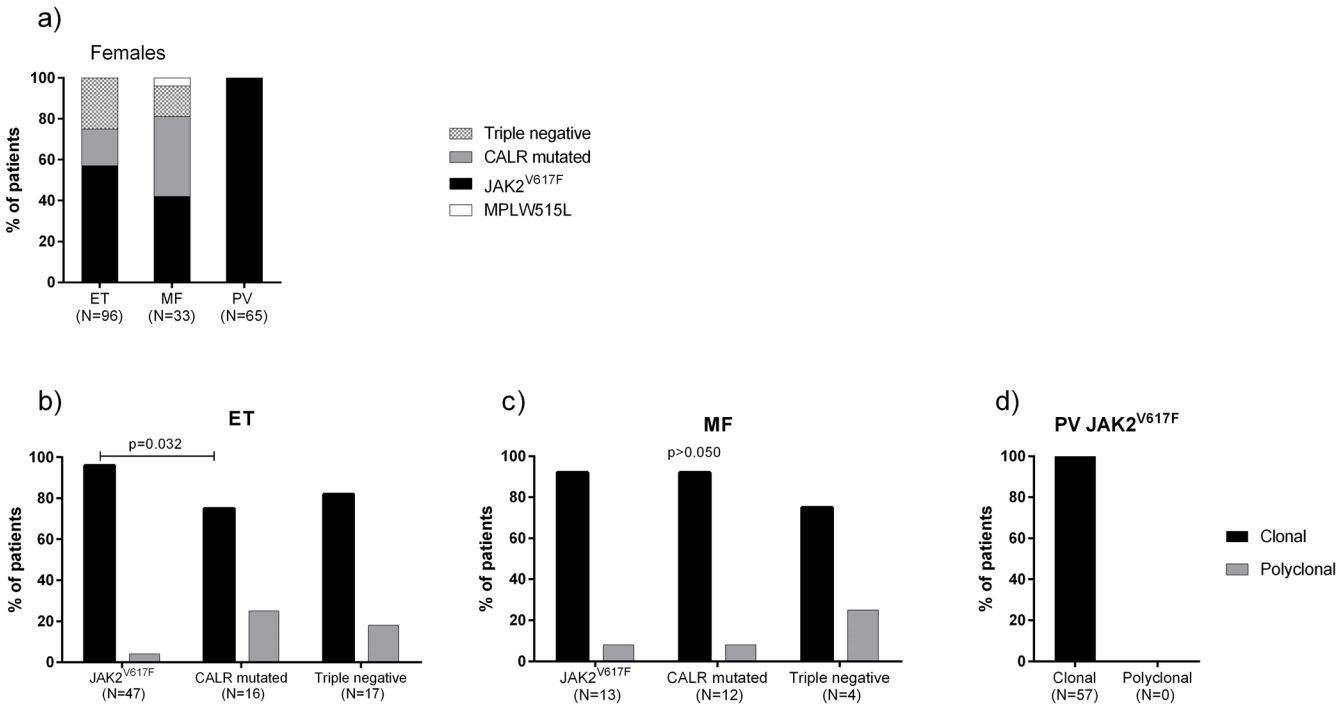

Figure 1.

Distribution of mutations in all females according the diagnosis (**a**); Clonality status in *JAK2*^{V617F}, *CALR*^{MUT} and triple negative MPN female patients: ET (**b**), PMF (**c**) and PV (**d**). Each bar represents the relative number of individuals. Frequencies were compared by Fisher's exact test.

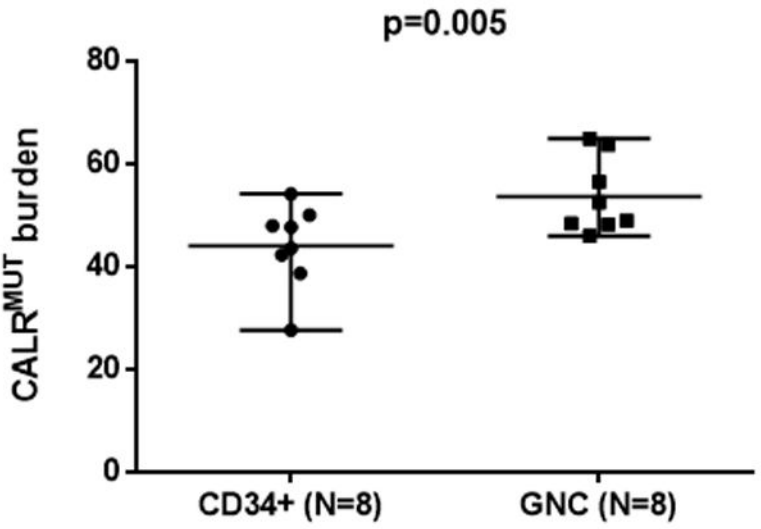

Figure 2.

$CALR^{MUT}$ burden in CD34+ cells and granulocytes (GNC). The lines represent mean and range. The paired t-test applied to compare the means of $CALR^{MUT}$ burden in the two matched groups.