

# PEAR1 Genetic Variants in Essential Thrombocythemia: A New Study of The Prevalence and Association of PEAR1 Variants with Hematological Parameters and ET Mutations

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## Research Article

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# Abstract

**Objective:** Essential thrombocythemia (ET) is a type of myeloproliferative neoplasm characterized by the expansion of the megakaryocytic/platelet line. Given the undeniable role of genetic variations in the pathogenesis of ET, as well as the proven effects of PEAR1 SNPs on platelet function, the innovative purpose of this study is to investigate the prevalence of PEAR1 variants (rs12041331 and rs12566888) and their relationship to hematological parameters and ET-related mutations.

**Materials and Methods:** We studied 105 ET patients and analyzed ET patients' mutational profiles, including JAK2 V617F mutation (detected by Allele-specific PCR), CALR, and MPL mutations (both through PCR amplification). Two SNPs of the PEAR1 gene were assessed through ARMS-PCR, and the Sanger method was used for the validation of ARMS-PCR amplification.

**Results:** The prevalence of rs12041331 and rs12566888 in ET patients were 43.9% and 38.5%, respectively, and rs12041331 was significantly associated with increased platelet counts (P-Value: 0.02). A significant relationship was also found between the rs12041331 and CALR mutation (P-Value: 0.03). Platelet count was higher in CALR<sup>+</sup> patients ( $934.45 \times 10^9/L \pm 265.35$  SD) than in JAK2<sup>+</sup> patients ( $790.11 \times 10^9/L \pm 265.35$  SD). Conversely, other hematological parameters and thrombosis were higher in JAK2<sup>+</sup> patients than the CALR<sup>+</sup> patients.

**Conclusions:** Our findings reinforce the idea that rs12041331 and rs12566888 may be associated with ET, and rs12041331 is significantly associated with increased platelet count. Besides, the prevalence of ET-related mutations in patients with rs12041331 and rs12566888 was almost similar; however, only CALR mutation had a significant relationship with rs12041331.

## Introduction

Essential thrombocythemia (ET) is a type of acquired stem cell-derived clonal disease associated with the expansion of megakaryocytic (MK)/platelet line. ET has been categorized as a myeloproliferative neoplasm (MPN) by Dameshech since 1954 (1, 2). This neoplasm's primary cause is the hyperproliferation of hematopoietic cells (HSCs) due to genetic driver mutations potentiating the myeloproliferative processes. Studies show that 100–205 individuals per 100,000 suffering from MPN or ET annually (2–4). Thrombotic events or thrombosis in the cerebral, hepatic, or coronary vessels due to the platelet defect is the leading cause of death in ET patients (5). In ET, the spectrum findings differ from symptomatic to asymptomatic: In asymptomatic ET patients, thrombocytosis and platelet counts  $> 450 \times 10^9/L$  along with giant megakaryocytes are common findings in the blood and bone marrow (BM); however, in symptomatic patients, the most common clinical symptoms include headache, erythromelalgia, and transient ischemic attacks (6, 7). Genetic factors play a significant role in platelet dysfunction as well as the pathogenesis and prognosis of ET patients. Genetic reports revealed that a somatic Janus kinase 2 mutation (JAK2 V617F) is present in 95% of Polycythemia Vera (PV) patients, 50% of ET patients, and nearly half of primary myelofibrosis (PMF) patients (8). Besides, in 2013, mutations in CALR exon 9 (the gene encoding calreticulin) were detected in approximately 20% of ET and PMF patients (but rarely in PV patients). A few

patients may have other mutations, including MPL exon 10 mutation, reported in approximately 3–15% of ET and PMF patients (9). Since about 20% of ET patients are negative for these three mutations (Triple-Negative patients), the absence of JAK2, CALR, and MPL mutations does not rule out the ET presence (2, 10).

Platelet endothelial aggregation receptor 1 (PEAR1), discovered in 2005 by genomics discovery, encodes the transmembrane tyrosine kinase receptors expressed on platelets and endothelial cells and plays an important role in biological platelet function/activation (11, 12). PEAR1 protein through the PI3K/PTEN pathway and continued activation of platelet aggregation via  $\alpha\text{IIb}\beta\text{3}$  could alter megakaryocytopoiesis (13, 14). Genetic studies have shown, two intronic variants (rs12041331 and rs12566888), are associated with platelet function/activation (15). Recently, researchers have investigated the effects of single nucleotide polymorphism (SNPs) in PEAR1 through in vitro megakaryocytes (MK) differentiation of HSCs and found that PEAR1 during MK differentiation affects both platelet aggregation and the number of mature platelets (15, 16). Recently, the role of PEAR1 SNPs has been highlighted in disorders such as coronary artery disease (CADs), acute coronary syndromes (ACS), sticky platelet syndrome (SPS), cardiovascular diseases (CVDs), deep vein thrombosis (DVT), and Kawasaki disease (KD) that are all associated with platelet activation/dysfunction. In the current survey for the first time, we examined rs12041331 and rs12566888 polymorphisms in the PEAR1 gene to investigate their association with hematological parameters, including platelet count, white blood cells (WBCs) counts, hemoglobin (Hb), and hematological symptoms such as thrombotic events and hemorrhage as well as their prevalence in ET patients. Additionally, we evaluated the prevalence of these polymorphisms in ET and their convergence with ET-related mutations. The innovative goals and challenging hypotheses of this study are classified below;

- Is the prevalence of rs12041331 and rs12566888 variants in ET patients significant?
- Is there a meaningful relationship between rs12041331/rs12566888 variants and platelet count, WBCs counts, and Hb levels?
- Is there a significant association between rs12041331/rs12566888 variants and hematological symptoms such as thrombotic events and hemorrhage in ET patients?
- Is there any significant relationship between the presence of rs12041331/rs12566888 variants and the occurrence of ET-related mutations?
- Investigating the effects of ET-related mutations on hematological findings and comparing these effects with rs12041331/rs12566888 variants?

## Materials And Methods

### Study subjects and Specimen collection

A total of 105 patients (50.0 % female and 42.1 % male) with a confirmed diagnosis of ET and Philadelphia chromosome-negative ( $\text{Ph}^-$ ) according to 2016 World Health Organization (WHO) criteria were entered in the present study between November 2018 and March 2020 that referred to the Ahvaz Baghaie 2 hospital (Table 1). The laboratory hematologic findings of ET patients (including platelet count,

WBC count, hemoglobin) along with clinical symptoms (thrombotic events and hemorrhage) and cytogenetic tests were under follow-up in Jundishapur University Department of Clinical Laboratory Sciences and recorded at Noor Genetics Lab of Ahvaz. The inclusion criteria in this study were considered 2016 WHO classification for ET (17, 18). Exclusion criteria were as follows: erythrocytosis, Philadelphia chromosome-positive (Ph+), leucoerythroblastic blood picture, morphological abnormalities compatible with PMF, PV, myelodysplastic syndromes (MDS), or other myeloid neoplasms, and evidence for reactive thrombocytosis. This study is based on the approval of the Medical Ethics Committee of Jundishapur Ahvaz University (**IR.AJUMS.REC.1398.571**). After obtaining written consent from all patients, 3 ccs of venous blood for analysis of ET-related mutations and PEAR1 SNPs (rs12041331 / rs12566888) was taken from them and collected into dipotassium ethylenediaminetetraacetic acid (K<sub>2</sub>EDTA)-anticoagulated tubes. Total DNA was extracted from venous blood specimens by using the QIAamp DNA Mini Kit (Germany) according to the manufacturer's instructions and preserved at - 70° C. Thermo NanoDrop One Microvolume ultraviolet-visible spectrophotometer (Thermo Fisher, USA) was used to determine DNA concentration and quality with a concentration of 100–200 ng/μl and 1.8-2.0 ratio in 260/280 nm (260/280 ratio). The samples with a concentration of less than 20 ng/μl were selected for this study. Besides, peripheral blood samples collected from 105 healthy donors were also used as controls in this study.

Table 1  
Demographic, hematologic/cytogenetic, and Clinical findings of ET patients.

Characteristics	ET patients
Age (mean ± SD), years	60.33 ± 12.31
Gender (M/F), n (%)	48/57 (42.1 / 50.0)
Plt (mean ± SD), ×10 <sup>9</sup> /L	835.33 ± 284.56
Hb (mean ± SD), g/dl	11.70 ± 2.40
WBCs count (mean ± SD), ×10 <sup>9</sup> /L	13.17 ± 10.85
Thrombotic events n (%)	34 (29.8)
Hemorrhage n (%)	3 (2.6)
Triple-Negative Patients n (%)	18 (15.8)
ET mutations / PEAR1 variants	64 (56.1%)
JAK2 V617F n (%)	
CALR exon 9 n (%)	20 (17.5%)
MPL exon 10 n (%)	4 (3.5%)
Triple-Negative n (%)	18 (15.8%)
rs12041331	<b>50 (43.9%)</b>
Homo n (%)	14 (12.3%)
Hetero n (%)	36 (31.6%)
rs12566888	<b>44 (38.5%)</b>
Homo n (%)	11 (9.6%)
Hetero n (%)	33 (28.9%)
<b>Abbreviation:</b> ET: Essential thrombocythemia; Hb: hemoglobin; Plt, platelet; WBCs: White Blood Cells; Homo: Homozygous; Hetero: Heterozygous.	

## ET diagnosis

In this study, patients were evaluated in terms of complete blood count, BM examination, and genetic testing. The criteria for ET diagnosis were according to the 2016 WHO criteria (Fig. 1). Platelet counts in all patients were  $\geq 450 \times 10^9/L$ , and the mean platelet count was  $835.33 \times 10^9/L \pm 284.56$  SD. Hematologists reviewed BM aspirates; an increased proliferation of megakaryocytic cell line along with increased enlarged/matured megakaryocytes was significant evidence in patients' aspirates (Fig. 1). Because most MPNs have overlap with each other or with other diseases, it is essential to rule out other causes of thrombosis, such as reactive thrombocytosis (in inflammation and iron deficiency) before ET is diagnosed

(5). For this purpose, thrombosis removal by iron replacement or the resolution of inflammation led to differentiate reactive thrombosis from ET. Given that ET is a type of Philadelphia-negative MPN, it was initially necessary to prove the Philadelphia chromosome's absence in all patients. Since sensitive and high-speed methods are needed to detect the BCR-ABL fusion gene, a multiplex reverse transcription-quantitative real-time PCR (Multiplex RT-qPCR) was performed in Ahvaz Noor Genetics Lab based on the approach published by Burmeister T. and Tong Y. et al. (19, 20). The genetic testing pattern (JAK2, CALR, and MPL mutations) was applied for all patients to diagnose ET based on 2016 WHO protocol, as shown in Fig. 2. Allele-specific polymerase chain reaction (PCR) was used to detect JAK2 V617F mutation (21). JAK2 V617F- negative patients were sequenced for CALR or MPL mutations through PCR amplification and Sanger sequencing (22). Allele-specific PCR for the detection of JAK2 V617F mutation (with a sensitivity defined as 0.5–2%) was performed with 5 µl of genomic DNA in the reaction mixtures contained 12.5 µL 2× TaqMan PCR Master Mix, TaqMan probe, primers (1 µL common reverse primer, and 0.5 µL each of forward primers), and distilled water. The primers sequences and probe used for PCR amplification of JAK2 V617F were as follows: Forward primer 1, 5'-AGCATTGGTTTAAATTATGGAGTATATT-3', Forward primer 2, 5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAG-3', Reverse primer, 5'-CTGAATAGTCCTACAGTGTTCAGTTTCA-3', and probe, (6FAM AATTATGGAGTATGTTTCTGMGBFNQ). Forward primer 1 (Forward wild-type-specific primer) only in the presence of a JAK2 V617F mutation generates 203 bp PCR product, while Forward primer 2 (Forward mutant-specific primer) anneals to both normal and mutant alleles and produces 364 bp PCR product, so it was used as an internal control. The PCR conditions included an initial denaturation of 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C (denaturing), 30 seconds at 58°C (annealing), 45 seconds at 72°C (elongation); and final extension of 72°C for 5 min.

The following forward and reverse primers were used for PCR amplification of CALR and MPL genes; CALR forward primer, 5'-CAT TCA TCC TCC AGG TCA AG-3'; CALR reverse primer, 5'-AGG GGA ACA AAA CCA AAA TC-3'; MPL forward primer, 5'-TGG GCC GAA GTC TGA CCC TTT-3'; and MPL reverse primer, 5'-ACA GAG CGA ACC AAG AAT GCC TGT-3'. PCR was performed as follows; 25–100 ng DNA in 100 µL PCR solution including, 10× MG Taq-HF buffer (10 µL), 2 mmol/L MG dNTPs mixture (10 µL), MG Taq-HF polymerase (1 µL), forward/reverse primers (0.2 µmol/L of each), and distilled water. PCR reactions were performed as follows; initial denaturation at 95 °C for 5 min followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and final extension of 72°C for 5 min. 2% agarose gel in 1X TBE buffer was used for electrophoresis, which was run for 50 min in 110 voltage (PAYA PAZHOOHESH, Iran). Then, the electrophoresis gel was visualized through UV light in a gel documentation system. Sanger sequencing was carried out via ABI-3130 XL (USA) using the above-described Forward/Reverse primers, and UGENE software was used for visualization of the sequences data (Fig. 5). The resultant sequences of CALR and MPL genes were compared with sequences on the GenBank website (<http://www.ncbi.nlm.nih.gov/sites/Entrez>). In every run (to detect JAK2, CALR, or MPL mutations) and amplification of the DNA templates, reactions with negative-control (included non-template control) and positive control were performed.

# Pear1 Variants Analysis

## Amplification Refractory Mutation System (ARMS-PCR)

Two intronic PEAR1 variants (rs12041331 and rs12566888) associated with platelet hyper-reactivity were selected according to literature, and the sequence was retrieved from the NCBI database (16). Because these polymorphisms are due to the single nucleotide changes in the alleles (rs12566888; G/T and rs12041331; G/A), the ARMS-PCR technique was considered a reliable method to detect these point mutations. This technique can also differentiate between heterozygous and homozygous individuals in terms of a single gene locus. For this purpose, two parallel reactions were performed in two separate tubes. In both of these reactions, a similar DNA sample was used. The first reaction (in tube 1) contained primers specific for normal DNA (normal forward/reverse primers) that could not replicate the mutated DNA at the gene locus. Conversely, the second reaction (in tube 2) contained a mutant allele-specific primer and normal primer (normal forward/mutant reverse), so it could not amplify normal DNA. We used three types of primers; Normal forward primer was constant, and its complementary sequence was present in both reactions. This primer was designed for regions of the gene that often lack mutation and serve as internal controls. The normal band was observed in all tubes containing normal primers. The normal tube (containing regular primers) is a kind of biological control in this study to verify ARMS-PCR results' accuracy. The other two types of primers are different at the 3' end. The first (normal reverse primer) is specific to the natural DNA sequence, and the second (mutant reverse primer) is specific to the mutated nucleotide. ARMS primers were designed by Batch Primer 3 software, which is freely available online at (<http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi>). The mismatch was deliberately added near the 3' end of the primer (at the terminal and proximal nucleotides) to prevent nonspecific replication of normal DNA by the mutant primer and subsequently increase the accuracy of the ARMS reaction. In this regard, if a strong mismatch (C-C, G-A, A-A) was observed at 3' end of the primer, a weak mismatch (T-T, T-C, T-G, G-G, A-C) was deliberately added to the nucleotide at -2 position and vice versa. Characteristics of the examined rs12041331/rs12566888 variants and sequences of associated primers are presented in Table 2. The PCR reaction was performed for both variants using the FlexCycler Thermocycler. ARMS PCR reaction for both rs12041331 and rs12566888 variants was optimized to amplify the desired region in two separate tubes (for each sample) as follows; 0.5 µl of DNA was used as a template in 10 µl reaction mixture (containing dNTPs, 1× PCR buffer, MgCl<sub>2</sub>, and Taq polymerase), 0.25 µl of forward and reverse primers and 14 µl of distilled water (the total volume of the reaction solution for each tube was 25 µl). The ARMS PCR procedure for rs12566888 variants consisted of denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 sec, 58 °C for 45 sec, 72 °C for 1 min, and a final extension at 72 °C for 3 min. Besides, the ARMS PCR procedure for rs12041331 variant was performed as follows; initial denaturation at 98 °C for 2 min was followed by 35 cycles of denaturation at 98 °C for 15 sec, annealing at 61 °C for 45 sec, and extension at 72 °C for 1 min, the final extension step was at 72 °C for 3 min. After the ARMS-PCR reactions, 6 µl of PCR product was loaded on 2 % agarose gel in ×1 tris-borate-EDTA buffer (PAYA PAZHOOHESH, Iran) for electrophoresis, which was stained with DNA safe stain. Electrophoresis was run in 115 voltage for 50 min. The gel was then inserted into a gel documentation

system (under UV light) and analyzed by UV-pro software. Schematic representation of ARMS-PCR assay along with gel electrophoresis of the normal, heterozygous, and homozygous subjects for both rs12041331/rs12566888 variants are shown in Fig. 3.

Table 2  
Characteristics of the selected PEAR1 SNPs, ET mutations and designed primer sequences.

Characteristics of PEAR1 SNPs	rs12566888		rs12041331
Chromosome	1		1
NC_000001.10	156869047		156869714
Location	Intron 1		Intron 1
HGVS names	c.-9-4663G > T		c.-9-3996G > A
Nucleotide change	TCCAG <b>[G/T]</b> ATAGG		CTTCC <b>[G/A]</b> TCACC
5'-Forward primer-3'	ATGGCTTTAGTGAGTGGATTGT		CGTTTTGGGGGATCAGGTTC
(N) 3'-Reverse primer-5'	CTCTCTCTCATCACTTCCTGTC		AAGCAGAGAGTAAGGGcGAC
(M) 3'-Reverse primer-5'	CTCTCTCTCATCACTTCCTGTA		AAGCAGAGAGTgAGGGTGAT
Characteristics of ET mutations	JAK2 V617F	CALR exon 9	MPL exon 10
Chromosome	9	19	1
NC_000001.10	NC_000009.12	NC_000019.10	NC_000001.11
Location	9p24.1	19p13.13	1p34.2
<b>Abbreviation:</b> SNP: single nucleotide polymorphism; HGVS: human genome sequence variation society; ET: Essential Thrombocythemia.			

Table 3

The association between rs12041331 / rs12566888 variants and ET-related mutations with hematological findings.

Variable	rs12041331	rs12566888	JAK2 V617F	CALR exon 9	MPL exon 10	TN
Mean Plt, ×10 <sup>9</sup> /L	910.500	920.100	790.11	934.45	534.250	890.31
	P <sup>*</sup> :0.02 df:2	P <sup>*</sup> :0.21 df:2	Sig: 0.928 P <sup>*</sup> :0.04 df:103	Sig: 0.569 P <sup>*</sup> :0.08 df:103	Sig: 0.489 P <sup>*</sup> :0.09 df:103	P <sup>*</sup> :0.02 df:103
Mean Hb, g/dl	11.9	10.6	12.30	9.52	12.2	9.7
	P <sup>*</sup> :0.39 df:2	P <sup>*</sup> :0.38 df:2	Sig: 0.376 P <sup>*</sup> :0.01 df:103	Sig: 0.062 P <sup>*</sup> :0.00 df:103	Sig: 0.723 P <sup>*</sup> :0.41 df:103	P <sup>*</sup> :0.21 df:103
WBCs, ×10 <sup>9</sup> /L	16.91	15.23	16.04	7.29	10.13	9.63
	P <sup>*</sup> :0.02 df:2	P <sup>*</sup> :0.02 df:2	Sig: 0.175 P <sup>*</sup> :0.01 df:103	Sig: 0.184 P <sup>*</sup> :0.06 df:103	Sig: 0.823 P <sup>*</sup> :0.32 df:103	P <sup>*</sup> :0.07 df:103
Thrombosis n (%)	50 (48.1)	44 (41.9)	28 (43.80)	3 (15.00)	0.00	3 (16.70)
	P <sup>*</sup> :0.08 df:2	P <sup>*</sup> :0.17 df:2	P <sup>*</sup> :0.02 df:1	P <sup>*</sup> :0.06 df:1	P <sup>*</sup> :0.39 df:1	P <sup>*</sup> :0.11 df:1
Hemorrhage n (%)	1 (2.80)	2 (6.30)	3 (4.70)	0 (0.00)	0 (0.00)	0 (.00)
	P <sup>*</sup> :0.16 df:2	P <sup>*</sup> :0.36 df:2	P <sup>*</sup> :0.16 df:1	P <sup>*</sup> :0.39 df:1	P <sup>*</sup> :0.72 df:1	P <sup>*</sup> :0.42 df:1
<b>Abbreviation:</b> TN: Triple-Negative Patients; Plt: platelet; Hb: Hemoglobin; WBCs: White Blood Cells; df: degrees of freedom; Sig: significance- value.						
P <sup>*</sup> : P-value was calculated through the $\chi^2$ test (for categorical variables) and Mann–Whitney U (for continuous variables).						

Table 4

The relationship between rs12041331/rs12566888 variants, the occurrence of ET mutations, and Triple-Negative patients.

Variable	Genotype	JAK2 V617F	CALR exon 9	MPL exon 10	TN
rs12041331	Homo	12.7 %	20.0 %	0.00 %	14.3 %
	Hetero	42.9 %	10.1 %	0.00 %	22.2 %
		P* :0.085 df: 2	P* :0.035 df: 2	P* :0.146 df: 2	P* :0.628 df: 2
rs12566888	Homo	12.5 %	5.0 %	0.00 %	18.2 %
	Hetero	31.3 %	25.0 %	0.00 %	27.3 %
		P* :0.864 df: 2	P* :0.419 df: 2	P* :0.223 df: 2	P* :0.152 df: 2
<b>Abbreviation:</b> TN: Triple-Negative Patients; Plt: platelet; Hb: Hemoglobin; WBCs: White Blood Cells; Homo: Homozygous; Hetero: Heterozygous.					
P* : P-value was calculated through the $\chi^2$ test (for categorical variables) and Mann–Whitney U (for continuous variables).					

Table 5

The association between PEAR1 variants, ET mutations and hematological symptoms with patients' gender.

Variant/Mutation	Male n (%)	Female n (%)	P-Value	OR	95% CI
rs12041331 <i>Homo</i>	6 (42.9)	8 (57.1)	0.962	1.882	0.633–5.594
rs12041331 <i>Hetero</i>	17 (47.2)	19 (52.8)			
rs12566888 <i>Homo</i>	4 (36.4)	7 (63.6)	0.790	1.882	
rs12566888 <i>Hetero</i>	15 (45.5)	18 (54.5)			
JAK2 V617F	31 (48.4)	33 (51.6)	0.484	0.796	0.278–2.285
CALR exon 9	11 (55.0)	9 (45.0)	0.354	0.571	
MPL exon 10	0 (0.00)	4 (100.0)	0.061	0.492	
TN	6 (31.4)	12 (68.2)	0.419	0.613	
Hematological symptoms	Male n (%)	Female n (%)	P-Value	OR	95% CI
Thrombosis	12 (35.3)	22 (64.7)	0.138	0.363	0.211–2.025
Hemorrhage	1 (33.3)	2 (66.7)	0.662	0.157	
<b>Abbreviation:</b> OR: Odds Ratio for gender (female / male); CI: Confidence Interval; Homo: Homozygous; Hetero: Heterozygous; TN: Triple-Negative Patients.					

## DNA sequencing for validation of ARMS-PCR amplification

The amplicons generated in dual PCR reactions were employed for direct sequencing using the indicated primers and ARMS-PCR conditions to validate ARMS-PCR amplification results. For this purpose, some representative genotypes of each variant were sequenced by the Sanger method; However, DNA sequences of only two samples are presented in Fig. 4. All the DNA samples were sequenced based on the following protocol: 0.7  $\mu$ L Big dye, 3  $\mu$ L H<sub>2</sub>O, 4  $\mu$ L buffer, 5  $\mu$ L of forward and reverse primers, and 5  $\mu$ L formamide. Sanger sequencing was performed on an ABI 3130XL Genetic Analyzer (USA), and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used for DNA analysis. After analyzing the sequence data by the UGENE software, the results obtained from DNA sequencing and ARMS-PCR assay were consistent with each other.

## Statistical analysis

This study's variables included quantitative and qualitative data, which were reported as mean  $\pm$  standard deviation (mean  $\pm$  SD) to describe the central tendency and interquartile range to describe data dispersion. In this research, the t-test and Mann–Whitney test were used to compare the scores of means, and ANOVA was employed to determine the correlation between PEAR1 variants (rs12041331 and rs12566888), platelet count, WBCs counts, and Hb levels. A chi-squared test was used to compare categorical variables and the determination of the relationship between the case and control data. In all the tests, the p-value was considered less than 0.05 (P-Value < 0.05) as statistically significant, and the Odds ratio (OR) with a 95% confidence interval (95%CI) was calculated. All the statistical analyses were processed by SPSS software (statistical package, version 24).

## Results

In this study, 105 ET patients with an average age of 60.33 years ( $\pm$  12.31 SD) and the same number of healthy controls were evaluated for the presence of rs12041331 and rs12566888 variants (Table 1). These two variants are localized in intron 1 of the PEAR1 gene (chromosome 1), which, according to previous studies, accounts strongly for platelet function phenotypes and aggregation (Table 2). The first investigation showed that 56.1% of ET patients in this study had JAK2 V617F mutation. The prevalence of CALR and MPL mutations was 17.5% and 3.5%, respectively, and 15.8% of patients were negative for three mutations (Triple-Negative). ARMS-PCR results showed that the rs12041331 variant was present in 50 patients (43.9%): 14 of these patients were homozygous (12.3%), and 36 were heterozygous (31.6%). The prevalence of the rs12566888 variant in these patients was 38.5%: 11 patients were homozygous (9.6%), and the remaining 33 patients were heterozygous (28.9%). It seems that just as the frequency of JAK2 mutation (56.1%) is higher than other ET-related mutations, the prevalence of rs12041331 (43.9%) is more prominent than rs12566888 (38.5%). Besides, the prevalence of rs12041331, rs12566888 variant and JAK2 mutation was higher in women (53.8%, 54.3%, and 51.6%) with a slight difference than in men (46.2%, 45.7%, and 48.4%). Also, the incidence of rs12041331 and rs12566888 variants in Triple-Negative patients were almost similar (17.3% and 17.1%, respectively), but no significant relationship was found between these variants and the absence of ET-related mutations. On the other hand, the frequency of ET-related mutations in rs12041331<sup>+</sup> patients were as follows: JAK2 (55.6%) > CALR (30%) > MPL (0.0%) and

consequently in patients with rs12566888 variant were: JAK2 (43.8%) > CALR (30%) > MPL (0.0%), which reveals that the prevalence of ET-related mutations is similar in both PEAR1 variants. Although 55.6% of rs12041331<sup>+</sup> patients had JAK2 mutation, no statistically significant relationship was observed (P-Value: 0.08, df: 2). Conversely, a significant relationship was observed between CALR mutation and rs12041331 variant in ET patients (P-Value: 0.03, df: 2).

In this study, the mean platelet count in ET patients was  $835.33 \times 10^9/L \pm 284.56$  SD; The mean platelet count in patients with rs12566888 and rs12041331 variant was  $920.100 \times 10^9/L \pm 272.10$  SD and  $910.500 \times 10^9/L \pm 270.02$  SD, respectively. The results reveal that between these two PEAR1 gene variants, rs12041331 is significantly associated with increased platelet count in ET patients (P-Value: 0.02, df: 2). Mutation analysis revealed the mean platelet count in JAK2<sup>+</sup> patients ( $790.11 \times 10^9/L \pm 265.35$  SD) was lower than in CALR<sup>+</sup> patients ( $934.45 \times 10^9/L \pm 275.09$  SD), and the presence of JAK2 mutation was significantly associated with a decreased platelet count in ET patients (P-Value: 0.04, Sig: 0.928, df: 103). Conversely, mean Hb levels were higher in JAK2<sup>+</sup> patients ( $12.30$  g/dl  $\pm 2.42$  SD) than in CALR<sup>+</sup> patients ( $9.52$  g/dl  $\pm 1.53$  SD): The results proved that JAK2 mutation was significantly associated with an increase in Hb levels (P-Value: 0.01, Sig: 0.376, df: 103) and CALR mutation was significantly associated with a decrease in Hb levels (P-Value: 0.00, Sig: 0.062, df: 103), while no significant relationship was found between Hb levels and PEAR1 variants. As in the previous hematological parameter (Hb), no significant relationship was found between patients' WBC counts and PEAR1 variants, but only JAK2 mutation had a significant relationship among ET-related mutations with increased WBC counts (P-Value: 0.01, Sig: 0.175, df: 103). It is noteworthy that the mean of total WBC counts in JAK2<sup>+</sup> patients ( $16.04 \times 10^9/L \pm 13.05$  SD) is higher compared to CALR<sup>+</sup> patients ( $7.29 \times 10^9/L \pm 1.45$  SD).

Thrombotic events were evaluated as a well-known hematological complication of ET in this study. The prevalence of thrombotic events in ET patients was 29.8%, which was more common in women (64.7%) than men (35.3%). Although thrombotic symptoms were detected in 48.1% of ET patients with rs12041331 variant, no significant association was found between thrombotic events and this variant (P-Value: 0.085, df: 2), which was probably due to the small statistical population. Similarly, the prevalence of thrombotic events in patients with rs12566888 variant was 41.9%, that no significant relationship was found between the thrombosis and rs12566888 variant (P-Value: 0.176, df: 2). The prevalence of thrombotic attacks in JAK2<sup>+</sup> patients was 43.8%, while this frequency in triple-negative and CALR<sup>+</sup> patients was 16.7% and 15.0%, respectively. In addition to the higher prevalence of thrombotic events in JAK2<sup>+</sup> patients compared to triple-negative patients and other ET-related mutations, only a significant relationship was observed between this mutation and thrombotic events (P-Value: 0.02, df: 1). Another hematological symptom in ET patients is hemorrhage, which occurs due to platelet dysfunction in the coagulation system despite the high platelet counts. Studies have revealed that in ET patients, hemorrhage is less frequent than thrombosis (3 to 18% of patients) (23); In the present study, the incidence of hemorrhage in ET patients was 2.6%, which was more in women (66.7%) than in men (33.3%).

## Discussion

ET is a Philadelphia-negative MPN characterized by increasing platelet counts in peripheral blood and clonal proliferation of the megakaryocytic lineage in BM (24, 25). Although this neoplasm is associated with longer overall survival, patients' life expectancy may be reduced due to the occurrence of thrombosis, hemorrhage, and the risk of hematological malignancies transformation (such as secondary myelofibrosis or acute myeloid leukemia) compared to the general population. (23, 26). The overall risk of arterial or venous thrombosis in these patients is estimated at 1–3% per patient-year; Therefore, thrombosis is a life-threatening risk factor in ET patients (27). Numerous acquired factors (advanced age, prior history of thrombosis, and vascular risk factors), genetic risk factors, and some changes in hematological parameters are involved in the pathogenesis of thrombosis in these patients (28). The presence of driver genetic mutations (such as JAK2 V617F) is engaged in ET pathogenesis and is considered one of the culprits of thrombotic attacks (29). On the other hand, some studies have revealed that leukocytosis (leukocyte count  $> 11 \times 10^9/L$ ) or abnormal platelet counts (platelet count  $\geq 450 \times 10^9/L$ ) were strongly associated with thrombosis/thrombohemorrhagic complications (5, 30, 31). Many platelet defects, including abnormal platelet aggregation, decreased functionality, acquired storage pool disease, and reduced levels of membrane adhesion molecules (i.e., GP Ib, IIb-IIIa, IV, and VI), have been identified in ET patients to increase the risk of thrombosis (28, 32). Recent studies have also revealed that PEAR1 protein as a transmembrane receptor (a type 1 receptor tyrosine kinase from the Epidermal Growth Factor family) could alter megakaryocytopoiesis through the PI3K/PTEN pathway (33). This protein can also enhance platelet aggregation by increasing the stability of  $\alpha IIb\beta 3$  on the platelet surface (34). This transmembrane receptor is present on the surface of resting platelets as well as  $\alpha$ -granules in platelets. Fc $\gamma$ RI $\alpha$  (IgE receptor) acts as a ligand for PEAR1, which their connection stimulates platelet aggregation and degranulation (12, 35, 36). Out of five intracellular domains of PEAR1, the one that is rich in proline (EMI domain) is responsible for platelet-platelet adhesion (34). The presence of PEAR1 polymorphisms during MK differentiation can lead to unexpected events in the size, platelet aggregation, and the number of mature platelets. Several polymorphisms have been identified in the PEAR1 gene, such as rs41299597, rs3737224, rs41273215, rs82242, rs11264579, rs12041331, and rs12566888, which increase PEAR1 expression and significantly affect platelet aggregation (37). Genetic researchers have discovered that more than 15% of platelet dysfunction cases are related to rs12041331 variant. Other studies have also revealed that rs12041331 and rs12566888 variants account for over 1% of platelet phenotypic variation (15, 38, 39). These two PEAR1 variants (rs12041331 and rs12566888) are located far from each other in the PEAR1 gene, but the GG allele in rs12041331 is closely related to the TT allele in rs12566888 and change the platelet activity with a similar mechanism (16, 40). However, the G allele in the rs12041331 variant is more strongly associated with increased platelet aggregation than the T allele in the rs12566888 variant and other PEAR1 variants (16, 41).

For the first time in this study, we surveyed 105 ET patients (and the same number of healthy donors as a control group) to assess the prevalence of rs12041331 and rs12566888 variants in these patients. Additionally, we evaluated the relationship between these variants and hematological parameters (platelet count, WBC counts, and Hb levels), hematological symptoms (thrombosis and hemorrhage), as well as their convergence with ET-related mutations. This study's innovative idea stems from the common presence of thrombosis/platelet aggregation in both ET patients and patients carrying PEAR1 variants in

platelet-related disorders, which has not been investigated before. We first identified ET patients based on a 2016 WHO classification through hematological/clinical findings and cytogenetic analysis (Fig. 2). Furthermore, the BM of all ET patients was examined to report the phenotype and number of megakaryocytes (Fig. 1). In the present study, ARMS-PCR was used to track rs12041331 and rs12566888 variant (then confirmed by DNA sequencing), and ET-related mutations were identified with the Allele-specific PCR and Sanger sequencing (Figs. 3 and 4). As expected, the frequency of JAK2 mutation in ET patients was 56.1%. The incidence of CALR and MPL mutations in these patients was 17.5% and 3.5%, respectively. The prevalence of JAK2 (f:m 1.02) and CALR (f:m 1.10) mutations was approximately the same among men and women, while all MPL<sup>+</sup> patients were female. Meanwhile, the prevalence of the rs12041331 variant (43.9%) was more prominent than rs12566888 (38.5%) in these patients. The incidence of ET-related mutations was almost similar in both rs12041331 (JAK2: 55.6% > CALR: 30% > MPL: 0.0%) and rs12566888 variant (JAK2: 43.8% > CALR: 30% > MPL: 0.0%). Despite the relatively high prevalence of JAK2 mutation in rs12041331<sup>+</sup> patients, no significant relationship was found, but a meaningful relationship was observed between CALR mutation and the presence of rs12041331 variant in these patients (P-Value: 0.03, df: 2). It is thought that the relationship between ET-related mutations and the presence of PEAR1 variants to be more significant in a larger statistical population; So, further research is required in this regard. Evaluation of the effects of PEAR1 variants on platelet count of ET patients revealed that the presence of rs12041331 is significantly associated with an increase in platelet count (P-Value: 0.02, df: 2). Considering the prominent influences of PEAR1 variants on the platelet function/activation and stimulating platelet aggregation in diseases related to platelet defects (such as SPS, DVT, KD, CADs, DVT, etc.), discovering the correlation between the presence of rs12041331 variant and increasing the platelet count in ET patients can be a cornerstone for new research on the effects of PEAR1 variants on platelet function/activation in ET or other platelet-related diseases. In contrast, no significant association was found between PEAR1 variants, WBC counts, and Hb levels. Follow-up of the prevalence of thrombotic events in ET patients indicated that thrombosis occurred in 57.1% of patients with homozygous rs12041331 and 43.8% of patients with rs12566888, but no statistically significant relationship was found between them. In parallel with the evaluation of PEAR1 variants, the results derived from the relationship between ET-related mutations and hematological parameters/symptoms in these patients underscored other recent studies' results. Recent studies on ET patients have proven that platelet counts in JAK2<sup>+</sup> patients are lower than in CALR<sup>+</sup> patients, while WBC counts, Hb levels, and thrombosis incidence in JAK2<sup>+</sup> patients is higher compared to CALR<sup>+</sup> patients (42). This study confirms the relationship between ET-related mutations with hematological parameters/symptoms as in previous studies (43, 44). Results of the present study, just like recent research, reveal that the mean platelet count in patients with JAK2 mutation ( $790.11 \times 10^9/L \pm 265.35$  SD) is lower than patients with CALR mutation ( $934.45 \times 10^9/L \pm 275.09$  SD), and the presence of JAK2 mutation is significantly associated with a decreased platelet count in these patients (P-Value: 0.04, Sig: 0.928, df: 103). Besides, WBC counts, Hb levels, and the prevalence of thrombosis was higher in JAK2<sup>+</sup> patients compared to CALR<sup>+</sup> patients, which also confirms the results of recent studies (43). Since the presence of JAK2 mutation is associated with increased WBC counts and a high prevalence of thrombosis, this mutation should be considered more than other ET-related mutations in the diagnosis, prognosis, and management of ET patients.

In recent years, researchers have focused on PEAR1 variants in various diseases, including SPS, CVDs, ACS, CADs, KD, and DVT (45–50). Some other studies have also investigated signaling, functional mechanisms, and protein expression of the PEAR1 gene. Faraday et al. (2011) sequenced the PEAR1 gene and discovered that the rs12041331 variant accounts for  $\leq 15\%$  of phenotypic variation in platelet function (16). Lei Pi et al. (2018) examined rs12566888 and rs12041331 variants for the risk of developing coronary artery aneurysm (CAA) in KD. Their results revealed a significant link between rs12041331 variant and CAA occurrence in KD, confirming the association between PEAR1 SNPs and cardiovascular disease, similar to previous studies (49). Keramati et al. (2018), who studied the effect of PEAR1 variants on platelet activation and atherosclerotic plaques in CAD patients, reported that the signaling of rs12566888 was not independent of rs12041331 variant. Conversely, their findings emphasized that in the presence of rs12041331 variant, the effect of the rs12566888 variant was less pronounced and that the rs12041331 variant could maintain its impact on platelet activation phenotype independently and more strongly (51). In the first large platelet function genome-wide association study (GWAS), John et al. (2010) demonstrated that two correlated intronic PEAR1 variants (rs12041331 and rs12566888) are linked with increased platelet count, ADP- and epinephrine associated platelet aggregation and platelet adhesion (38). In this study, the rs12041331 variant was associated with an increase in platelet count, which, like previous studies, indicates that this variant could be a threat to stimulate platelet aggregation and thrombosis. On the other hand, some further genetic investigations, including targeted re-sequencing, exome sequencing, and targeted SNP genotype studies, have failed to find a convincing association between PEAR1 variants and platelet activation traits, concluded that rs12041331 variant is the most likely a platelet functional variant (16, 52, 53). Criel et al. and Vandenbriele et al. in 2016 demonstrated that while the PEAR1 variants in the mice models reflected features of platelet activation, platelet function in humans was less affected by these variants (54, 55). Therefore, due to the novelty of the PEAR1 gene, there is still no consensus regarding its effect on platelets functional in many diseases and malignancies. We designed this study in ET patients before large-scale quantitative researches to evaluate the prevalence of PEAR1 variants, their effect on the hematological parameters/symptoms, and their relationship to ET-related mutations. This experiment was also performed to avoid wasting time and money on an inadequately designed project, and it can be a cornerstone for more comprehensive research. Suppose we consider the rs12041331 variant as a co-factor for thrombosis progression (along with JAK2 mutation) and increasing platelet counts. In that case, it may be argued that the presence of this variant could be a risk factor for thrombocytopenia and platelet defects in ET patients. However, more extensive research is needed to substantiate such claims. Finally, we point out some of the limitations of this study; First, in this research, we only focused on two of the potentially functional PEAR1 SNPs, which may overlook other important PEAR1 variants. Second, influences of other external factors that may affect platelet counts, including lifestyle behaviors and therapy methods, have not been considered. Third, the small statistical population is another limitation of this study, and we recommend that further research be conducted with a larger target community.

## Conclusions

The results of this study revealed for the first time that the rs12041331 variant was significantly associated with increased platelet count in ET patients. As the prevalence of thrombosis is high in JAK2 + patients, the rs12041331 variant was significantly related to thrombotic events. Also, this study, like recent studies, emphasized that platelet counts in JAK2 + patients are lower than in CALR + patients. In contrast, WBC counts, Hb levels, and thrombosis incidence in JAK2 + patients are higher than CALR + patients. The prevalence of ET-related mutations in patients with PEAR1 variants was almost similar to the order of these mutations' frequency in ET patients; however, only CALR mutation had a significant relationship with rs12041331 variant. This study could be a cornerstone for more extensive research, namely a larger ET cohort, together with clinical indexes and comprehensive analysis of genomics, which will be needed further to investigate the association of PEAR1 variants and thrombocytopenia in ET patients and their impacts on the hematological findings.

## Declarations

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**Ethical approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Local ethics code of the committee of Ahvaz Jundishapur University of Medical Sciences: **IR. AJUMS.REC.1398.571**.

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

The authors declare no conflict of interest.

### Authors' Contributions

Dr. Najmaldin Saki conceived the manuscript and revised it. Mohsen Maleknia and Dr. Mohammad Taha Jalali wrote the manuscript and prepared Tables. Dr. Gholam Abbas Kaydani and Dr. Ahmad Ahmadzadeh were included in qualifying the concept and design. Mohsen Maleknia critically evaluated the intellectual contents. All authors participated in preparing the final draft of the manuscript, revised the manuscript, and critically assessed the academic contents. All authors have read and approved the manuscript's content and confirmed the accuracy or integrity of any part of the work.

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## Figures

# Diagnosis of ET

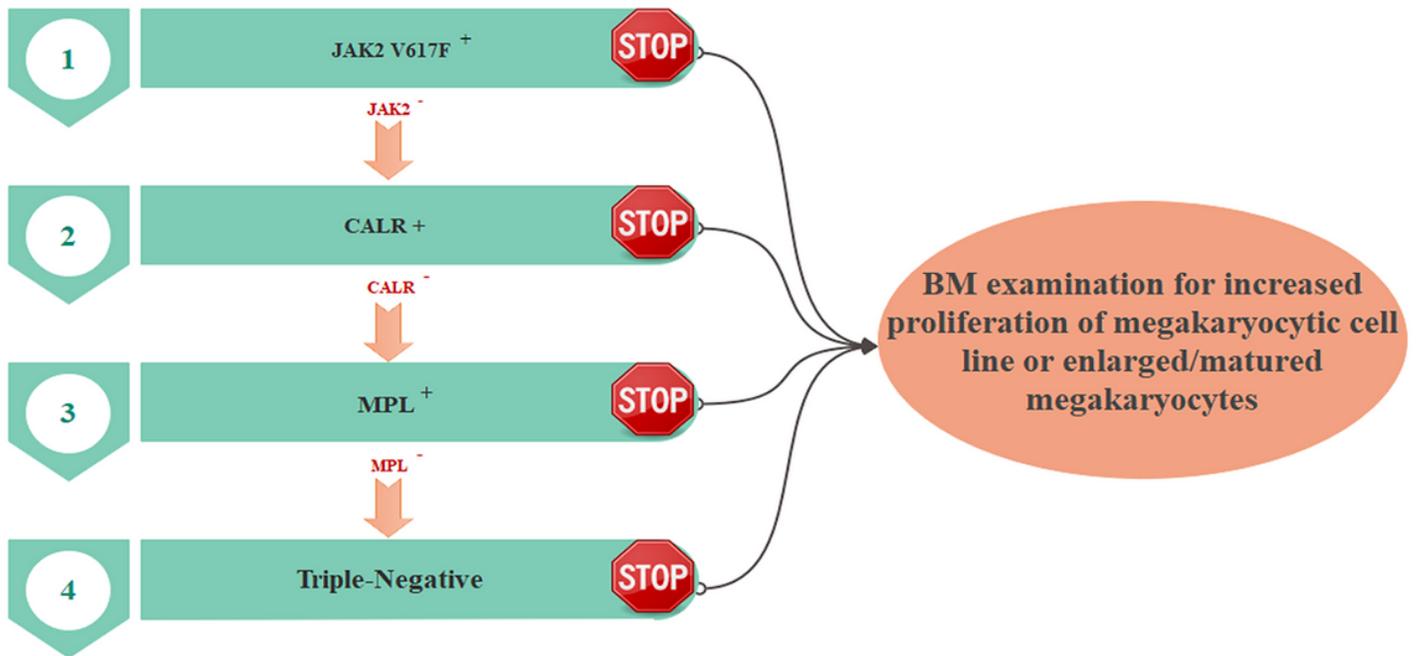
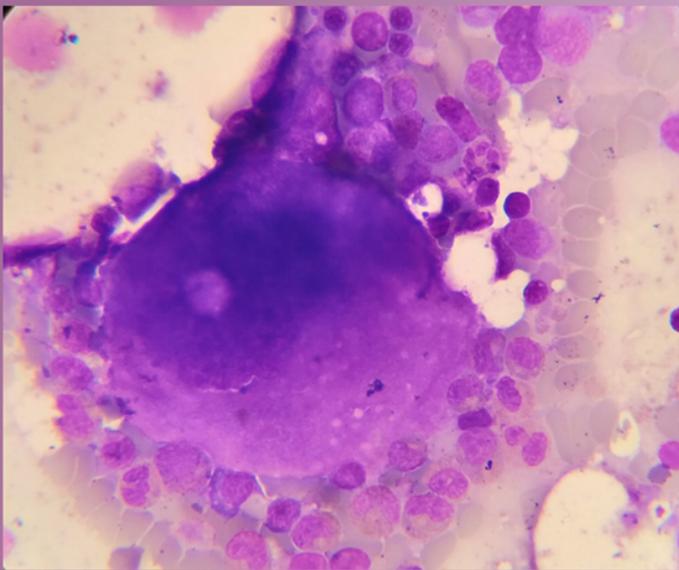
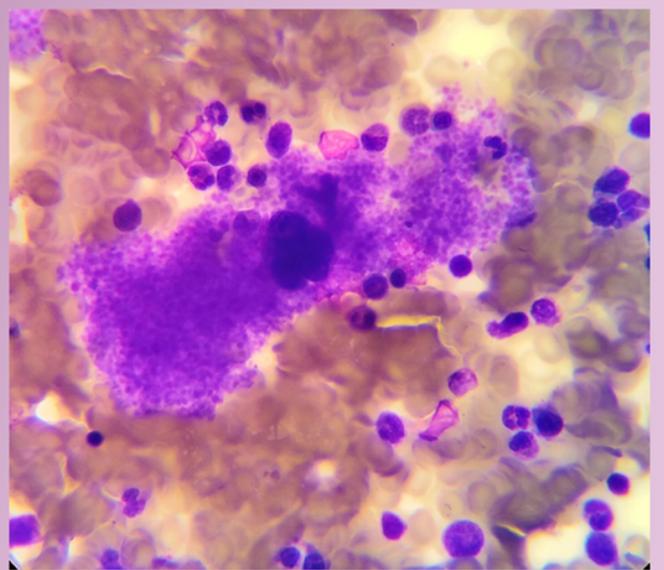


Figure 1

BM aspirate and megakaryocytic characteristics in two cases of ET patients (Wright Giemsa stain, 100 $\times$ ). a, Giant megakaryocyte with enlarged nucleus. b, Atypical megakaryocyte with abundant mature cytoplasm and nuclear fragmentation. Abbreviations: ET: Essential thrombocythemia; BM: Bone marrow.



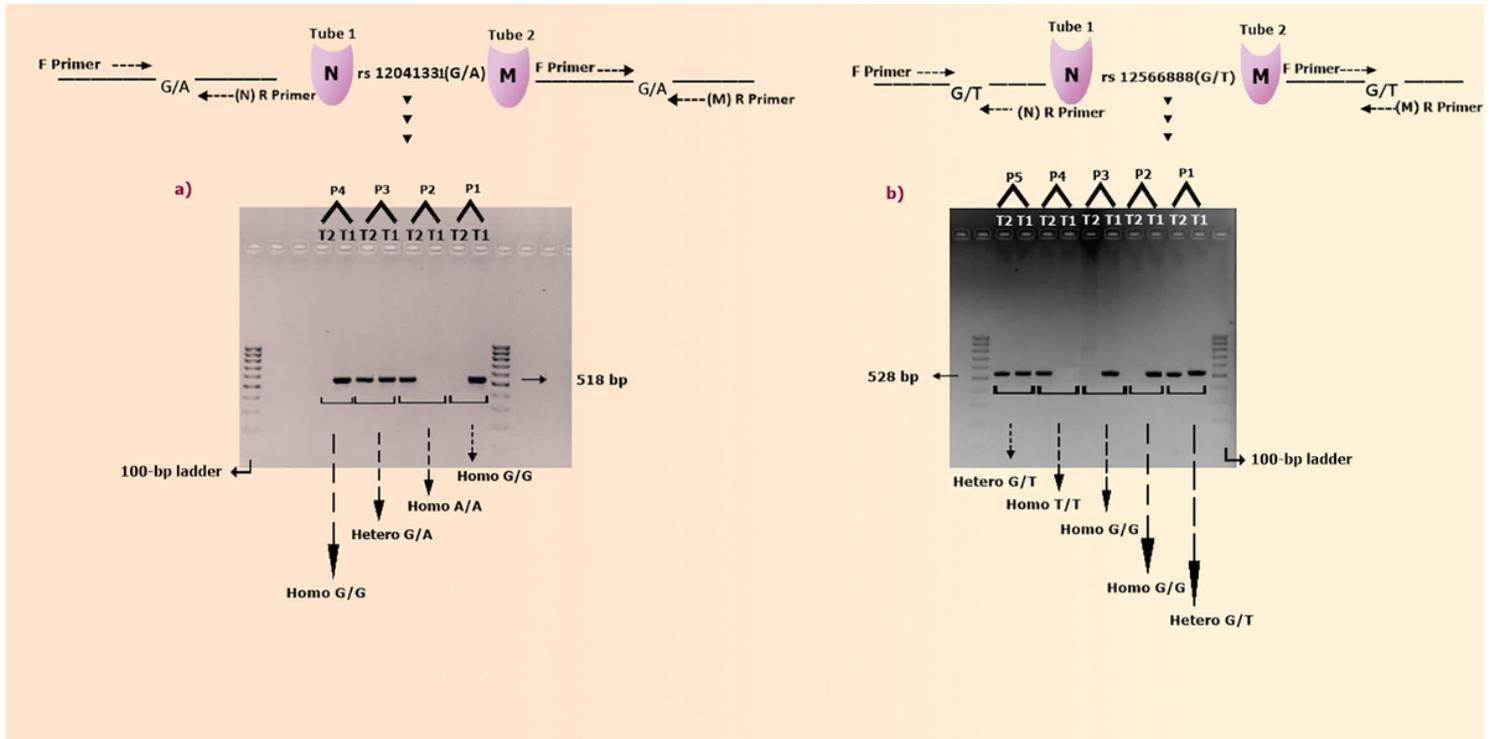
(a)



(b)

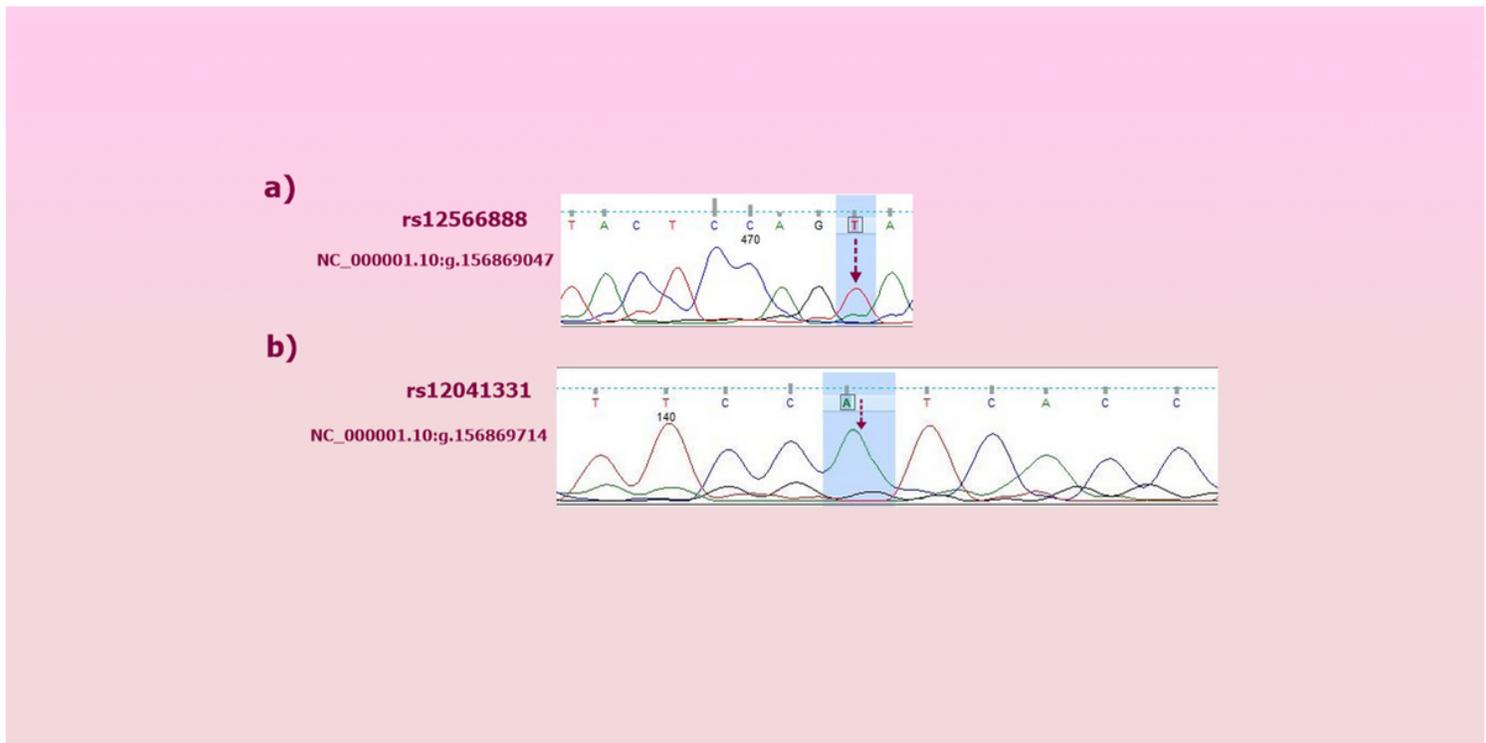
## Figure 2

Differential diagnosis of ET through genetic analysis and BM examination. PCR amplification was used for detection of JAK2 V617F mutation: JAK2 V617F – patients were then sequenced to identify CALR or MPL mutations. It is noteworthy that the bone marrow of ET patients was examined in parallel with the identification of ET-related mutations. Abbreviations: BM: Bone marrow.



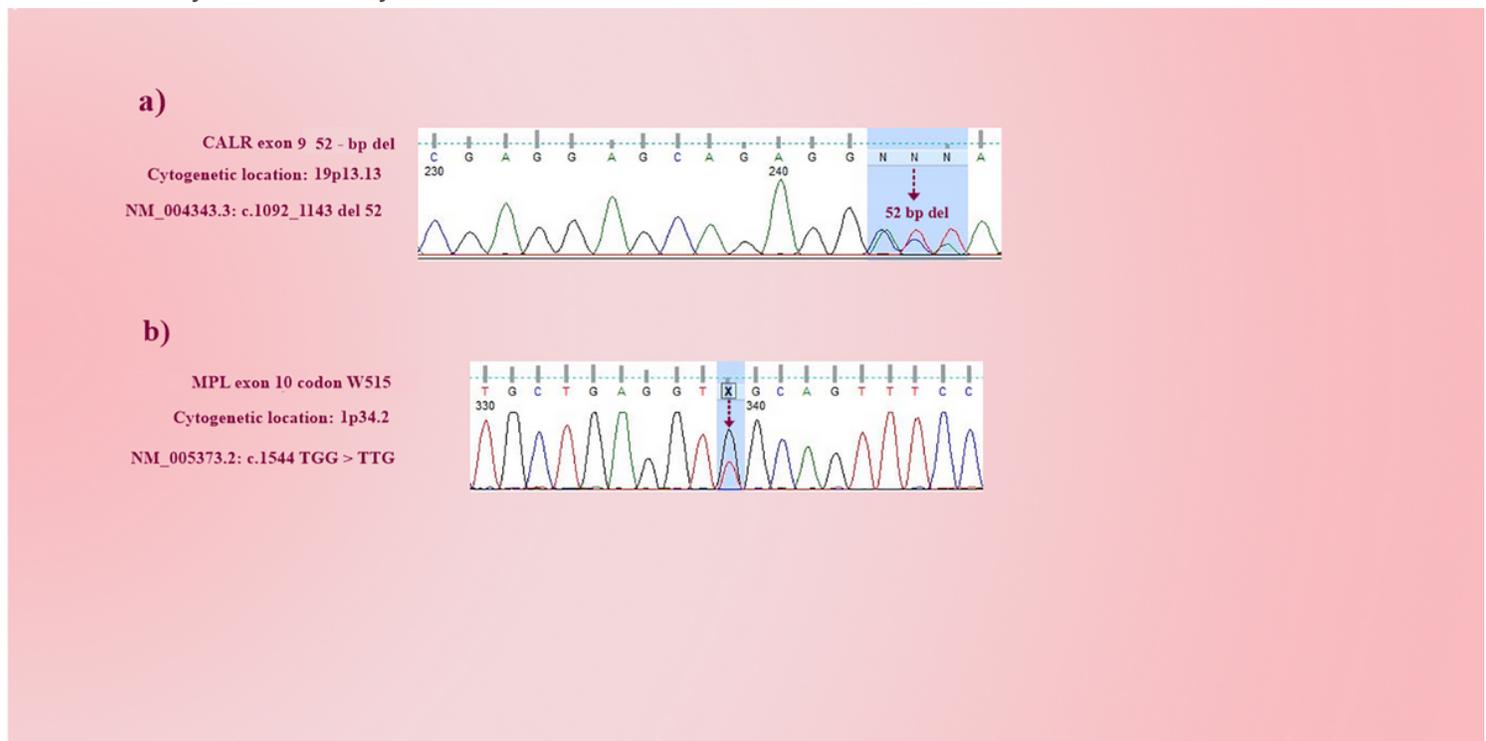
## Figure 3

Schematic diagram of the ARMS-PCR assay and agarose gel electrophoresis. ARMS-PCR was performed using three types of primers (normal forward, normal reverse, and mutant reverse primer) in two separate tubes (a total of four primers for each sample). a, Agarose gel electrophoresis of four samples, shows G/G homozygote, an A/A homozygote, G/A heterozygote, and G/G homozygote individuals for the rs12566888 variant, respectively. b, Agarose gel electrophoresis of five samples, indicates G/T heterozygote, G/G homozygote, G/G homozygote, T/T homozygote, and G/T heterozygote individuals for the rs12041331 variant, respectively. A 100-bp DNA ladder was used in all electrophoresis. Abbreviations: F: Forward; R: Reverse; N: Normal; M: Mutant; P: Patient; T: Tube; Homo: homozygote; Hetero: heterozygote.



**Figure 4**

Sanger sequencing of PEAR1 single-nucleotide variation in ET patients. In the chromatogram Red, Black, Blue, and Green peaks indicate T, G, C, and A nucleotides, respectively. The site of PEAR1 variants is represented in the highlighted section. a, Showing T/T genotype in the PEAR1 gene for rs12566888 variant. b, Showing A/A genotype in the PEAR1 gene for rs12041331 variant. Abbreviations: A: Adenine; G: Guanine; C: Cytosine; T: Thymine.



## Figure 5

Sequence analysis of CALR exon 9 (52-bp del) and MPL exon 10 (codon W515) in ET patients. Sequence analysis of CALR exon 9 and MPL exon 10 in ET patients. a, 52-base pair deletion is evident in the CALR locus (19p13.13). b, the mutated nucleotide at the codon W515 of MPL locus is visible (1p34.2).

Abbreviations: A: Adenine; G: Guanine; C: Cytosine; T: Thymine; del: deletion; bp: base pair.