Investigation of T-cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3) polymorphisms in essential thrombocythaemia (ET)

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ABSTRACT

Objectives: T-cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3) is preferentially expressed on terminally differentiated Th1 cells and inhibits their IFN-γ production. It has been reported that chronic inflammation may be an important driving force for myeloproliferative neoplasms (MPNs). Therefore, we hypothesized that as an important inflammation regulator, TIM-3 may be involved in essential thrombocythaemia (ET). The goal of this study was to investigate whether the −1516G > T, −574G > T and +4259T > G single-nucleotide polymorphisms (SNPs) within the TIM-3 gene contribute to the genetic susceptibility of individuals to ET.

Methods: Genotyping of the TIM-3 −1516G > T, −574G > T and +4259T > G SNPs was performed in 175 patients with ET and in 151 controls via a polymerase chain reaction-restriction fragment length polymorphism assay. We also investigated the relationships between the genotypes of each SNP and the risk factors of ET such as routine blood indexes, age and JAK2 V617F mutation.

Results: The genotype and allele frequencies of the −1516G > T SNP (p = 0.016 and 0.019, respectively), the −574G > T SNP (p = 0.035 and 0.038, respectively) and the +4259T > G SNP (p = 0.036 and 0.038, respectively) of the ET patients and the controls were significantly different. A haplotype analysis found that the GGT and TGT haplotypes had significantly different distributions between ET and controls (p = 0.041 and 0.041, respectively). However, no significant differences were detected between the genotypes of all SNPs and routine blood indexes, age and JAK2 V617F mutation.

Conclusion: The −1516G > T, −574G > T and +4259T > G SNPs within TIM-3 gene might play an important role as a genetic risk factor in the pathogenesis of ET.

Introduction

Essential thrombocythaemia (ET) is an acquired stem cell-derived clonal disease with expansion of the megakaryocytic/platelet line characterized by a sustained increase in the platelet count, and a tendency to develop thrombosis or bleeding [1]. ET shares phenotypic and pathogenetic similarities with other myeloproliferative neoplasms (MPN), particularly polycythaemia vera (PV) and primary myelofibrosis (PMF) [2].

Recent studies have provided evidence suggesting that genetic factors may influence the development and prognosis of ET. The seminal report in this regard was first published in early 2005 and described a somatic Janus kinase 2 (JAK2) mutation (JAK2V617F; an exon 14 somatic 1849G > T mutation) in PV, ET and PMF, which is estimated at 50% of patients with ET, along with half of those with PMF and 95% of those with PV [3]. An important addition to our knowledge was the finding of a new mutation, the CALR mutation, the gene encoding calreticulin, in 2013. It is present in about 20% of patients with ET and PMF but very rarely in PV, and in ET it is (with very few exceptions) not present in JAK2- or MPL-positive patients [4,5]. Therefore, CALR mutation appears to be relatively specific to ET and PMF. A small percentage of ET patients have other mutations, especially MPL mutations, which is reported in around 5% of those with ET or PMF [6]. However, there is still no reliable singular molecular marker for ET.

Further studies showed that the MPNs were associated with chronic inflammatory states due to the continuous release of inflammatory products from in vivo activated leucocytes and platelets [7–9]. Chronic inflammation is typically associated with sustained myeloproliferation (reactive leucocytosis and thrombocytosis) and the activation of a number of cellular pathways, which may ultimately trigger DNA damage in haematopoietic cells through the accumulation of mutation, the gene encoding calreticulin, in 2013. It is present in about 20% of patients with ET and PMF but very rarely in PV, and in ET it is (with very few exceptions) not present in JAK2- or MPL-positive patients [4,5]. Therefore, CALR mutation appears to be relatively specific to ET and PMF. A small percentage of ET patients have other mutations, especially MPL mutations, which is reported in around 5% of those with ET or PMF [6]. However, there is still no reliable singular molecular marker for ET.

Further studies showed that the MPNs were associated with chronic inflammatory states due to the continuous release of inflammatory products from in vivo activated leucocytes and platelets [7–9]. Chronic inflammation is typically associated with sustained myeloproliferation (reactive leucocytosis and thrombocytosis) and the activation of a number of cellular pathways, which may ultimately trigger DNA damage in haematopoietic cells through the accumulation of
reactive oxygen species (ROS) [10]. The inflammatory factor – IFN-γ has been reported to stimulate the proliferation of relatively mature megakaryocytic progenitors independent of thrombopoietin [11].

The TIM gene family located in chromosome 5q33.2 consists of three different genes in humans, TIM-1, TIM-3 and TIM-4, which regulate T-cell proliferation and Th1/Th2 differentiation with based on common structural motifs [12,13]. As an immune regulatory molecule, TIM-3 plays important roles in inflammatory diseases. TIM-3 is a transmembrane protein that is preferentially expressed on Th1 cells. TIM-3 can interact with its ligand-galectin-9, to downregulate the response of Th1 cells, thus, downregulating the inflammatory response. Moreover, several other cell types may also express TIM-3, such as cytotoxic CD8+ T cells, Th17 cells, regulatory T cells, monocytes, dendritic cells, microglia and mast cells [14–17].

TIM-3 gene may influence the susceptibility of cells to Th1-mediated conditions, and several studies have showed that polymorphisms in TIM-3 gene might be associated with autoimmune diseases, including allergic asthma, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [18,19]. However, it is unknown whether the polymorphisms of the TIM-3 gene are associated with ET. In the present study, we detected three polymorphisms in the TIM-3 gene in patients with ET: rs10053538 (G>T) and rs10515746 (G>T) single-nucleotide polymorphisms (SNPs) in the promoter region of the TIM-3 gene at positions −1516 and −574, respectively, and the rs1036199 (T>G) SNP mapped to exon 3 of the TIM-3 gene at position 4259, resulting in an amino acid substitution (Arg→Leu). The primary objective of the present study was to identify TIM-3 polymorphisms in ET patients and to determine the association between TIM-3 and ET. The secondary objective was to examine the relationship between TIM-3 polymorphisms and specific risk factors such as routine blood indexes, age, and the JAK2 mutational status.

**Subjects and methods**

**Study subjects**

A total of 326 subjects which collected in Shandong Provincial Hospital Affiliated to Shandong University from January 2014 to April 2015 were included in the study, including 175 ET patients and 151 normal healthy adults who were all Han Chinese. All subjects had no history of ET or any complications that might be related to ET prior to enrollment. All routine blood parameters in patients with ET were collected before treatment. The diagnosis of ET is based on the 2008 World Health Organization (WHO) criteria [20]: (1) PLT ≥450 × 10⁹/l. (2) BM biopsy showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes. No significant increase of left-shift of neutrophil granulopoiesis or erythropoiesis. (3) Not meeting WHO criteria for BCR-ABL+CML, PV, PMF, MDS or other myeloid neoplasm. (4) JAK2V617F or other clonal marker or in the absence, no evidence for reactive thrombocytosis. Bone marrow aspirations and biopsies were independently reviewed by two haematopathologists who were blinded to the patient’s mutation profile. Discrepant cases were resolved by consensus between the two haematopathologists. The characteristics of the study subjects are presented in Table 1. Informed consent was obtained from all ET patients and controls. The hospital-based ethics committee approved this study.

**DNA extraction**

Ethylenediaminetetraacetic acid (EDTA) anti-coagulated venous blood samples were collected from all subjects. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Cat. No. A1125, Madison, WI), according to the manufacturer’s protocol. The extracted genomic DNA samples were preserved at −80°C.

**TIM-3 genotyping**

The TIM-3 −1516G>T, −574G>T and 4259T>G polymorphisms were analysed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers and PCR conditions are listed in Table 2. Solutions for the PCR assay included 50–150 ng of genomic DNA, 10 μM of each primer, and Taq PCR MasterMix (Tiangen Biotech, Cat. No. KT201, Beijing, China) in a total volume of 25 μl. After an initial denaturation at 95°C for 5 minutes, the
DNA was amplified for 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension for 10 minutes at 72°C. Cleavage was performed using restriction enzymes (New England BioLabs, Ipswich, MA), according to the manufacturer’s instructions. Digested fragments were separated on 2% agarose gels and RFLP bands were visualized under UV light with ethidium bromide.

**DNA sequencing analysis**

Fifteen per cent of the PCR products were randomly selected and then purified with a PCR purification kit. The purified products were sequenced (GeneCore Biotechnologies Co. Ltd, Shanghai). The results between the PCR and DNA sequencing analysis were 100% concordant.

**Statistical analysis**

Statistical analyses were performed using the SPSS 19.0 software package. For numerical data, normally distributed parameters are presented as mean ± S.D while abnormally distributed parameters are presented as the median (interquartile range). Categorical variables are shown as frequencies. The genotype and allele frequencies were obtained by direct count. Genotype and allele frequencies were analysed using the χ² test or Fisher’s exact test with one or more variables (<5). Odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated using logistic regression analyses. p value <0.05 (two-tailed) was considered statistically significant.

**Result**

**Demographic characteristics of the study subjects**

The demographic characteristics of the patients and controls are listed in Table 1. The sex ratio was matched between the patients and controls without a significant difference (χ² = 1.661, p = 0.197). Significant difference was detected in age between ET patients and controls (χ² = 4.131, p = 0.042), which indicated that age older than 60 was a risk factor for ET. The JAK2 V617F mutation rate was 54.9% in 175 patients with ET, which was consistent with 54.28% of Han Chinese detected in the previous study [22]. The primary information relating to the TIM-3 −1516G > T, −574G > T and +4259T > G SNPs is summarized in Table 3. For these three SNPs, the genotyping success rate was more than 98% in all samples. The genotype frequencies for the −1516G > T, −574G > T and +4259T > G SNPs among the controls and were in HWE (p = 0.738, p = 0.869 and p = 0.935, respectively).

**Genotype and allele frequencies of the TIM-3 (−1516G > T, −574G > T and +4259T > G) polymorphisms**

To identify TIM-3 polymorphisms in ET patients and to determine the association between TIM-3 and ET, we evaluated TIM-3 −1516G > T, −574G > T and +4259T > G genotypes and allele distributions between all patients with ET and 151 controls. No deviations from Hardy–Weinberg equilibrium were observed in either

**Table 2. PCR primers and restriction enzymes for SNP assays.**

| Polymorphism | Primer sequence (5′ → 3′) | Restriction enzymes | Annealing temperature (°C) |
|--------------|---------------------------|---------------------|---------------------------|
| −1516G > T   | F:GCTCTGACCAAGTTCATGCT   | Bst1                | 60                        |
|              | R:ACACCGCCGGGATATTGT      |                     |                           |
| −574G > T    | F:AGAGGAGGATGAGGGAGGAGG   | Taq1                | 60                        |
|              | TTGCAGGTTAGCTGGGAGGTTTC    |                     |                           |
| +4259T > G   | F:CACTCTACGTTGCTGCAGCAGCAG| Pst1                | 60                        |
|              | R:GCATCTGGGAAAGGCCAGCAG    |                     |                           |

Notes: F: forward; R: reverse; PCR: polymerase chain reaction; SNP: single-nucleotide polymorphism.

**Table 3. Primary information for TIM-3 polymorphisms (rs100535338 G > T, rs10515746 G > T and rs1036199 T > G).**

| Genotyped | TIM-3 (rs100535338 G > T) | TIM-3 (rs10515746 G > T) | TIM-3 (rs1036199 T > G) |
|-----------|---------------------------|---------------------------|--------------------------|
| SNPs      | 5                         | 5                         | 5                        |
| Chr Pos   | nearGene-5 156470088       | nearGene-5 156469146      | missense 156464314       |
| Function  | Y                         | Y                         | Y                        |
| Splicing  | –                         | –                         | –                        |
| ESE or    | ESS                       | ESS                       | ESS                      |
| miRNA     | –                         | –                         | –                        |
| mRNAs     | –                         | –                         | –                        |
| rsSNP     | –                         | –                         | –                        |
| Global MAF| 0.074                     | 0.151                     | 0.117                    |
| MAF in our | 0.026                     | 0.013                     | 0.007                    |
| controls (n = 151) |                     |                           |                           |
| p value for HWE in our controls | 0.738 | 0.869 | 0.935 |
| Genotyping method | PCR-RFLP | PCR-RFLP | PCR-RFLP |
| % genotyping value | 98.32% | 98.65% | 99.03% |

Notes: TFBS: transcription factor binding site; ESE: exon splicing enhancer; ESS: exon splicing silencer; MAF: minor allele frequency; HWE: Hardy–Weinberg equilibrium; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.
the controls or the patients with ET for each polymorphism. Linkage disequilibrium was calculated independently by using the software SHEsis and no strong linkage disequilibrium (\(r^2 > 0.33\)) was detected among the three polymorphisms. Genotype and allele frequencies of the TIM-3 \(-1516G > T\), \(-574G > T\) and \(+4259T > G\) polymorphisms in ET cases and controls are summarized in Table 4. There were significant differences in the genotype and allele frequencies between the patients with ET and the controls for the \(-1516G > T\) (\(p = 0.016\) and \(p = 0.019\), respectively), \(-574G > T\) (\(p = 0.035\) and \(p = 0.038\), respectively) and \(+4259T > G\) (\(p = 0.036\) and \(p = 0.038\), respectively) polymorphisms. We also analysed the haplotypes of the TIM-3 \(-1516/-574/+4259\) polymorphisms, which are listed in Table 4. The haplotypes comparison analysis suggested that the TGT haplotype frequency was significantly increased in ET patients (\(p = 0.041\)) in comparison to that in the control group. In addition, the GGT haplotype was found to confer a decreased risk of ET (\(p = 0.041\)). These results strongly suggest that these three SNPs might be associated with ET susceptibility.

### The relationship between the genotypes of the TIM-3 SNPs and blood counts in ET patients

To further confirm the association between TIM-3 gene polymorphisms and specific risk factors of ET, we analysed the relationships between each polymorphic site of the TIM-3 gene and routine blood parameters. As shown in Table 5, none of the four blood parameters were significantly different in each genotype of the three SNPs.

### The relationship between the genotypes of the TIM-3 SNPs and the age or JAK2 V617F mutational status of ET patients

We analysed the relationship between the genotypes of the TIM-3 SNPs and age in the ET patients. We divided ET patients into two groups, <60 years old and \(\geq\)60 years old. No significant differences were detected between the genotypes of all SNPs and the two groups. Furthermore, we determined the JAK2 V617F mutational status of each genotype in the ET patients. There were no significant differences between the genotypes of all SNPs and the patients’ JAK2 V617F mutational status (Table 6).

### Discussion

In recent years the evidence is increasing that chronic inflammation may be an important driving force for clonal evolution and disease progression in the myeloproliferative neoplasms (MPNs), essential thrombocythaemia (ET), polycythaemia vera (PV) and myelofibrosis (MF). Abnormal expression and activity of a number of proinflammatory cytokines are related to MPNs, in which immune dysregulation is pronounced as evidenced by dysregulation of several immune and inflammation genes [9,23]. In addition, chronic inflammation has recently been described as a potentially very important facilitator not only of...
premature atherosclerosis, but also of clonal evolution and second cancer [9]. Getting enlightenment from the above results, we hypothesized that as an important inflammation regulator, TIM-3 might play an important role in the pathogenesis of ET.

TIM-3 gene polymorphisms may have roles in innate and adaptive immunity. Several studies have reported that the −574G>T SNP of TIM-3 is significantly associated with asthma, allergic rhinitis and rheumatoid arthritis [18,24,25]. The +4259T>G SNP has also been shown to be significantly associated with rheumatoid arthritis and allergic rhinitis [18,24]. Another report also observed that the genotype and allele frequencies of the −574G>T and −1516G>T SNPs were significantly higher in gastric cancer patients than in controls [26]. Meanwhile, researchers have found that the distribution frequencies of the +4259 T>G SNP in the TIM-3 gene of patients with pancreatic cancer or renal cell carcinomas are significantly different than in those of healthy people [27,28]. Based on this evidence, we speculated that as an important inflammation regulator, TIM-3 might contribute to the susceptibility of patients to ET. Therefore, the primary objective of the present study was to identify TIM-3 polymorphisms in ET patients and to determine the association between TIM-3 and ET. The secondary objective was to examine the relationship between TIM-3 polymorphisms and specific risk factors such as routine blood indexes, age and the JAK2 mutational status.

As the first purpose of the study, we evaluated the distributions of the TIM-3 –1516G>T, −574G>T and +4259T>G polymorphisms in ET patients and in healthy controls to explore the relationship between variations in TIM-3 and the susceptibility of patients to ET. As shown in Table 4, the genotype and allele frequencies of the −1516G>T, −574G>T and +4259 T>G polymorphisms in ET patients were all significantly different from those of the controls. In addition, haplotype analysis found that GGT and TGT had significantly different frequencies between patients with ET and controls. These results suggested that the three SNPs within the TIM-3 gene may be associated with an increased susceptibility to ET.

There is a lack of good quality prospective data concerning long-term survival in ET. From retrospective studies, the classical risk factors in the stratification are age over 60 years, history of prior thrombosis and platelets >1500 × 10^9/l (for bleeding). New risk factors of potential value have been suggested: leukocytosis, the JAK2V617F mutation and cardiovascular (CV) risk factors [29,30]. Therefore, as the second purpose of the study, we investigated the relationships between the genotypes of each SNP and routine blood parameters, age and JAK2 V617F mutational status (Table 5). Furthermore, we

| Polymorphic sites | Genotype | n (137) | RBC Mean SD | p value | HB Mean SD | p value |
|-------------------|----------|---------|-------------|---------|-----------|---------|
| TIM-3 (−1516G/T)  | GG       | 116     | 4.25 0.69   | 0.541   | 125 23.39 | 0.114   |
|                   | TG + TT + CG | 21     | 4.35 0.60   | 0.005   | 133 19.39 | 0.235   |
| TIM-3 (−574G/T)   | GG       | 124     | 4.30 0.66   | 0.085   | 127 23.12 | 0.868   |
|                   | TG       | 13      | 3.96 0.75   | 0.283   | 119 20.84 | 0.686   |
| TIM-3 (+4259 T/G) | TT       | 128     | 4.25 0.69   | 0.005   | 126 23.52 | 0.235   |
|                   | TG       | 9       | 4.50 0.35   | 0.283   | 127 13.54 | 0.235   |

Notes: p values were determined by t-test. RBC: red blood cell; HB: haemoglobin; PLT: platelet; WBC: white blood cell.

| Polymorphic sites | Genotype | n (137) | PLT Mean SD | p value | WBC Mean SD | p value |
|-------------------|----------|---------|-------------|---------|-------------|---------|
| TIM-3 (−1516G/T)  | GG       | 116     | 912 404.88  | 0.607   | 8.55 3.28   | 0.255   |
|                   | TG + TT + CG | 21     | 865 256.45  | 0.031   | 10.16 6.15  | 0.319   |
| TIM-3 (−574G/T)   | GG       | 124     | 915 393.35  | 0.331   | 8.9 3.84    | 0.194   |
|                   | TG       | 13      | 805 293.53  | 0.562   | 8.3 3.86    | 0.208   |
| TIM-3 (+4259 T/G) | TT       | 128     | 910 389.35  | 0.562   | 8.69 3.86   | 0.208   |
|                   | TG       | 9       | 832 336.25  | 0.103   | 10.37 4.02  | 0.582   |

Notes: p values were determined by χ^2 test.

### Table 5. The relationship between genotypes of TIM-3 SNPs and age as well as JAK2 V617F mutational status in ET patients.

| Polymorphic sites | Genotype | Age<60 (n = 73) (%) | ≥60 (n = 102) (%) | p value | Mutant (n = 96) (%) | Wild type (n = 79) (%) | p value |
|-------------------|----------|---------------------|-------------------|--------|---------------------|-----------------------|--------|
| TIM-3 (−1516G/T)  | GG       | 66 (90.4)           | 86 (84.3)         | 0.239  | 82 (85.4)           | 70 (88.6)             | 0.534  |
|                   | TG + CG + TT | 7 (9.6)           | 16 (15.7)         |        | 14 (14.6)           | 9 (11.4)              |        |
| TIM-3 (−574G/T)   | GG       | 67 (91.8)           | 94 (92.2)         | 0.928  | 86 (89.6)           | 75 (94.9)             | 0.194  |
|                   | TG + TT   | 6 (8.2)             | 8 (7.8)           |        | 10 (10.4)           | 4 (5.1)               |        |
| TIM-3 (+4259 T/G) | TT       | 69 (94.5)           | 96 (94.1)         | 1.000  | 89 (92.7)           | 76 (96.2)             | 0.322  |
|                   | TG       | 4 (5.5)             | 6 (5.9)           |        | 7 (7.3)             | 3 (3.8)               |        |

Note: p values were analyzed by χ^2 test.
divided ET patients into two groups, <60 years old and ≥60 years old. No significant differences were detected between the genotypes of all SNPs and the two groups (Table 6). Similarly, no apparent association existed between each genotype of the three SNPs and JAK2V617F mutation. These results suggest that TIM3 polymorphism has not yet been associated with major thrombosis and a poorer survival rate of ET. However, the above results were preliminary due to the small sample size in our present study. Further research should be performed in larger samples and different ethnic groups. It will be helpful to elucidate the impact of TIM-3 gene polymorphisms on the prognosis of patients with ET.

These findings of the present study are only preliminary results. Some limitations need to be overcome in the future study. The first limitation is the small sample size. Further research should be performed with larger samples. We will continue to study the mechanism of how TIM-3 polymorphisms regulate the secretion of IFN-γ, thus, affecting the occurrence of ET. The second one is that ET patients were comprehensively judged by bone marrow morphology, JAK2 mutation status, routine blood index and treatment effect. We should measure other markers such as the CALR and MPL mutations. The third one is that not all patients in this study had precise records of complications. Therefore, we have not yet carried out further study on the relationship between TIM3 polymorphisms and the prognosis of patients with ET. We will strengthen the follow-up of complications for the next steps of this research, to further observe the relationship between TIM-3 polymorphisms and the complications of thrombosis and haemorrhages, as well as the prognosis of ET patients.

In conclusion, the study first evaluated the distributions of the −1516G>T, −574G>T and +4259 T>G SNPs within the TIM-3 gene in ET patients and indicated that they might play an important role as a genetic risk factor in the pathogenesis of ET.

Disclosure statement
No potential conflict of interest was reported by the authors.

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