## Title

# Hereditary thrombocythemia due to splicing donor site mutation of *THPO* in a Japanese family


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**Abstract**

Thrombopoietin (THPO) is an essential factor for platelet production. Hereditary thrombocythemia (HT) is caused by a germline mutation of *THPO*, *MPL* or *JAK2* and is inherited in an autosomal-dominant manner. We identified a Japanese family with HT due to a point mutation of the splicing donor site of the *THPO* gene (*THPO* c.13+1G>A). Bone marrow biopsy showed increased megakaryocytes mimicking essential thrombocythemia. One affected family member developed chronic myeloid leukemia. We cloned the mutation and developed mutated and wild type THPO expression vectors. Molecular analysis showed that the mutation causes an exon 3 skipping transcript of *THPO* that abrogates a suppressive untranslated upstream open reading frame. Although the transcript levels of *THPO* mRNA were comparable, mutated transcripts were more efficiently translated and THPO protein expression was significantly higher than that of the wild type.

**Introduction**

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm due to sustained proliferation of megakaryocytes, which results in increased numbers of circulating platelets, thrombotic or hemorrhagic episodes, and occasional leukemic transformation. A somatic mutation of *JAK2*, *MPL* or *CALR* is known to contribute to the development of ET. Hereditary thrombocythemia (HT) with autosomal-dominant transmission has been shown to have manifestations that are similar to those of sporadic ET. Thrombopoietin (THPO) is a key growth factor for megakaryocytes and platelets. MPL is a receptor of THPO located on the surface of megakaryocytes and platelets. MPL stimulates the JAK-STAT pathway through activating JAK2 [1,2]. Germline mutations of *THPO, MPL*, and *JAK2* have been reported to be responsible mutations [3-28]. We identified a novel family with HT caused by *THPO* c.13+1G>A mutation. One of the affected family members developed chronic myeloid leukemia (CML) that was treated by a tyrosine kinase inhibitor (TKI). We investigated the clinical manifestations of the pedigree and revealed the mechanisms of increased production of THPO that contributes to the development of HT.

**Patients and Methods**

Pedigree

The patients were a 22-year-old Japanese female (iii1 in Fig. 1A) and her brother (iii2 in Fig. 1A). Hematological examination revealed elevated platelet counts (1050 x $10^9$/L and 847 x $10^9$/L, respectively) (Fig. 1B). The father of the two patients also showed an elevated platelet count and was diagnosed with CML in another hospital. He had been treated with a TKI and elevated platelet counts (1200-1600 x $10^9$/L) persisted even after the acquisition of major molecular remission. He died from pneumonia during major molecular remission of CML at the age of 49 years. The paternal grandmother of the two patients also had thrombocythemia, but a medical record could not be obtained. Family members with thrombocythemia showed increased levels of THPO (Fig. 1B). None of the family members had congenital abnormalities.

Genetic analysis

Genomic DNA was prepared from peripheral blood of the two affected family members (iii1 and iii2) and a non-affected member (ii2) in the pedigree (Fig. 1A). Sanger sequencing of *CALR, JAK2* exon12, *MPL,* and *THPO* and fluorescence in situ hybridization (FISH) of *BCR::ABL1* were performed for the female patient (iii1). Sanger sequencing of *THPO* was also performed for family members (iii2 and ii2).

Cloning and transfection assay

*THPO* cDNA was cloned from the human hepatocellular carcinoma cell line HepG2. *THPO* genome parts including the mutation site were amplified by polymerase chain reaction (PCR) from the female patient's genome (iii1). Genome-templated *THPO* exons 2-4 and cDNA-templated *THPO* exons 4-7 were cloned into a mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA), using an In-Fusion HD Cloning Kit (TAKARA, Kusatsu, Japan). The construct is shown in Fig. 2A. Both wild type (wt) and *THPO* c.13+1G>A were cloned from the female patient (iii1). A GFP-expressing plasmid, pmaxGFP (Lonza, Basel, Switzerland), was used as a negative control. Plasmids were transfected to HEK293T by TransIT-293 Reagent (TAKARA, Kusatsu, Japan), and the cells and medium were collected 72 hours after transfection.

Western blot analysis

Western blotting was performed for analyzing the expression of THPO protein. Transfected cell lysates were prepared with RIPA buffer (Nakalai tesque, Kyoto, Japan). Secretion of THPO in the transfected cell culture medium was also investigated by Western blot analysis. Anti-thrombopoietin (THPO) antibody (#ab196026, Abcam, Cambridge, UK),

anti-β-actin antibody (AC-15, Sigma-Aldrich, MO), and anti-BSA antibody (66201-1-Ig, Proteintech, IL, USA) were used as primary antibodies. After incubation with a secondary antibody, antigens were detected and visualization was carried out by using an enhanced chemiluminescence detection system (Image Lab software, Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

We used t-tests to compare the averages of continuous variables between the groups. *P*-values < 0.05 were considered as indicating statistical significance. Statistical analyses were performed by using Easy R version 1.55 [29].

**Results**

The peripheral blood of the female patient (iii1) showed increased platelets with giant platelets (Fig. 1C). A bone marrow biopsy showed megakaryocytic hyperplasia with platelet aggregation (Fig. 1D). Immunostaining for CD61 further revealed dysmegakaryopoiesis reminiscent of myeloproliferative neoplasms including ET (Fig. 1E). Bone marrow fibrosis was not observed (MF-0). Genetic analysis revealed a single nucleotide substitution (G>A) at the splicing donor site at intron 3 of *THPO* (c.13+1G>A) of the affected members (iii1 and iii2) (Fig. 1F). The mutation was on the same allele of SNP rs956732 located 150 bp upstream. There was no mutation of *CALR, JAK2*V617F, *JAK2* exon12, *MPL*, or *BCR::ABL1*. G-banding showed a normal karyotype. Echo sonography of the female patient (iii1) revealed slight splenomegaly (109.4 mm x 36.6 mm).

To verify the mechanisms of thrombocythemia, we cloned the mutation on the mammalian expression vector. *THPO* exons 2-4 (c.13+1G>A and wild type) were cloned from the patient's genome and connected with *THPO* exons 4-7 templated from HepG2 cDNA (Fig. 2A). We made pcDNA3/THPO wt and pcDNA3/THPO c.13+1G>A and verified the sequences by Sanger sequencing. Reverse transcription-PCR (RT-PCR) of transient transfection of the vectors into HEK293T showed a shorter band lacking exon 3 of *THPO* for pcDNA3/THPO c.13+1G>A (Fig. 2B). After transient transfection of pmaxGFP, pcDNA3/*THPO* wt and pcDNA3/THPO c.13+1G>A into HEK293T cells, THPO concentrations in the supernatant culture media were measured by the ELISA method. Real-time quantitative PCR (RQ-PCR) showed a comparable level of *THPO* transgene, but the THPO protein levels in the media of HEK293T cells transfected with pcDNA3/THPO c.13+1G>A were significantly higher than the level of pcDNA3/THPO wt (Fig. 2C, D). Western blot analysis of THPO protein in the transfected cell lysate and culture media showed that the cells transfected with pcDNA3/THPO c.13+1G>A expressed much higher

THPO protein levels than those in cells transfected with pcDNA3/THPO wt (Fig. 2E, F). Original THPO was detected as 35 kDa and 55 kDa bands in the cell lysate, and glycosylated large THPO protein (90 kDa) was secreted into the culture media. These results suggested that the transcription levels of mutated *THPO* and wild type *THPO* were almost the same, but the mutation contributed to higher levels of production and secretion of THPO protein.

**Discussion**

HT was previously reported to be caused by a *THPO* mutation that resulted in increased secretion of THPO, an *MPL* mutation that resulted in constitutional active form of the receptor, or a *JAK2* mutation that resulted in constitutional active signal transduction [30]. THPO measurement is considered to be a key component in the evaluation of familial thrombocytosis [31]. Affected individuals in our pedigree showed an increased THPO level in serum, which is the hallmark of HT with *THPO* mutation. We identified a *THPO* c.13+1G>A mutation that is identical to that reported in a family in Korea [14]. Since both pedigrees were found in East Asia, they might be genetically related. The mutation was on 150 bp downstream of the SNP rs956732 allele in our family, but this SNP was not mentioned in the previous report. In addition to identifying the *THPO* c.13+1G>A mutation, we showed by an in vitro experiment that this mutation leads to exon 3 skipping of *THPO* mRNA and overexpression of THPO protein. A bone marrow biopsy showed increased megakaryocytes in our case. It is difficult to distinguish HT from sporadic ET by clinical or pathologic features. Without a familial history, the affected member of HT could be diagnosed as having ET. Careful history-taking is important to avoid unnecessary treatment with cytotoxic agents.

Reported *THPO* mutations in HT were clustered at the 5'-untranslated region (UTR) [5,8,10] and the splice donor site of intron 3 [4,6,9,11-14] (Fig. 3A). Mutations at the splicing site abrogated the splicing donor site, resulted in an exon 3 skipping transcript. *THPO* has several upstream open reading frames (uORFs) in the 5'-UTR, and these uORFs suppress translation of THPO starting from the main ORF (ORF8). Exon 3 skipping abrogates suppressive uORF7 and connects ORF5 directly to the main ORF9 in-frame, resulting in effective translation (Fig. 3B). Increased levels of THPO were reported in individuals with HT. However, comparison of serum THPO levels in affected and non-affected individuals does not directly reflect the difference of production level of THPO because THPO is saturated by MPL, the receptor of THPO expressed, on increased platelets and megakaryocytes in vivo [32,33]. We verified increased efficiency of mutated THPO translation in vitro, in which platelets did not interfere with THPO protein. We

observed 36 kDa and 55 kDa THPO protein bands in the Western blot of the cell lysate and a 90 kDa band in the supernatant. THPO is synthesized as a precursor protein with a molecular weight of 36 kDa, and THPO undergoes gradual glycosylation following removal of an N-terminus signal peptide [34]. Zhang et al. showed that a 55 kDa THPO protein in the cell lysate and a 94 kDa secreted THPO protein were both glycosylated THPO [35]. *THPO* mutations reported in HT affect the structure of the N-terminus signal peptide of THPO protein, but the hydrophobic domain, which is critical for the function of the signal peptide, is preserved in the mutated THPO protein. Because the signal peptide fraction is removed in the process of maturation, the sequence of the amino acid residues of the mature THPO protein is identical with that of the wildtype THPO protein [4]. Our data showed that the THPO protein was increased in each maturation process both in the cell lysate and the secreted fraction.

HT is polyclonal thrombocytosis that selectively affects the megakaryocytic lineage [36]. Most individuals with *THPO* mutation showed benign reactive thrombocytosis; however, hematological malignancies such as multiple myeloma [12], acute myeloid leukemia [37], cutaneous B-cell malignant lymphoma [38] and myelofibrosis [19,37] developed in some cases. In our pedigree, one patient developed CML. Since the emergence of a CML clone in patients with ET has been repeatedly reported [39-41], sustained stimulation of the JAK-STAT pathway might trigger the emergence of CML clones in HT. On the other hand, no hematologic malignancies have so far been reported in patients with HT associated with germline mutations of *MPL* and *JAK2* [15-28].

Thrombosis and/or bleeding complication in HT associated with hereditary *THPO* mutation have been reported [9,19,42]. Schlemper et al. reported an HT pedigree with 11 affected members among whom 6 members showed vaso-occlusive symptoms and 2 members showed bleeding symptoms [42]. Later, the first *THPO* mutation (c.13+1G>C) was identified in this pedigree [4]. There was no direct correlation between the number of platelets and thrombotic or bleeding events, on the other hand 5 of the 6 members with veno-occlusive symptoms were more than 40 years of age [42]. Some patients have been treated with low-dose aspirin [9,14,19]. Although thrombotic and bleeding events were rare in patients with HT associated with *MPL* [15,18,20,21,23], transmembrane domain mutations of *MPL* (S505N and L502G) were shown to be associated with a high thrombotic risk and myelofibrosis [19,22]. Teofilli et al. reported 7 pedigrees with HT caused by MPLS505N, and 8 of the 21 affected members had bone marrow fibrosis and 4 of those 8 members had thrombotic events [19]. As for HT associated with *JAK2*, Mead et al. reported a pedigree with HT in which 3 of the 6 affected members showed thrombotic events and all those 3 members were more than 40 years of age [24]. From these reports, it might be

better to consider low-dose aspirin or an antiplatelet drug for older patients (>40 years old) and patients with a transmembrane mutation of *MPL* as primary prophylaxis for thrombotic events. Our patients have not developed thrombosis or bleeding complication without any intervention. Optimal management for HT has yet to be established.

In conclusion, we identified a novel pedigree with HT due to a germline mutation of *THPO* c.13+1G>A. Our data suggested that the mutation abrogates the splicing donor site, resulting in production of a mutated *THPO* transcript lacking exon 3. Although the levels of the mutated transcripts were comparable to those of wild type THPO, mutated transcripts were more efficiently translated to THPO protein, resulting in the development of HT. Careful history-taking and evaluation of serum THPO protein level would be useful to distinguish HT from sporadic ET. Because of the risk of hematological malignancies, myelofibrosis, and thrombotic and hemorrhagic complications, careful follow-up is recommended.

**Figure legends**

Figure 1.

(A) Pedigree with hereditary thrombocythemia. A circle indicates a female and a square indicates a male. Filled symbols indicate family member with thrombocytosis. Blank symbols indicate non-affected family members. The arrow indicates the proband.

(B) Complete blood counts and THPO serum concentrations of the pedigree. Data for (ii1) were obtained during TKI therapy and the patient achieved a major molecular response.

(C) Peripheral blood smear of the affected individual (iii1) showed increased platelets with giant platelets (arrow).

(D) Bone marrow biopsy of patient iii1 stained with hematoxylin and eosin, showing increased megakaryocytes.

(E) Immunostaining for CD61 revealed dysmegakaryopoiesis of bone marrow.

(F) *THPO* mutation

Sanger sequencing showed *THPO* c.13+1G>A mutation in affected family members. The mutated allele had SNP rs95672 at 150 bp upstream.

Figure 2. Molecular analysis of *THPO* c.13+1G>A.

(A) Construct of expression vectors of wild type and mutated *THPO*.

(B) RT-PCR of a cell line transfected with pcDNA3/THPO wt or pcDNA3/THPO c.13+1G>A. Each band on gel electrophoresis was sequence-verified by Sanger sequencing. The short band was confirmed to be an exon 3 (158 bp) skipping transcript.

(C) RQ-PCR of *THPO* mRNA targeting exons 4 to 6.

(D) THPO in the supernatant of transfected cells was analyzed by ELISA.
(E) Western blot of the transfected cell lysate showed 35 kDa and 55 kDa THPO bands. The expression level of THPO of pcDNA3/THPO c.13+1G>A is much higher than that of pcDNA3/THPO wt.
(F) Western blot of transfected cell culture media showed a 90 kDa secreted mature THPO. The expression level of THPO of pcDNA3/THPO c.13+1G>A is much higher than that of pcDNA3/THPO wt.

Figure 3.
(A) Previously reported *THPO* mutations contributing to the development of HT. Mutations were clustered at the 5'-UTR and splicing donor site of exon 3 of *THPO*. Superscripts represent reference number.
(B) In wild type THPO, upstream untranslated ORFs suppress translation starting from main ORF8. Exon 3 skipping abrogated ORF 7 and 8 and resulted in a direct connection of downstream of URL5 to the main ORF9 in-frame.

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**Author contributions**

H.K. and M.O. designed the study, analyzed the data, and wrote the manuscript. H.K., J.H. and D.H. performed experiments. H.O. and Y.M. verified pathological finding of bone marrow biopsy. M.K., T.M., and K.T. were involved in the critical evaluation of the manuscript and data presentation. T.T. revised and approved the manuscript.

**Compliance with Ethical Standards**

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**Disclosure of conflict of interest:** The all authors declare that they have no conflict of interest.

**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. This study was approved by the Institutional Review Board of Hokkaido University Faculty of Medicine (IRB#17-037).

**Informed consent:** Informed consent was obtained from all individual participants included in the study. Written informed consent was obtained from each family member in accordance with the Declaration of Helsinki.

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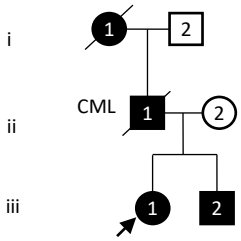
158

**Table 1. Heredirary thrombocytosis**

| | THPO mutation | Function | Associated findings | References |
|---|---|---|---|---|
| 1 | *THPO* c.13+1G>C | exon 3 skipping | Thrombocytosis<br>High thrombopoietin level<br>Microcirculation disturbance and thrombosis<br>Mild splenomegaly | Wiestner et al (Nature Genetics 1998)<br>Liu et al (Haematologica 2008) |
| 2 | *THPO* c.-31G>T | Early stop codon was created shortening the uORF 7 and translational reinitiation | Thrombocytosis<br>High thrombopoietin level<br>Skeletal abnormalities | Ghilardi and Skoda (BJH 1999)<br>Graziano et al (Blood 2009) |
| 3 | *THPO* c.-47delG | uORF 7 in frame with THPO coding sequence and translation initiation at uORF 7 | Thrombocytosis<br>High thrombopoietin level | Kondo et al (Blood 1998) |
| 4 | *THPO* c.13+5G>A | exon 3 skipping | Thrombocytosis<br>High thrombopoietin level<br>Mild splenomegaly | Jorgensen et al (Blood 1998 ASH mtg abstract) |
| 5 | *THPO* c.13+2T>C | exon 3 skipping | Thrombocytosis<br>High thrombopoietin level | Zhang et al (Blood 2011) |
| 6 | *THPO* c.13G>A | exon 3 skipping | Thrombocytosis<br>High thrombopoietin level | Prouzet-Mauleon V et al. (BJH 2020) |
| our case | *THPO* c.13+1G>A | exon 3 skipping | Thrombocytosis<br>High thrombopoietin level<br>Skeltal abnormalities<br>Mild splenomegaly, CML | Jung Nani et al (Annals of Lab Med 2020) |

# Figure 1

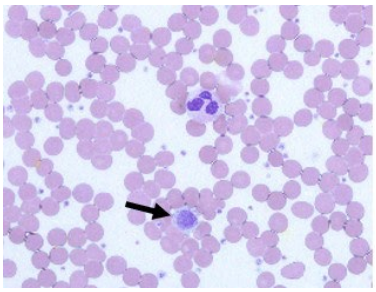
A



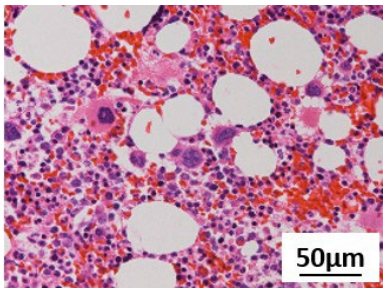

B

| Family member | Gender | WBC (x10$^9$/L) | Hb (g/dL) | Platelets (x10$^9$/L) | THPO (Fmol/mL) |
|---|---|---|---|---|---|
| ii1 | M | 7.3 | 12.9 | 1,558 | NA |
| ii2 | F | 6.6 | 15.2 | 331 | 1.04 |
| iii1 | F | 10.1 | 14.7 | 1,050 | 5.04 |
| iii2 | M | 7.0 | 17.1 | 847 | 6.56 |

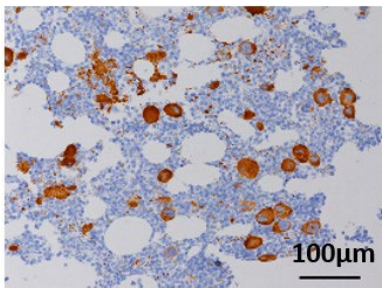
C



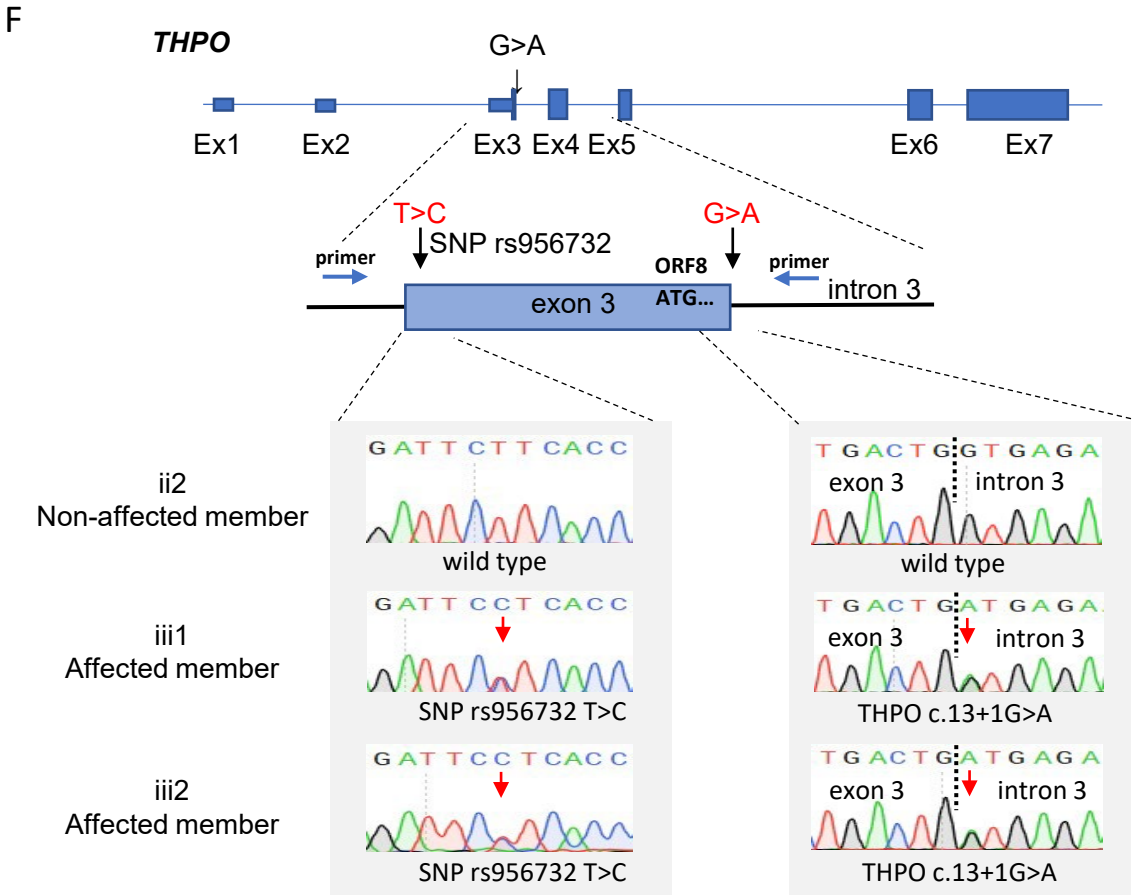
D



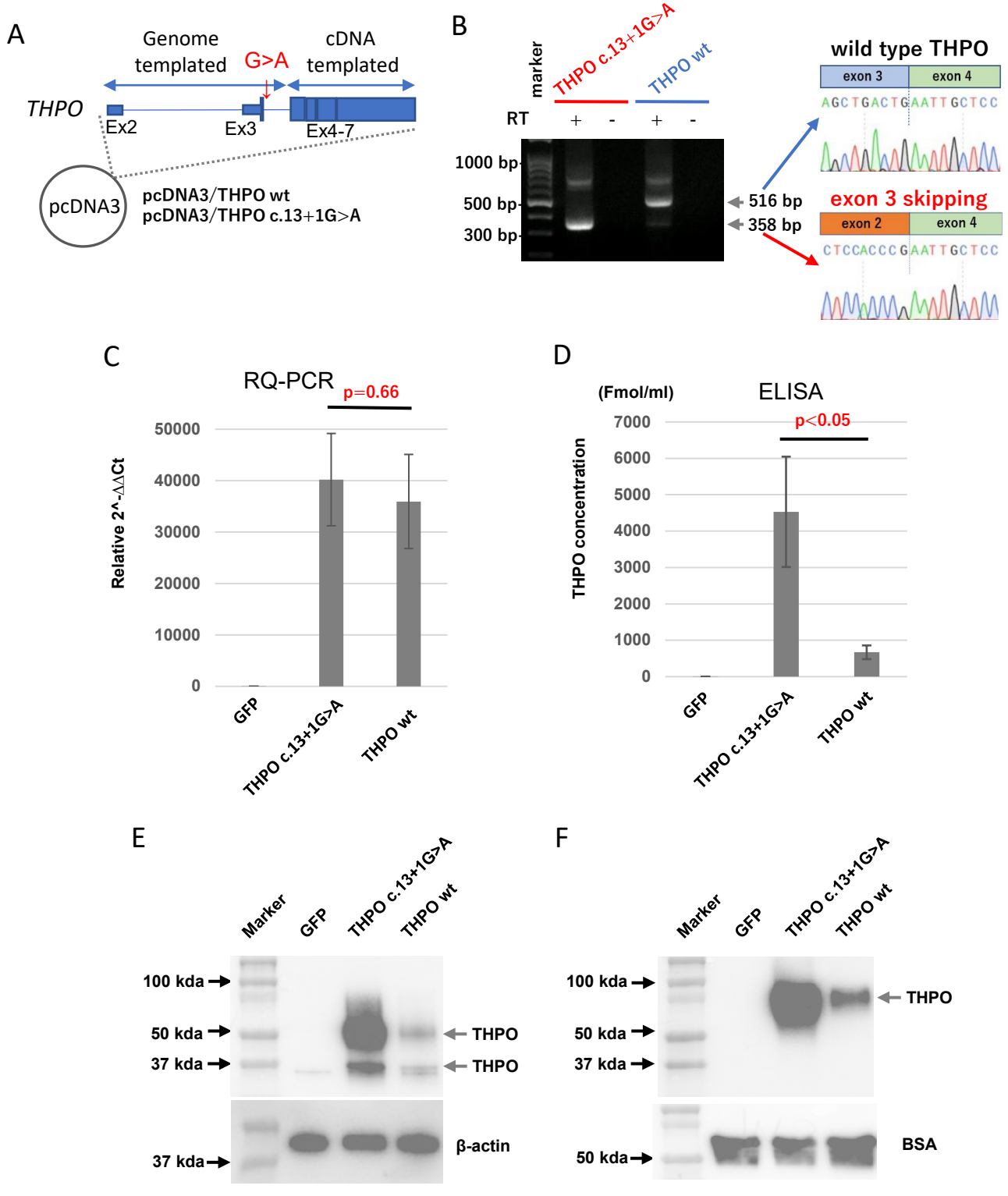

E



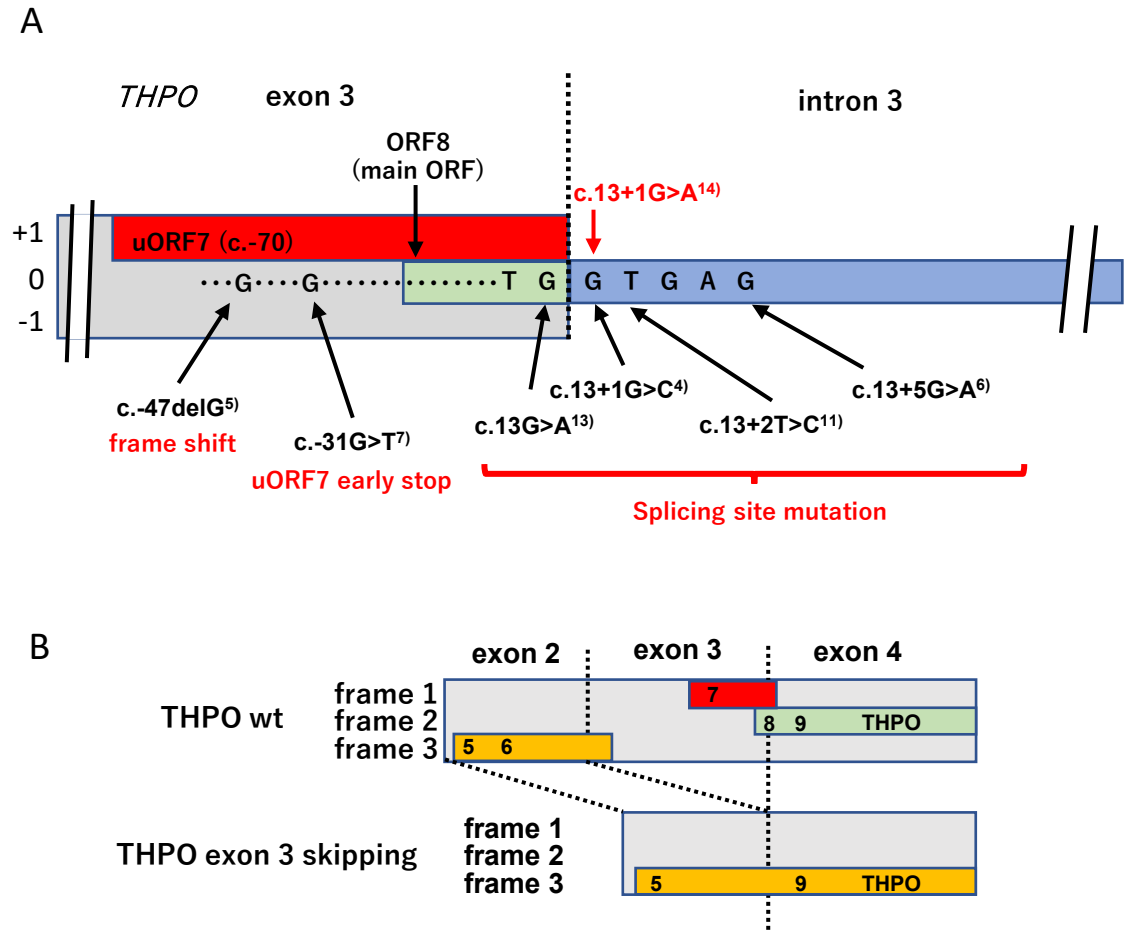

F



# Figure 2


A
Genome templated
G>A
cDNA templated
THPO
Ex2
Ex3
Ex4-7
pcDNA3
pcDNA3/THPO wt
pcDNA3/THPO c.13+1G>A
B
marker
THPO c.13+1G>A
THPO wt
RT
+ - + -
1000 bp
500 bp
300 bp
516 bp
358 bp
wild type THPO
exon 3
exon 4
AGCTGACTGAATTGCTCC
exon 3 skipping
exon 2
exon 4
CTCCACCCGAATTGCTCC
C
RQ-PCR
p=0.66
Relative 2^-ΔΔCt
50000
40000
30000
20000
10000
0
GFP
THPO c.13+1G>A
THPO wt
D
(Fmol/ml)
ELISA
p<0.05
THPO concentration
7000
6000
5000
4000
3000
2000
1000
0
GFP
THPO c.13+1G>A
THPO wt
E
Marker
GFP
THPO c.13+1G>A
THPO wt
100 kda
50 kda
37 kda
THPO
THPO
37 kda
β-actin
F
Marker
GFP
THPO c.13+1G>A
THPO wt
100 kda
50 kda
37 kda
THPO
50 kda
BSA

# Figure 3


A
THPO
exon 3
intron 3
ORF8
(main ORF)
c.13+1G>A14)
+1
0
-1
uORF7 (c.-70)
···G····G·····T G
G T G A G
c.-47delG5)
frame shift
c.-31G>T7)
uORF7 early stop
c.13G>A13)
c.13+1G>C4)
c.13+2T>C11)
c.13+5G>A6)
Splicing site mutation
B
exon 2
exon 3
exon 4
THPO wt
frame 1
frame 2
frame 3
7
8 9 THPO
5 6
THPO exon 3 skipping
frame 1
frame 2
frame 3
5 9 THPO