

# Hypomegakaryocytic thrombocytopenia (HMT): an immune-mediated bone marrow failure characterized by an increased number of PNH-phenotype cells and high plasma thrombopoietin levels

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**Hypomegakaryocytic thrombocytopenia (HMT): An immune-mediated bone marrow failure characterized by an increased number of PNH-phenotype cells and high plasma thrombopoietin levels**

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**Summary**

Patients with mild hypomegakaryocytic thrombocytopenia (HMT) that does not meet the diagnostic criteria for a definite disease entity may potentially progress to aplastic anaemia (AA) refractory to therapy. To clarify the clinical pictures of HMT, we prospectively followed 25 HMT patients that fulfilled

the following criteria: a WBC count of  $>3.0 \times 10^9/l$ , an Hb level of  $>10g/dl$ , and a platelet count of  $<100.0 \times 10^9/l$  in the absence of morphological and karyotypic abnormalities in the bone marrow.

Glycosylphosphatidylinositol-anchored protein-deficient blood cells (PNH-type cells) were detected in 7 of the 25 (28%) patients and elevated plasma thrombopoietin (TPO) levels ( $>320$  pg/ml) were observed in 11 (44%) patients. Five (four PNH+ and one PNH-) of six TPO<sup>high</sup> patients who were treated with cyclosporine (CsA) showed improvement. Among the 21 patients who were followed without treatment, thrombocytopenia progressed in four of ten TPO<sup>low</sup> patients and four of 11 TPO<sup>high</sup> patients. The three-year failure-free survival rate of the TPO<sup>high</sup> patients who were treated with CsA (100%) was significantly higher than that of the untreated TPO<sup>high</sup> patients (20%). These results suggest that a significant population of HMT patients has an immune pathophysiology that is similar to AA and may be improved by early therapeutic intervention with CsA.

**Keywords:** hypomegakaryocytic thrombocytopenia, PNH-type cells, thrombopoietin, cyclosporine

## **Introduction**

Mild thrombocytopenia without anaemia and leukopenia is associated with various haematologic diseases, including immune thrombocytopenic purpura (ITP) (Provan, *et al* 2010) and myelodysplastic syndromes (MDS) (Cheson, *et al* 2006, Valent, *et al* 2007). There is, however, a considerable number of cases that do not meet the diagnostic criteria for such established diseases, due to megakaryocytic hypoplasia in patients with ITP and the absence of dysplastic signs in the bone marrow (BM) cells. When their thrombocytopenia is mild ( $\geq 50 \times 10^9/L$ ) (Provan, *et al* 2010), these patients are usually followed without therapy. However, some patients with hypomegakaryocytic thrombocytopenia (HMT) may progress to aplastic anaemia (AA), which is refractory to immunosuppressive therapy (IST), after long-term observation, just like the patients with moderate AA who were followed without IST (Nishio, *et al* 2009). It is therefore important to elucidate the pathophysiology of HMT at the time of diagnosis, even in patients without severe thrombocytopenia.

We previously conducted a retrospective analysis of 29 HMT patients that

fulfilled the following criteria: a WBC count of  $>3.0 \times 10^9/l$ , an Hb level of  $>10g/dl$ , and a platelet count of  $<100.0 \times 10^9/l$  in the absence of morphological and karyotypic abnormalities in BM with megakaryocytic hypoplasia, and found that 55% of the patients possessed an increased number of glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP)-deficient blood cells (PNH-type cells). PNH-type cells, which are often detected in patients with AA, are associated with a better response to IST than that which is observed in patients without PNH-type cells (Kulagin, *et al* 2014, Sugimori, *et al* 2006, Wang, *et al* 2001, Wang, *et al* 2002). In this retrospective analysis, 10 of the 16 patients with PNH-type cells (PNH[+] patients) received cyclosporine (CsA) monotherapy; 60% of them responded to the therapy (unpublished observation). On the other hand, thrombocytopenia patients with megakaryocyte deficiency have high plasma thrombopoietin (TPO) levels (Emmons, *et al* 1996) and our previous study demonstrated that low-risk MDS patients with high ( $>320$  pg/ml) TPO levels are more likely to respond to IST and that they show a better prognosis than patients with low ( $<320$  pg/ml) TPO levels (Seiki, *et al* 2013). All of these findings suggest the presence of immune mechanisms in a subset of patients

with HMT. To further characterize the clinical pictures and the prognosis of HMT, we conducted a multicenter prospective observational study (UMIN-CTR 000002729). We herein report the results of an interim analysis of this study.

## **Patients and Methods**

### *Patients*

A nationwide observational study of patients with HMT was started in January 2010. The inclusion criteria were as follows: a white blood cell (WBC) count of  $> 3 \times 10^9/L$ , a haemoglobin (Hb) level of  $>10$  g/dL and a platelet (Plt) count of  $<100 \times 10^9/L$  with a decreased number of megakaryocytes in the BM. The megakaryocyte cellularity was determined by a pathologist on trephine biopsy sections, and only those patients who showed a decrease in the number of megakaryocytes were enrolled in this study. The decrease in the number of megakaryocytes was defined as 1 megakaryocyte per 5 to 10 low-power fields or  $<10/mm^2$ . The patients who met the diagnostic criteria for well-established hematological diseases, including ITP (Provan, *et al* 2010), AA (Marsh, *et al* 2009), MDS (Cheson, *et*

*al* 2006, Valent, *et al* 2007) and PNH (Parker, *et al* 2005) were excluded from the present study. The therapeutic strategies were left to each physician's discretion. This study was approved by the institutional review boards of all of the institutions that participated this study (no. 796), and all patients provided written informed consent prior to sampling.

#### *Detection of PNH-type cells*

PNH-type cells were detected by a high-sensitivity flow cytometry (FCM) assay, which was performed at the time of diagnosis and annually thereafter. The analysis was performed according to a previously described method (Araten, *et al* 1999, Sugimori, *et al* 2006) . The cutoff values of the assay were >0.003% for granulocytes and >0.005% for erythrocytes (Kulagin, *et al* 2014, Parker, *et al* 2005, Sugimori, *et al* 2009).

#### *Measurement of plasma TPO*

TPO levels were determined at the time of enrollment. All peripheral blood samples were sent to SRL Inc. (Tokyo, Japan) to measure the plasma TPO levels using a Chemiluminescent Enzyme Immunoassay (CLEIA). Briefly,

standard rhTPO (FUJIREBIO, Tokyo, Japan) and plasma samples were incubated in plate wells that were coated with mouse anti-human anti-rhTPO antibody (FUJIREBIO, Tokyo, Japan). Biotinylated rabbit anti-human rhTPO antibodies (FUJIREBIO, Tokyo, Japan) were added as the primary antibody. After incubation with streptavidin-alkaline phosphatase-labeled secondary antibodies (DAKO, Glostrup, Denmark), Substrate/Enhancer solution (Life Technologies, Carlsbad, CA, USA) was added, and the relative chemiluminescence intensity was measured using a luminometer (Molecular Device, Sunnyvale, CA, USA).

### *Statistical methods*

The laboratory parameters were compared between subgroups with different TPO levels. Fisher's exact test was used to analyze the prevalence of increased PNH-type cells and the Mann-Whitney U test was used for the analyses of the other clinical parameters. *P* values of <0.05 were considered to indicate statistical significance. All of the *P*-values were two-sided. The overall and failure-free survival rates were calculated according to the Kaplan-Meier method and the findings were compared using the log-rank

test. Treatment failure was defined as death or the progression of thrombocytopenia. We defined the progression of thrombocytopenia as a decrease in the platelet count by  $10 \times 10^9/L$  from the count at the time of enrollment. All of the statistical analyses were performed using the EZR software package (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for the R software program (The R Foundation for Statistical Computing version 2.13.0).

## Results

### *Patient characteristics*

Twenty-five HMT cases (male/female, 14/11) were enrolled in this study from August 2010 to March 2014, and the data for this interim analysis were collected in July 2015. The median follow-up period was 41 months (range, 19-59 months). The patient characteristics are shown in Table I. The median age was 65 years (range, 25-79 years). The mean values of the complete blood cell counts were as follows: WBC count,  $4.1 \times 10^9/L$  (range,  $2.77-8.50 \times 10^9/L$ ); Hb level, 11.9 g/dl (range, 10.0-14.8 g/dl), and platelet count,  $40.0 \times 10^9/L$  (range,  $1.0-95.0 \times 10^9/L$ ). PNH-type cells were detected in 7 of the 25 cases

(28%); the median clone sizes of the PNH-type cells were 0.073% (range, 0.015% - 20.5%) in granulocytes and 0.069% (range, 0% - 3.8%) in erythrocytes. Figure 1 shows representative scattergrams of PNH-type cells in three PNH(+) patients.

*The plasma TPO levels and their correlation with other laboratory parameters*

The median TPO level was 290 pg/ml (range, 14-1510 fmol/ml). Eleven (44%) patients had TPO levels of >320 pg/ml (TPO<sup>high</sup> patients), which was the lowest TPO level of patients with low-risk MDS whose pathophysiology was similar to that of AA (Seiki, *et al* 2013). Table II shows the relationships between the TPO levels and the clinical characteristics. When the clinical data in the TPO<sup>high</sup> patients and the patients with TPO levels of <320 pg/ml (TPO<sup>low</sup> patients) were compared, the TPO<sup>high</sup> patients showed significantly lower Hb levels (11.0 vs 12.4, P<0.01) and a higher prevalence of increased PNH-type cells (64% vs 0%, P<0.001) in comparison to the TPO<sup>low</sup> patients.

*Clinical course*

The clinical courses could be followed in 11 TPO<sup>high</sup> and 10 TPO<sup>low</sup> patients for a median of 27 months (range, 12 to 48 months) after their enrollment. Six of the 11 TPO<sup>high</sup> patients were treated with CsA while all of the TPO<sup>low</sup> patients were left untreated. Five of the six (PNH[+], n=4; PNH[-], n=1; 83%) TPO<sup>high</sup> patients responded well to CsA. The remaining patient who did not respond to CsA therapy was negative for PNH-type cells. The changes in the blood cell counts of the five patients are shown in Table III.

Thrombocytopenia recurred in one TPO<sup>high</sup> with PNH(+) patient in association with a dose-reduction of CsA. No toxicities greater than grade 2 developed in any of the patients who were treated with CsA in this study.

Thrombocytopenia progressed in 4 of the 5 (80%) TPO<sup>high</sup> (three PNH+ and two PNH-) patients who were followed without IST. Notably, two of the four progressive patients developed moderate AA at 12 and 15 months after the diagnosis of HMT (Figs 2 and S1A) and one died of sepsis at 24 months.

Among the 10 TPO<sup>low</sup> patients, two were treated with prednisolone (PSL) after being enrolled in this study; both improved. The remaining seven patients were followed throughout the observation period without treatment; one patient was treated with vitamins D and K. Thrombocytopenia remained

stable in three, spontaneously resolved in one, and progressed in four. One of the progressive patients eventually developed acute myeloid leukemia, which led to the patient's death at 22 months after the diagnosis of HMT (Fig. S1B).

As of July 2015, 19 of the 21 patients were alive. There was no difference in the four-year overall survival rates of the TPO<sup>high</sup> and TPO<sup>low</sup> patient groups (88% vs. 89%) (Fig. 3A), while the three-year failure-free survival rate of the CsA-treated TPO<sup>high</sup> patients (100%) was significantly higher than that of the untreated TPO<sup>low</sup> patients (20%, Fig. 3B).

## **Discussion**

This prospective analysis revealed that 44% of HMT patients were positive for markers that were suggestive of immune pathophysiology; 28% had increased PNH-type cells and 44% had high TPO levels. These results were consistent with our previous observation that 15 of 29 patients (55%) with HMT showed increased percentages of PNH-type cells and a good response to CsA (unpublished observation). All the of the patients who were positive for PNH-type cells in the current study had high TPO levels and the TPO<sup>high</sup>

patients tended to show lower Hb levels than the TPO<sup>low</sup> patients. Given the fact that two of the five TPO<sup>high</sup> patients who were followed without treatment developed AA, the lower Hb levels in the TPO<sup>high</sup> patients may be a feature of pre-AA.

The immune-mediated destruction of haematopoietic stem or progenitor cells plays a central role in the pathophysiology of AA and inflammatory cytokines, such as IFN- $\gamma$ , TNF- $\alpha$ , and TGF- $\beta$ , which are secreted from activated immune cells, are thought to inhibit haematopoiesis (El Mahgoub, *et al* 2014, Li, *et al* 2011, Nakao, *et al* 1997, Serio, *et al* 2011, Zeng and Katsanis 2015). Recent studies have shown that TGF- $\beta$  has suppressive effects on megakaryocyte/erythroid progenitor (MEP) proliferation (Akel, *et al* 2003, Xie, *et al* 2015, Zermati, *et al* 2000). Similar mechanisms to AA may exist in patients with HMT that manifests as thrombocytopenia alone, possibly because the levels of myelosuppressive cytokines that are present in the BM of patients with HMT are lower than those in patients with AA.

Half of the TPO<sup>high</sup> patients in the current study who were treated with CsA showed improvement while two of the five TPO<sup>high</sup> patients who were followed without IST progressed to moderate AA. These findings also

indicate that TPO<sup>high</sup> HMT patients have a similar pathophysiology to patients with AA. Previous reports showed that some patients with chronic ITP that was refractory to PSL or  $\gamma$ -globulin responded well to CsA (Emilia, *et al* 2008, Emilia, *et al* 2002). These studies did not refer to the megakaryocyte cellularity in the BM of their patients. The megakaryocyte number is generally increased or normal in patients with ITP (Harker and Finch 1969, Houwerzijl, *et al* 2004, Louwes, *et al* 1999, Wintrobe and Greer 2009). However, the recent diagnostic criteria proposed by the American Society Haematology and the British Society of Haematology do not necessitate a BM examination to assess megakaryocyte cellularity (2003, George, *et al* 1996). It is therefore possible that HMT patients who are responsive to CsA may be diagnosed as having ITP.

Half of the TPO<sup>low</sup> HMT patients who were followed without treatment showed the progression of cytopenia. It is known that both ITP and MDS patients show low TPO levels due to an increase in the number of megakaryocytes as well as the number of TPO receptors on megakaryocytes (Hou, *et al* 1998, Ogata and Tamura 2000, Tamura, *et al* 1998). In reality, two of our TPO<sup>low</sup> HMT patients responded well to PSL – similarly to

patients with typical ITP - suggesting that the megakaryocyte cellularity in the BM may have been underestimated in the TPO<sup>low</sup> HMT patients.

Considering the fact that one of the 10 TPO<sup>low</sup> HMT patients progressed to AML, TPO<sup>low</sup> HMT patients should be followed carefully. Their BM should be examined if their platelet counts show further decreases in order to rule out MDS and AML.

This interim analysis revealed that about half of the patients with HMT had a similar pathophysiology to AA, and suggested that early treatment with CsA may improve their prognosis. The evaluation of the number of PNH-type cells and the plasma TPO level might be useful for the appropriate management of patients with HMT. A long-term follow-up of a larger number of patients is necessary to clarify the impact of early CsA treatment on the prognosis of HMT.

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### **Author contributions**

C.S. and K.I. contributed equally to this work by performing the research and writing the paper. K.I designed the study. C.S. analyzed the data. H.Y., Y.Z. and S.N. managed the samples and collected the clinical data. All of the authors approved of the final version of this manuscript.

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## Figure legends

**Figure 1.** The detection of PNH-type cells in HMT patients. Scattergrams of three representative cases are shown. The numbers in the left upper quadrant represent PNH-type cell percentages.

**Figure 2.** Changes in the platelet count with time in nine TPO<sup>high</sup> patients. Five of the six patients who received cyclosporine (CsA) therapy showed platelet recovery. None of the patients who were followed without therapy showed an increased platelet count.

**Figure 3.** The overall survival rates (A) and failure-free survival rates in the TPO<sup>high</sup> patients (B).

**Figure S1.** The treatment outcomes of the HMT patients. (A) The TPO<sup>high</sup> patient group. \*Treatment with iron or anabolic steroids. (B) The TPO<sup>low</sup> patient group. TPO, thrombopoietin; CsA, cyclosporine; w/o, without; Tx, treatment; PSL, prednisolone; Vit., vitamin; AML, acute myeloid leukemia.

Table I. Patient characteristics

	Median (range)
Age (year)	65 (25-79)
male/female	14/11
WBC (x10 <sup>9</sup> /L)	4.1 (2.77-8.50)
Hb (g/dl)	11.9 (10.0-14.8)
Platelet (x10 <sup>9</sup> /L)	40.0 (1.0-95.0)
Positivity for PNH-type cells	7/25
Clone size of granulocytes (%)	0.073 (0.015-20.5)
Erythrocyte (%)	0.069 (0.0-3.8)
Plasma TPO level (fmol/ml)	8.4 (0.4-42.8)

WBC, white blood cell count; Hb, hemoglobin; PNH-type cells, paroxysmal nocturnal hemoglobinuria-type cells; TPO, thrombopoietin.

Table II. Relationships between plasma TPO levels and laboratory parameters.

	TPO <sup>high</sup> (n=11)	TPO <sup>low</sup> (n=14)	<i>P</i> value
WBC (x10 <sup>9</sup> /L)	3.37 (3.3-8.5)	5.5 (2.8-7.8)	0.13
Hb (g/dl)	11.2 (10.0-13.0)	12.4 (10.7-14.8)	< 0.01
Platelet (x10 <sup>9</sup> /L)	37.0 (6.0-75.0)	49.0 (1.0-95.0)	0.23
MCV (fl)	104.6 (85.0-115.0)	96.8 (85.0-110.3)	0.19
Positivity for PNH-type cells	7 (64%)	0 (0%)	< 0.001

WBC, white blood cell count; Hb, hemoglobin; PNH-type cells, paroxysmal nocturnal hemoglobinuria-type blood cells; TPO, thrombopoietin.

Table III. Changes in the blood cell counts of TPO<sup>high</sup> patients responsive to CsA at 12 months.

	WBC (x10 <sup>9</sup> /L)		Hb (g/dl)		Platelet (x10 <sup>9</sup> /L)	
	before	after	before	after	before	after
Case 1	3.7	3.9	12.7	12.6	22.0	75.0
Case 2	8.5	7.3	11.3	11.6	7.0	159.0
Case 3	3.3	3.8	10.7	11.2	51.0	108.0
Case 4	3.3	4.4	10.2	10.3	45.0	67.0
Case 5	4.6	5.0	11.1	11.3	50.0	73.0

WBC, white blood cell count; Hb, hemoglobin.

Fig 1

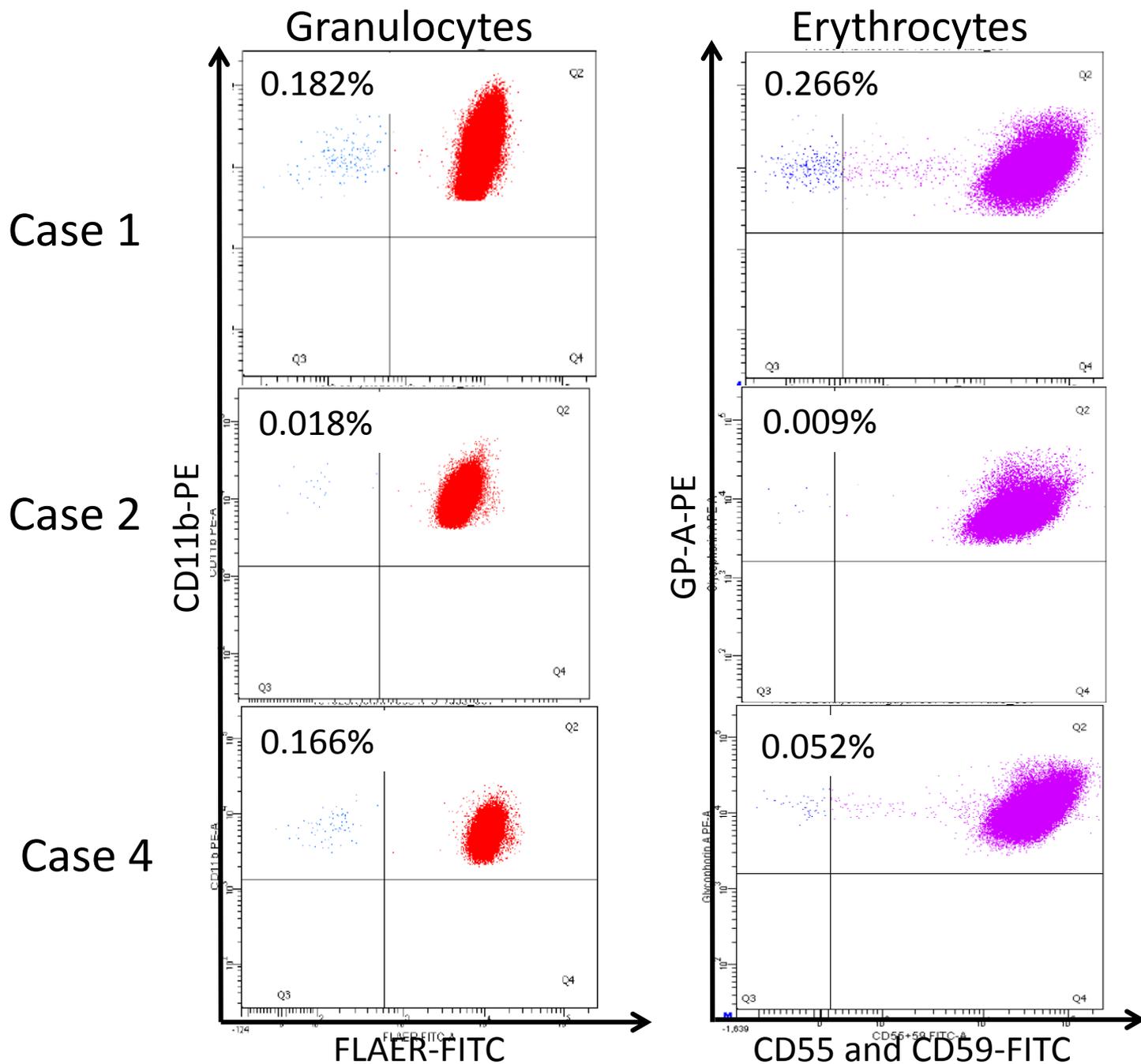
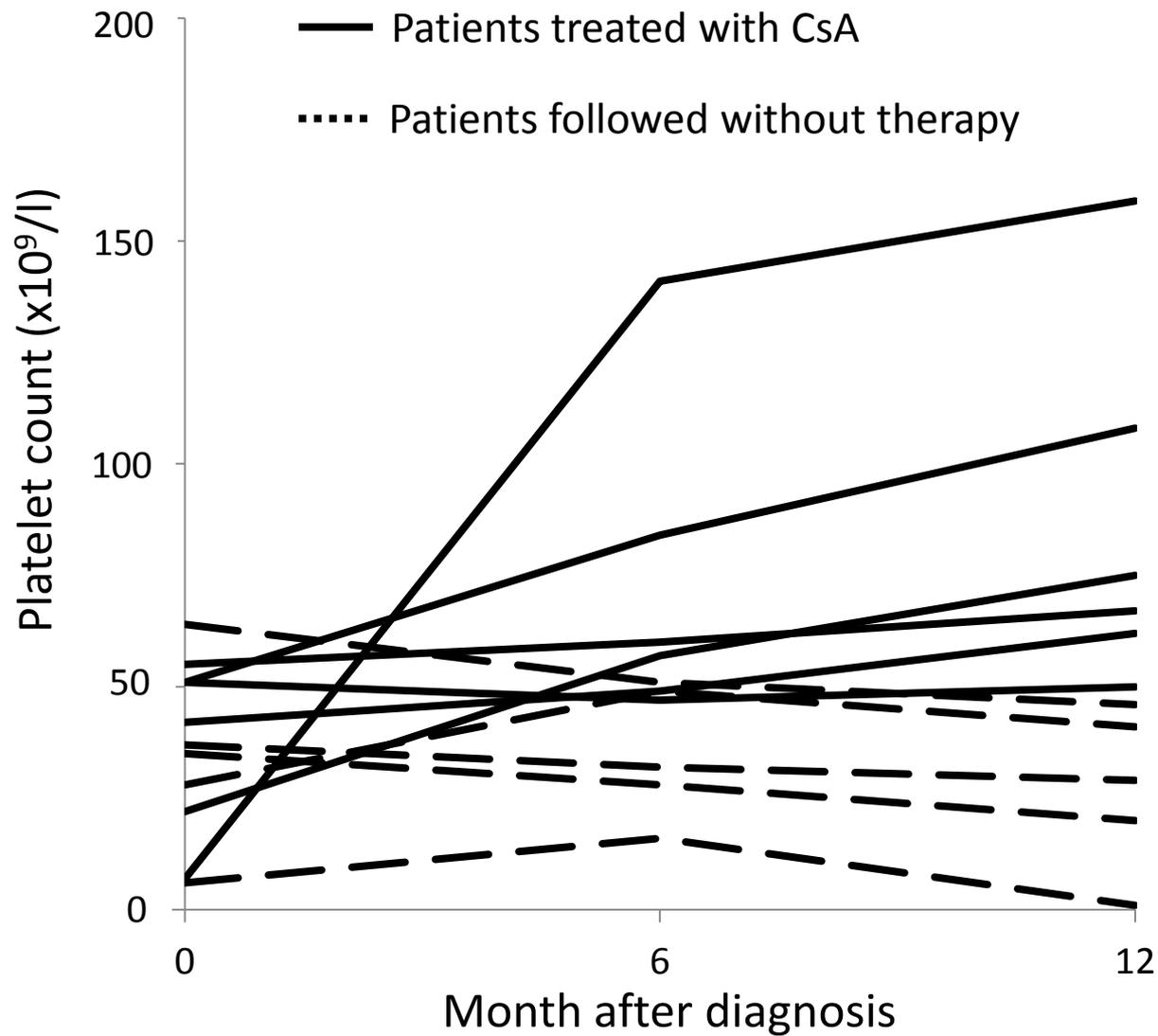
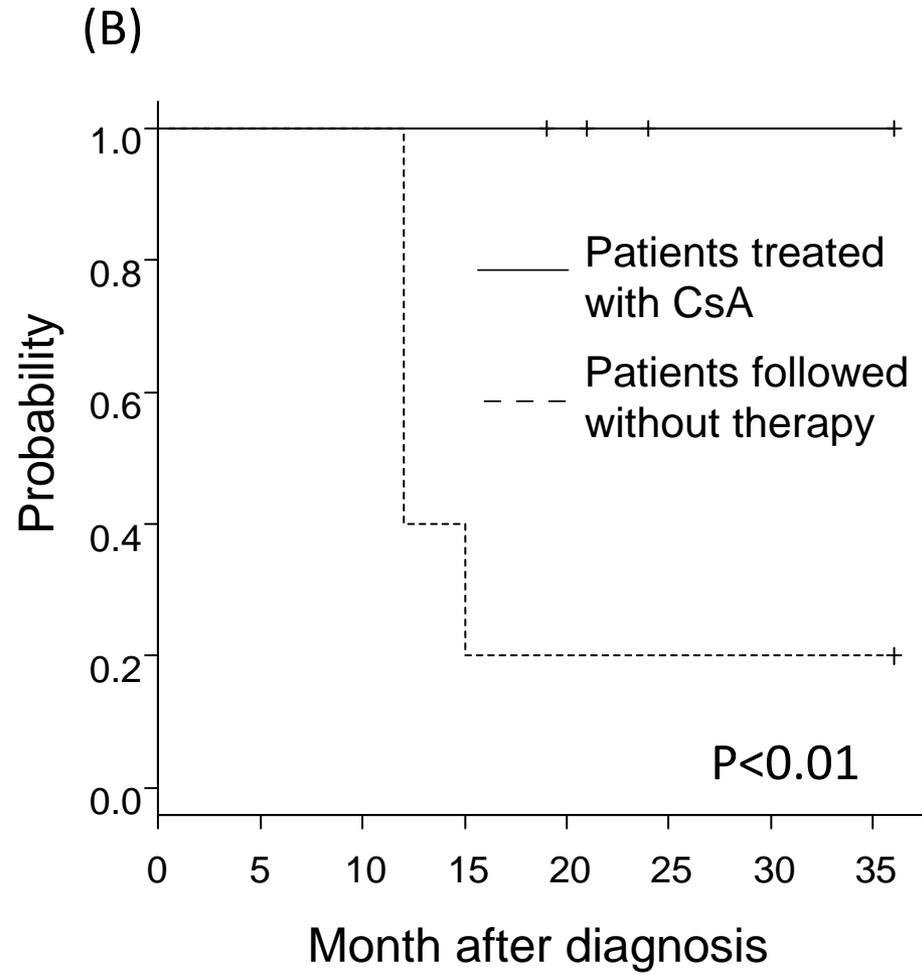
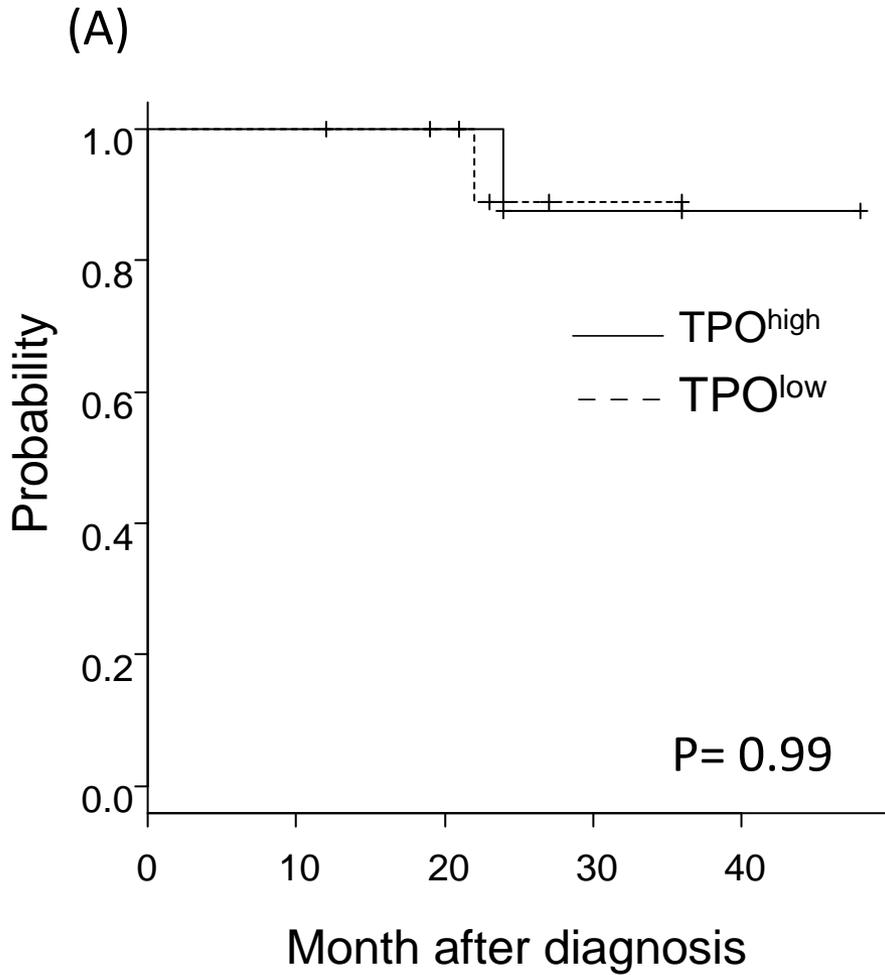


Fig 2

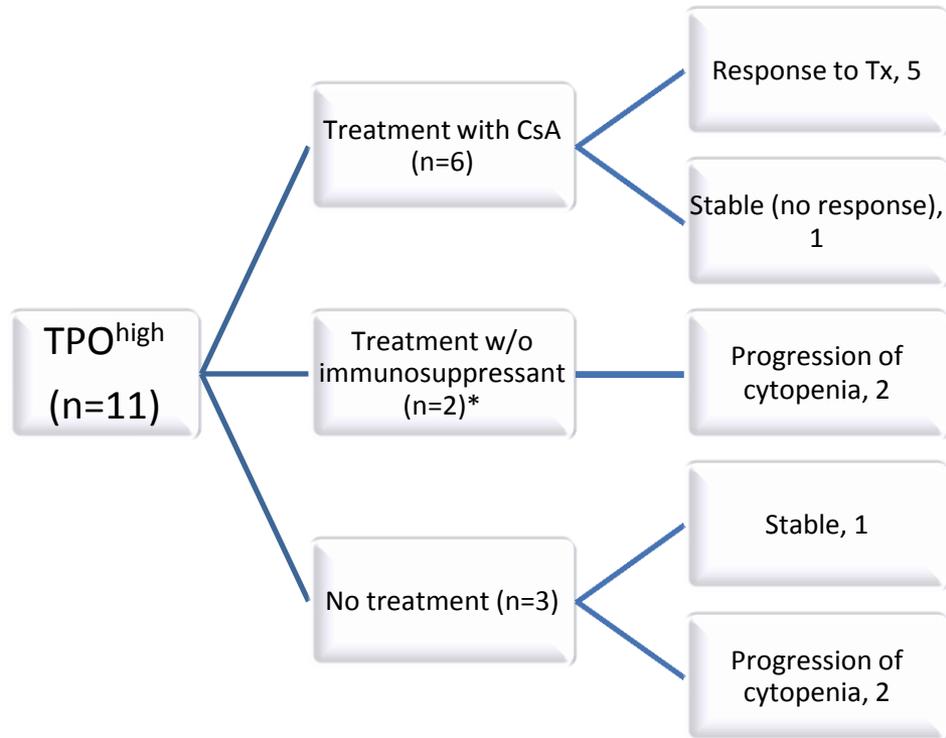


# Fig 3



# Supplementary Fig S1

(A)



(B)

