Rapidity and Severity of Hemoglobin Decreasing Associated with Erythrocyte Inosine Triphosphatase Activity and ATP Concentration during Chronic Hepatitis C Treatment

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The purpose of this study was to evaluate the association between therapy-induced hemoglobin (Hb) decreasing rapidity and severity with erythrocyte inosine triphosphatase (ITPase) activity and ATP concentration in chronic hepatitis C patients receiving chronic hepatitis C (HCV) treatment. Forty-three Japanese patients were included in the study. Erythrocyte ITPase activity before therapy was determined by HPLC-UV. Erythrocyte ATP concentrations before and during therapy were determined by luciferase assay. Genotyping for ITPA 94C>A (rs1127354) and IVS2+21A>C (rs7270101) was conducted using TaqMan probes. The median ITPase activity (µmol/h/g hemoglobin) of ITPA 94CC, CA, and AA genotypes was 136.8 (range, 80.4–289.6), 41.1 (24.3–93.1), and 11.8, respectively. ITPase activity and Hb decreasing showed a significantly inverse relationship at therapeutic weeks 2, 4, and 6 (p<0.01). Erythrocyte ATP concentration was decreased by therapy, and Hb decreasing was significantly and inversely correlated with erythrocyte ATP concentration at week 4 and after week 8 (p<0.001 and 0.05, respectively). ATP concentration for patients with ITPA 94CA was significantly lower than ITPA 94CC at week 4 (p=0.045). We concluded that ITPase activity plays an important function and that ATP concentration changes due to therapy are related to the Hb decreasing mechanism in the early period of therapy with HCV treatment.

Key words inosine triphosphatase activity; ATP; hemoglobin decreasing; chronic hepatitis C

Severe hemolytic anemia is an important limiting adverse effect associated with peg-interferon (Peg-IFN) and ribavirin (RBV) therapy for chronic hepatitis C. The mechanism of anemia induction has not been fully elucidated in vivo. To the best of our knowledge, therapy-induced anemia is caused by RBV-induced hemolysis, while bone marrow suppression is caused by protease inhibitors (PI) and Peg-IFN treatment. Fellay et al. and Thompson et al. reported that genetic variation in ITPA encoding inosine triphosphatase (ITPase) was a protective factor against hemolytic anemia in patients receiving Peg-IFN and RBV therapy.1–5 Some researchers reported that ITPA genotypes associated with severity of RBV induced anemia in Japanese.1–7 ITPA code ITPase, catalyzed the pyrophosphohydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP), and A variant of ITPA 94C>A (rs1127354) and IVS2+21A>C (rs7270101) was associated with reduced ITPase activity.8,9 Simone et al. reported that ITPase structural change by 94C>A variant and protein instability cause ITPase deficiency.10 Although ITPA variant is identical, hemoglobin (Hb) decreasing level was wide range. Peltenburg et al. reported that ITPase was more accurate predictor for RBV-induced anemia in hepatitis C patients than ITPA genotype.11 Although some patients with ITPA wild-type have been reported to have low ITPase activity value to that of patients with ITPA genetic mutation in healthy individuals and childhood patients with acute lymphoblastic leukemia,8,12–14 the association between ITPA genotype and ITPase activity has not been reported in patients who have chronic hepatitis C. Therefore, we hypothesize that ITPase activity might be affected rapidly of RBV-induced Hb decreasing.

Some researchers reported that ATP reduction was occurred by RBV therapy15,16 and decreasing ATP concentration may induce sensitivity for oxidative stress and hemolysis.18–20 RBV therapy-induced-decreasing ATP level is one of the mechanisms of RBV-induced hemolysis. Hitomi et al. investigated that ITPA genotype affect decreasing ATP concentration in vitro.21 They reported that erythrocyte ATP reduction was more severe in the wild-type ITPA genotype than in the variant genotype in vitro. However, it has been unknown that erythrocyte ATP concentration affect Hb decreasing level in clinical.

In the present study, we evaluated whether ITPase activity and ATP concentrations are associated with the rapidity of hemoglobin decreasing. We measured ITPase activity and ATP concentrations in erythrocytes of patients with chronic hepatitis C, and analyzed their relationship with Hb decreasing.

PATIENTS AND METHODS

Patients receiving therapy with Peg-IFN, RBV and PI were recruited into this study. A total of 43 patients with chronic hepatitis C were enrolled at Kitasato University Medical Center, Japan. The protocol was approved by the Ethics Committee of Kitasato University Medical Center (approval no. 26-12). The study was conducted after approval had been obtained from the institutional ethics committees.

Blood samples were collected in heparinized tubes, following which erythrocytes were separated from plasma and leukocytes by centrifugation and washed three times with saline.

ITPase activity was measured in erythrocyte lysates using a HPLC procedure based on the conversion of ITP to IMP, as previously described.19 The enzymatic reaction was stopped

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using perchloric acid and saturated dipotassium hydrogen phosphate. IMP was quantified by measuring the absorbance at 262 nm, after separation on a C18 column using 20 mmol/L phosphate buffer (pH 2.5) as the mobile phase. Within-day and between-day imprecision values of <3% and <7%, respectively, and an inaccuracy value of <1.2% were verified using pooled erythrocytes.

ATP concentrations were determined by luciferase assay method. Erythrocyte samples from patients were washed three times with saline and added to the ATP assay kit for Blood (Toyo B-Net Co., Ltd., Tokyo, Japan), then measured luminescence using GENE LIGHT GL-200 (Microtec Co., Ltd., Chiba, Japan). For evaluation of ATP concentration, we calculated the ATP concentration per 10^6 erythrocytes (pmol/10^6 RBC).

Genotyping was performed before therapy. DNA was extracted from buffy coat samples using the QIAamp Blood Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. Total genomic DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies, U.S.A.). Real-time TaqMan allelic discrimination polymerase chain reaction (PCR, StepOne; Applied Biosystems, U.S.A.) was used for ITPA 94C>A (rs1127354) and IVS2 +21 A>C (rs7270101) genotyping. Primers and probes were obtained from Applied Biosystems.

Statistical analysis was conducted using SPSS Statistics, version 19 (IBM Corp., NY, U.S.A.). The differences in ITPase activity between wild-type ITPA and the variant was compared using the Mann–Whitney U test. Statistical correlation between ITPase activity, decreased Hb concentration, and ATP concentration were evaluated using Spearman’s rank-order correlation coefficient test. The difference in ATP concentration during therapy was evaluated using the Kruskal–Wallis test and Wilcoxon signed rank test. Differences were defined statistically significant at p<0.05.

RESULTS

**ITPase Activity and Hb Concentration** In total, 43

Japanese patients with hepatitis virus C (HCV) were evaluated in this study. The patients’ characteristics were shown in Table 1 and Supplementary Table. We did not detect the ITPA IVS2+21 A>C polymorphism in any of our patients. The A allele frequency for ITPA 94C>A was 0.16 and not different from database of The National Center for Biotechnology Information.19 The median ITPase activities (µmol/L/h/gHb) of the 94 CC, CA, and AA genotypes were 136.8 (range, 80.4–289.6), 41.1 (24.3–93.1), and 11.8, respectively (Fig. 1). The ITPase activity of the 94CC was significantly higher than that of the 94CA and AA (p<0.001), and ITPase activity exhibited wider variation especially in CC genotype.

To evaluate the relationship between ITPase activity and rapidity of Hb decreasing, we investigated the correlation between ITPase activity in erythrocytes and the degree of decreasing Hb concentration after 2, 4, 6, 8, and 12 weeks of therapy. At therapeutic weeks 2, 4, and 6, ITPase activity and decreasing Hb concentration showed a significant inverse relationship (p<0.01), and the values for the correlation coef-
ATP Concentration Association with Hemoglobin Decreasing

Preliminary, we evaluated tendency of therapy induced changing erythrocyte ATP concentration during therapy every 4 weeks in 14 patients. ATP concentration was showed minimum value at 4 weeks and then achieved a steady concentration after 8 weeks of therapy (Supplementary Figure). Therefore, we used ATP concentration after 8 weeks as value at 8 weeks to evaluate association with Hb decreasing level in this study. We evaluated ATP concentration for 22 patients who could be measured ATP concentration at all three points, which were before therapy, at 4 weeks, and after 8 weeks of therapy. In 22 patients, ATP concentration at 4 weeks and after 8 weeks was significantly lower than before therapy ($p<0.001$, Table 2). The ATP concentration was significantly correlated with Hb decreasing level at 4 weeks ($r=-0.709$, $p<0.001$, Fig. 3) and at 8 weeks ($r=-0.443$, $p<0.05$, respectively, Fig. 3). Then, we evaluated the association between ATP concentration and Hb decreasing level in each $ITPA$ genotype. ATP concentration for patients with $ITPA$ 94CA was significantly lower than $ITPA$ 94CC at 4 weeks ($p=0.045$, Table 2), and ATP concentrations for $ITPA$ 94CC and CA were significantly correlated with Hb decreasing levels at 4 weeks.

### Table 2. Erythrocyte ATP Concentration

<table>
<thead>
<tr>
<th>$N$</th>
<th>Erythrocyte ATP concentration (pmol/10^6 RBC, range)</th>
<th>$p$-Value (vs. before therapy)</th>
<th>Change rate (from before therapy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before therapy</td>
<td>4 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>All</td>
<td>22</td>
<td>110 (41–225)</td>
<td>71 (25–174)</td>
</tr>
<tr>
<td>$ITPA$ 94CC</td>
<td>15</td>
<td>116 (62–225)</td>
<td>73 (62–174)</td>
</tr>
<tr>
<td>$ITPA$ 94CA</td>
<td>7</td>
<td>10.4 (41–122)</td>
<td>58 (25–79)</td>
</tr>
</tbody>
</table>

![Fig. 2. The Correlation of ITPase Activity with the Reduction in Hemoglobin (Hb) Concentration at Therapeutic Week 2, 4, and 6 Compared with the Start of Therapy](image1)

![Fig. 3. The Correlation of Erythrocyte ATP Concentration with the Reduction in Hemoglobin (Hb) Concentration at 4 Weeks and 8 Weeks of Therapy](image2)
ATP decreasing rates were not different between ITPA 94CC and CA (Table 2). We evaluated the association between ATP concentrations and Hb decreasing levels in each sexes and Pls, and there were significant association at week 4 ($p<0.05$).

**DISCUSSION**

ITPA variant is protective factor for RBV-induced anemia during HCV treatment with PI.\(^1\)\(^2\) We found a correlation between ITPAase activity and rapidity of Hb decreasing in the early period of therapy, with high ITPAase activity related with a rapid Hb decrease during HCV treatment with PI. The mechanism by which low activity variants in the ITPA gene protect against the ATP reduction observed during treatment with RBV *in vitro*.\(^\text{17}\) In present clinical study, as association between ATP and Hb decreasing, high ATP concentration showed severe Hb decreasing, and erythrocyte ATP concentrations were significantly correlated with Hb decreasing levels at 4 weeks and 8 weeks.

RBV-induced anemia is a limiting factor of HCV treatment with PI clinically. Some studies have reported risk factors for RBV-induced anemia in HCV treatment with PI.\(^3\)\(^4\) At baseline, age, hemoglobin level, renal function were risk factor for anemia.\(^5\) Genetic risk factors for anemia have been reported genotype of ITPA, SLC28A2, and SLC29A1.\(^6\)\(^7\) In these factors, ITPA genotype was major factor for severe anemia during RBV therapy.\(^7\) Although ITPA variant is identical, Hb decreasing level was wide range, especially ITPA 94CC. As ITPAase was encoded by ITPA and distributed over a wide range, we hypothesized that ITPAase activity more accurately predict rapidity and severity of therapy-induced Hb decreasing. Peltenburg et al. reported in 106 Dutch HCV patients that patients with low ITPAase activity were less frequently anemia than normal activity (21% vs. 78%).\(^1\) They concluded that ITPAase activity predicts RBV-induced anemia more accurately than ITPA genotype. The correlation between ITPAase activity value and Hb decreasing has not been shown. Our study demonstrated significant correlation between ITPAase activity and of Hb decreasing level in the early stages of therapy. This wide distribution of ITPAase activity could be responsible for the difference in decreasing Hb concentrations in the wild-type genotype.

It is generally assumed that ATP deficiency and the rise in susceptibility to oxidative damage is one of the mechanisms of RBV-induced anemia. Some researchers found that RBV reduces ATP levels in *in vitro* erythrocytes. Hitomi *et al.* reported that ATP deficiency was also protective mechanism of erythrocytes. Hitomi reduces ATP levels in *in vitro* regimen of RBV-induced anemia. Some researchers found that RBV susceptibility to oxidative damage is one of the mechanisms and of Hb decreasing level in the early stages of therapy. This demonstrated significant correlation between ITPAase activity value and Hb decreasing has not been shown. Our study demonstrated significant correlation between ITPAase activity and of Hb decreasing level in the early stages of therapy. This wide distribution of ITPAase activity could be responsible for the difference in decreasing Hb concentrations in the wild-type genotype.

High erythrocyte RBV concentration has been reported one of the factors of severe anemia.\(^2\)\(^3\)\(^4\)\(^6\)\(^8\) De Franceschi *et al.* reported *in vitro* that erythrocyte ATP reduction was more severe in 1 mmol/L RBV stimulation.\(^5\) Therefore, some researchers assumed that high RBV concentration decrease ATP concentration.\(^6\)\(^8\) but there was no report about association between RBV concentration and ATP concentration in patients with RBV therapy. In our study, although we did not evaluate erythrocyte RBV concentration, patients with severe Hb decreasing were found small decreasing or increased ATP concentration from start of therapy in each ITPA genotype and sex. Therefore, erythrocyte ATP changing effect for Hb decreasing in patients with HCV treatment was different tendency from *in vitro*. Mechanisms of Hb decreasing related with ITPAase activity have been unclear in patients receiving therapy yet in clinical setting. In the future, we need to evaluate the association between ATP concentration, RBV concentration, and ITPAase activity in patients’ erythrocyte during therapy.

ITPAase activity value is responsible for the decreasing Hb concentration in the early period of therapy with HCV treatment with PI. And higher erythrocyte ATP concentration was significantly associated with severity of Hb decreasing at 4 therapeutic weeks. We concluded that ITPAase activity plays an important function and ATP concentration change by therapy relates to Hb decreasing mechanism in the early period of therapy with HCV treatment.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**REFERENCES**


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