Post-therapeutic recovery of serum interleukin-35 level might predict positive response to immunosuppressive therapy in pediatric aplastic anemia

Zhen Huang\textsuperscript{a}, Hongfei Tong\textsuperscript{b}, Yuan Li\textsuperscript{b}, Haixia Zhou\textsuperscript{a}, Jiangchao Qian\textsuperscript{a}, Juxiang Wang\textsuperscript{a} and Jichen Ruan\textsuperscript{a}

\textsuperscript{a}Department of Hematology, Yuying Children’s Hospital, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China; \textsuperscript{b}Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

ABSTRACT

Background: The predictive value of interleukin-35 (IL-35) on efficacy of immunosuppressive therapy (IST) in aplastic anemia (AA) has not been well investigated. The aim of the study was to evaluate the association between serum IL-35 level and response to IST in pediatric AA.

Methods: A total of 154 children with AA and 154 controls were included between January 2012 and December 2013. Blood and bone marrow fluid specimens were collected. Serum level of IL-35 was determined by enzyme-linked immunosorbent assay. Patients were treated with IST, and response to therapy was evaluated during 180-day follow-up period after starting therapy.

Results: Serum levels of IL-35 at admission decreased significantly in patients compared with that in controls (10.9 ± 5.5 pg ml\textsuperscript{-1} and 45.3 ± 8.8 pg ml\textsuperscript{-1}, \(p<0.001\)). After starting IST, serum levels of IL-35 in patients recovered 30.7 ± 9.7 pg ml\textsuperscript{-1} in the first 28 days (\(p<0.001\)). During the follow-up period, increased range of serum IL-35 level ≥30.7 pg ml\textsuperscript{-1} in the first 28 days was associated with effective response to therapy (odds ratio 7.97, 95% confidence interval 3.82–16.79). In addition, Fas/FasL protein expression in bone marrow mononuclear cells dropped significantly in the same group of patients in the first 28 days (\(p<0.05\)).

Conclusion: The study revealed that post-therapeutic recovery of circulating IL-35 concentration might be an independent predictor for effective response to IST in pediatric AA. Moreover, apoptosis might be involved in such a forecasting process.

1. Introduction

Acquired aplastic anemia (AA) is an immune-mediated blood system disease characterized by hypocellular bone marrow and peripheral pancytopenia [1]. In Western countries, incidence of AA is approximately two per million people each year [2], but is several times higher in Asian countries [3]. Certain drugs, radiation, viral infection and immune disease are common causes of AA. However, many patients have no clear cause [4]. Previous studies have revealed that immunological tolerance loss and excessive apoptosis in bone marrow stem cells might play an essential role in onset of AA [5,6].

Immunosuppressive therapy (IST) including antithymocyte globulin (ATG) and cyclosporine A (CsA) is a widely used therapy for AA [7]. Pharmacological mechanisms include inhibition of T-cell proliferation, reconstruction of immune tolerance and inhibition of bone marrow stem cell apoptosis [8,9]. The hematopoietic response rate to IST in AA patients is 42–74%, and the long-term overall survival rate is about 90% [10,11]. Therefore, response to IST in patients showed heterogeneity. Such heterogeneity might be related to diverse apoptosis levels in bone marrow stem cells.

As we all know, early assessment of response to IST is meaningful. Several potential predictors such as age, gender, interval between diagnosis and therapy, white blood cell (WBC) count, absolute reticulocyte count (ARC), absolute neutrophil count (ANC) and absolute lymphocyte count (ALC) have been proposed, but still cannot meet the clinical needs [12–14].

Interleukin-35 (IL-35) is a heterodimeric cytokine, which is first identified in 2007 [15]. More and more evidence have proved that IL-35 plays a critical role in anti-inflammatory and immunotolerance processes [16,17]. Previous studies have demonstrated the relationships between serum IL-35 level and several immune-related diseases [18–22]. Furthermore, Yu et al. have preliminarily explored the role of serum IL-35 level in AA and have suggested that decreased serum level of IL-35 might contribute to the loss of immune tolerance in AA patients [23].

At present, potential relationship between serum IL-35 level and response to IST in acquired AA children has not been well elucidated. Therefore, we conducted a prospective observational study to clarify the clinical significance of circulating IL-35 concentration in response to IST and to further reveal the association...
of response to IST with bone marrow stem cell apoptosis in acquired AA children.

2. Materials and methods

2.1. Participants

From 1 January 2012 to 31 December 2013, a total of 154 children with acquired AA from Yuying Children’s Hospital were enrolled in the study. Inclusion criteria were predefined as follows: (1) meet the international study group criteria for AA [24]; (2) meet severe aplastic anemia (SAA) or very severe aplastic anemia (VSAA) criteria; (3) less than 18 years old; (4) lack of available donors; (5) obtain consent to IST and (6) never receive an IST before.

SAA was defined as follows: (1) bone marrow cellularity <25%, or 25–50% with <30% residual hemopoietic cells; (2) two-thirds of the following: neutrophil count <0.5 × 10^9 l^-1, platelet count <20 × 10^9 l^-1 or reticulocyte count <20 × 10^9 l^-1 [25]. VSAA was defined as follows: as for SAA but neutrophil count <0.2 × 10^9 l^-1 [26].

Exclusion criteria were as follows: (1) fail to meet SAA or VSAA criteria; (2) older than or equal to 18 years old; (3) receive a hematopoietic stem cell transplantation; (4) suffer from paroxysmal nocturnal hemoglobinuria or Fanconi anemia; (5) suffer from other hematological diseases or autoimmune diseases; (6) suffer from severe sepsis or malignant tumour and (7) receive a hematopoietic stem cell transplant before.

The control group included 154 volunteers who were randomly selected from children with ocular trauma in Yuying Children’s Hospital. Written informed consents were obtained from all participants and their guardians. The study was approved by the ethics committees of Yuying Children’s Hospital.

2.2. Therapy and response evaluation

Each patient received a standard IST, including rabbit ATG (Thymoglobulin; Genzyme, Cambridge, MA, USA) and CsA [24]. After an allergy test, ATG was given intravenously for 5 days with methylprednisolone and chlorphenamine (daily dose of ATG was 3.75 mg kg^-1). Meanwhile, oral CsA was given at 5 mg kg^-1 daily. Adjust oral dose continuously and keep the trough serum level of CsA between 100 and 150 μg l^-1. Oral CsA therapy would be continued for at least one year.

Response to IST was evaluated in the first 180 days after starting therapy according to established criteria described by Camitta in 2000 [27]. The criteria had classified the overall response into no response (NR), partial response (PR) and complete response (CR). Both PR patients and CR patients were defined as responders, and NR patients were defined as non-responders.

2.3. Data collection

Demographic and other necessary information of each participant was obtained from medical records.

Blood specimens were collected from all participants including AA children and controls at admission. In a preliminary study, we discovered that serum IL-35 levels in patients with effective response to IST had the most significant increase in the first 28 days. However, after this period of time, the increase in trend became more gentle (data not showed). Therefore, blood specimens were also collected from AA children at the 28th day after starting IST. The specimens were centrifuged and stored at −80°C until laboratory analysis. The serum level of IL-35 was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biolegend, San Diego, CA, USA).

Meanwhile, two bone marrow fluid specimens were obtained from each AA patient separately at admission and at the 28th day after starting IST. Bone marrow mononuclear cells (BMMCs) were isolated by Ficoll-Hypaque (1.077 g ml^-1) density gradient centrifugation. BMMCs were cultured at 37°C with 5% CO2 atmosphere for 7 days, and serum-free cell culture mediums (TexMACS Medium; Miltenyi Biotec Co, Bergisch Gladbach, Germany) supplemented with penicillin (2 mmol l^-1) were adopted for cell culture. BMMCs and culture supernatants were collected for latter analysis.

Distribution of T-cell subsets and expression of Fas/FasL proteins in BMMCs were detected using flow cytometry. BMMCs were stained with CD4/IFN-γ, CD4/IL-4, CD4/CD25, CD3/CD8, CD34/Fas, CD8/FasL and their isotype control monoclonal antibodies (Biolegend) according to the manufacturer’s instructions. FACSCalibur flow cytometer was adopted for further detection (Becton Dickinson & Co, San Antonio, TX, USA). Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) levels in culture supernatants were measured using a commercial ELISA kit (Boshide Biotech Co, Wuhan, China).

2.4. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences, version 17.0 (SPSS, Chicago, IL, USA). Difference of continuous variables was measured by an independent specimen t-test, and difference of dichotomous variables was determined by the χ^2 test. Difference of response rates to
IST was evaluated using survival analysis. The $p$ value of $<0.05$ was considered statistically significant. To estimate relative risk, multivariate logistic regression analysis was used to calculate odds ratio (OR) with 95% confidence interval (CI). If CI did not include value 1, it was considered to be significant statistically.

### 3. Results

In Table 1, 154 AA patients and 154 controls were well matched in age, gender and race ($p = 0.619, 0.135$ and 0.258). Most patients were idiopathic AA cases (81.2%). In addition, 70 patients met SAA criteria and 84 patients met VSAA criteria.

At admission, serum levels of IL-35 decreased significantly in patients compared with that in controls ($10.9 \pm 5.5$ and $45.3 \pm 8.8$ pg ml$^{-1}$, $p < 0.001$). Serum levels of IL-35 in VSAA patients were even lower than those in SAA patients ($6.3 \pm 2.3$ and $15.5 \pm 3.3$ pg ml$^{-1}$, $p < 0.001$). At the 28th day after starting IST, serum levels of IL-35 in patients were $41.6 \pm 7.5$ pg ml$^{-1}$ and remarkably increased $30.7 \pm 9.7$ pg ml$^{-1}$ compared with that at admission ($p < 0.001$).

In Table 2, patients were divided into the elevated group ($\geq 30.7$ pg ml$^{-1}$, $n = 72$) and the non-elevated group ($<30.7$ pg ml$^{-1}$, $n = 82$) according to the increased range of IL-35 in the first 28 days. Only ARC was significantly higher in the elevated group than that in the non-elevated group ($p < 0.001$). During the follow-up period of 180 days, 89 patients were defined as responders and 65 patients were defined as non-responders. In Table 3, increased range of IL-35 $\geq 30.7$ pg ml$^{-1}$ in the first 28 days was associated with effective response to IST (OR: 7.97, 95% CI: 3.82–16.79). Excluding the patients with ARC at admission, serum levels of IL-35 increased in the elevated group as well as in the non-elevated group ($p < 0.001$), which might show the improvement of hematopoietic function. At admission, there were no differences in culture supernatants levels of IFN-$\gamma$ and TNF-$\alpha$ between the elevated group and the non-elevated group ($p = 0.591$ and 0.631). At the 28th day, the TNF-$\alpha$ level in the elevated group was significantly lower than that in the non-elevated group ($p < 0.001$), but such a decline trend was not observed in IFN-$\gamma$ ($p = 0.578$). It was suggested that the apoptosis level was significantly decreased in the elevated group compared with that in the non-elevated group.

### Table 1. Characteristics of participants in the study.

| Category                  | AA group | Control group | $p$ value |
|---------------------------|----------|---------------|-----------|
| Number of participants    | 154      | 154           | –         |
| Age (years, mean ± SD)$^a$| 8.3 ± 3.6| 7.9 ± 3.9     | 0.619     |
| Gender                    |          |               |           |
| Boys                      | 94       | 81            | 0.135     |
| Girls                     | 60       | 73            |           |
| Race                      |          |               |           |
| Han                       | 146      | 141           | 0.258     |
| Other                     | 8        | 13            |           |
| Etiology                  |          |               |           |
| Idiopathic                | 125      | –             |           |
| Hepatitis-associated      | 29       | –             |           |
| Severity                  |          |               |           |
| Severe                    | 70       | –             |           |
| Very severe               | 84       | –             |           |

$^a$SD, standard deviation; AA, aplastic anemia.

### Table 2. Differences of several potential predictors between the elevated group and the non-elevated group.

| Category                  | Elevated group | Non-elevated group | $p$ Value |
|---------------------------|----------------|--------------------|-----------|
| Number of patients (n)    | 72             | 82                 | –         |
| Etiology                  |                |                    |           |
| Idiopathic                | 56             | 69                 | 0.313     |
| Hepatitis-associated      | 16             | 13                 |           |
| Severity                  |                |                    |           |
| Severe                    | 29             | 41                 | 0.227     |
| Very severe               | 43             | 41                 |           |
| IBDAT                     |                |                    |           |
| <30 days                  | 33             | 45                 | 0.263     |
| $\geq$30 days             | 39             | 37                 |           |

### Table 3. Association of serum IL-35 level with effective response to immunosuppressive therapy in pediatric aplastic anemia.

| Category                  | Effective response to IST (n) | Total (n) | Multivariate analysis$^a$ OR (95% CI) |
|---------------------------|-------------------------------|-----------|--------------------------------------|
| IL-35 at admission        |                               |           |                                      |
| <25 × 10$^9$ $\Gamma^{-1}$| 31                            | 73        | Reference 3.45 (1.78, 6.71)          |
| $\geq$25 × 10$^9$ $\Gamma^{-1}$ | 58                   | 81        |                                      |
| IL-35 at admission        |                               |           |                                      |
| <10.9 pg ml$^{-1}$        | 40                            | 78        | Reference 1.73 (0.92, 3.37)          |
| $\geq$10.9 pg ml$^{-1}$   | 49                            | 76        |                                      |
| IL-35 increased in the first 28 days $^b$ | 30              | 82        | Reference 7.97 (3.82, 16.79)         |
| <30.7 pg ml$^{-1}$        | 59                            | 72        |                                      |
| $\geq$30.7 pg ml$^{-1}$   | 54                            | 66        | 12.45 (3.45, 46.01)                   |

$^a$OR, odds ratio; CI, confidence interval; IL-35, interleukin-35; IBDAT, interval between diagnosis and therapy.

$^b$Excluding patients with absolute reticulocyte count at admission $<25 \times 10^9 \Gamma^{-1}$.
In Table 4, T-cell subsets at admission were evenly distributed between the elevated group and the non-elevated group ($p > 0.05$). At the 28th day after starting IST, distribution of T-cell subsets had no change compared with that at admission ($p > 0.05$). In Figure 3, expression levels of CD34/Fas and CD8/FasL were separately equivalent between the elevated group and the non-elevated group ($p = 0.959$ and 0.425). In the elevated group, expression levels of CD34/Fas and CD8/FasL remarkably dropped at the 28th day after starting IST compared with that at admission ($p = 0.012$ and 0.001). However, no such differences for CD34/Fas and CD8/FasL were discovered in the non-elevated group ($p = 0.362$ and 0.090). It was also suggested that the apoptosis level was significantly decreased in the elevated group compared with that in the non-elevated group.

### 4. Discussion

A previous study published in 2015 demonstrated that decreased serum level of IL-35 in AA contributed to loss of immune tolerance and also revealed that the serum level of IL-35 was closely correlated with disease progression.
severity [23]. However, it did not determine the clinical significance of serum IL-35 level on response to IST in AA patients.

In the present study, serum levels of IL-35 remarkably dropped in children with AA, and severe cases showed the lowest levels of IL-35. This was consistent with the previous study mentioned above [23]. A similar relationship was also discovered in inflammatory bowel disease [20], primary immune thrombocytopenia [21] and systemic lupus erythematosus [28]. However, in systemic sclerosis [18], rheumatoid arthritis [19] and malignant tumour [22,29], serum levels of IL-35 were unexpectedly elevated and fell back to normal after therapy [19,29]. Therefore, IL-35 might play a complex role in those diseases. Further research should be conducted to determine underlying mechanisms involved.

The present study had demonstrated that increased trend of circulating IL-35 concentration in the first 28 days, but not circulating IL-35 concentration at admission, had an ability to predict IST efficacy. First, serum levels of IL-35 significantly recovered in the first 28 days after starting therapy. Second, increased range of IL-35 was not associated with several predictors for IST efficacy, except ARC. Third, multivariate regression analysis excluded the impacts of ARC and other confounding factors and reported a sixfold increased possibility of effective response to IST in AA patients with increased range of serum IL-35 \( \geq 30.7 \text{ pg ml}^{-1} \) in the first 28 days. However, the relationship between IL-35 level at admission and effective response to IST has not been proved statistically. Fourth, survival analysis revealed that patients with increased range of serum IL-35 \( \geq 30.7 \text{ pg ml}^{-1} \) after therapy had more satisfactory response rate to IST. Therefore, increased range of serum IL-35 level might be an independent predictor of response to IST in AA patients.

TNF-\( \alpha \) and IFN-\( \gamma \) were common hematopoietic negative regulators, and TNF-\( \alpha \) was also a cytokine involved in the apoptosis process. In the study, levels of both cytokines dropped after IST, and levels of TNF-\( \alpha \) showed more decline in the elevated group. Fas and its ligand FasL were apoptosis-related membrane surface molecules [30,31]. At admission, expression levels of Fas/FasL in the elevated group were similar to that in the non-elevated group. After IST, expression levels of Fas/FasL in the elevated group dropped significantly, but was unchanged in the non-elevated group. Taken together, such results gave us a hint that apoptosis might be a bridge between post-therapeutic serum IL-35 level and response to IST. However, more in-depth researches should be conducted in the future.

In contrast, there were no differences in distribution of T-cell subsets between the elevated group and the non-elevated group. Percentages of T-cell subsets were also not changed before and after IST. Long life-time of T cell might be partly explained the result mentioned above, which also limited the clinical significance of T-cell subset in IST efficacy.

All patients received combination therapy of rabbit ATG and CsA. Each patient was also treated with antibiotic therapy, liver protection therapy and supportive therapy. However, hemopoietic growth factors were not adopted due to poor efficacy and potential possibility of worsening illness [32,33]. In addition, no patient received a blood transfusion before the last blood specimen collection, because blood transfusion might directly affect the serum level of IL-35.

In conclusion, the present study revealed that post-therapeutic recovery of circulating IL-35 concentration, but not circulating IL-35 concentration at admission, might be an independent predictor of effective
response to IST in AA children. We also obtained some interesting, but not sufficient evidence that apoptosis might be involved in the forecasting process of IL-35 on response to IST.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Notes on contributors**

Dr Huang Zhen is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of hematology.

Dr Tang Hongfei is a researcher at the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of hematology.

Dr Li Yuan is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. He is an expert in the field of epidemiology.

Dr Zhou Haixia is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of hematology.

Dr Qian Jiangchao is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of epidemiology.

Dr Ruan Jichen is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of hematology.

Dr Qian Jiangchao is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of epidemiology.

Dr Wang Juxiang is a researcher at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. She is an expert in the field of hematology.

Dr Ruan Jichen is a professor at Yuying Children’s Hospital and the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China. He is an expert in the field of hematology.

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