Investigation of the correlation between immune thrombocytopenia and T cell activity-regulated gene polymorphism using functional study

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Thrombocytopenia is a condition where the platelet count is under $100 \times 10^9/L$, which is caused by various disorders. However, the mechanism of thrombocytopenia is still unclear. Hence, we tried to investigate the correlation between immune thrombocytopenia (ITP) and single nucleotide polymorphisms (SNPs) of genes related to T cell activation. There were 32 ITP patients and 30 healthy controls enrolled in this study. PCR and sequencing were used to find out the significant SNPs, which we focused on the promoter region of CTLA4 and CD28. In this study, the ITP cases were divided into primary ITP group, secondary ITP group, and the combination of the two to the follow-up analysis. Moreover, dual-luciferase reporter assay was used to evaluate the transcription activity of the significant SNP. We found the $-1765$ rs11571315 of CTLA4 gene was associated with primary ITP ($p = 0.006$), secondary ITP ($p = 0.008$), and the combination of the two ($p = 0.003$). Moreover, the $-318$ rs5742909 also had statistical significance in secondary ITP group that was only caused by autoimmune disease ($p = 0.019$). In functional study, the rs5742909 would decrease 19% of the transcription activity when it carried a T-allele at this position ($p = 0.040$). It was noted that CTLA4 gene polymorphism was related to ITP but not CD28. According to our results, we surmised that CTLA4 is involved in the pathogenesis of ITP, and the secondary ITP result from the lower CTLA4 expression that leads to T cell over-activation.

Platelets are derived from megakaryocytes, and their production and maturation are regulated by thrombopoietin in the bone marrow. In addition to hemostasis and thrombosis, platelets are also involved in immune response and cancer biology. The cut-off value of platelets in patients with thrombocytopenia is set to $100 \times 10^9/L$. When the counts of platelets are less than the cut-off value, it might increase the risk of bleeding, purpura and may even be life-threatening.

People with low platelet count are referred to as thrombocytopenia, which is caused by many disorders. The immune-related thrombocytopenia (ITP) may be caused by excessive destruction of platelets or inhibition of platelet production. The excessive destruction of platelets is resulted from platelet antibody (PAIgG) production or other immunological dysfunction, including type 1 helper T cells (Th1) polarization, regulatory T cells (Treg) scarcity, and autoreactive T cells activation and then leading to immune tolerance loss, megakaryocyte apoptosis, and platelet lysis. The mechanism of over-destroyed platelets is resulted from the absence of immune-tolerance, leading to autoreactive T cells attacking their own platelets. In addition, PAIgG will adhere to the surface of platelets, which leads to platelets over-engulfed by macrophages. Therefore, the differentiation and activation of immune cells play a vital role in the pathogenesis of ITP. Moreover, it is noted that B-T cell interaction is

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a necessary to induce ITP\textsuperscript{12}. The costimulatory molecules, such as CTLA4 (cytotoxic T-lymphocyte-associated protein 4) and CD28, are involved in T-cell activation\textsuperscript{13–15}, and they are the research subjects of most immune-related studies. CD28 is continually expressed on T cells\textsuperscript{16}, and it can provide signal to promote T cell activation\textsuperscript{17}. CTLA4 is continually expressed on Tregs or expressed on activated T cells after being induced by CD28 and T cell receptor signals\textsuperscript{18,19}. In addition, CTLA4 would compete with CD28 for CD80/CD86 and block the stimulatory signal of T cell activation\textsuperscript{20}. Therefore, both CTLA4 and CD28 genes were considered here.

As far as my knowledge is concerned, the mechanism of ITP is still unclear yet. In recent years, it had been shown that thrombocytopenia was associated with the transcription level of CTLA4 and regulation of T-cell activation\textsuperscript{21,22}. Moreover, the transcription level was related to gene mutation located in the promoter region\textsuperscript{23}. Hence, we supposed that primary ITP would be associated with abnormal immunity through T cell activity-regulated gene mutation. In this study, we investigated the correlation between single nucleotide polymorphisms (SNPs) in the promoter region of CTLA4, CD28, and ITP, and further explored the effects of the significant SNP on the gene expression. Additionally, immune-related thrombocytopenia includes primary ITP caused by abnormal immunity disorders less than 30\%\textsuperscript{29}, and the prevalence of thrombocytopenia caused by autoimmune disorders was less than 30\%\textsuperscript{29}. Thus, primary ITP and secondary ITP were both explored in this study.

**Results**

The characteristics of patients were shown in Table 1. Five of the 32 patients with ITP were men (15.6\%) and twenty-seven were women (84.4\%), with a median age of 60 years old. The 11 patients were suffered from severe ITP with platelet counts below 5 \times 10^9/L. Among the ITP patients, 53.1\% (17/32) cases were diagnosed as idiopathic thrombocytopenic purpura (primary ITP), and the other cases with low platelet count were caused by autoimmune disease (secondary ITP), such as systemic lupus erythematosus and rheumatoid arthritis.

| Characteristic                  | Cases, no. (%) (n = 32) | Controls, no. (%) (n = 30) |
|--------------------------------|-------------------------|---------------------------|
| Median age of the patients     | 60 (56.7 ± 3.7)         | 22 (22.8 ± 0.3)           |
| Gender                         |                         |                           |
| Male                           | 5 (15.6)                | 10 (33.3)                 |
| Female                         | 27 (84.4)               | 20 (66.7)                 |
| Bleeding                       |                         |                           |
| Yes                            | 10 (31.3)               |                           |
| No                             | 22 (68.7)               |                           |
| Severity of thrombocytopenia   |                         |                           |
| < 5 \times 10^9/L              | 11 (34.4)               |                           |
| < 100 \times 10^9/L            | 21 (55.6)               |                           |
| Diagnosis                      |                         |                           |
| Primary ITP                    | 17 (53.1)               |                           |
| Secondary ITP                  | 15 (46.9)               |                           |

Table 1. Characteristics of patients and controls.

Allele and genotype frequencies remain constant because of Hardy–Weinberg equilibrium (HWE) (p > 0.05) (Table 2). The allele frequency of all SNPs had no significant difference between cases and controls (Table 2). The completely statistical data of genotype frequency were shown in Table 3 and Supplementary Table S1. In association analysis of CTLA4 and CD28 gene polymorphisms with ITP (primary ITP + secondary ITP), only the genotype frequency of −1765_rs11571315 of CTLA4 had a significant difference between ITP cases and healthy controls (CC vs. CT vs. TT, p = 0.003). In addition, the control group had significantly higher heterozygous genotype frequency than ITP cases (p = 0.002).

Furthermore, the ITP cases were divided into primary ITP group without underlying disease and secondary ITP group caused by autoimmune disease to investigate their correlation with CTLA4 SNPs. We found that the rs11571315 had statistical significance in terms of primary ITP group (p = 0.006), secondary ITP group (p = 0.008), and all ITP cases (p = 0.003). Additionally, the rs11571315 also had a statistical significance when it was analyzed using the dominant model (TT vs. CT+CC), in which the p-value was 0.041 in the primary ITP group and 0.007 in the secondary ITP group. Moreover, there was only one extra significant SNP (rs5742909, p = 0.019) only in the secondary ITP caused by autoimmune disease (Table 4).

We further investigated the biological effects of the significant SNPs (rs11571315 and rs5742909). In the reporter assay, C\textsubscript{rs11571315} and C\textsubscript{rs5742909} were considered as wild type to compare with the sequences of T\textsubscript{rs11571315} and T\textsubscript{rs5742909}. The raw data and the bar chart were shown in supplementary raw data & Supplementary Fig. S1. The transcription activity was evaluated by calculating the luciferase ratio of NanoLuc to Firefly. Then, the luminescence value of the wild type was assumed as a reference for the relative light unit (RLU). The RLU was not different between the rs11571315 with T-allele and C-allele (p = 0.095). However, the RLU of the rs5742909 had statistical significance between the rs5742909 with T-allele and C-allele (p = 0.040). When the rs5742909 carried T-allele, it would decrease 19\% of transcription activity.
to CD28. In addition, B-T (follicular helper T) cell interaction is necessary for ITP induction12. The main biological function of CTLA4 is to down-regulate the activity of helper T cells, and the differentiation of follicular helper T cells is controlled by CTLA4 through the participation of CD28, while the main biological function

| SNP          | Allele major/minor | Minor allele frequency | HWE p value | Odds ratio 95% CI | p value |
|--------------|--------------------|------------------------|-------------|-------------------|---------|
| CTLA4        |                    |                        |             |                   |         |
| -1765_rs11571315 | T/C               | 0.129 0.183            | 1.000       | 0.660 (0.245–1.775) | 0.408   |
| -1722_rs733618   | C/T               | 0.435 0.450            | 0.792       | 0.943 (0.461–1.927) | 0.872   |
| -1661_rs4553808  | A/G               | 0.032 0.083            | 0.883       | 0.367 (0.068–1.968) | 0.269   |
| -1577_rs11571316 | G/A               | 0.145 0.100            | 0.365       | 1.528 (0.509–4.593) | 0.448   |
| -1478_rs62182959 | G/A               | 0.032 0.100            | 0.831       | 0.360 (0.058–1.550) | 0.160   |
| -1147_rs16840252  | C/T               | 0.022 0.017            | 0.770       | 0.252 (0.050–1.268) | 0.092   |
| -318_rs5742909    | C/T               | 0.065 0.133            | 0.701       | 0.448 (0.129–1.576) | 0.202   |
| rs49_rs313775     | G/A               | 0.333 0.283            | 0.449       | 1.204 (0.556–2.611) | 0.637   |
| CT60_rs3087643    | G/A               | 0.222 0.150            | 0.897       | 1.619 (0.622–4.211) | 0.321   |
| CD28           |                    |                        |             |                   |         |
| -1198_rs1879877  | T/G               | 0.391 0.350            | 0.967       | 1.190 (0.573–2.471) | 0.640   |
| -1066_rs3181096  | C/T               | 0.250 0.233            | 0.932       | 1.059 (0.485–2.495) | 0.829   |
| -1059_rs3181097  | A/G               | 0.438 0.483            | 0.340       | 0.831 (0.410–1.687) | 0.609   |
| -1042_rs3181098  | G/A               | 0.266 0.233            | 0.932       | 1.188 (0.526–2.687) | 0.678   |
| rs56228674       | C/T               | 0.063 0.050            | 0.959       | 1.267 (0.271–5.910) | 1.000   |
| 17+3_rs3116496   | T/C               | 0.125 0.133            | 0.701       | 0.929 (0.325–2.654) | 0.890   |

Table 2. Statistical analysis of CTLA4 and CD28 allele frequency. HWE Hardy–Weinberg equilibrium, 95% CI 95% confidence interval; pα values were counted from Chi-square test or Fisher’s exact test.

Discussion

There are various causes of thrombocytopenia, and the purpose of this study was to investigate the association between immune regulatory genes that are involved in T cell activation and ITP. The subjects with thrombocytopenia caused by organ diseases (such as lesion of liver, kidney, or spleen), drugs, chemoradiotherapy, or HIV were excluded in this research. The inclusion criteria in this study were not only limited to the low platelet counts (< 100 × 109/L) without underlying disease, also known as primary ITP30, but also the patients suffered from thrombocytopenia triggered by autoimmune diseases were also accepted. The promotion or inhibition of T cell activation is determined by the balance of CD28 and CTLA4 signals31. In addition, the SNPs located in the promoter region might affect the gene expression, and they would influence the level of protein expression and lead to the pathogenesis of disease32. Thus, we focused on the promoter region of CTLA4 and CD28 in this study. Based on our results, we found that the transcription activity was significantly different between rs5742909 with T-allele and C-allele in the functional assay. The previous studies also demonstrated that the gene polymorphism of rs5742909 was associated with autoimmune disease, cancer, and transfusion reaction33–36. Thus, patients with secondary ITP have an underlying autoimmune disease, and the polymorphisms found in the CTLA4 promoter could be related to the underlying autoimmune disease, and not necessarily to the development of ITP. Moreover, we suggested that the rs11571315 might lead to thrombocytopenia via another pathway rather than affecting CTLA4 expression.

This was the first study in the literature showed that CTLA4 gene polymorphism was related to ITP. The previous ITP-related studies only suggested that CTLA4 gene polymorphism may affect the severity of chronic thrombocytopenia39, or SNPs of CTLA4 were related to the mRNA transcription level. In Yao’s study, they showed that patients with primary ITP had a lower expression level of CTLA4 in Chinese Han children (5.43 ± 3.21 years old) when compared to controls, but the rs11571315 was not susceptible to primary ITP30. However, our data demonstrated that rs11571315 was a susceptible SNP for primary ITP in the Taiwan population. According to these two research pieces, we suggested that environmental factors may play an important role in ITP39, such as infection, cytokine, vaccines, and so on. These environmental factors would affect the changes of epigenetic markers that include DNA methylation, histone modification, micro-RNA regulation, etc. Zhao et al. indicated that the polymorphisms found in the CTLA4 promoter region of CTLA4 and CD28 in this study. Based on our results, we found that the transcription activity was significantly different between rs5742909 with T-allele and C-allele in the functional assay. The previous studies also demonstrated that the gene polymorphism of rs5742909 was associated with autoimmune disease, cancer, and transfusion reaction33–36. Thus, patients with secondary ITP have an underlying autoimmune disease, and the polymorphisms found in the CTLA4 promoter could be related to the underlying autoimmune disease, and not necessarily to the development of ITP. Moreover, we suggested that the rs11571315 might lead to thrombocytopenia via another pathway rather than affecting CTLA4 expression.

Our result indicated that only CTLA4 was associated with ITP but not CD28. It may be explained that CTLA4 has a higher affinity to its ligand (B7) than CD2842. Additionally, CTLA4 could have the ability to inhibit T-cell activation without binding B743. Thus, CTLA4 had a greater impact on the pathway of T-cell activation compared to CD28. In addition, B-T (follicular helper T) cell interaction is necessary for ITP induction42. The main biological function of CTLA4 is to down-regulate the activity of helper T cells, and the differentiation of follicular helper T cells is controlled by CTLA4 through the participation of CD28, while the main biological function

https://doi.org/10.1038/s41598-022-10631-z
| SNP           | Genotype frequency | Genotype | Odds ratio 95% CI | p value |
|--------------|--------------------|----------|-------------------|---------|
| −1765_rs11571315 | CC vs. CT vs. TT    |          |                   |         |
| T>C          | TT                 | 27       | 20                | Ref     |
|              | CT                 | 0        | 9                 | NA      |
|              | CC                 | 4        | 1                 | 2.963 (0.307–28.574) | 0.637 |
|              | TT vs. CT+CC       |          |                   | 0.296 (0.081–1.083) | 0.058 |
|              | TT+CT vs. CC       |          |                   | 4.296 (0.451–40.890) | 0.354 |
| −1722_rs733618  | CC vs. CT vs. TT    |          |                   |         |
| T>C          | CC                 | 12       | 10                | Ref     |
|              | CT                 | 11       | 13                | 0.705 (0.221–2.253) | 0.555 |
|              | TT                 | 8        | 7                 | 0.952 (0.255–3.553) | 0.942 |
|              | CC vs. CT+TT       |          |                   | 0.792 (0.278–2.258) | 0.662 |
|              | CC+CT vs. TT       |          |                   | 1.143 (0.356–3.673) | 0.823 |
| −1661_rs4553808 | AA vs. AG vs. GG   |          |                   |         |
| A>G/A>T       | AA                 | 29       | 25                | Ref     |
|              | AG                 | 2        | 5                 | 345 (0.061–1.935) | 0.255 |
|              | GG                 | 0        | 0                 | NA      |
|              | AA vs. AG+GG       |          |                   | 345 (0.061–1.935) | 0.255 |
|              | AA+AG vs. GG       |          |                   | NA      |
| −1577_rs11571316 | AA vs. AG vs. GG  |          |                   |         |
| G>A/G>C       | GG                 | 24       | 25                | Ref     |
|              | AG                 | 5        | 4                 | 1.302 (0.312–5.436) | 1.000 |
|              | AA                 | 2        | 1                 | 2.083 (0.177–24.506) | 1.000 |
|              | GG vs. AG+AA       |          |                   | 1.458 (0.407–5.230) | 0.561 |
|              | GG+AG vs. AA       |          |                   | 2.000 (0.172–23.293) | 1.000 |
| −1478_rs62182595 | AA vs. AG vs. GG  |          |                   |         |
| G>A          | GG                 | 29       | 24                | Ref     |
|              | AG                 | 2        | 6                 | 0.276 (0.051–1.494) | 0.147 |
|              | AA                 | 0        | 0                 | NA      |
|              | GG vs. AG+AA       |          |                   | 0.276 (0.051–1.494) | 0.147 |
|              | GG+AG vs. AA       |          |                   | NA      |
| −1147_rs16840252 | CC vs. CT vs. TT  |          |                   |         |
| C>T          | CC                 | 29       | 23                | Ref     |
|              | CT                 | 2        | 7                 | 0.227 (0.043–1.197) | 0.081 |
|              | TT                 | 0        | 0                 | NA      |
|              | CC vs. CT+TT       |          |                   | 0.227 (0.043–1.197) | 0.081 |
|              | CC+CT vs. TT       |          |                   | NA      |
| −318_rs5742909  | CC vs. CT vs. TT    |          |                   |         |
| C>T          | CC                 | 27       | 22                | Ref     |
|              | CT                 | 4        | 8                 | 0.407 (0.108–1.534) | 0.211 |
|              | TT                 | 0        | 0                 | NA      |
|              | CC vs. CT+TT       |          |                   | 0.407 (0.108–1.534) | 0.211 |
|              | CC+CT vs. TT       |          |                   | NA      |
| +49_rs231775   | AA vs. AG vs. GG   |          |                   |         |
| A>G/A>T       | GG                 | 15       | 14                | Ref     |
|              | AG                 | 10       | 15                | 0.622 (0.211–1.836) | 0.358 |
|              | AA                 | 5        | 1                 | 4.667 (0.483–45.045) | 0.207 |
|              | GG vs. AG+AA       |          |                   | 0.875 (0.318–2.410) | 0.796 |
|              | GG+AG vs. AA       |          |                   | 5.808 (0.853–53.012) | 0.195 |
| CT60_rs3087243  | AA vs. AG vs. GG   |          |                   |         |
| G>A          | GG                 | 16       | 22                | Ref     |
|              | AG                 | 10       | 7                 | 1.964 (0.615–6.271) | 0.251 |
|              | AA                 | 1        | 1                 | 1.375 (0.080–23.667) | 1.000 |
|              | GG vs. AG+AA       |          |                   | 1.891 (0.620–5.768) | 0.260 |
|              | GG+AG vs. AA       |          |                   | 1.115 (0.066–18.748) | 1.000 |
of CD28 is to modulate the activation of cytotoxic T cell\(^{44-46}\). Therefore, the effect of CTLA4 on ITP was more significant than CD28. When the amount of CTLA4 is too few to compete with CD28 for B7, the helper T cells would not be suppressed, which deeply affects the interaction between follicular helper T and B cell\(^{20}\) and then results in an autoimmune response. However, a recent study showed that the rs1980422 located downstream of CD28 was related to ITP\(^{47}\). Therefore, in the future, we should expand the region of gene to explore the SNP associated with ITP.

In conclusion, the present study indicated that CTLA4 gene polymorphism was related to the susceptibility of ITP but not CD28. Helper T cells are majorly regulated by CTLA4, which is involved in the mechanism of ITP, while CD28 is involved in cytotoxic T cell regulation. In addition, based on the results of our study and the literature\(^{30}\), we suggested that the environmental factors would also play an important role in ITP, and the mechanism of ITP might be different between adults and children. However, the relatively small sample size was a limitation in this study, it should be verified by increasing the number of including subjects.

### Materials and methods

#### Study subjects.

The Institutional Review Board of Chang Gung Memorial Hospital has reviewed and approved the study. The approval ID was 201901246B0. All study subjects signed informed consent and performed in accordance with relevant guidelines and regulations. In the present study, we focused on immune thrombocytopenia, and subjects with thrombocytopenia (platelet counts less than 100 \(\times\) 10\(^9\)/L) without receiving chemotherapy, radio therapy, bone marrow related disorder (leukemia, myelodysplastic syndromes, and aplastic anemia), liver, kidney, and spleen disorders. Seventeen of thirty-two patients were diagnosed as idiopathic thrombocytopenic purpura (ITP) in Chang Gang Memorial Hospital, while others were caused by autoimmune disorders. These inclusion criteria were based on the definition proposed by the International Working Group (IWG). Thirty healthy volunteers who had no bleeding disorders and with a normal platelet count (150 \(\times\) 10\(^9\)/L–400 \(\times\) 10\(^9\)/L) participated in this study.

#### DNA extraction.

The blood samples were collected in EDTA-coated vacuum tubes and genomic DNA was extracted via using QIAamp® DNA Mini kit (Qiagen GmbH, Hilden, Germany) in the light of the manufacturer’s instructions. Then, DNA concentration was assessed by measuring the optical density at 260 and 280 nm through a UV spectrometer.

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**Table 3.** Statistical analysis of CTLA4 SNPs and ITP. \(p\) values were counted from Chi-square test or Fisher’s exact test, where “*” means that it had significant difference between test group and control group. 95% CI 95% confidence interval; NA not applicable.

|          | Immune thrombocytopenia (n = 32) | Primary ITP (n = 17) | Secondary ITP (n = 15) |
|----------|----------------------------------|----------------------|------------------------|
|          | Case vs. Control                 | Case vs. Control     | Case vs. Control       |
|         | 0.008*                           | 0.003*               | 0.006*                 |
| 0.008*   | 0.003*                           | 0.006*               | 0.008*                 |
| 0.058    | 0.041*                           | 0.007*               |                        |
| 0.019*   | 0.019*                           | 0.019*               |                        |

**Table 4.** The immune thrombocytopenia (ITP) cases were divided into primary ITP without underlying disease and secondary ITP caused by autoimmune disease to confirm the correlation between CTLA4 SNPs and each ITP group. “*” means that it had significant difference between test group and control group. The same group of controls (n = 17) was used to analyze the correlation between controls and the primary ITP and secondary ITP.
candidate SNPs, because they were hot points in research about immune diseases. (exon 1) and the rs3087243 (3'UTR) of CTLA-4 and the rs3116496 (intron 3) of CD28 were also selected to be rs5742909, rs1879877, rs3181096, rs3181097, rs3181098, rs56228674, and rs3116496. Additionally, the rs231775 on the promoter region of CTLA-4 and CD28, in which we found 12 candidate SNPs in Taiwanese through searching thoroughly, including rs11571315, rs733618, rs4553808, rs11571316, rs62182595, rs16840252, rs5742909, rs1879877, rs3181096, rs3181097, rs3181098, rs56228674, and rs3116496. Additionally, the rs231775 (exon 1) and the rs3087243 (3’UTR) of CTLA-4 and the rs3116496 (intron 3) of CD28 were also selected to be candidate SNPs, because they were hot points in research about immune diseases.

**PCR amplification.** The PCR mixtures were 25 µL in total, consisting of 1 µg DNA, 10 µL HotStar Taq DNA Polymerase (Qiagen GmbH, Hilden, Germany), 1 µL of forward primer (10 Mµ), 1 µL of reverse primer (10 Mµ), and 12 µL of ddH2O. The primers used in here were shown in Table 5. The PCR program for CTLA-4 primers was 1 cycle of 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 65.5 °C for 30 s, and 72 °C for 1 min. The final elongation step was 3 min at 72 °C and then soaking at 10 °C forever. The PCR program for CD28 primers was 95 °C for 3 min at initial denaturation step and 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min. The final extension step was 10 min at 72 °C and finally soaking at 10 °C indefinitely. For gel electrophoresis visualization, 5 µL of the PCR products was pipetted onto a 1.5% agarose gel and run at 100 V for 30 min. After that, the gel was visualized un-der UV illumination and ensured the correctness.

**Purifying and SNPs analysis.** The PCR products were purified by enzyme, each containing 1.25 µL of shrimp alkaline phosphatase and 0.025 µL exonuclease I (New England Biolabs, UK) and worked at 37 °C for 30 min, 80 °C for 15 min and then subsequently storage at 4 °C. Next, the purified PCR products were sequenced using ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The SNPs analysis mainly focused on the promoter region of CTLA-4 and CD28, in which we found 12 candidate SNPs in Taiwanese through searching thoroughly, including rs11571315, rs733618, rs4553808, rs11571316, rs62182595, rs16840252, rs5742909, rs1879877, rs3181096, rs3181097, rs3181098, rs56228674, and rs3116496. Additionally, the rs231775 (exon 1) and the rs3087243 (3’UTR) of CTLA-4 and the rs3116496 (intron 3) of CD28 were also selected to be candidate SNPs, because they were hot points in research about immune diseases.

**Functional analysis.** The wild type reporter was constructed first. We amplified the promoter region of CTLA4 by PCR to construct the promoter-reporter that was digested with HindIII and ScaI. Subsequently, the reporter was transferred to the pNL1.1 expression vector (Promega) that was with NanoLuc luciferase and transfected into DH5α competent cells. Then, the site-directed mutagenesis was used to produce the gene fragment with rs11571315 C>T by Quick-Change mutagenesis kit (Strata-gene) with the mutagenesis forward primer 5’-GTT ATC AGA TTA C-3’ and the reverse primer was 5’-GCT TCA TGT TCA CTT TAA GGA TCT GGA TAA C-3’. Next, these fragments were transferred to pNL1.1 expression vector to construct a reporter with site-directed mutation. 1 µg of the reporter and 1 µg of the pGL 4.5 [Luc2/TK] vector (Promega) was used to measure the luciferase activity according to the manufacturer’s protocol. After culturing for 24 h, the Luciferase Assay System (Nano-Glo® Dual-Luciferase® Reporter Assay System, Promega) was used to measure the luciferase activity according to the manufacturer’s instructions.

**Statistical analysis.** Statistical analysis was performed via SPSS 17.0. Allele frequency of each SNP of control was analyzed by the HWE analysis to reduce sampling bias in case–control studies. If the p-value of HWE is higher than 0.05, we can say that the control group that was enrolled in this study can represent the entire population. Genotype frequency of CTLA-4 and CD28 gene were compared between the control group and test group using chi-square test or Fisher’s exact test to appraise the association between ITP and gene polymorphism. The SNPs data were not all available, because of the insufficient DNA samples and unclear sequencing results. In the functional study, the luciferase activity was detected by the luminescence ratio of NanoLuc luciferase to Firefly luciferase (NanoLuc/ Firefly). The luminescence ratio of the wild type was corrected to 1, being a reference of RLU. And the Mann–Whitney U test was used to analyze the difference of RLU between the reporter with wild type and with site-directed mutation to evaluate the transcription level affected by SNP that was located in the promoter region of the gene.

| primer for CTLA-4 promoter and rs231775 | GC content | Tm (°C) | Base pair |
|----------------------------------------|------------|---------|----------|
| F: 5’ GGC AAG AGA GAC CCC ACC GTC TTG 3’ | 21/13(62%) | 65.3    | 1234     |
| R: 5’ GAG GAC CTT CTA TAA ATC TGG AGA G 3’ | 25/12(48%) | 65.8    | 1170     |
| F: 5’ CTC TCC AGA TTT AAG GAA GGT CCT C 3’ | 25/12(48%) | 65.8    | 1170     |
| R: 5’ GGA ATA CAG AGC CAG CCA AGC G 3’ | 22/13(59%) | 65.8    | 1170     |

Table 5. Primers used for amplifying the target region of CTLA4 and CD28. F forward primer, R reverse primer.

| primer for CD28 promoter and rs3116496 | GC content | Tm (°C) | Base pair |
|----------------------------------------|------------|---------|----------|
| F: 5’ - GGC TGG TAA GAA GGA TGT GGA TAT C-3’ | 24/11 (46%) | 63.6    | 1542     |
| R: 5’ - CAA GGC ATC TGG ACT GCA GCA-3’ | 21/12 (57%) | 63.2    | 1542     |
| F: 5’ - AAG GAT GCA GGT TAG GGT CTA CAT T-3’ | 25/10 (40%) | 62.5    | 886      |
| R: 5’ - GAT CAA GCC AAC ATT GTG CAT TGG-3’ | 24/11 (46%) | 63.6    | 886      |
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Acknowledgements
The excellent consulting assistance and sample resources from molecular diagnosis laboratory of Chang-Gung Memorial Hospital are gratefully acknowledged.

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D.-P. C. conceived and designed the experiments and wrote draft of the manuscript. Y.-H. W. reviewed literature. W.-T. L. performed the experiments and analyzed the data. W.-T. W. performed the experiments and reviewed literature. All authors read and approved the final manuscript.

Funding
This work was supported by the Chang Gung Memorial Hospital grants CMRPG3I0261, CMRPG3I0262 and CMRPG3I0263 to D.P. Chen.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-10631-z.

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