Association of STAT4 and PTPN22 polymorphisms and their interactions with type-1 autoimmune hepatitis susceptibility in Chinese Han children

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ABSTRACT

Aims: To investigate the impact of signal transducer and activator of transcription 4 (STAT4) and the protein tyrosine phosphatase N22 (PTPN22) gene single nucleotide polymorphisms (SNPs), gene–gene interactions and haplotype on type-1 Autoimmune Hepatitis (AIH) risk.

Results: Logistic regression analysis showed that type 1 AIH was significantly higher in carriers of T allele of rs7574865 than those with GG genotype (P-value less than 0.001), higher in carriers of C allele of rs7582694 than those with GG genotype (P-value < 0.001), and lower in carriers of T allele of rs2476601 than those with CC genotype (P-value < 0.001). GMDR model indicated a significant two-locus model (p = 0.0100) involving rs7582694 and rs2476601. Participants with GC or CC of rs7582694 and CC of rs2476601 genotype have the highest type 1 AIH risk (P-value < 0.001), after covariates adjustment. Haplotype containing the rs7582694-C and rs7574865-T alleles were associated with a statistically increased type 1 AIH risk (P < 0.001).

Materials and Methods: Logistic regression was performed to investigate association between SNPs within STAT4 and PTPN22 gene and susceptibility to type 1 AIH. Generalized multifactor dimensionality reduction (GMDR) was used to screen the best interaction combinations among the 4 SNPs.

Conclusions: We conclude that rs7574865 and rs7582694 in STAT4 gene minor alleles, interaction between rs7582694 and rs2476601, and haplotype containing the rs7582694-C and rs7574865-T alleles are associated with increased type 1 AIH risk, but rs2476601 in PTPN22 gene minor allele is associated with decreased type 1 AIH risk.

INTRODUCTION

Autoimmune hepatitis (AIH) was a kind of chronic inflammation of the liver, hypergammaglobulinemia and autoantibodies production [1]. A recent study in pediatric patients reported an incidence of 0.4 case per 100 000 children [2]. Another study from Poland reported an incidence of 3 to 4 per 100 000 children [3]. This disease displays female predominance and is considered rare in childhood, although it may occur in very young children [4]. The pathogenesis of AIH was not well known, but could be influenced by both genetic and environmental factors [5]. Mutations in the human leukocyte antigen (HLA) region have been reported associations with some types of autoimmune diseases, including AIH [6, 7]. Signal transducer and activator of transcription 4 (STAT4) gene has important role in dendritic cells and macrophages, activated peripheral blood monocytes [8]. The association between STAT4 and some autoimmune diseases, including rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) has been reported in several previous studies [9, 10], and in a mouse model, STAT4 is considered important of Th1-dependent liver injury [11]. A recent study suggested that STAT4 polymorphism was positively associated with type-1 AIH. rs2476601 in PTPN22 was a missense single nucleotide polymorphism (SNP), which has been more studied previously [12], and has been reported associations with some types of autoimmune diseases, including RA and SLE [12–15]. A Japanese population base study [16] indicated that...
PTPN22 SNPs play an important role in the genetic resistance to autoimmune liver disease. In consideration of the limited number of study on association between STAT4 and PTPN22 gene and AIH risk, particularly in Children. In this study, we aimed to investigate the impact of STAT4 and PTPN22 gene SNPs, additional gene–gene interaction and haplotype combination on type 1 AIH risk based on Chinese Han population.

RESULTS

A total of 542 participants (98 boys, 444 girls) consist of 180 AIH patients and 362 normal participants were included in this study. The mean age of all participants is 8.1 ± 3.9 years. The clinical characteristics for cases and controls were shown in Table 1. The distribution of gender and the mean age were not significantly different between cases and controls. The mean of total Bilirubin was 3.87 ± 4.14 mg/ml, albumin was 3.89 ± 0.75 g/L, the rate of SMA and cirrhosis at entry were 56.7% and 30.6 respectively.

All genotypes are distributed according to HWE in controls (all p values are more than 0.05). The frequencies for rs7574865- T allele and rs7582694- C allele were significantly higher in type 1 AIH cases than control group (30.6% vs 19.3%, 32.5% vs 20.0%), and the frequencies for rs2476601- T allele was significantly lower in type 1 AIH cases than control group (19.7% vs 30.0%). Logistic regression analysis showed that type 1 AIH was significantly higher in carriers of T allele of rs7574865 than those with GG genotype (GT + TT versus GG), adjusted OR (95% CI) = 1.63 (1.28–98), and higher in carriers of C allele of rs7582694 than those with GG genotype (GC + CC versus GG), adjusted OR (95% CI) = 1.73 (1.38–2.19). In addition, we also found type 1 AIH risk was significantly lower in carriers of T allele of rs2476601 than those with CC genotype (CT + TT versus CC), adjusted OR (95% CI) = 0.65 (0.44–0.93). However, we did not find any significant association between rs2488457 and type 1 AIH risk after covariates adjustment. (Table 2).

GMDR were used to screen the best interaction combinations among 4 SNPs within STAT4 and PTPN22 gene on type 1 AIH risk (Table 3). We found that there is a significant two-locus model (p = 0.0100) involving rs7582694 and rs2476601. Overall, the cross-validation consistency of this two-locus model was 10/10, and the testing accuracy was 60.72%. We also conducted stratified analysis for interaction between rs7582694 and rs2476601 by using logistic regression. We found that participants with GC or CC of rs7582694 and CC of rs2476601 genotype have the highest type 1 AIH risk, compared to participants with GG of rs7582694 and CT or TT of rs2476601 genotype, OR (95% CI) = 3.12 (2.04–4.29), after covariates adjustment (Figure 1).

Pairwise LD analysis was performed on relationship with type 1 AIH risk, this study indicated that STAT4 represents a transcription factor, which involved in Th1/Th2 cytokine balance regulation [18]. The association between STAT4 and some autoimmune diseases, including rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) has been reported in several previous studies [9, 10]. Taylor et al. [19] concluded that STAT4 polymorphism played an important role on susceptibility to SLE. Another two Japanese studies [20, 21] also suggested that STAT4 is a common autoimmune diseases related genetic risk factor, including RA and SLE. A recent meta-analysis [22, 23] demonstrated a significant association between rs7574865- T allele within STAT4 gene and susceptibility to SLE, RA, T1D and so on. AIH pathogenesis were complex, study indicated that STAT4 represents a transcription factor, which involved in Th1 and Th17 differentiation [24]. STAT4 was an important genetic factor for IL-22 production, which plays a pathological role in IL-17-dependent hepatitis [25].

In terms of PTPN22, although previous study has showed no significant association between PTPN22 and AIH [26], to date, just one study [16] have been performed on relationship with type 1 AIH risk, this study...
suggested that \textit{PTPN22} gene SNP play a protective role on AIH risk. \textit{rs2476601} in \textit{PTPN22} was a missense SNP, which has been known as a factor associated with several autoimmune diseases, including RA and SLE [12–14], but this functional SNP was not associated with these autoimmune diseases in other studies [27–29].

In this study, type 1 AIH risk was influenced by both \textit{STAT4} and \textit{PTPN22} gene, so it is interesting to investigate the impact of gene–gene interaction between the two genes on type 1 AIH risk. We found a significant gene–gene interaction between rs7582694 and \textit{rs2476601}, participants with GC or CC of rs7582694 and CC of \textit{rs2476601} genotype have the highest type 1 AIH risk, compared to participants with GG of rs7582694 and CT or TT of \textit{rs2476601} genotype. To our knowledge this is the first study for investigating impact of interaction

\begin{table}[h]
\centering
\caption{General characteristics of 542 study participants in case and control group}  
\begin{tabular}{llll}
\hline
\textbf{Variables} & \textbf{Case group (n = 180)} & \textbf{Normal group (n = 362)} & \textbf{p-values} \\
\hline
Age (year) & 7.8 ± 4.1 & 8.3 ± 3.7 & 0.154  \\
Girls, N(%) & 146 (81.11) & 298 (82.3) & 0.730  \\
Total Bilirubin (mg/ml) & 3.87 ± 4.14 &  \\
Albumin (3.5–5.0 g/L) & 3.89 ± 0.75 &  \\
IgG (870–1700 mg/dl) & 3085 (2407–3875) &  \\
IAIHG score & 16.4 ± 2.3 &  \\
SMA, n (%) & 102 (56.7) &  \\
ANA + (≥ 1:40), N (%) & 151 (83.9) &  \\
Baseline laboratory values & &  \\
AST (< 40 IU/L) & 432.4 ± 444.1 &  \\
ALT (< 40 IU/L) & 484.3 ± 490.5 &  \\
ALP (< 112 IU/L) & 463.7 ± 210.3 &  \\
\hline
\end{tabular}
\footnotesize{Note: Means ± standard deviation for age, total Bilirubin, albumin and IAIHG score; Median (inter-quartile range) for IgG.}
\end{table}

Figure 1: Interaction analysis for rs7582694 and rs2476601 by using logistic regression.
Table 2: Genotype and allele frequencies of 4 SNPs between case and control group

| Gene/ SNP | Genotypes and Alleles | Frequencies N (%) | OR (95% CI)* | P-values | P-values for HWE test in controls |
|-----------|-----------------------|-------------------|-------------|----------|----------------------------------|
| STAT4 gene | Co- dominant          |                   |             |          |                                  |
| rs7574865 | GG                    | 89 (49.4)         | 237 (65.5)  | 1.00     | 0.622                            |
|           | GT                    | 72 (40.0)         | 110 (30.4)  | 1.56 (1.24-1.87) | < 0.001                     |
|           | TT                    | 19 (10.6)         | 15 (4.1)    | 2.13 (1.46-2.97) | < 0.001                     |
|           | Dominant              |                   |             |          |                                  |
|           | GG                    | 89 (49.4)         | 237 (65.5)  | 1.00     |                                  |
|           | GT + TT               | 180 (50.6)        | 125 (34.5)  | 1.63 (1.28–1.98) | < 0.001                     |
|           | Allele, T (%)         | 110 (30.6)        | 140 (19.3)  |          |                                  |
| rs7582694 | Co- dominant          |                   |             |          |                                  |
|           | GG                    | 84 (46.7)         | 232 (64.1)  | 1.00     | 0.875                            |
|           | GC                    | 75 (41.7)         | 115 (31.8)  | 1.64 (1.31–2.04) | < 0.001                     |
|           | CC                    | 21 (11.7)         | 15 (4.1)    | 2.21 (1.68–2.92) | < 0.001                     |
|           | Dominant              |                   |             |          |                                  |
|           | GG                    | 84 (46.7)         | 232 (64.1)  | 1.00     |                                  |
|           | GC + C                | 96 (53.3)         | 130 (35.9)  | 1.73 (1.38–2.19) | < 0.001                     |
|           | Allele, C (%)         | 117 (32.5)        | 145 (20.0)  |          |                                  |
| PTPN22 gene | Co- dominant          |                   |             |          |                                  |
| rs2488457 | GG                    | 105 (58.4)        | 192 (53.0)  | 1.00     | 0.958                            |
|           | GC                    | 65 (36.1)         | 143 (39.5)  | 0.85 (0.57–1.19) | 0.528                     |
|           | CC                    | 10 (5.6)          | 27 (7.5)    | 0.77 (0.52–1.23) | 0.476                     |
|           | Dominant              |                   |             |          |                                  |
|           | GG                    | 105 (58.4)        | 192 (53.0)  | 1.00     |                                  |
|           | GC + C                | 75 (41.7)         | 170 (47.0)  | 0.83 (0.44–1.20) | 0.507                     |
|           | Allele, C (%)         | 85 (23.6)         | 197 (27.2)  |          |                                  |
| rs2476601 | Co- dominant          |                   |             |          |                                  |
|           | CC                    | 118 (65.6)        | 184 (50.8)  | 1.00     | 0.105                            |
|           | CT                    | 53 (29.4)         | 139 (38.4)  | 0.68 (0.47–0.93) | 0.002                     |
|           | TT                    | 9 (5.0)           | 39 (10.8)   | 0.56 (0.26–0.91) | < 0.001                     |
|           | Dominant              |                   |             |          |                                  |
|           | CC                    | 118 (65.6)        | 184 (50.8)  |          |                                  |
|           | CT + TT               | 62 (34.4)         | 178 (49.2)  | 0.65 (0.44–0.93) | < 0.001                     |
|           | Allele, T (%)         | 71 (19.7)         | 217 (30.0)  |          |                                  |

*Adjusted for gender and age. Bonferroni correction threshold: \( P_c < 0.0083 \).
between STAT4 and PTPN22 gene on type 1 AIH risk in Chinese population. The underlying mechanisms for this interaction may due to that both SNP were associated with AIH or other autoimmune diseases. We also conducted haplotype analysis in STAT4 and PTPN22 gene respectively. We found that haplotype containing the rs7582694-C and rs7574865-T alleles within STAT4 gene were associated with a statistically increased type 1 AIH risk. But we did not find any haplotype combination within PTPN22 gene associated with type 1 AIH risk.

The current study also has some limitations. Firstly, limited number of SNP in STAT4 and PTPN22 gene are included in current study, and in the future, more SNPs should be included in analysis. Secondly, some environment risk factors should be included in the gene–environment interaction analysis. In addition, the selection bias existed in the participant inclusion and exclusion, so the frequency for the T allele of rs2476601 was higher than that in HapMap data. Thirdly, the results obtained in current study should be checked in other populations, for example, the gender and race difference of this relationship. Lastly, we do not resolve the question of STAT4 SNPs and AIH, Which SNP is dominant or is this purely a haplotype association because of strong LD.

In conclusion, the results of current study indicated that rs7574865 and rs7582694 in STAT4 gene minor alleles, interaction between rs7582694 and rs2476601, and haplotype containing the rs7582694-C and rs7574865-T alleles are associated with increased type 1 AIH risk, but rs2476601 in PTPN22 gene minor allele is associated with decreased type 1 AIH risk.

**MATERIALS AND METHODS**

**Study population**

All participants in this study are consecutively recruited between January 2008 and November 2015 from the Third Affiliated Hospital of Sun Yat-sen University. All AIH patients had been diagnosed according to the scoring system of the International Autoimmune Hepatitis Group [30] and were classified as having type 1 AIH based on antibody profiles. Those patients with clinical evidence of cholangitis or non-alcoholic steatohepatitis, positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA, and with other causes of liver disease were excluded from the study, controls are those who are free of liver related diseases and matched by sex, age and ethnic background in the same regions and nearly 1:2 matched to cases on the basis of age (± 3 years), and control participants with other immune diseases were excluded. At last, a total of 542 participants (98 boys, 444 girls) consist of 180 AIH patients and 362 normal participants were included in this study (Figure 2). The mean age of all participants is 8.1 ± 3.9 years. The racial background of all individuals was Chinese Han. Data on demographic information, lifestyle and history of disease for all participants were obtained using a questionnaire administered by trained staffs, including data included

| Locus no. | Best combination | Cross-validation consistency | Testing accuracy | p-values* |
|-----------|------------------|-------------------------------|------------------|-----------|
| 2         | rs7582694 rs2476601 | 10/10                         | 0.6072           | 0.0100    |
| 3         | rs7582694 rs2476601 rs7574865 | 8/10                         | 0.5399           | 0.1719    |
| 4         | rs7582694 rs2476601 rs7574865 rs2488457 | 7/10                         | 0.4958           | 0.3770    |

*Adjusted for gender and age. Bonferroni correction threshold: Pc < 0.0125.

| Haplotype | rs7582694 | rs7574865 | Frequencies | OR (95%CI) | p-values* |
|-----------|-----------|-----------|-------------|------------|-----------|
|           | Case group | Control group |             |            |           |
| H1        | G         | G         | 0.4732      | 1.00       | —         |
| H2        | C         | G         | 0.2236      | 1.14 (0.82–1.64) | 0.592 |
| H3        | G         | T         | 0.1935      | 1.28 (0.91–1.76) | 0.602 |
| H4        | C         | T         | 0.1097      | 1.85 (1.36–2.47) | < 0.001 |

*Adjusted for gender and age. Bonferroni correction threshold: Pc < 0.0125.
coexisting autoimmune diseases, serum levels of alkaline phosphatase and bilirubin and serum levels of ALT, AST and so on. Both anti-nuclear antibodies (ANA) and anti-smooth muscle antibodies (ASMA) were measured by indirect immunofluorescence on HEp-2 cells and cut-off titers for positivity were 1:40. Written informed consent was obtained from all participants. The protocol of this study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

Genomic DNA extraction and genotyping

We selected SNPs within the \textit{STAT4} and \textit{PTPN22} gene according to the following methods, including: 1) which have been reported associations with autoimmune diseases or risk factors of AIH; 2) minor allele frequency (MAF) greater than 5%. Taking into account the limitations of human, material and financial resources, a total of two SNPs of \textit{STAT4} gene and two SNPs of \textit{PTPN22} gene were selected for genotyping in the study: rs7574865 and rs7574865 within STAT4, rs2476601 and rs2488457 within \textit{PTPN22}. Genomic DNA is extracted from EDTA-treated whole blood, using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The four SNPs were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [31]. PCR primer sequences for each polymorphism are shown in Table 5. The PCR reactions were carried out in a final volume of 20 μl containing: 10 × PCR buffer,

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**Table 5: Description and Primer sequences for 4 SNPs used for PCR analysis**

| SNP ID   | Chromosome | Functional Consequence | Major/Minor | Restriction enzyme | Primers sequence |
|----------|------------|-----------------------|-------------|--------------------|------------------|
| STAT4 gene |            |                       |             |                    |                  |
| rs7574865 | 2:191099907| Intron variant         | G/T         | \textit{HpaI}      | F: 5′-AAAGAAGTGGAATAAAAAAAGGTTTG-3′<br>R: 5′-CCACTGAAATAAGATAACCACTGT-3′ |
| rs7582694 | 2:191105394| Intron variant         | G/C         | \textit{HpyCH4III} | F: 5′-ATCCAACCTCTTCTCAGCCCT-3′<br>R: 5′-TCATAATCAGGAGAGGAG-3′ |
| PTPN22 gene |            |                       |             |                    |                  |
| rs2488457 | 1:113872746| Intron variant, upstream variant 2KB | G/C         | \textit{SacI}       | F: 5′-CCATTGAGAGGTTATGCGAGCT-3′<br>R: 5′-CGCCACCTTGCTGACAACAT-3′ |
| rs2476601 | 1:113834946| Intron variant, missense | C/T         | \textit{XcmI}       | F: 5′-CCACGTTCCTCAACCACAATAATG-3′<br>R: 5′-CACTGCTCCAAGGATAGATGTA-3‘ |

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Figure 2: A flowchart on study population selection and exclusion.

All participants in this study are consecutively recruited between January 2008 and November 2015 from the Third Affiliated Hospital of Sun Yat-sen University.

Those who had histological evidence of cholangitis or non-alcoholic steatohepatitis, positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA, and patients with other causes of liver disease were excluded.

Controls are those who are free of liver related diseases and matched by sex, age and ethnic background, in the same regions and nearly 1:2 matched to cases on the basis of age (±3 years) and sex.

Control participants with other immune diseases were not included in the control group.

A total of 180 AIH patients were included in the case group.

A total of 362 volunteers were included in the control group.
4.5 mM MgCl₂ (Roche, Germany), 0.4 mM of each dNTP (Fermentas, Germany), 10 pmol of each primer, 32 ng template DNA, 1 U Taq DNA polymerase (Roche, Germany) and sterile distilled water up to 20 μl.

For the two SNPs within STAT4 gene, rs7574865 was a 147-bp PCR product and was digested with restriction enzyme and electrophoresed on a 2.5% polyacrylamide gel. Rs7574865 was a 338-bp PCR product was digested with restriction enzyme and electrophoresed on a 3.0% polyacrylamide gel. For the two SNPs within PTPN22 gene, the PCR products were incubated with restriction enzymes for 1 or 16 hours. 4% agarose gel with Gold View (SBS Genentech, Beijing, China) was used to visualize the obtained digestion products. 20% of PCR-amplified DNA samples were examined by direct sequencing to confirm the genotyping results, which was 100% concordant. Amplification conditions started with an initial denaturation step of 6 min at 94°C, followed by 35 cycles of 40 s denaturation (94°C), 30 s annealing (56°C) and 40 s extension (72°C), ended by a final extension for 5 min (72°C).

Statistical analysis

The means and standard deviations (SDs) were calculated for normally distributed continuous variables and were analyzed using Student’s t test or one-way analysis of variance. Percentages were calculated for categorical variables and were analyzed using χ² test. Departure from Hardy-Weinberg equilibrium (HWE) in cases and controls was tested using Pearson χ² goodness-of-fit test. Haplotype analysis and Pairwise LD analysis were investigated by using SNPStats (available online at http://bioinfo.iconcologia.net/SNPstats). Logistic regression was performed to investigate association between SNPs within STAT4 and PTPN22 gene and susceptibility to type 1 AIH. Bonferroni correction was applied in case of multiple comparisons using the formula \(pc = \frac{p}{n}\) (pc represents corrected value where n is the number of comparisons made). Generalized multifactor dimensionality reduction (GMDR) [32] was used to screen the best interaction combinations among the 4 SNPs, some parameters such as cross-validation consistency, the testing balanced accuracy, and the sign test were calculated. Permutation testing is also conducted to gain empirical \(P\) values of prediction accuracy as a benchmark based on 10,000 shuffles. A sign test or a permutation test (providing empirical \(p\)-values) for prediction accuracy can be used to measure the significance of an identified model.

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CONFLICTS OF INTEREST

There is no conflicts of interest.

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