**HLA-B*35:01 Allele Is a Potential Biomarker for Predicting Polygonum multiflorum-Induced Liver Injury in Humans**

Chaopeng Li,1,3,6 Tai Rao,1 Xiaoping Chen,1 Zhengsheng Zou,2 Aiwu Wei,4 Jinfo Tang,4 Peng Xiong,2 Pengyan Li,2 Jing Jing,2 Tingting He,2 Zhaofang Bai,2 Jiye Yin,1 Zhirong Tan,1 Peng Yu,3,5 Honghao Zhou,1 Jiabo Wang,2 Xiaohua Xiao,2 and Dongsheng Ouyang1,3

From the 1Department of Clinical Pharmacology, Xiangya Hospital, Central South University; Institute of Clinical Pharmacology, Central South University, Hunan Key Laboratory of Pharmacogenetics; Engineering Research Center of Applied Technology of Pharmacogenomics, Ministry of Education; National Clinical Research Center for Geriatric Disorders, Changsha, Hunan, P.R. China; 2The Fifth Medical Center, General Hospital of PLA, Beijing, P.R. China; 3Hunan Key Laboratory for Bioanalysis of Complex Matrix Samples, Changsha, Hunan, P.R. China; 4The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, Zhengzhou, Henan, P.R. China; 5School of Pharmaceutical Science, Central South University, Changsha, Hunan, P.R. China; and 6The First Affiliated Hospital of the Medical College, Shihezi University, Shihezi, Xinjiang, P.R. China.

*Corresponding authors*

Dongsheng Ouyang

Department of Clinical Pharmacology, Xiangya Hospital, Central South University; Institute of Clinical Pharmacology, Central South University, Hunan Key Laboratory of Pharmacogenetics; Engineering Research Center of Applied Technology of Pharmacogenomics, Ministry of Education

110 Xiangya Rd., Changsha 410008, Hunan, China

E-mail: ouyangyj@163.com

Tel.: +86 731 8480 5380

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Xiaohe Xiao  
The Fifth Medical Center, General Hospital of PLA  
100 West 4th Ring Rd., Beijing, China  
E-mail: pharmacy302@126.com  
Tel.: +86 10 6693 3322

Jiabo Wang  
The Fifth Medical Center, General Hospital of PLA  
100 West 4th Ring Rd., Beijing, China  
E-mail: pharm_sci@126.com  
Tel.: +86 10 6693 3323

**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; DILI, drug-induced liver injury; GWAS, genome-wide association studies; HLA, human leukocyte antigen; IDILI, idiosyncratic DILI; MHC, major histocompatibility complex; OR, odds ratio; PM, *Polygonum multiflorum*; PCR-SBT, polymerase chain reaction-sequence based typing; RR, relative risk; RUCAM, Roussel Uclaf Causality Assessment Method; TBil, total bilirubin; ULN, upper limit of normal.

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Abstract

Polygonum multiflorum (PM) is a well-known Chinese herbal medicine that has been reported to induce inflammation-associated idiosyncratic liver injury. This study aimed to identify the genetic basis of susceptibility to PM-drug-induced liver injury (PM-DILI) and to develop biological markers for predicting the risk of PM-DILI in humans. The major histocompatibility complex (MHC) regions of 11 patients with PM-DILI were sequenced, and all human leukocyte antigen (HLA)-type frequencies were compared to the Han-MHC database. An independent replication study that included 15 patients with
PM-DILI, 33 patients with DILI (other drug-DILI), and 99 population controls was performed to validate the candidate allele by HLA-B polymerase chain reaction-sequence based typing (PCR-SBT). A prospective cohort study that included 72 outpatients receiving PM for 4 weeks was designed to determine the influence of the risk allele on PM-DILI. In the pilot study, the frequency of HLA-B*35:01 was 45.4% in PM-DILI patients compared with 2.7% in the Han Chinese population (odds ratio [OR], 30.4; 95% confidence interval [CI], 11.7-77.8; P = 1.9 × 10⁻¹⁰). In the independent replication study and combined analyses, a logistic regression model confirmed that HLA-B*35:01 is a high-risk allele of PM-DILI (PM-DILI vs. other drug-DILI: OR, 86.5; 95% CI, 14.2-527.8; and PM-DILI vs. population controls: OR, 143.9; 95% CI, 30.1-687.5). In the prospective cohort study, an asymptomatic increase in transaminase levels was diagnosed in 6 patients, representing a significantly higher incidence (relative risk [RR], 8.0; 95% CI, 1.9-33.2; P < 0.02) in the HLA-B*35:01 carriers (37.5%) than in the noncarriers (4.7%). Conclusion: The HLA-B*35:01 allele is a genetic risk factor for PM-DILI and a potential biomarker for predicting PM-DILI in humans.
*Polygonum multiflorum* Thunb. (PM) is a popular herbal medicine that is known as He Shou Wu in Asia and Fo-ti in the United States. The use of PM and processed PM products as medicines for nourishing the liver and kidney have been recorded for nearly 1,000 years in China. Currently, many herbal medicine prescriptions, dietary supplements, and natural medicine products still contain PM in some Asian, European, and American countries. In 2013-2016, the annual consumption of PM reached 6,000 tons in China. In recent years, however, the increasing incidence of PM-drug-induced liver injury (PM-DILI) has resulted in great concern worldwide\(^{(1)}\) especially in China where 30% of herbal hepatotoxicity cases are reported to be related to PM-DILI\(^{(2,3)}\).

Recently, reliable diagnostic methods have been established based on integrated evidence from the chain-based identification of Chinese herbal medicine\(^{(4)}\). The China Food and Drug Administration officially published the *Guidance for the Clinical Evaluation of Traditional Chinese Medicine-Induced Liver Injury* in 2018. Unfortunately, the prevention, diagnosis, and treatment of PM-DILI are still difficult problems, and challenges remain in the clinic due to a lack of specific biomarkers and limited understanding of PM-DILI mechanisms. Several groups have found that PM-DILI is an unpredictable, dose-independent, and typical delayed-type adverse reaction\(^{(5-8)}\). In addition, individuals with diseases associated with the immune system activation might be more susceptible to PM-DILI\(^{(9)}\). Liver biopsies
revealed that some patients had marked mixed inflammatory cell infiltration, eosinophils, and Kupffer cell activation.\(^{(10,11)}\) These studies suggest that PM-DILI may be a type of immune-mediated idiosyncratic DILI (IDILI).

In the past decade, increasing genome-wide association studies (GWAS) have supported that human leukocyte antigens (HLAs) are associated with IDILI susceptibility to several drugs\(^{(12)}\) (e.g., HLA-B*57:01 with flucloxacillin,\(^{(13)}\) HLA-B*35:02 with minocycline,\(^{(14)}\) HLA-DRB1*16:01-DQB1*05:02 with flupirtine\(^{(15)}\)). HLA alleles have been important genetic markers for IDILI. However, there are currently no reports describing the association of specific HLA polymorphisms and PM-DILI. Given that PM-DILI possesses the typical features of immune-mediated IDILI, we focused on the role of HLA alleles in PM-DILI in this study. The aim of the current work is to investigate pharmacogenetic variants that might predict the risk of PM-DILI based on HLA genotypes.

**Participants and methods**

**Study Design.** The study protocol was approved by the medical ethics committee of Xiangya Hospital, Central South University (Changsha, China). All participants were self-reported as Han Chinese and provided written informed consent. Blood samples were collected from each of the participants, who were recruited at 302 Military Hospital (Beijing, China); Xiangya Hospital, Central South University.
(Changsha, China); The First Affiliated Hospital, Henan University of Traditional Chinese Medicine (Zhengzhou, China); and The First Affiliated Hospital of the Medical College, Shihezi University (Shihezi, China). A pilot study, a replication study, and a prospective cohort study were included in this work. A total of 230 subjects were enrolled based on eligibility criteria\(^{(16,17)}\) The overall study design is shown in Fig. 1.

**Pilot Study.** A total of 11 patients hospitalized from 2012 to 2015 who were diagnosed with “drug-induced liver injury,” “drug-induced liver disease,” “drug-induced hepatitis,” or “drug-induced liver failure” attributed to PM were enrolled in the study. All patients met one of the following predefined laboratory criteria: (1) aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) levels ≥5× the upper limit of normal (ULN); (2) ALT or AST level ≥3× ULN and total bilirubin (TBil) level ≥2 ULN with clinically apparent jaundice; (3) serum alkaline phosphatase (ALP) level ≥2× ULN; (4) pathological diagnosis of liver injury. The serological markers of viral hepatitis A, B, and C and those of other viruses mimicking hepatitis, such as cytomegaloviruses and Epstein-Barr viruses, were negative in these subjects. Subjects with other nondrug etiologies, such as autoimmune liver diseases, alcoholic liver diseases, and nonalcoholic liver diseases, were also excluded.
Various drugs containing PM have been widely prescribed for different medicinal purposes, and PM was extensively used as liver and kidney "tonic" in healthy individuals in China. Therefore, we selected 10,689 healthy Han Chinese individuals from the Han-major histocompatibility complex (MHC) database as study controls.\(^{(18)}\)

**Replication Study.** In this stage, 15 patients were diagnosed with PM-DILI by at least two hepatologists. Inclusion and exclusion criteria were consistent with those in the pilot study. Thirty-three other drug-DILI patients who were diagnosed with "drug-induced liver injury," "drug-induced liver disease," "drug-induced hepatitis," or "drug-induced liver failure" attributed to drugs other than PM, amoxicillin-clavulanate, or flucloxacillin from 2015 to 2018 were enrolled in the replication study. A total of 99 ancestry-, age-, and gender-matched control individuals from The Health Management Center of Xiangya Hospital, Central South University, were included. The sample size was calculated to detect a specified odds ratio (OR) >30, with a given power of 0.9 and a type I error of 0.05 with a module of tests for two proportions in PASS 15.\(^{(19)}\)
**Prospective Cohort Study.** A total of 81 female infertility outpatients were suitable for PM treatment as prescribed by traditional Chinese medicine. All participants were administered traditional Chinese herbal compound medicinal decoctions that mainly contain *P. multiflorum* Radix, *Atractylodis macrocephala* Rhizoma, *Dioscoreae* Rhizoma, and *Moutan* cortex for tonifying kidney and participated in a follow-up examination between 2015 and 2016. After excluding participants who did not complete blood collection (*n* = 9), there were 72 patients involved in the prospective cohort study. All patients had normal ALT/AST, bilirubin, and ALP levels according to the exclusion criteria before starting to consume PM-containing products. The patients were prescribed a PM decoction for 4 weeks, which then continued for up to 12 weeks. Patients who had (1) ALT/AST level $\geq 2\times$ ULN with or without TBiL level $\geq 2\times$ ULN or (2) ALP level $\geq 2\times$ ULN after the administration of PM were diagnosed with asymptomatic transaminase elevation. To protect the safety of participants, all subjects diagnosed with asymptomatic transaminase elevation immediately ceased the PM treatment. The sample size was calculated to detect a specified $P_1 = 0.5$, $P_2 = 0.05$, $R = 8$, with a type I error of 0.05 and a given power of 0.8 by tests for two proportions module of PASS 15.
**Causality Assessment.** The DILI cases were evaluated by a panel of two hepatologists. All patients had a Roussel Uclaf Causality Assessment Method (RUCAM) score of 3 or greater.\(^{(20)}\) RUCAM scores were grouped into the following likelihood levels: “excluded” (≥0), “unlikely” (1-2), “possible” (3-5), “probable” (6-8), and “highly probable” (≥9).

**Liver Histopathology.** An experienced histopathologist reviewed available liver biopsies. All samples were scored for multiple histological features as well as an overall pattern of liver injury.

**MHC-Targeted Sequencing.** Genomic DNA extraction from peripheral blood samples was performed using the EasyPure Blood Genomic DNA Kit (TransGen Biotech) and quantified using agarose gel electrophoresis. In the pilot study, the HLA sequences (chr6:28477797-33448354) were efficiently enriched in 1.0 μg genomic DNA as determined by using SeqCap EZ Choice Enrichment Kits (NimbleGen) according to the manufacturer's protocol. The fragments between 180 and 280 base pairs (bp) in length were extracted and sequenced using the Illumina HiSeq X Ten system. We sequenced the target sequence to 100-fold with a coverage of 97% in 11 Han Chinese patients in the pilot study.
We predicted classical HLA alleles using the Han-MHC database as a reference panel. HLA typing was performed by comparing contigs with currently known HLA gene sequences in the ImMunoGeneTics (IMGT)/HLA database (Release 3.30.0). The following filtering criteria were applied in the association study: (1) average sequencing depth $\geq 100\times$, (2) a major allele frequency $\geq 0.005$, and (3) GC content of 42% to 48%.

**HLA-B High-Resolution Genotyping.** The HLA-B locus was typed by the four-digit resolution HLA SBT method$^{[21]}$ in the replication study and prospective cohort study. To obtain the four-digit alleles, ambiguous results were resolved by a sequence-specific primer using allele-specific HLA typing.$^{[22]}$

**Statistical Analysis.** Statistical analyses were performed using R (3.4.2) and RStudio (1.1.383) for Windows. The Mann-Whitney U test was used to determine whether two independent samples came from the same population. $P < 0.05$ (2-tailed) was considered statistically significant. A logistic regression model was used to investigate the association of HLA alleles with PM-DILI. ORs were calculated by a 2×2 contingency table, which added 0.5 to all fields to accommodate possible zero
counts. We used chi-squared or Fisher’s exact test to compare genotype frequencies between groups.

Confidence intervals (CIs) and $P$ values were calculated using R package “exact 2x2” (1.5.2), with Haldane’s modification. In the pilot study, the association significance threshold of HLA alleles was adjusted to $P < 1 \times 10^{-5}$ by Bonferroni correction. Power analysis and sample size programs (PASS 15, NCSS) were used to compute the sample sizes of the replication study and prospective cohort study.

Results

Clinical feature. The clinical details of patients hospitalized with PM-DILI in the pilot study and replication study are summarized in Supporting Table S1. Nineteen patients self-administered PM as a supplementary food for health, and 7 patients took traditional Chinese medicinal prescriptions containing PM.

The baseline demographic characteristics of patient cohorts in the pilot and replication studies are summarized in Table 1. Patients consumed PM for a total of 11.4 (2-55) weeks. The main symptoms of liver injury were jaundice, nausea, anorexia, and oil avoidance, and 2 patients had signs of fever.

Twenty-one patients were symptomatic at presentation, with the most common symptoms being
jaundice (n = 26), fatigue (n = 21), nausea (n = 3), fever (n = 2), and itching (n = 3); 17 cases (73.9%) presented with hepatocellular injury, 1 case (4.4%) presented with cholestatic injury, and 5 cases (21.7%) presented with mixed injuries.

The liver biopsies obtained from 12 patients with PM-DILI suggested that acute hepatitis and acute cholestatic hepatitis were two major types of histological patterns. The histological features of patients with PM-associated DILI are shown in Fig. 2. The histological presentation of patients with acute hepatitis usually involved focal lytic necrosis, focal confluent necrosis, and scattered lymphocytic infiltration in sinuses in zone 3 (Fig. 2A,B), whereas the liver histology of patients with cholestatic hepatitis commonly included mild to moderate lobular necroinflammation and cholestasis (Fig. 2C,D), including focal necrosis, canalicular cholestasis, hepatocellular cholestasis, Kupffer cells cholestasis, and bile duct injury. In the patients with PM-DILI, the histological presentations were consistent with drug- or toxin-induced acute liver injury.

No significant difference in the distribution of population characteristics was observed between the PM-DILI group and the population control group or the PM-DILI group and the other drug-DILI group (Supporting Table S2). No patients underwent liver transplantation or died during our study. PM-DILI patients had an optimal outcome after stopping PM. The average hospitalization time of all patients
was 19.9 ± 8.5 (9-37) days. Within 6 months of follow-up, the laboratory tests of 26 patients returned to normal, and the longest restoration time was 4 months.

Association testing for complete HLA alleles in the pilot study. In the pilot study, MHC-targeted sequencing was performed on the 11 PM-DILI patients. The entire HLA region located at hg19_chr6: 28477797-33448354 contains 230 genes. In this study, 27 gene loci were sequenced, including the classical HLA genes HLA-A, HLA-B, HLA-C, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, and HLA-DRB1; the nonclassical HLA genes HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DRA1, HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-E, HLA-F, HLA-G, HLA-H, HLA-J, HLA-K, and HLA-L; and the non-HLA genes MICA, MICB, TAP1, and TAP2. A total of 124 four-digit resolution HLA alleles were acquired, and no novel HLA allele was detected.

The complete genotypes for HLA-A, HLA-B, HLA-C, DRB1, DQB1, and DPB1 in the 11 initial cases are provided in Supporting Table S3. HLA-B, which included 11 alleles, showed the greatest variation. However, HLA-B*35:01 was present in 90.9% (10/11) of the patients. The frequencies of HLA-B*35:01
were 45.4% in the patients; 2.7% in the Han-MHC database, which consisted of 10,689 healthy control subjects\(^{(18)}\); and 2.8% in Chinese volunteers from the Chinese Bone Marrow Donor Program.\(^{(23)}\) In Fisher’s exact test analysis, \(H\text{LA-B}^*35:01\) (OR, 30.4; 95% CI, 11.7-77.1; \(P = 1.91 \times 10^{-10}\), after adjusting \(P = 2.37 \times 10^{-8}\)) was more significantly associated with PM-DILI than were the other HLA alleles (Fig. 3). \(H\text{LA-DRB1}^*15:01\) was reported to be a risk factor for increased susceptibility to amoxicillin-clavulanic acid–induced liver injury.\(^{(24)}\) In the pilot study, 6 cases carried \(H\text{LA-DRB1}^*15:01\). However, the adjusted \(P\) value did not reach statistical significance.

**Independent Replication Study and Combined Analyses.** To validate whether \(H\text{LA-B}^*35:01\) is a risk allele of PM-DILI, a replication study that included 15 cases of PM-DILI, 33 cases of other drug-DILI (the medication information of the other drug-DILI cases is listed in Supporting Table S4), and 99 matched population controls was performed. High-resolution genotyping of the \(H\text{LA-B}\) of all subjects was performed. There were 51 \(H\text{LA-B}\) alleles among the 139 subjects. The three most common \(H\text{LA-B}\) alleles in population controls were \(H\text{LA-B}^*46:01\), \(H\text{LA-B}^*40:01\), and \(H\text{LA-B}^*15:02\), which was consistent with the Han Chinese population data in the HLA Allele Frequency Net Database (http://www.allelefrequencies.net). However, 13 of 15 (86.7%) PM-DILI patients carried \(H\text{LA-B}^*35:01\),
whereas only 4 of 33 (12.1%) other drug-DILI patients and 5 of 99 (5.05%) Han population controls carried the allele. The results confirmed that HLA-B*35:01 was an independent risk factor for PM-DILI compared to other drug-DILI and population controls (PM-DILI vs. other drug-DILI: OR, 77.9; 95% CI, 9.9-614.8; \( P = 3.6 \times 10^{-5} \) and PM-DILI vs. population: OR, 131.7; 95% CI, 19.7-879.0; \( P = 4.7 \times 10^{-7} \)) in the independent study (Table 2).

The combined analysis of the 26 PM-DILI patients (11 discovered cases plus 15 replication cases), 33 other drug-DILI patients, and 99 population controls showed that the frequency of HLA-B*35:01 in PM-DILI patients was significantly higher than in other drug-DILI patients (\( P = 4.5 \times 10^{-9} \)) and population controls (\( P < 2.2 \times 10^{-16} \)). After adjusting for the covariates, age and gender, a logistic regression model showed that HLA-B*35:01 was a risk factor (PM-DILI vs. other drug-DILI: OR, 86.5; 95% CI, 14.2-527.8 and PM-DILI vs. population: OR, 143.9; 95% CI, 30.1-687.5). In our study, 23 of 26 PM-DILI patients carried HLA-B*35:01, and 29 of 33 other drug-DILI patients did not carry this allele. The sensitivity and specificity of the PM-DILI clinical diagnosis were 88.5% and 87.9%, respectively.

**Prospective Cohort Study.** To explore the value of using HLA-B*35:01 as a prediagnostic test for PM-DILI, we designed a prospective cohort study with 72 female patients. All patients had normal ALT/AST, bilirubin, and ALP levels according to the exclusion criteria before starting to consume
PM-containing products. Patients who received PM treatment were observed for at least 12 weeks after the therapy began. The population characteristics are shown in Supporting Table S5. Six patients had ALT levels ≥2× ULN, and no patient had a TBil level ≥2× ULN. A total of 37.5% (3/8) of HLA-B*35:01 carriers showed asymptomatic transaminase elevation (relative risk [RR], 8.0; 95% CI 1.2-110.6; \( P < 0.02 \)), compared with 4.7% (3/64) of noncarriers (Table 3). All patients who had abnormal liver enzyme elevation stopped their medication and were monitored until they recovered.

The presence of the allele had a sensitivity value of 50.0% and a specificity of 92.4% (Table 4).

**Discussion**

Numerous cases of PM-DILI have emerged worldwide since the earliest case was recorded in 1996 in China.\(^{(25)}\) Recent studies have indicated that PM-DILI might be an IDILI related to immune reaction.\(^{(3,8)}\)

Previous studies have attempted to find biomarkers for the diagnosis and prevention of PM-DILI by evaluating cytochrome 450 (CYP450) polymorphisms\(^{(26)}\) and metabolomics\(^{(7,27)}\) in various models. Unfortunately, no satisfactory biomarkers are currently available to identify human populations susceptible to this injury. Moreover, conventional animal models\(^{(28-31)}\) in which only high doses that significantly exceed the typical PM dose that can induce liver injury are not suitable for mimicking dose-independent PM-induced idiosyncratic hepatotoxicity. Recently, however, a rat model of immune
action-mediated liver injury induced by cotreatment with a nontoxic dose of lipopolysaccharide (LPS) and a therapeutic dose of PM exhibited liver injury characteristics similar to those shown in humans and induced a state of immune activation and inflammatory stress.\(^{32}\) In addition, the average latency to develop DILI is 15 to 30 days after the first exposure to PM but only a few days after reexposure.

Histopathologic examinations of liver biopsies also suggested that some of the PM-DILI patients displayed immune reaction-associated features, such as mixed inflammatory cell infiltration, eosinophil elevation, and Kupffer cell activation,\(^{(10,11)}\) which are consistent with our observations in PM-DILI (Fig. 2). This evidence indicated that an immune-mediated idiosyncratic hepatotoxicity was induced by PM.

Delayed adverse drug reactions (ADRs) are driven by the inappropriate activation of T cells.\(^{(33)}\) The HLA molecules encoded in the MHC region on chromosome 6 are thought to primarily be key proteins that activate T cells.\(^{(34,35)}\) Several genetic studies of DILI have shown high-risk factors in both HLA class I and II genes,\(^{(14,15,36-40)}\) which suggests that HLA genetic polymorphisms play a crucial role in adaptive immunity in DILI. Overall, we hypothesized that the HLA genotype might be related PM-DILI.

The current study successfully identified \textit{HLA-B*35:01} as a potential genetic high-risk factor for PM-DILI that is specific for PM rather than other drugs (such as amoxicillin-clavulanate or flucloxacillin).

Three studies were designed to assess the association between PM-DILI and HLA alleles. In the pilot
study, we isolated and deeply sequenced the entire HLA region and analyzed the associations of all high-resolution HLA alleles with PM-DILI. *HLA-B*35:01 was significantly associated with PM-DILI.

*HLA-B*35:01 is a common *HLA-B* allele in Han Chinese, with a frequency of 2% to 5% according to the Allele Frequency Net Database (http://www.allelefrequencies.net). However, 90.9% (10/11) PM-DILI patients carried the allele. The association between the PM-DILI and *HLA-DRB1*15 reported to be related with amoxicillin-clavulanate–induced hepatotoxicity was also evaluated.⁴¹) Although in the present study, 8 of the 11 cases carried *HLA-DRB1*15 (combined *HLA-DRB1*15:01 and *HLA-DRB1*15:02) and the allele frequency was 45.5% (15.8% in control), the *P* value for *HLA-DRB1*15 adjusted through Bonferroni did not reach statistical significance. The results indicated that *HLA-B*35:01 plays a much more important role in PM-DILI.

To validate *HLA-B*35:01, we genotyped *HLA-B* alleles at a four-digit resolution in an independent sample (15 PM-DILI patients, 33 other drug-DILI patients, and 99 population controls). A total of 11 *HLA-B* alleles were identified in the 15 PM-DILI patients (Supporting Table S3). *HLA-B*35:01 was the most significant independent signal of the other *HLA-B* alleles. In total, 87% of PM-DILI patients (13/15) carried the *HLA-B*35:01 allele, whereas 2 patients carried the *HLA-B*15:18 allele, and only 1 patient carried another *HLA-B* allele. Therefore, we consider *HLA-B*35:01 to be the only risk factor. The
combined analysis results demonstrated that the \textit{HLA-B*35:01} allele had high specificity and sensitivity for diagnosed PM-DILI. \textit{HLA-B*35:01} testing might be useful as a diagnostic tool and anti-diastole aid for PM-DILI, especially in distinguishing PM-DILI from other drug-DILI and hepatic disorders.

In the pilot and replication studies, some of the PM-DILI patients were concomitantly exposed to other drugs. Information on the herbs or drugs used by all PM-DILI patients is listed in Supporting Table S1. Based on current knowledge, the confounding contributions of these concomitantly used herbs and drugs to liver injury could be excluded. In addition, reliable screening methods based on integrated evidence of chain-based causality identification in herb-induced liver injury also helped to diagnose PM-DILI.\textsuperscript{(10)}

In the cohort study, compared with noncarriers, individuals carrying \textit{HLA-B*35:01} had an 8-fold increased risk of developing PM-related liver injury. Our study now provides strong evidence suggesting that prospective \textit{HLA-B*35:01} screening may identify patients with the greatest risk of PM-DILI, such as HLA-A*31:01 testing for carbamazepine-induced hypersensitivity\textsuperscript{(42,43)} and \textit{HLA-B*57:01} screening for hypersensitivity to abacavir.\textsuperscript{(36,38)} Despite the small sample sizes of subjects included in this work, our data were significant and in line with other GWAS demonstrating an
association of HLA genotype and DILI susceptibility.\(^{44,45}\) Based on the assumption that the approximate prevalence of PM-DILI is 8.3\% (6/72) in Han Chinese, the \textit{HLA-B*35:01} allele increases the risk of DILI to 37\% (3/8), whereas the risk of DILI in noncarriers is 4.7\% (3/64). Considering the protection of the patients' right to safety, we did not administer PM long term (more than 1 month) in the prospective cohort study. Most of the PM-DILI occurred between 1 and 2 months\(^{9,10}\); hence, the current data might underrepresent PM-DILI occurrence, which might provide an explanation for the 5 \textit{HLA-B*35:01}-positive individuals who were exposed to PM but did not develop asymptomatic transaminase elevation in the cohort study. Another limitation was that the cohort consisted only of women with infertility who were suitable for PM treatment according to traditional Chinese medicine. Although the homogeneity of the existing health conditions of the cohort allowed for comparisons to be made, this homogeneity also limited the extrapolation of this allelic association to male subjects. In addition, patients in China are more cautious about taking medicines containing PM today than before the China Food and Drug Administration's official announcement about the potential risk of liver injury induced by PM in July 2014. Given that the small sample size of PM-DILI might bring uncertainties and variations to our cohort study, a larger, independent cohort of patients with PM-DILI is needed to further validate these findings. According to our current results, \textit{HLA-B*35:01} “is necessary but not
sufficient by itself\textsuperscript{(38)} for PM-DILI. Nevertheless, \textit{HLA-B*35:01} may be useful as a diagnostic aid when PM-DILI is suspected and as a prospective screening tool to predict PM-DILI risk.

Although DILI caused by PM is often due to the solo use of this herb, there are some cases associated with the use of PM preparations. Clinical observations and the published PM-DILI literature do not reveal any clustering characteristics of specific preparations or formulas of PM; PM-DILI cases occur with the use of general preparations or formulas of PM. Additionally, our previous studies and published data showed that either PM or preparations containing PM could induce liver injury, but the concomitant components in the preparations and formulas did not\textsuperscript{(9,10,46,47)} Thus, we consider the allele found in this study to be primarily associated with PM.

Notably, \textit{HLA-B*35:01} might be a high-risk allele of PM-DILI rather than the other drugs (PM-DILI vs. other drug-DILI: OR, 63.0; 95% CI, 10.2-387.3), suggesting that the specific HLA allele and resulting MHC I structure may be involved in the recognition of antigens modified by specific PM compounds through metabolic activation. The toxic components in PM and the mechanism linking \textit{HLA-B*35:01} with PM-DILI remain unknown. However, HLA allotypes are key proteins that regulate the T-lymphocyte response. HLA class I alleles are usually associated with specific CD8+ cytotoxic T-cell reactions\textsuperscript{(48)} Several studies have found that \textit{HLA-B*35:01} is associated with nevirapine-induced
cutaneous hypersensitivity in Caucasian, Asian, and Thai populations\(^{(49)}\) and that CD8+ and CD4+ T cells are implicated in this pathogenesis.\(^{(50)}\) One possibility is that the \textit{HLA-B*35:01} antigens are directly or indirectly involved in the activation of T lymphocytes. Further study is needed to identify and validate the PM components or their \textit{in vivo} metabolites that lead to hepatotoxicity.

In summary, our study reported an HLA genotype as a potential novel biomarker of PM-DILI for the first time, presenting a new insight into the mechanism of PM-DILI. \textit{HLA-B*35:01} may have potential clinical value as a biomarker to reduce the risk of PM-DILI in humans. Our group is planning additional \textit{in vitro} experiments on immunocytes from PM-DILI patients and healthy volunteers with and without the \textit{HLA-B*35:01} allele to identify the specific PM components leading to DILI and to elucidate the molecular mechanisms of PM-DILI.

\textbf{References}


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30) Li DK, Chen J, Ge ZZ, Sun ZX. Hepatotoxicity in rats induced by aqueous extract of Polygoni Multiflori Radix, root of Polygonum multiflorum related to the activity inhibition of CYP1A2 or CYP2E1. Evid Based Complement Alternat Med 2017;2017:9456785.


Figure legends

**FIG. 1.** The overall study design.

**FIG. 2.** Examples of the two most common injury patterns in patients with PM-DILI (stained with H&E).

(A,B) Acute hepatitis injury. The apoptosis body, the focal lytic necrosis, and scattered lymphocytic infiltration are in the lobules (black arrow). (C,D) Cholestatic hepatitis injury. There is hepatocellular cholestasis (blue arrow) and Kupffer cell cholestasis (black arrow). Abbreviations: H&E, hematoxylin and eosin.

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FIG. 3. Association tests between all HLA alleles and PM-induced DILI. Circles denote alleles with an OR <1, whereas squares denote alleles with an OR >1. HLA-B*35:01 (OR, 30.4; 95% CI, 11.7-77.2; \( P = 1.9 \times 10^{-10} \)) showed the strongest signal in PM-induced liver injury versus popular Han Chinese controls. HLA allele that exceeded a \( P \) value of \( 10^{-5} \) threshold.
### TABLE 1. Demographic characteristic of the PM-DILI cases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pilot Stage Study (n = 11)</th>
<th>Replication Stage Study (n = 15)</th>
<th>Combined Analysis (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, years</td>
<td>46 (34-64)</td>
<td>44 (23-60)</td>
<td>45 (23-64)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>4 (36)</td>
<td>9 (60)</td>
<td>13 (50)</td>
</tr>
<tr>
<td>BMI (mean ± SD)</td>
<td>23.0 ± 3.0</td>
<td>22.1 ± 2.5</td>
<td>22.5 ± 2.7</td>
</tr>
<tr>
<td><strong>Total weeks on PM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8 ± 6.8</td>
<td>10.4 ± 12.0</td>
</tr>
<tr>
<td><strong>Clinical feature</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>691 ± 446</td>
<td>1105 ± 544</td>
<td>930 ± 537</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>524 ± 390</td>
<td>823 ± 465</td>
<td>697 ± 453</td>
</tr>
<tr>
<td>ALP (U/L), n</td>
<td>184 ± 60 (11)</td>
<td>201 ± 106 (13)</td>
<td>193 ± 87 (24)</td>
</tr>
<tr>
<td>TBil (mg/dL), n</td>
<td>211 ± 195 (7)</td>
<td>165 ± 123 (14)</td>
<td>181 ± 147 (21)</td>
</tr>
<tr>
<td>DBil (mg/dL), n</td>
<td>173 ± 145 (6)</td>
<td>151 ± 128 (14)</td>
<td>158 ± 130 (20)</td>
</tr>
<tr>
<td><strong>Injury type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocellular</td>
<td>9</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>Cholestatic</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>RUCAM score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-5 (Possible)</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>6-8 (Probable)</td>
<td>7</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>9 (Highly probable)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index.
### TABLE 2. Association test results for HLA-B*35:01 in the case-control study

<table>
<thead>
<tr>
<th></th>
<th>PM-DI</th>
<th>Other Drug-DILI</th>
<th>Population Controls</th>
<th>Other Drug-DILI</th>
<th>PM-DI vs. Population Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Replication study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B*35:01(+)</td>
<td>13 (15)</td>
<td>4 (33)</td>
<td>5 (99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odds Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(95% CI)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77.9</td>
<td>(9.9- 614.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*P Value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6 x 10^{-5}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odds Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(95% CI)</td>
<td></td>
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</tr>
<tr>
<td>131.7</td>
<td>(19.7-879.0)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PM-DI vs. Other Drug-DILI</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Odds Ratio</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(95% CI)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>143.9</td>
<td>(30.1-687.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| *ORs, 95% CIs, and P values were from logistic regression.
### TABLE 3. 4-fold contingency table of cohort study

<table>
<thead>
<tr>
<th>Group</th>
<th>PM-DILI</th>
<th>PM-Tolerant</th>
<th>Total</th>
<th>Incidence Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carriers</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>37.5</td>
</tr>
<tr>
<td>Noncarriers</td>
<td>3</td>
<td>61</td>
<td>64</td>
<td>4.7</td>
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<tr>
<td>Total</td>
<td>6</td>
<td>66</td>
<td>72</td>
<td>8.3</td>
</tr>
<tr>
<td>Performance Characteristic</td>
<td>Number of Patients</td>
<td>Percent% (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td><strong>PM-DILI</strong></td>
<td>3</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>PM-Tolerant</strong></td>
<td>5</td>
<td>61</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td><strong>Sensitivity:</strong></td>
<td></td>
<td></td>
<td>50.0  (13.9-86.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Specificity:</strong></td>
<td></td>
<td></td>
<td>92.4  (82.5-97.1)</td>
<td></td>
</tr>
<tr>
<td><strong>PPV</strong>:</td>
<td></td>
<td></td>
<td>37.5  (10.2-74.1)</td>
<td></td>
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<tr>
<td><strong>NPV</strong>:</td>
<td></td>
<td></td>
<td>95.3  (86.0-98.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Positive predictive value.

**Negative predictive value.