HLA
Immune Response Genetics

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A low-cost, sensitive and specific PCR-based tool for rapid clinical detection of *HLA-B*35* alleles associated with delayed drug hypersensitivity reactions

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HLA (HLA) alleles are risk factors for CD8+ T-cell-mediated drug hypersensitivity reactions. However, as most HLA associations are incompletely predictive and/or involve risk alleles at low frequency, costly sequence-based typing can elude an economically productive cost: benefit ratio for clinical validation studies and diagnostic and/or preventative screening. Hence rapid and low-cost detection assays are now required, both for single alleles but also across risk loci associated with broader multi-disease risk; exemplified by associations with diverse alleles in HLA-B*35, including *HLA-B*35:01* and green tea- or co-trimoxazole-induced liver injury. Here, we developed a cost-effective (<$10USD) qPCR assay for rapid (<2.5 h) clinical detection of *HLA-B*35* alleles. The assay was validated using 430 DNA samples with previous American society for histocompatibility and immunogenetics-accredited sequence-based high-resolution HLA typing, positively detecting all *HLA-B*35* allelic variants in our cohort, and as expected by primer design, the six samples that expressed low-frequency *B*78:01*. The assay did not result in positive detection for any negative control allele. With expected detection of *B*35* and *B*78*, our assay sensitivity (95% CI, 95.07%–100.00%) and specificity (95% CI, 98.97%–100.00%) of 100% using as low as 10 ng of DNA provides a reliable *HLA-B*35* screening tool for clinical validation and HLA–risk-based prevention and diagnostics.

KEYWORDS
drug hypersensitivity reactions, HLA (HLA), Immunogenetics, real-time quantitative polymerase chain reaction

1 | INTRODUCTION

Diverse alleles from the highly polymorphic HLA (HLA) complex are associated with predisposition to CD8+ T-cell-mediated drug hypersensitivity reactions.[1–3] HLA class I

alleles are critical to immunopathogenesis as a requirement for antigenic presentation of drug- or drug-modified self-antigen to T-cells, culminating in cytotoxic activation and patient tissue-directed cytotoxicity.[4–7] Resulting reactions often target the skin and liver and range from mild skin rash to drug-induced liver injury (DILI), associated with acute liver failure and <10% mortality, and Stevens

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Johnson syndrome and toxic epidermal necrolysis (SJS/TEN), a severe cutaneous manifestation associated with blistering and 50% mortality.[8–11] For strongly predictive associations, including those first reported between the anti-retroviral drug abacavir and *HLA-B*57:01*, and the anti-convulsant carbamazepine and *HLA-B*15:02*,[1,12,13] HLA-typing is successfully utilised in clinic and high-risk populations for preventative pre-treatment screening. However, as most HLA allele associations to date are incompletely predictive and/or involve risk alleles at low population frequency, costly sequence-based typing dependant on specialist expertise and equipment can elude an economically productive cost:benefit ratio for (i) clinical validation studies of proposed risk HLA, required before recommending clinical implementation of preventative measures, and (ii) preventative or diagnostic screening.[1,13–15] Hence robust, rapid and low-cost detection assays suited to non-specialist on-site clinical laboratories are now required to navigate these economic pressures, both for single-alleles but also across risk loci associated with broader evidence-based risk. This is exemplified by recent associations with different culprit drug-induced reactions and diverse alleles in *HLA-B*35*, a common gene in haplotypic association with *HLA-C*04* and broad distribution in European Caucasian (11%), African American (13%), Hispanic (14%), Japanese (17%) and Chinese (4%) populations (http://www.allelefrequencies.net/). Key associations now include *HLA-B*35:01* and green tea-,[16] polygonum multiflorum[17] or co-trimoxazole-induced liver injury,[18] *HLA-B*35:02* and minocycline-induced hepatotoxicity,[19] and *HLA-B*35:05* with nevirapine-induced skin reactions.[20] While high similarity between the >550 reported *B*35* alleles is prohibitive to PCR-based sequence differentiation of single-alleles without full HLA sequencing, as B35 alleles share peptide-binding specificities with shared position 2 preference for proline and dichotomic preference for tyrosine or smaller hydrophobic residues at position 9, we developed a sensitive *B*35*-specific PCR-based assay. The assay is suited for rapid (2.5 h) clinical validation or preventative and/or diagnostic screening at low reagent cost (<$10USD) compared with time-consuming sequence-based HLA typing, which for a single allele can cost approximately $110 in a clinical setting.[21]

2 | MATERIAL AND METHODS

2.1 | Primers and TaqMan probes

Exon 2 and 3 sequences of all *HLA-B* alleles were obtained from the IMGT/HLA database v3.45.1 (https://www.ebi.ac.uk/ipd/imgt/HLA/), sequence aligned, and *B*35* selective regions searched against reference for *HLA-B*35:01* using BioEdit Sequence Alignment Editor 3.0. Both forward and reverse primers (Table 1) were designed as locked nucleic acid (LNA) primers to increase sensitivity,[22,23] performance and amplification success as previously reported.[24,25] The forward primer was designed to target exon 2 of *HLA-B*35:01* at position 117–133, sharing specificity with *HLA-B*18*, *B*35*, *B*37*, *B*51*, *B*52*, *B*53*, *B*58* and *B*78*. The sequence is specific to the *HLA-B* locus and differs from several alleles including *B*13*, *B*15* and *B*44* by just one base pair at position 133, hence LNA is required to prevent primer hydrolyzation and ensure specificity. The reverse primer inversely complements *HLA-B*35:01* exon 2 at positions 229–252, a sequence observed in multiple *HLA-B* alleles (*B*07 B*08 B*14 B*15 B*18 B*35 B*39 B*40 B*41 B*42 B*46 B*47 B*48 B*49 B*50 B*54 B*55 B*56 B*59 B*67 B*73 B*78-B*81 B*82 B*83*) but differing from *HLA-B*37*, *B*51*, *B*52* and *B*53* at position 229. Thus, the primer combination narrows the amplicon specificity to *HLA-B35*, *B78* and *B18* alleles only. To further increase specificity given *HLA-B*18* is one of the most abundant *HLA-B* antigens in Caucasian populations, we designed a fluorescein amidite (FAM)-labelled probe to reverse complement the *HLA-B*35:01* sequence at position 178–204 in exon 2, which differs from *HLA-B*18* sequences at position 199 (Table 1). The final combination of primers and probe are thus specific to detection of *HLA-B*35* and *B*78* alleles only (Figure 1). Importantly, of the 552 *HLA-B*35* alleles listed at up to six-digit resolution by the IMGT/HLA database as of July 2021, only 51 low-frequency *HLA-B*35* variants do not align with our primer sequences and thus are not proposed to be detected by this assay (Figure S1). We also searched allele sequences to six-digit resolution across more prevalent *HLA-B*35:01*, :02 and :05 alleles recently associated with drug hypersensitivity. Within *HLA-B*35:01*, 52/54 alleles at six-digit resolution aligned with our target sequence excluding *B*35:01:20 and *B*35:01:53, while just *B*35:02:09 was excluded from the 13 different variants of *B*35:02 (Figure S1). All five variants of *B*35:05 are detected by our assay. Importantly, with sequence similarity to *HLA-B*35*, *HLA-B*78* alleles are also likely detected except *B*78:03, *B*78:05 and *B*78:06, but are present at a low frequency across global populations (http://www.allelefrequencies.net/, last accessed 6 July 2021) (Table S1). Previously described primers and Hexachloro-fluorescein (HEX)-labelled probe were also included specific to the housekeeping endonuclease gene, ribonuclease P (RNAseP), as positive amplicon control (Table 1).

2.2 | DNA samples

To validate specificity and sensitivity of this assay, 430 DNA samples expressing diverse HLA-B alleles

TABLE 1 Sequences of primers and probes

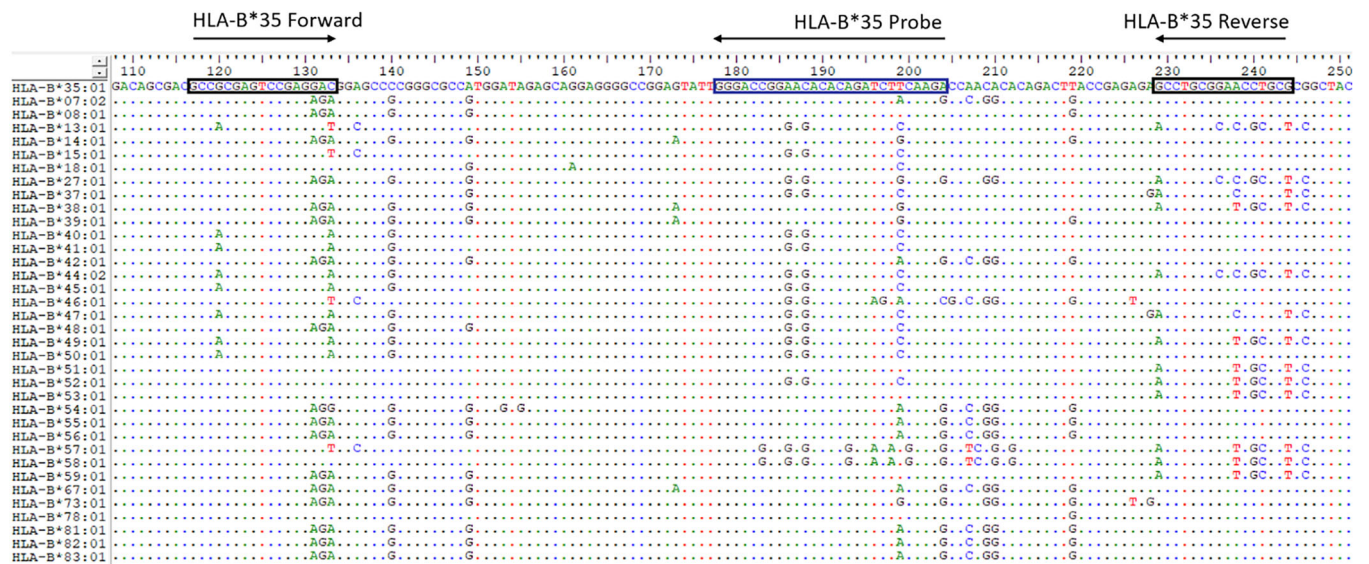| Primer/probe	Sequences	Target
B35 Forward	5′-GCCGCGAGTCCGAGGAC LC-3′	*HLA-B*35*
B35 Reverse	5′CGCAGGTTCCGCAGG LC-3′	*HLA-B*35*
B35 Probe	5′/56-FAM/TCTTGAAGA/ZEN/TCTGTGTGTTCCGGTCCC/3IABkFQ/−3′	*HLA-B*35*
RNAse P Forward	AGATTTGGACCTGCGAGCG	*RNAse P*
RNAse P Reverse	GAGCGGCTGTCTCCACAAGT	*RNAse P*
RNAseP Probe	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	*RNAse P*

FIGURE 1 Targeted sequence design for discrimination of *HLA-B*35* from other HLA-B alleles at four-digit resolution. Binding sites of *HLA-B*35* primers/probe aligned with *HLA-B*35:01* allele. The *HLA-B* sequences from IMGT/HLA database were aligned with *HLA-B*35:01* using Bioedit 3.0. Primer and probe positions are indicated by the boxes, with the arrows indicating direction. The forward primer spans Exon 2 at positions 117–133 and is locked at position 133. The reverse primer is complementary to Exon 2 at positions 229–244 and is locked complementary to position 229. The *HLA-B*35* probe is complementary to Exon 2 at positions 178–204, with the targeted region between the dye (FAM) and quencher (ZEN), which allows optimization

including representation of *HLA-B*35* (*n* = 67), *B*78* (*n* = 6) or *B*18* (*n* = 22) were selected for analysis from North American population cohorts of the International HLA DNA Exchange with American Society for Histocompatibility and immunogenetics accredited sequence-based high-resolution HLA typing; all data of which was de-identified. Where cell stocks were stored, DNA was extracted from thawed peripheral blood mononuclear cells stored in liquid nitrogen using Qiagen automated DNA purification kit (Qiagen, Valencia, CA) and genotype blinded to the operator during assay validation. All samples met minimum quality specifications with a 260/280 ratio over 1.7 and mean DNA concentration of 50 ng/μl prior to normalisation to 25 ng/μl with sterile deionised water (Cat# W3500; Sigma-Aldrich, Australia).

2.3 | Real-time TaqMan qPCR for *HLA-B*35* detection

Each real-time qPCR reaction was performed in a final volume of 10 μl consisting of 2 μl (50 ng) DNA and 8 μl mastermix, consisting of 1× Gotaq mastermix (Promega, Madison, Wisconsin, USA), 2 pmol of forward and reverse primer, 1 pmol of *HLA-B*35* probe, and 1× RNaseP Primer-Probe (VIC) mix (Applied Biosystems, Waltham, Massachusetts, United States*)*. The master mix was dispensed on 96- or 384-well qPCR plates using a high-volume chip on the Mantis Liquid Handler (Formulatrix, Bedford, MA) and DNA samples stamped to the qPCR plates straight from DNA storage using a Biomek FX liquid handler (Beckman Coulter, Carlsbad, CA 92010, USA). The real-time qPCR reactions were performed using a
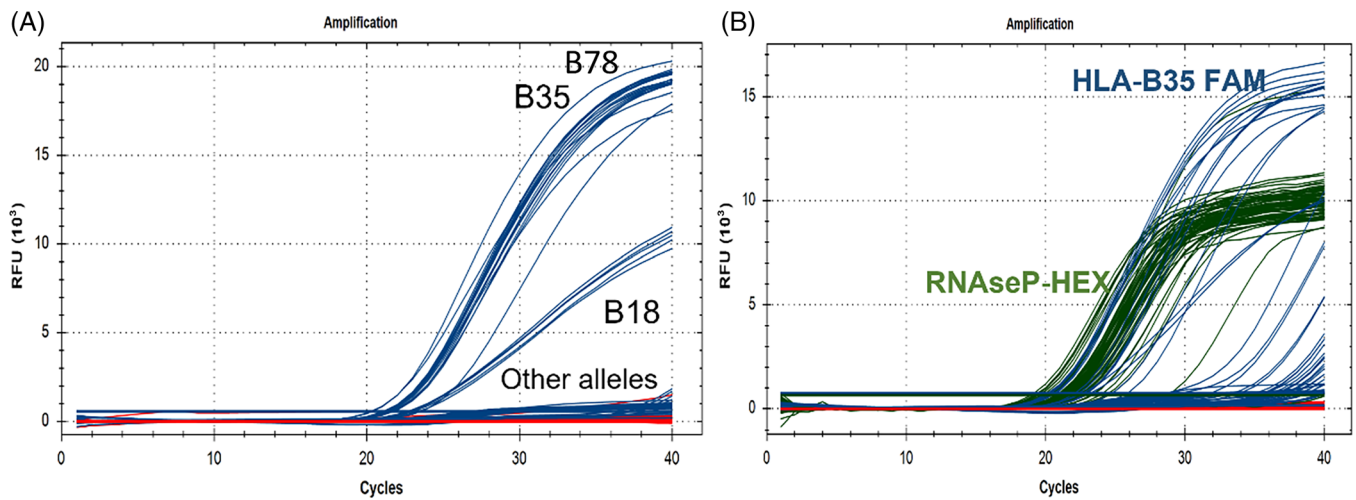
FIGURE 2 *HLA-B*35* assay real time PCR result. (A). *HLA-B*35* positive and negative samples were distinguished by the *HLA-B*35* assay. Fluorescence of *HLA-B*35* (and *B*78*) positive samples (100 ng) plateaued at higher levels (>18 × 10^3 RFU) after 40 cycles of PCR. *HLA-B*18* alleles that are distinguished by one base pair within the probe showed elevated but lower-level fluorescence (10 × 10^3 RFUs). (B). Amplification of housekeeping gene RNAseP showed HEX fluorescence (plateaued at 10 × 10^3 RFUs) after 30 cycles of PCR

Bio-Rad CFX96/384 qPCR machine (Bio-Rad Laboratories, Hercules, CA, USA). The optimised thermocycling conditions used were as follows: initial hot start at 96°C for 6 min for polymerase activation, followed by 39 cycles of denaturing at 96°C for 30 s, and annealing at 62°C for 30 s. The results were read by Biorad CFX manager 3.0 software to provide the relative fluorescence unit (RFU) of each reaction. Assay sensitivity and specificity were calculated using MedCalc statistic software (https://www.medcalc.org/calc/diagnostic_test.php).

3 | RESULTS

To validate assay sensitivity and specificity, we analysed 430 DNA samples previously genotyped with high-resolution sequence-based HLA typing. Positive and negative samples were distinguished by the level of FAM channel fluorescence after 40 cycles of PCR, with *HLA-B*35* or *B*78* positive samples emitting a higher RFU (17 × 10^3–25 × 10^3) than the *B*18*-positive samples (10 × 10^3 –12 × 10^3) selected out within the probe targeting sequence (Figure 2A). In comparison, the control HEX-labelled RNAseP probe plateaued at 10 × 10^3 RFU (Figure 2B). Although not all *B*35* alleles were found in our cohort those of highest population frequency were identified and included. As expected by design, the assay positively detected all 12 *HLA-B*35* variants found across 67 samples in our cohort, including *B*35:01*, *B*35:02*, *B*35:03*, *B*35:05*, *B*35:08*, *B*35:12*, *B*35:14*, *B*35:17*, *B*35:21*, *B*35:43*, *B*35:47* and *B*35:48*, and the six samples that expressed *B*78*:01 (Table 2). In contrast, the

assay did not detect any negative control HLA alleles including those expressing the closely related *B*53:01* allele (Table 2). Moreover, positive response was not detected for the samples expressing *B*18:01* (*n* = 22) or indeed any other control alleles selected by the forward primer (*B*37*, *n* = 9; *B*51*, *n* = 40; *B*52*, *n* = 15; *B*58*, *n* = 26), nor *B*13* (*n* = 11), *B*15* (*n* = 94) and *B*44* (*n* = 78), which differ at the forward primer targeting sequence by one base pair, demonstrating selectivity of the LNA primer. With expected detection of *B*35* and *B*78*, our results indicate assay sensitivity (95%CI, 95.07%–100.00%) and specificity (95%CI 98.97%–100.00%) of 100%. Highest fluorescence for *B*35/B*78* alleles was observed using 10–150 ng of DNA, with reduced RFUs observed out of this range (Figure S2A). Moreover, the optimised annealing temperature was 58.8°C (27 × 10^3 RFU) with similar fluorescence in the range 56–61.5°C. Reduced fluorescence was observed out of this temperature range (Figure S2B).

4 | DISCUSSION

*HLA-B*35* is expressed across global populations and high frequency *B*35:01*, *B*35:02* and *B*35:05* variants are associated with life-threatening delayed drug hypersensitivity reactions.[16–20] Indeed *HLA-B*35:01*, the most prevalent global *B*35* variant, is recently associated with predisposition to co-trimoxazole-, polygonum multiflorum- and green tea-induced liver injuries, with expression in 50%, 45.4% and 72% of the respective affected patient populations,[16–18] and also potentially

TABLE 2 Presence or absence of known HLA-B alleles in our DNA cohort when tested using the B*35 assay

| HLA-B35 carriers | | | Other HLA-B alleles expressed in non-HLAB35 carriers | | | | | | | | | | | | | | | | | | | | | | | |
Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-	Allele	n	+/-
35:01	32	+	07:02	34	−	15:08	1	−	15:27	1	−	37:01	9	−	40:01	28	−	42:01	14	−	51:01	31	−	56:02	1	−
35:02	3	+	07:05	3	−	15:09	1	−	15:30	1	−	38:01	10	−	40:02	21	−	42:02	1	−	51:02	3	−	56:04	1	−
35:03	7	+	08:01	34	−	15:10	4	−	15:31	1	−	38:02	9	−	40:03	1	−	44:02	23	−	51:06	2	−	57:01	21	−
35:05	2	+	08:12	1	−	15:11	1	−	15:35	4	−	38:09	1	−	40:05	1	−	44:03	39	−	51:08	1	−	57:02	1	−
35:08	2	+	13:01	8	−	15:12	1	−	15:39	2	−	39:01	4	−	40:06	8	−	44:77	1	−	51:13	2	−	57:03	11	−
35:12	8	+	13:02	11	−	15:13	3	−	15:54	1	−	39:02	4	−	40:08	2	−	45:01	17	−	51:78	1	−	57:39	1	−
35:14	3	+	14:01	13	−	15:15	7	−	18:01	21	−	39:05	5	−	40:10	2	−	46:01	13	−	52:01	15	−	58:01	21	−
35:17	5	+	14:02	15	−	15:16	1	−	18:03	1	−	39:06	7	−	40:11	1	−	47:01	1	−	53:01	24	−	58:02	5	−
35:21	1	+	15:01	29	−	15:17	2	−	27:02	3	−	39:08	2	−	40:20	1	−	48:01	14	−	54:01	6	−	59:01	2	−
35:43	3	+	15:02	4	−	15:21	6	−	27:04	3	−	39:10	2	−	40:56	1	−	49:01	19	−	55:01	8	−	73:01	2	−
35:47	1	+	15:03	18	−	15:24	1	−	27:05	17	−	39:11	3	−	41:01	4	−	50:01	13	−	55:02	1	−	78:01	6	+
35:48	1	+	15:04	1	−	15:25	4	−	27:06	2	−	39:20	1	−	41:02	2	−	50:02	2	−	56:01	5	−	81:01	2	−

Note: DNA sample cohort utilised with American Society for Histocompatibility and Immunogenetics-accredited, sequence-based, high-resolution, full allelic HLA typing. n = number of DNA samples expressing the indicated allele and without B*35 or B*78 expression.

with liver injury to *Garcinia cambogia*.[26] However, while pre-treatment HLA screening has proven successful for *HLA-B*57:01 and abacavir hypersensitivity syndrome, a complete predictive value is not generalizable across all ethnicities and drugs.[1,2] Providing a cost-effective pharmacogenetic strategy to enable clinical validation studies and HLA risk based prevention and diagnostics, we have developed a fast, sensitive, specific, and low-cost TaqMan-based assay incorporating LNA primers and FAM-labelled probe to screen for high-frequency *HLA-B*35 alleles, suited to standard clinical labs with qPCR facilities. Within our DNA sample cohort (n = 430) we demonstrate reliability for safe clinical use with 100% sensitivity and specificity for detection of prevalent *HLA-B*35 alleles, and *B*78 alleles at low frequencies across the most prevalent global ethnicities: 0.98% in African Americans, 0.63% in Hispanics and < 0.01% in European Caucasian and Asian populations. Importantly, the assay was designed not to detect the *B*35-related *B*53:01 allele, which presents in 20% of African population and associated with predisposition to raltegravir-induced drug reaction with eosinophilia and systemic symptoms.[27] Despite just four polymorphic differences at the side of the antigen binding cleft in the alpha 1 helix, raltegravir does not similarly bind *HLA-B*35:01, highlighting the importance of being able to dissect between these two similar-sequence high-prevalence alleles to increase the proportion of patients correctly identified as 'at risk'. Moreover, *HLA-B*18 alleles with similar primer-targeting sequence are effectively distinguished from *B*35 alleles by specificity of the FAM labelled probe at position 199 in exon 2 (5′/56-FAM/TCTTGAAGA/ZEN/TCTGTGTGTTCCGG TCCC/3IABkFQ/−3′). Indeed, despite elevated fluorescence level after 20 cycles, the emitted RFU of the *HLA-B*18 amplifications were still clearly distinguishable from *HLA-B*35 alleles. The reagent cost per reaction is <$10, including reagents for DNA extraction, GoTaq master mix, primers and FAM-labelled probe. Moreover, the complete assay including DNA extraction, qPCR, and analysis can be performed in 2.5 h and thus suited for day return of clinical sample. The assay is designed with alignment to a shared sequence of the most frequent *HLA-B*35 alleles, excluding only a minority that present in low frequencies. Indeed, the sequence for >90% (501/552) of *HLA-B*35 alleles reported at up to six-digit resolution as of July 2021 aligned with our targeted sequences.

With high sequence similarity of individual *B*35 alleles a hindrance to development of an allele-specific PCR assay, we envisage this tool to provide cost-effective (i) means to narrow in on a *B*35-expressing interest demographic during clinical validation study, with full HLA sequencing on this limited cohort as required, and

(ii) pharmacogenetic risk-based prevention and diagnostics, of particular utility for green-tea-induced hepatic injury. Indeed, data suggest that herbal drugs are the most common cause of DILI, and in China, Chinese herbal medicine accounted for 54% of liver injury in hospitalised patients.[28] Furthermore, high concentration green tea is now being studied as an adjunctive cancer treatment. In the Minnesota Green Tea Trial (MGTT) observing breast cancer biomarkers after 12-month daily green tea catechin supplementation, 6%–7% of patients developed hepatitis, in keeping with the prevalence of *HLA-B*35:01* in this population.[29,30] Testing for *HLA-B*35* in this context may help identify patients at risk for rechallenge hepatitis or indeed other drug-induced hypersensitivity reactions and guide advice on future safe practices.

AUTHOR CONTRIBUTIONS

Yueran Li, Pooja Deshpande, Abha Chopra, Linda Choo performed the investigation, formal analysis, and validation. Yueran Li, Pooja Deshpande, Abha Chopra analysed the data. Abha Chopra, Andrew Gibson, Elizabeth J. Phillips conceptualised and designed the study. Yueran Li, Pooja Deshpande, Andrew Gibson wrote the manuscript. Andrew Gibson, Abha Chopra, and Elizabeth J. Phillips provided project administration and supervision. All the authors have read and approved the final manuscript.

CONFLICT OF INTEREST

Elizabeth J. Phillips receives royalties from UpToDate and consulting fees from Biocryst, Janssen and Vertex. She is co-director of IIID Pty Ltd. that holds a patent for *HLA-B*57:01* testing for abacavir hypersensitivity, and she holds a patent with AC for detection of *HLA-A*32:01* in connection with Diagnosing Drug Reaction with Eosinophilia and Systemic Symptoms without financial remuneration. All the other authors have no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.