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RESEARCH ARTICLE

Identification of regulatory variants of carboxylesterase 1 (CES1): A proof-of-concept study for the application of the Allele-Specific Protein Expression (ASPE) assay in identifying *cis*-acting regulatory genetic polymorphisms

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Abstract

It is challenging to study regulatory genetic variants as gene expression is affected by both genetic polymorphisms and non-genetic regulators. The mRNA allele-specific expression (ASE) assay has been increasingly used for the study of *cis*-acting regulatory variants because *cis*-acting variants affect gene expression in an allele-specific manner. However, poor correlations between mRNA and protein expressions were observed for many genes, highlighting the importance of studying gene expression regulation at the protein level. In the present study, we conducted a proof-of-concept study to utilize a recently developed allele-specific protein expression (ASPE) assay to identify the *cis*-acting regulatory variants of CES1 using a large set of human liver samples. The CES1 gene encodes for carboxylesterase 1 (CES1), the most abundant hepatic hydrolase in humans. Two *cis*-acting regulatory variants were found to be significantly associated with CES1 ASPE, CES1 protein expression, and its catalytic activity on enalapril hydrolysis in human livers. Compared to conventional gene expression-based approaches, ASPE demonstrated an improved statistical power to detect regulatory variants with small effect sizes since allelic protein expression ratios are less prone to the influence of non-genetic regulators (e.g., diseases and inducers). This study suggests that the ASPE approach is a powerful tool for identifying *cis*-regulatory variants.

KEYWORDS
allele-specific protein expression, carboxylesterase 1, pharmacogenetics, proteomics, regulatory variants

Abbreviations: ASPE, allele-specific protein expression; CES1, carboxylesterase 1; HLM, human liver microsome; HLS9, human liver S9 fraction.

1 | INTRODUCTION

Regulatory genetic variants can be classified into *cis*- and trans-acting variants. A *cis*-acting regulatory variant is located in the proximity of the gene being regulated, such as the promoter of the gene, while a *trans*-acting variant and the gene being regulated are usually located on different chromosomes [1, 2]. Consequently, *cis*-variants affect gene expression in an allele-specific manner, whereas trans-variants regulate gene expression in both alleles. *Trans*-variants typically have weaker effects on gene expression than *cis*-variants [2–4]. Gene expression can also be heavily influenced by non-genetic factors (e.g., diseases and inducers), resulting in impaired statistical power when the gene expression level is used as a phenotype for regulatory genetic variant identification. Accordingly, measuring gene allele-specific expression (ASE) has been increasingly used as a powerful means to identify *cis*-acting polymorphisms because allelic expression ratios are less prone to the influence of non-genetic regulators [5–7].

ASE was traditionally studied at the mRNA level. A study simultaneously quantified mRNA and protein expressions of many drug-metabolizing enzymes (DMEs) and transporters in human livers and revealed that, while there was a strong correlation between mRNA and protein levels for CYP3A4, CYP2B6, CYP2C8, and OATP1B1, mRNA expression was either moderately or poorly correlated with protein levels for the most tested DMEs and transporters [8]. Moreover, a study of transcriptomics and proteomics in a large set of human cell lines identified a class of *cis*-acting variants that affected protein expression without significantly altering mRNA or ribosome levels [9]. The discordant mRNA and protein expressions are likely caused by post-transcriptional processes, such as protein translation, post-translational modification, and degradation [10]. Genetic variants affecting such post-transcriptional processes could not be identified by conventional mRNA expression and ASE approaches. Thus, a protein expression-based strategy is needed to comprehend all regulatory genetic variants, including those affecting gene expression at the post-transcriptional level.

A targeted proteomics method was developed by our laboratory and others to quantify allele-specific protein expression (ASPE) of a gene using heavy stable isotope-labeled QconCAT internal standards [11–13]. We expect that this ASPE approach is superior to the conventional ASE method because it has the potential to detect genetic variants that regulate gene expression at the post-transcriptional level. The ASPE assay could also be advantageous over conventional protein expression-based assays because allelic protein expression ratios are less likely affected by non-genetic regulators.

The expression of a DME can vary markedly between individuals, which is a major contributing factor to the interindividual variability in the pharmacokinetics (PK) and pharmacodynamics (PD) of its substrate drugs. Genetic polymorphisms have been well established as a determinant of varied DME expressions and can affect the therapeutic outcomes of many medications. Despite extensive research in the past several decades, regulatory genetic variants identified to date can only explain a small portion of inheritable variability in DME expression. The inability to identify regulatory variants is a major obstacle hindering the full potential of DME pharmacogenetics in optimizing pharmacotherapy. Thus, identifying and characterizing hidden genetic variants capable of regulating DME expression is essential for advancing DME pharmacogenetics-based precision pharmacotherapy.

SIGNIFICANCE OF THE STUDY

Targeted proteomics methods have been established to measure allele-specific protein expression (ASPE) by our group and others. This study was the first to utilize the ASPE assay to identify cis-acting regulatory variants. Unlike previous protein expression-based association study methods, the ASPE assay reduces the confounding effects caused by non-genetic factors, resulting in improved statistical power. We demonstrated its utility by identifying two cis-acting regulatory variants of the CES1 gene, suggesting that the method could be widely used to study cis-regulatory variants.

In this proof-of-concept study, we applied this ASPE assay to identify *cis*-acting variants of the CES1 gene. CES1 encodes for the carboxylesterase 1 (CES1) protein, the most abundant hepatic hydrolase in humans. CES1 is responsible for the metabolism of many drugs, endogenous substances, and environmental toxins [14]. CES1 expression and activity differ significantly among individuals, which is associated with the interindividual variability in response to drugs metabolized by the enzyme [15–17]. The CES1 gene is highly polymorphic with numerous genetic variants in both regulatory and coding regions. CES1 nonsynonymous variants have been extensively studied in the past decade. Among those identified CES1 nonsynonymous variants, the loss-of-function variant G143E (rs71647871) markedly altered the PK and clinical outcomes of several CES1 substrates, such as methylphenidate and clopidogrel [18, 19]. However, these nonsynonymous variants can only explain a small portion of CES1 interindividual variability because of their low frequencies and not accounting for the expression variation. Given the marked interindividual variability in CES1 protein expression in human livers, much effort has been devoted to identifying CES1 regulatory variants [20–31]. However, none of the studied variants showed consistent effects on CES1 expression or clinical outcomes across different studies. Therefore, in the present study, instead of using conventional pharmacogenetic approaches, we measured the ASPE ratios of CES1 in normal human livers using the CES1 nonsynonymous variant S75N (rs2307240) as the marker for CES1 allelic expression (Figure 1). S75N marker was used because the variant does not affect CES1 protein expression but allows us to distinguish the protein expressed from each allele [16]. We further performed whole-genome genotyping of these samples and conducted an association analysis using the ASPE ratios as the phenotype. The study discovered two novel *cis*-acting variants that were significantly associated with CES1 protein expression and activity in human livers. This investigation

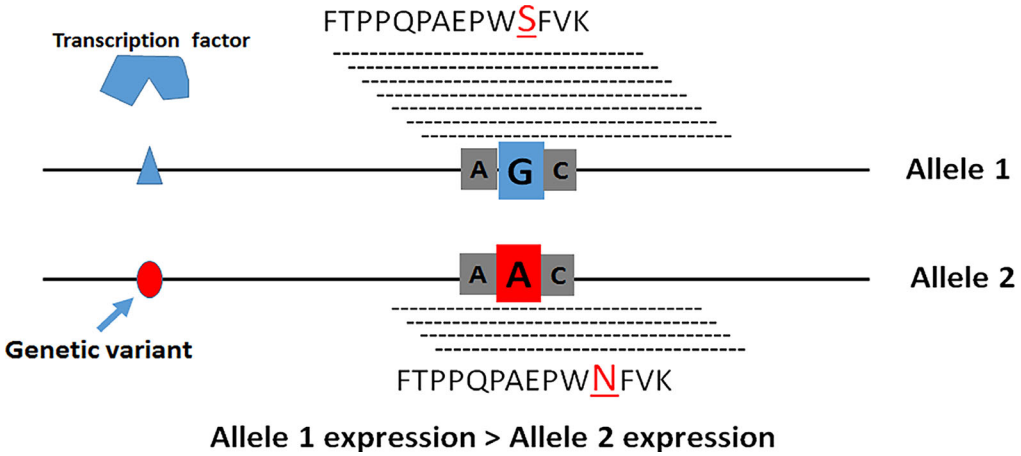

FIGURE 1 Concept of Allele-Specific Protein Expression (ASPE). The ASPE assay measures protein expression from each allele, and the ratios of protein expression from two alleles are used as a phenotype for the identification of cis-acting regulatory variants. One of the major advantages of the ASPE assay is that the ASPE ratios are not affected by non-genetic regulatory factors. The expression from one allele would serve as the control for another allele to filter out the background noise caused by non-genetic regulators. In this schematic, there is a genetic variant impairing transcriptional factor binding, resulting in reduced CES1 protein expression from Allele 2. By using the ASPE ratio as a phenotype, this genetic variant can be detected with higher statistical power. We used a benign nonsynonymous SNP (a genetic variant that does not affect the expression level and activity) S75N (rs2307240) as a marker to differentiate CES1 protein expression from each allele.

was the first to use this novel ASPE assay to identify regulatory genetic polymorphisms of a gene.

2 | MATERIALS AND METHODS

2.1 | Materials

Amino acids, acetonitrile, benzonase nuclease, calcium chloride hexahydrate, formic acid, glucose, M9 salts, magnesium sulfate, imidazole, isopropyl-D-1-thiogalactopyranoside disodium phosphate, sodium chloride, thiamine, and trifluoroacetic acid were purchased from Sigma-Aldrich (Saint Louis, MO). $^{13}C_6$ arginine and $^{13}C_6$ and $^{15}N_2$ lysine were products from Cambridge Isotope Laboratories (Tewksbury, MA). Lysyl endopeptidase was purchased from Wako Chemicals (Richmond, VA). Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ). Lysozyme solution (50 mg/mL), slide-A-Lyzer G2 dialysis cassettes (3.5K MWCO), Pierce BCA protein assay kit, urea, and dithiothreitol were purchased from Fisher Scientific Co. (Pittsburgh, PA). Iodoacetamide and ammonium bicarbonate were purchased from Acros Organics (Morris Plains, NJ). Water Oasis HLB columns were from Waters Corporation (Milford, MA). Synthetic iRT standards were purchased from Biognosys AG (Cambridge, MA). *Escherichia coli* strain BL21(DE3) and BugBuster protein extraction reagent were products of EMD Millipore (Burlington, MA). HisTrap HP histidine-tagged protein purification columns were from GE Healthcare (Pittsburgh, PA). Two hundred eighty-seven normal human liver samples were obtained from XenoTech LLC (Lenexa, KS, USA), the University of Minnesota Liver Tissue Cell Distribution System, and the Cooperative Human Tissue Network (CHTN), and the demographic information was reported in our previously published studies [11, 32]. The gender and ethnicity of the human liver samples are included in Table S1.

2.2 | Human liver microsome and S9 fraction preparation

Human liver microsomes (HLM) and human liver S9 fractions (HLS9) were prepared using previously published methods [15, 33]. Briefly, a 200 mg human liver tissue was cut into approximately one by one mm pieces and homogenized in 600 μL PBS buffer (pH 7.4) using a tissue grinder. The sample was centrifuged at 10,000 $\times$ *g* for 30 min, and the supernatant (i.e., HLS9) was collected. To prepare HLM, the supernatant was transferred to Beckman ultracentrifuge tubes and centrifuged at 300,000 $\times$ *g* for 20 min. The pellets were resuspended in PBS using a tissue grinder and collected (i.e., HLM). Protein concentrations were determined using a Pierce BCA protein assay kit. Both HLS9 and HLM samples were stored at −80°C until use. Of note, the same set of liver samples was used for the preparation of both HLM and HLS9 samples; however, different proteomics methods were used for CES1 protein quantifications (i.e., heavy stable isotype internal standard-based assay for HLM vs. label-free quantification method for HLS9) [34].

2.3 | QconCAT internal standard preparation

A QconCAT DNA construct was synthesized de novo to generate the heavy stable isotope-labeled QconCAT internal standard for the CES1 ASPE analysis using a method similar to that we previously

reported [11]. The construct contains the DNA sequences encoding for both the wild-type CES1 tryptic peptide FTPPQPAEPWSFVK and the S75N variant FTPPQPAEPWNFVK. The S75N is a benign nonsynonymous variant without significant effects on CES1 expression and activity [27, 29] and was chosen as a biomarker to differentiate CES1 allelic expression. The minor allele frequency (MAF) S75N is around 5% across different populations [29]. Both peptides are flanked by 15 native amino acids to ensure the same trypsin digestion efficiency [11]. The DNA construct also includes three CES1 surrogate peptides (AISESGVALTSVLVK, TAMSLLWK, and ELIPEATEK) for total CES1 protein quantification. The amino acid sequences of the CES1 QconCAT construct can be found in Figure S1.

The QconCAT DNA construct was transformed to *E. coli* strain BL2L and cultured in the medium supplemented with $^{13}C_6$ arginine, $^{13}C_6$, and $^{15}N_2$ lysine. QconCAT protein expression was induced by adding 1 mM isopropyl-D-1-thiogalactopyranoside. After 5 h of growth at 37°C, the cells were lysed, and QconCAT proteins were extracted using affinity chromatography, followed by three rounds of dialysis against 50 mM ammonium bicarbonate containing 1 mM dithiothreitol.

2.4 | Proteomic sample preparation

HLS9 and HLM samples were prepared for proteomics analysis using the method we previously published [35]. Briefly, 80 μg HLS9 or HLM proteins were mixed with 0.2 μg bovine serum albumin. For the HLM samples, the QconCAT internal standard (172 ng) was also added. The sample was mixed with one ml of pre-cooled acetone, and the mixture was briefly vortexed and stored at −20°C for at least 2 h to precipitate proteins. The mixture was then centrifuged at 17,000 × *g* for 15 min at 4°C, and the supernatant was removed. The pellet (i.e., precipitated proteins) was air-dried and then resuspended in 100 μl of 4 mM dithiothreitol/8 M urea/100 mM NH_4HCO_3 solution for reduction at 37°C for 45 min. A 100 μl of 20 mM iodoacetamide/8 M urea/100 mM NH_4HCO_3 solution was added, and the mixture was incubated at room temperature in the dark for 30 min for alkylation. Following the incubation, 56.6 μl of 50 mM NH_4HCO_3 was added to reduce the urea concentration to 6 M.

A two-step protease digestion protocol was used to digest the proteins. The first digestion was with lysyl endopeptidase (protein: lysyl endopeptidase = 100:1) in an orbital incubator shaker at 220 rpm and 37°C for 6 h. Then, 733 μl of 50 mM NH_4HCO_3 was added to further reduce the urea concentration to 1.6 M. The second digestion was carried out with TPCK-treated trypsin (protein: trypsin = 50:1) in an orbital incubator shaker at 220 rpm and 37°C for overnight. One μl trifluoroacetic acid was added to terminate the digestion. Waters Oasis HLB columns were utilized to clean and extract the digested peptides. The eluted peptides were dried in a SpeedVac SPD1010 vacuum concentrator and resuspended in 3% acetonitrile solution containing 0.1% formic acid. The eluted peptides were then centrifuged, and half of the supernatant was transferred to an autosampler vial and mixed with 1 μl of the synthetic iRT standard solution prior to LC-MS/MS analysis.

2.5 | LC-MS/MS-based proteomics analysis

The proteomic analysis was conducted using the previously published method [34] on a TripleTOF 5600+ mass spectrometer (AB Sciex, Framingham, MA) coupled with an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). A trap-elute configuration was adopted for the analysis, which included a trapping column (ChromXP C18-CL, 120 Å, 5 μm, 0.3 mm cartridge, Eksigent Technologies, Dublin, CA) and an analytical column (ChromXP C18-CL, 120 Å, 150 × 0.3 mm, 5 μm, Eksigent Technologies, Dublin, CA). Six μg of digested proteins were injected, and peptides were trapped and cleaned on the trapping column with the mobile phase A (water with 0.1% formic acid) at a flow rate of 10 μl/min for 3 min before being separated on the analytical column with gradient elution at a flow rate of 5 μl/min. The gradient time program was set as follows for the phase B (acetonitrile containing 0.1% formic acid): 0 to 68 min: 3% to 30%, 68 to 73 min: 30% to 40%, 73 to 75 min: 40% to 80%, 75 to 78 min: 80%, 78 to 79 min: 80% to 3%, and finally 79 to 90 min at 3% for column equilibration. A blank sample was injected between each analysis to prevent carryover. The mass spectrometer was operated in a positive ion mode with an ion spray voltage floating at 5500 v, ion source gas one at 28 psi, ion source gas two at 16 psi, curtain gas at 25 psi, and ion source temperature at 280°C.

Both HLS9 and HLM samples were analyzed using a data-independent acquisition (DIA) method we previously reported, which included a 250-ms TOF-MS scan from 400 to 1250 Da and MS/MS scans from 100 to 1500 Da [36]. The MS/MS scans of all precursors were performed in a cyclic manner using a 100-variable isolation window scheme. The accumulation time was 25 ms per isolation window, resulting in a total cycle time of 2.8 s.

For the S75N heterozygous HLM samples, the expression levels of each of two CES1 alleles (i.e., ASPE) were determined based on the peak ratios of the light peptides FTPPQPAEPWNFVK (S75N mutant peptide) and FTPPQPAEPWSFVK (S75N wild-type peptide) to the corresponding heavy internal standards. Total CES1 protein expression levels in the HLM were determined based on the ratios of the peak areas of the CES1 signature peptides AISESGVALTSVLVK, TAMSLLWK, and ELIPEATEK to their heavy isotope-labeled internal standard counterparts. The Skyline software (University of Washington, Seattle, WA) was used for the HLM proteomics data analysis. The HLS9 DIA data were analyzed using the Spectronaut Pulsar software (version 11.0; Biognosys AG, Schlieren, Switzerland) with default settings (precursor Q value < 0.01, protein Q value < 0.01) and its internal reference spectral library "Human – Liver (fractionated)". Absolute CES1 protein levels in the HLS9 samples were estimated using the DIA-TPA algorithm [34].

2.6 | CES1 activity measurements

Enalapril is a selective substrate of CES1, and enalapril hydrolysis was determined in the HLS9 samples as a surrogate maker for CES1 activity [15]. Briefly, 100 μl of enalapril solution (500 μM in PBS) was mixed with 100 μl of 0.2 mg/mL of HLS9. After incubation at 37°C for 10 min,

the reactions were terminated by adding a 4-fold volume of methanol containing the analytical internal standard 5-hydroxyomeprazole (10 ng/mL). Then, the samples were vortexed and centrifuged at 13,200 rpm at 4°C for 20 min to remove the precipitated proteins. The supernatant was collected and analyzed for the concentrations of the hydrolytic metabolite enalaprilat utilizing an established LC-MS/MS method [15].

2.7 | Data analysis and statistics

The genotype data of the human liver samples ($n = 287$) were retrieved from a study recently published by our group [32], which contained 1,779,819 genetic markers. Thirty out of the 287 subjects were identified as S75N heterozygotes. The subsequent quality control (QC) analysis was performed to remove single nucleotide polymorphisms (SNPs) with MAF < 0.01 or deviating from Hardy-Weinberg equilibrium ($p < 0.0001$) [32]. The genotype data were phased during genotype imputation [32] to detect the allele-specific effects of *cis*-regulatory variants. Previous literature suggested that *cis*-acting regulatory variants are usually located approximately 5000 base pairs (bp) upstream and downstream of the gene [37, 38]. Thus, a total of 856 SNPs located 5000 bp upstream and downstream of the CES1 gene were included in this study. SNPs were removed if their MAFs were less than 10% in the 30 S75N heterozygous liver samples. Moreover, when several SNPs were in complete linkage disequilibrium, only one SNP was used as the tag SNP for data analysis to alleviate the multiple testing burden. Overall, 72 SNPs were retained for data analysis.

The CES1 ASPE ratios of 75S to 75N were log2 transformed to normalize the effect size of regulatory SNPs, given that the SNPs can reside on either the S or the N allele. For each of the 72 SNPs, two linear regression models were created to identify CES1 *cis*-regulatory variants. The first model used the S75N ASPE ratios as the phenotype to test the associations between the genotypes and the ASPE ratios in the S75N heterozygous samples ($n = 30$). The second model used the total CES1 protein expression (i.e., the CES1 expression from both alleles) to test the associations between the genotypes and the CES1 expression in all 287 liver samples. *p*-values from the two linear models were then combined using a Fisher's combined probability test, and the Benjamini-Hochberg method was used for the multiple testing correction.

A conventional genome-wide association study (GWAS) was performed to detect genetic variants associated with hepatic CES1 protein expression using the previously published method [32]. The HLM and HLS9 expression data and the genotype dataset from the same human liver samples were used for the GWAS analysis. The *p*-value threshold was set at 5×10^{-8} to account for multiple comparisons.

3 | RESULTS

We successfully measured CES1 ASPE and protein expression levels in a large set of human liver samples. We also genotyped the whole genomes and determined CES1 catalytic activity in the liver samples. The study revealed two novel *cis*-acting variants associated with CES1 expression and catalytic activity in the liver, demonstrating that ASPE could be a powerful approach to identify *cis*-regulatory genetic variants.

3.1 | CES1 ASPE and CES1 protein expression in HLM and HLS9

We measured the CES1 ASPE in 30 S75N heterozygous liver samples using a QconCAT internal standard. The ASPE ratios of 75S to 75N ranged from 0.80 to 1.47 (log2 transformed values: -0.3 to 0.5) (Figure 2), indicating the presence of *cis*-regulatory genetic variants of the CES1 gene. Relative CES1 expression levels in HLM were determined using a heavy isotype-labeled QconCAT standard, and absolute CES1 protein levels in HLS9 were estimated using the DIA-TPA algorithm we previously established [34]. CES1 expressions varied markedly in both HLM and HLS9 samples. Neither HLM nor HLS9 CES1 levels were significantly correlated with the ASPE ratios in the 30 S75N heterozygous samples.

3.2 | GWAS result

We conducted a GWAS in both HLM and HLS9 samples with a total of 1,671,387 genotyped and imputed SNPs. The Manhattan plot showed the P-value of the association of each SNP with CES1 protein expression in HLS9 (Figure 3A) and HLM (Figure 3B). The GWAS significant P-value threshold was set at 5×10^{-8} to account for multiple comparisons. No genetic variants were found to be significantly associated with CES1 protein expression at the genome-wide significant level (Figure 3).

3.3 | Identification of *cis*-acting CES1 regulatory variants

The ASPE-based statistical model revealed that two CES1 regulatory variants, rs6499788 and rs35918553, were significantly associated with CES1 ASPE in the HLM samples (Figure 4). Moreover, the rs6499788 A allele and the rs35918553 G allele were associated with 15.9% ($p = 0.01$) and 14.9% ($p = 0.01$) reductions of total CES1 protein expression in HLM, respectively (Figure 4). The effect of the two variants on CES1 protein expression was also evaluated in HLS9 prepared from the same human liver samples. Consistent with the findings from HLM, the rs6499788 A allele and the rs35918553 G allele were associated with 10.3% and 11.8% reductions, respectively, in CES1 protein expression in HLS9 (Figure 5).

The activity study confirmed that the rs6499788 A allele and the rs35918553 G allele reduced CES1 activity on hydrolyzing the CES1 selective substrate enalapril by 27.9% ($p = 0.04$) and 26.6% ($p = 0.08$), respectively, in the HLS9 samples (Figure 5). The associations of the

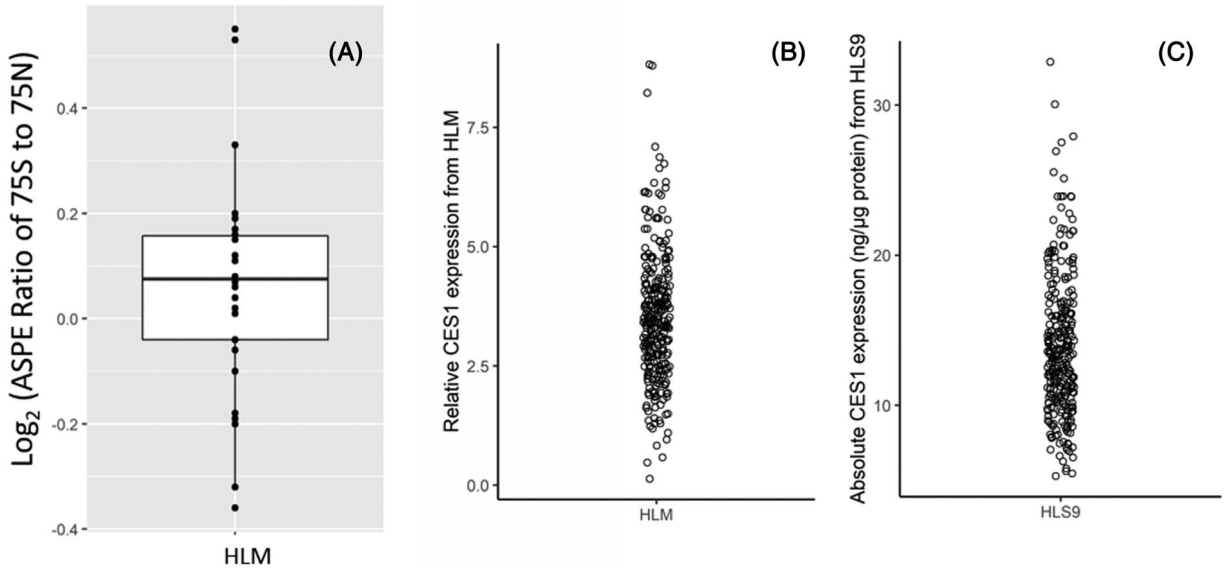

FIGURE 2 Distribution of CES1 ASPE ratios (A), CES1 protein expression levels in HLM (B), and HLS9 (C) in human liver samples. The ASPE ratios of 75S to 75N were determined in 30 S75N heterozygous HLM samples using a heavy stable isotype-labeled QconCAT internal standard (Figure 2A). Relative CES1 expression levels in HLM were determined using the same QconCAT standard, and the values in the Y-axis are the ratios of the light-to-heavy CES1 peptides (Figure 2B). CES1 in the HLS9 samples was measured using a label-free DIA method, and the absolute CES1 protein levels were estimated using an established DIA-TPA algorithm (Figure 2C).

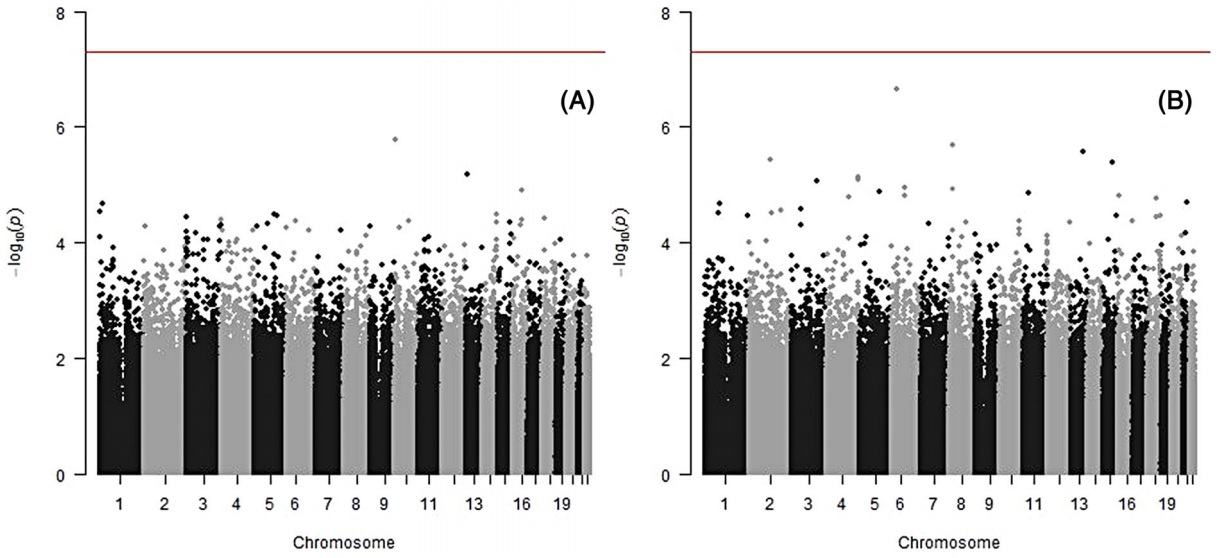

FIGURE 3 Manhattan plots of the genome-wide association study (GWAS) of CES1 protein expression in HLS9 (A) and HLM (B). The Y-axis represents the *p* values of the association between the SNPs and CES1 protein levels, and the X-axis represents the genomic coordinates of the tested SNPs. No SNP was found to reach the statistically significant threshold (2.99×10^{-8}, the red horizontal line).

two SNP with CES1 protein expression and activity in human livers were summarized in Table 1.

4 | DISCUSSION

For the first time, we used an ASPE approach to identify *cis*-regulatory genetic variants and revealed two *cis*-acting genetic variants (rs6499788 and rs35918553) associated with CES1 ASPE and protein expression in HLM. The findings were further validated with the CES1 protein expression and CES1 activity data obtained from HLS9 prepared from the same set of liver samples. Rs6499788 and rs35918553 are located within 5000 bp upstream of the CES1 gene. Both SNPs are common variants (MAF: rs6499788: 27.7% and rs35918553: 27.2%) and are in high linkage disequilibrium (D = 1, Ensembl genome database). We performed an in-silico analysis to eval-

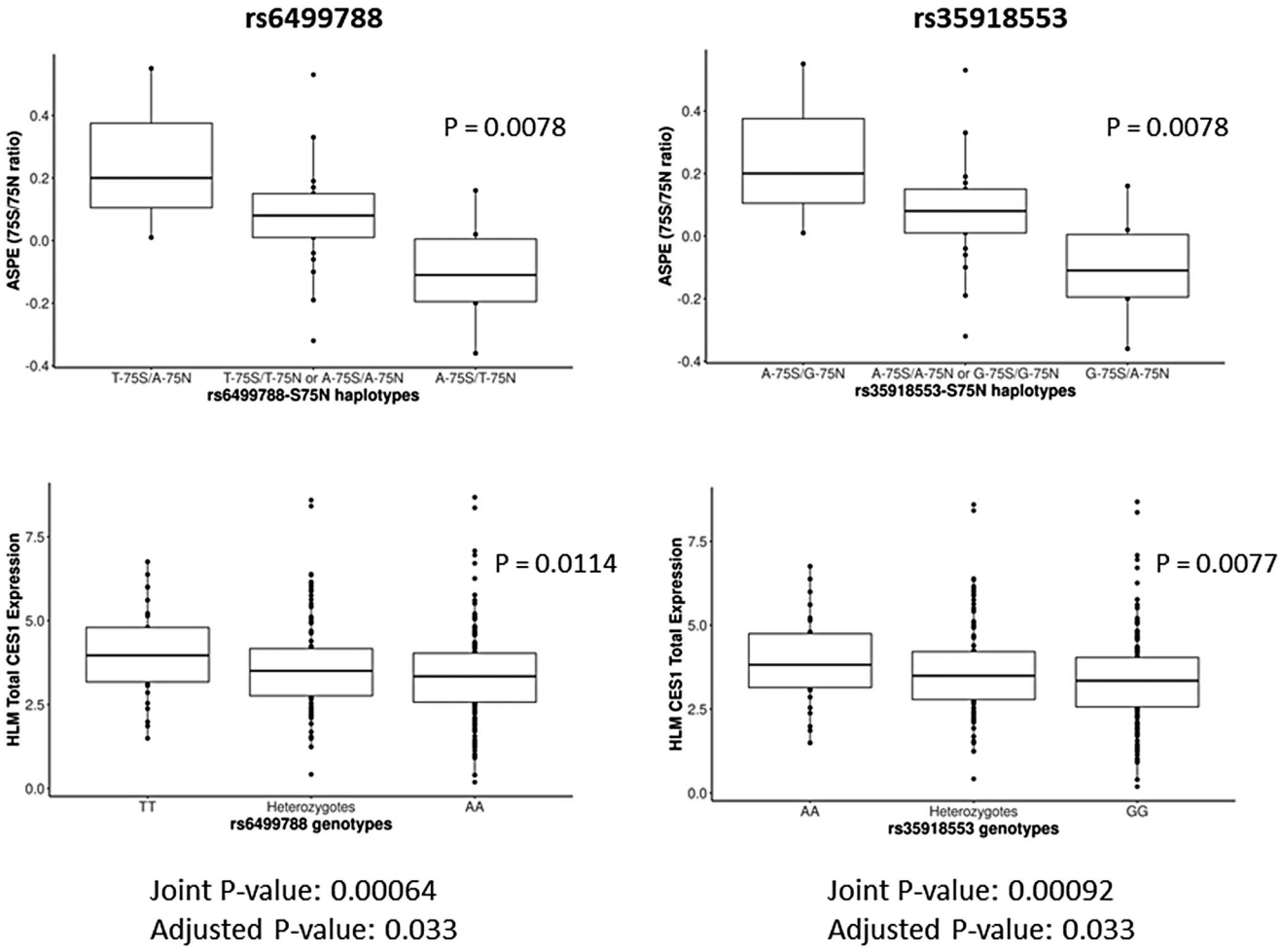

FIGURE 4 Two regulatory variants (rs6499788, rs35918553) showed a significant association with CES1 ASPE (upper panels) and protein expression in HLM (lower panels). A linear regression t-test was performed for the ASPE and CES1 expression models. The tests of the ASPE and CES1 expression models were performed independently, and *p*-values from the two models were combined using Fisher's combined probability test for each SNP (i.e., Joint *p*-value). Joint *p*-value was corrected for multiple comparisons using the Benjamini-Hochberg method (i.e., adjusted *p*-value). The adjusted *p*-value at the bottom represents the final *p*-value for each SNP.

TABLE 1 Impact of rs6499788 and rs35918553 on CES1 protein expression and catalytic activity on enalapril hydrolysis in human livers

	rs6499788					rs35918553				
	TT	Heterozygotes	AA	R^2	*p*-value	AA	Heterozygotes	GG	R^2	*p*-value
Relative CES1 expression in HLM ($n = 287$)	4.01 ± 1.34	3.66 ± 1.42	3.37 ± 1.31	0.02	0.01	3.95 ± 1.31	3.69 ± 1.44	3.37 ± 1.31	0.02	0.01
CES1 expression in HLS9 (ng/μg protein) ($n = 287$)	14.99 ± 5.46	14.24 ± 4.89	13.44 ± 4.24	0.01	0.05	15.23 ± 5.39	14.20 ± 4.93	13.43 ± 4.22	0.01	0.03
Enalapril Hydrolysis Rate (pmol/min/mg protein) ($n = 102$)	69.08 ± 44.70	64.94 ± 50.55	49.77 ± 29.82	0.03	0.04	69.08 ± 44.70	63.01 ± 49.36	50.68 ± 31.14	0.02	0.08

Relative CES1 expression in HLM was quantified using a heavy stable isotope-labeled QconCAT internal standard, and the quantification values are the ratios of the light-to-heavy CES1 peptides.
Absolute CES1 expression in HLS9 was quantified using a label-free quantification method [34].
Enalapril hydrolysis rate was used as a surrogate marker for CES1 activity. Enalapril is a selective substrate of CES1 [15].

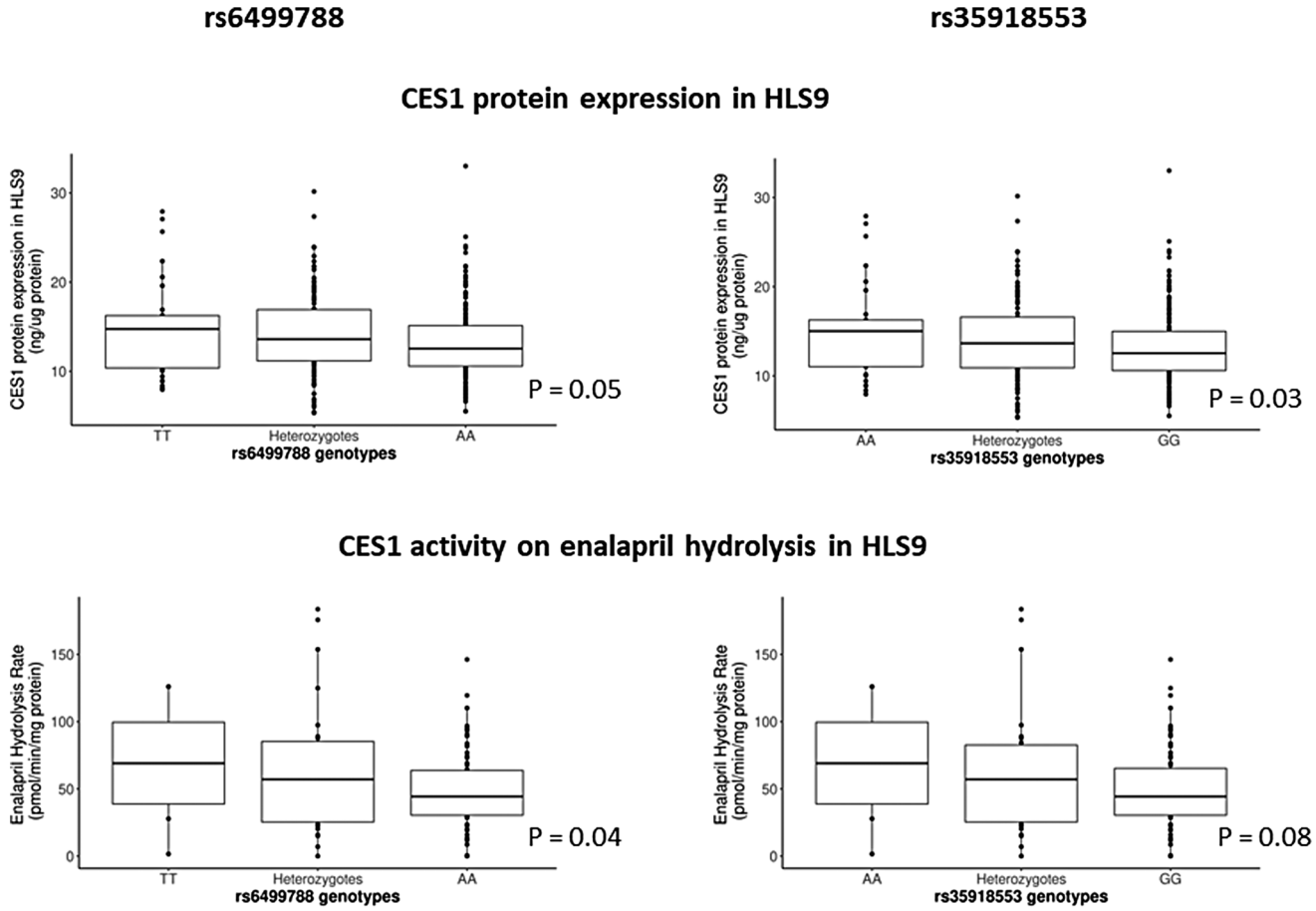

FIGURE 5 Association of the identified regulatory variants (rs6499788, rs35918553) with CES1 protein expression and catalytic activity in HLS9. To validate the association of the two identified SNP, the associations of the two variants with CES1 protein expression and activity on enalapril hydrolysis were determined in HLS9. The same set of liver samples was used for the preparation of HLM and HLS9 samples; however, different proteomics methods were used for CES1 protein quantification (i.e., heavy stable isotype internal standard-based targeted assay for HLM vs. label-free quantification method for HLS9).

uate the potential function of the two SNPs using the online annotation tool HaploReg [39]. The results indicate that the variant rs35918553 is located in a region with the enhancer histone marks BLD, LIV, and LNG. Moreover, the SNP rs35918553 region binds to the transcription factor MafK. Thus, it is plausible that the observed association between the two variants and the CES1 ASPE is due to that rs35918553 regulates the CES1 enhancer activity. Further experiments are needed to validate this speculation.

4.1 | Importance of studying CES1 regulatory variants

CES1 is an important DME responsible for 80% to 95% of total hydrolytic activity in the liver [40]. CES1 plays a key role in metabolizing endogenous compounds, environmental toxins, and numerous therapeutic agents [15, 41]. CES1 expression and activity vary significantly among individuals, which is a major factor contributing to interindividual variability in response to medications metabolized by CES1. Though considerable efforts have been devoted to the study of functional CES1 genetic variants, to date, the nonsynonymous variant G143E is the only clinically significant loss-of-function variant identified for CES1 [20–31]. However, considering the MAF of G143E is 2% to 4% [15], G143E can only explain a small portion of the interindividual variability of the CES1 function.

While nonsynonymous polymorphisms can directly affect the catalytic function of an enzyme by altering amino acid sequences in regions critical to the protein function, regulatory variants can affect the function of an enzyme by regulating gene expression [42]. Similar to many other DMEs, CES1 protein expression correlates poorly to its mRNA expression in the liver [43]. For example, Sanford et al. reported that a translocation CES1 variant reduced mRNA expression of CES1 by 30% using a conventional mRNA ASE method. However, this regulatory variant was not associated with CES1 protein expression or CES1 activity in human livers [43]. Thus, it is essential to study CES1 expression regulation at the protein level. To the best of our knowledge, the two CES1 regulatory variants identified in the present study are the first found to be associated with both CES1 protein expression level and CES1 activity in the human liver. Identification of CES1 regulatory genetic variants will lead to a better understanding of hepatic CES1

expression variability and allow for a better prediction of the PK/PD of CES1 substrate drugs.

4.2 | Novelty and significance of the ASPE assay

Relative to conventional protein expression-based association analysis (e.g., GWAS), the main advantage of the ASPE assay is its improved statistical power, enabling the detection of small effect size variants by filtering out confounding effects caused by non-genetic regulators. In addition, the ASPE method could detect genetic variants regulating gene expression at the post-transcription level. To date, the most common method to identify regulatory genetic variants is to determine the association between the mRNA expression level and genetic variations. Unfortunately, many genes, especially those encoding for DMEs, have a poor correlation between mRNA and protein expression levels, which is likely due to the presence of various regulatory elements that affect post-transcriptional processing, such as protein translation and degradation [8, 9, 16, 43]. Recently, protein expression level has been increasingly used as the phenotype to identify regulatory genetic variants [9, 44]; however, the statistical power of this approach is compromised by the fact that, in addition to genetic variants, non-genetic factors (e.g., diseases and inducers) can also affect protein expression. Unlike conventional protein expression methods that measure total protein expression from both alleles of a gene, the ASPE assay enables accurate quantification of protein expression from each allele of the gene. Since *cis*-regulatory variants influence gene expression in an allele-specific manner while *trans*-acting regulatory elements and non-genetic factors affect gene expression on both alleles, the observation of an allelic expression imbalance would suggest the existence of *cis*-acting regulatory elements. Thus, ASPE is more accurate, sensitive, and robust than conventional approaches (e.g., GWAS) for the discovery of regulatory variants, given that the expression of each of the two alleles is measured simultaneously in the same individual under the same experimental conditions, and each allele can serve as a control for the other. Although the present study focused on CES1 genetic variants, we expect that this ASPE assay could be widely used to identify regulatory variants of other genes.

Some limitations of the study include a small sample size of the heterozygous S75N samples ($n = 30$) and a lack of true biological replicates for the validation study as both HLS9 and HLM were prepared from the same liver samples.

In summary, the ASPE approach enabled the detection of regulatory variants with a small effect size ($R^2 = 0.01 \sim 0.03$, Table 1) that the conventional GWAS was not able to detect (Figure 3), which is likely due to the fact that GWAS is more prone to the influence of the expression variability caused by non-genetic regulators. The two small effect size yet common variants (MAF = 0.277) could play an important role in building a comprehensive model to better predict the PK and PD of CES1 substrate drugs. Future clinical studies are warranted to examine the effect of rs6499788 and rs35918553 on the PK and PD of drugs metabolized by CES1. In addition, future investigations involving more S75N heterozygous liver samples might allow researchers to detect additional *cis*-acting CES1 regulatory variants.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw data and complete MS datasets have been uploaded to the ProteomeXchange database (Dataset identifier PXD019169) and are freely available to the public.

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