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A comprehensive functional assessment of carboxylesterase 1 nonsynonymous polymorphisms

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Running title: Pharmacogenetics of CES1 nonsynonymous polymorphisms

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Abstract

Carboxylesterase 1 (CES1) is the predominant human hepatic hydrolase responsible for the metabolism of many clinically important medications. CES1 expression and activity vary markedly among individuals; and genetic variation is a major contributing factor to CES1 interindividual variability. In the present study, we comprehensively examined the functions of *CES1* nonsynonymous single nucleotide polymorphisms (nsSNPs) and haplotypes using transfected cell lines and individual human liver tissues. The 20 candidate variants include *CES1* nsSNPs with a minor allele frequency (MAF) > 0.5% in a given population or located in close proximity to the CES1 active site. Five nsSNPs including L40Ter (rs151291296), G142E (rs121912777), G147C (rs146456965), Y170D (rs148947808), and R171C (rs201065375) were found to be loss-of-function variants for metabolizing the CES1 substrates clopidogrel, enalapril, and sacubitril. Additionally, the A158V (rs202121317), R199H (rs2307243), E220G (rs200707504), and T290M (rs202001817) decreased CES1 activity to a lesser extent in a substrate-dependent manner. Several nsSNPs including the L40Ter (rs151291296), G147C (rs146456965), Y170D (rs148947808), and R171C (rs201065375) significantly reduced CES1 protein and/or mRNA expressions in the transfected cells. Functions of the common nonsynonymous haplotypes D203E-A269S and S75N-D203E-A269S were evaluated using cells stably expressing the haplotypes and a large set of human livers. Neither CES1 expression nor activity were affected by the two haplotypes. In sum, the study revealed several functional nsSNPs with impaired activity on the metabolism of CES1 substrate drugs. Clinical investigations are warranted to determine whether these nsSNPs can serve as biomarkers for the prediction of therapeutic outcomes of drugs metabolized

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Introduction

Carboxylesterase 1 (CES1) is the most abundant drug-metabolizing enzyme in human livers (Achour et al., 2017), contributing to 80%-95% of total hepatic hydrolytic activity (Imai, 2006). CES1 is responsible for the metabolism of a wide range of therapeutic agents, endogenous compounds, and environmental toxins (Laizure et al., 2013). Of particular clinical relevance, CES1 catalyzes the hydrolysis of numerous clinically important medications, such as angiotensin-converting enzyme (ACE) inhibitor prodrugs (Wang et al., 2016b), clopidogrel (Plavix®) (Zhu et al., 2013), sacubitril (Entresto®) (Shi et al., 2016b), methylphenidate (Ritalin®) (Zhu et al., 2008), oseltamivir (Tamiflu®) (Shi et al., 2016a), and dabigatran etexilate (Pradaxa®) (Laizure et al., 2014; Shi et al., 2016c). Marked interindividual variability in CES1 expression and activity has been well documented (Hosokawa et al., 1995; Shi et al., 2006; Yoshimura et al., 2008; Hagihara et al., 2009; Yang et al., 2009; Zhu et al., 2009a; Ross et al., 2012), which may affect the pharmacokinetics and pharmacodynamics of drugs metabolized by CES1. Genetic polymorphisms are increasingly recognized as an important factor contributing to CES1 variability and varied responses to CES1 substrate drugs (Geshi et al., 2005; Zhu et al., 2008; Nemoda et al., 2009; Sai et al., 2010; Rasmussen et al., 2015; Tarkiainen et al., 2015a). For example, the *CES1* nonsynonymous single nucleotide polymorphism (nsSNP) G143E (rs71647871) was associated with diminishing CES1 activity on metabolizing several CES1 substrate drugs, including methylphenidate (Zhu et al., 2008; Nemoda et al., 2009; Stage et al., 2017), oseltamivir (Zhu and Markowitz, 2009; Tarkiainen et al., 2012), enalapril (Tarkiainen et al., 2015b; Wang et al., 2016b), clopidogrel (Lewis et al., 2013; Tarkiainen et al., 2015a), dabigatran etexilate (Shi et al.,

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2016c), and sacubitril (Shi et al., 2016b). These findings indicate that *CES1* genetic variants may significantly impact responses to drugs metabolized by CES1.

The *CES1* gene is located on chromosome 16q13-q22.1 , and is highly polymorphic with over 600 nsSNPs across its 14 exons. The frequencies of *CES1* nsSNPs vary markedly among different ethnic groups. Several nsSNPs were found to be rare in Caucasian, but common in other populations, which include the variants T167S (rs147694791, MAF=5.48% in African), R186P (rs60054861, MAF=12.39% in African), A158V (rs202121317, MAF=6.69% in South Asian), and E220G (rs200707504, MAF=3.1% in Korean). A recent *in silico* structure-based analysis predicted that eight *CES1* nsSNPs including the G142E (rs121912777), G143E (rs71647871), G147C (rs146456965), Q169P (rs143718310), Y170D (rs148947808), R171C (rs201065375), G173D (rs4784575), and E220G (rs200707504) reside within 5 Å from the enzyme active site (Nzabonimpa et al., 2016), and thus are likely to alter enzyme-substrate binding affinity. However, except for a very few variants that have a relatively high MAF in Caucasian (e.g. G143E), the majority of *CES1* nsSNPs have not been thoroughly studied for their functional consequences.

In the present study, we conducted a comprehensive *in vitro* functional study using transfected cell lines and individual human liver tissues to evaluate the functions of *CES1* nsSNPs that have a MAF > 0.5% in a given population and the common haplotypes D203E-A269S and S75N-D203E-A269S. Furthermore, nsSNPs located in close proximity to the CES1 active site were also subjected to the functional study. The study revealed that several nsSNPs significantly impaired CES1 activity on the metabolism of the CES1 substrates enalapril, clopidogrel, and sacubitril. The findings reinforce the

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notion that CES1 genetic variants play an important role in interindividual variability in response to medications metabolized by CES1.

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Materials and Methods

Materials

QuikChange Lightning Multi Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA, USA). The products purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA) include Flp-In™-293 cell line, pOG44 plasmid, S.N.A.P.™ Plasmid DNA MiniPrep Kit, Lipofectamine® 2000 Transfection Reagent, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hygromycin B in phosphate buffered saline (PBS, 50mg/ml) and 100× antibiotics mixture containing penicillin (100 IU/mL) and streptomycin (100 μg/mL), Trypsin-EDTA (0.25%), the SILAC Protein Quantitation Kit-DMEM containing SILAC DMEM (deficient in arginine and lysine), $^{13}C_6$ l-lysine-2HCl, $^{13}C_6{}^{15}N_4$ l-arginine-HCl, dialyzed FBS, Urea, dl-dithiothreitol (DTT), trifluoroacetic acid (TFA), TRIzol RNA isolation reagent and acetonitrile. Iodoacetamide (IAA) was the product of Acros Organics (Morris Plains, NJ, USA). TPCK-treated trypsin was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA). Water Oasis HLB columns were from Waters Corporation (Milford, MA, USA). Recombinant CES1 (purity >95%) was the product of R&D Systems (Minneapolis, MN, USA).

Enalapril maleate was purchased from Sigma-Aldrich (St. Louis, MO, USA). The enalapril hydrolytic metabolite enalaprilat dehydrate was the product from Sellechchem (Houston, TX, USA). S-(l)-clopidogrel and clopidogrel carboxylate were obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). Sacubitril was purchased from MedKoo Biosciences (Chapel Hill, NC, USA). The hydrolytic active metabolite of sacubitril, LBQ657, was obtained in our laboratory following incubation of 100 μM

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sacubitril with 50 ng/μl recombinant human CES1 at 37 °C for 2 h (Shi et al., 2016b). Sacubitril was completely hydrolyzed to LBQ657 after incubation as determined by LC-MS/MS analysis (Shi et al., 2016b). High-Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were the products from Applied Biosystems (Foster City, CA, USA).

A total of 104 individual normal human liver samples were obtained from XenoTech LLC (Lenexa, KS, USA) and Cooperative Human Tissue Network (CHTN, Columbus, OH, USA). The liver samples consist of 48 males and 56 females with ages ranging from 22 to 81 years. The donors included 94 Caucasians, 6 African-Americans, 2 Hispanics and 2 classified as 'others'.

Establish cell lines stably expressing wild type and mutant CES1

A total of twenty *CES1* variants were selected for an *in vitro* functional study based on the criteria that the SNPs or haplotypes have a MAF greater than 0.5% in a population or the variants are located within a 5Å distance from the CES1 active site (Table 1). Mutant *CES1* plasmids were generated by a site-directed mutagenesis assay with specific mutagenic primers (Supplementary Table S1) and transfected to human embryonic kidney cells (Flp-In^{TM}-293) based on the methods that we previously published (Zhu et al., 2008). The desired sequences of WT and mutant *CES1* plasmids were confirmed by DNA sequencing analysis. Verified *CES1* plasmids were co-transfected with a pOG44 plasmid at a ratio of 1:10 into Flp-In-293 cells with Lipofectamine 2000. Six hours after transfection, cells were gently rinsed to remove transfection reagents and were then cultured in complete medium (Dulbecco's modified Eagle's medium containing 10% fetal-bovine serum). After 12 hours, the culture medium was replaced with a complete

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medium supplemented with the selecting antibiotic hygromycin B (100 μg/mL). Stable WT and mutant CES1 expressing cell lines were established after culturing with hygromycin B for at least three weeks. All transfected cell lines were validated by DNA sequencing analysis.

Preparation of S9 fractions from transfected cells and individual human livers

For the preparation of S9 fractions from the transfected cell lines, cells were cultured in 175cm^2 flasks until reaching approximately 95% confluence. The cells were rinsed twice with ice-cold phosphate-buffered saline before being harvested in 0.5 ml of the same buffer. The cells were then lysed by sonication, followed by centrifugation at 9,000 g at 4°C for 30 min. The supernatant (S9 fraction) was collected in 1.5 ml protein low binding tubes and stored at -80°C until use. Individual human liver S9 fractions were prepared according to a previous publication (Wang et al., 2016b).

Enzymatic activity assays

In vitro incubation studies were conducted to determine CES1 activities of the prepared S9 fraction samples on hydrolysis of the CES1 substrates enalapril, clopidogrel and sacubitril. Enalapril incubation was carried out in 4 ml silanized glass vials because of significant non-specific bindings of enalapril and its hydrolytic metabolite enalaprilat to plastic Eppendorf (EP) tubes (Wang et al., 2016b). For clopidogrel and sacubitril, incubations were performed in 1.5 ml EP tubes. Enalapril (500 μM), clopidogrel (100 μM), and sacubitril (200 μM) were incubated with S9 fractions at final protein concentrations of 0.2 mg/ml, 0.1 mg/ml and 0.05 mg/ml, respectively, at 37°C for 10 min. The hydrolysis reactions were terminated by addition of a four-fold volume of methanol containing the internal standard (IS) 5-hydroxy omeprazole (20 ng/ml) for

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enalapril, a two-fold volume of acetonitrile with the IS d4-clopidogrel carboxylic acid (25 ng/ml) for clopidogrel, and a three-fold volume of acetonitrile with the IS ritalinic acid (7.2 μg/ml) for sacubitril. After centrifugation at 17,000 g at 4°C for 20 min, the supernatant was collected and analyzed for tmetabolite concentrations using the HPLC-MS/MS methods described previously (Zhu et al., 2013; Shi et al., 2016b; Wang et al., 2016b). Additionally, S9 fractions from vector-transfected cells served as a blank control in the CES1 activity studies.

Quantification of CES1 protein in human livers and WT and mutant *CES1* transfected cells

Absolute CES1 protein expression levels in individual human liver samples were quantified using a SILAC-based LC-MS/MS assay that we previously established (Wang et al., 2016a).

Relative CES1 protein quantifications in S9 fractions from transfected cells were conducted using a similar SILAC-based LC-MS/MS assay without having CES1 calibration curves. The intensities of several selected CES1 unique peptides were normalized by the SILAC counterpart peptides (i.e. IS), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference protein for quality control. Relative CES1 protein expression in each mutant cell line was calculated by comparison to the CES1 expression level in WT *CES1* transfected cells.

Analysis of *CES1* mRNA expressions in WT and mutant *CES1* transfected cells

WT and mutant *CES1* transfected cells were cultured in 6-well plates. One μg of RNA extracted from the cells was reversely transcribed to cDNA using oligo dT primers.

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Quantitative real-time polymerase chain reactions (qRT-PCR) were performed on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR green fluorescence. GAPDH was detected as the internal control for normalization. *CES1* gene expressions in mutant cell lines relative to that in WT CES1 cells were determined by the $2^{-\Delta\Delta CT}$ method. Additionally, RNA isolated from vector-transfected cells was included in the qRT-PCR study as a blank control. The primers and experimental conditions for the qRT-PCR experiments are described in Supplementary Table S1 and S2, respectively.

cDNA sequencing of human liver samples

Total RNA was isolated from 50 mg of frozen individual human liver tissues using a TRIzol™ reagent according to manufacturer instructions. cDNA was synthesized from one μg total RNA using the reverse transcription protocol described above. Nested PCR was subsequently applied to amplification of whole length of the CES1 cDNA. Final PCR products were purified and subjected to bi-directional sequencing with four pairs of primers. The primers and nested PCR conditions for the CES1 cDNA amplification are listed in Table S1 and S2, respectively.

HPLC-MS/MS analysis

The HPLC-MS/MS analysis was performed on a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) coupled with an Applied Biosystems API 4000 triple quadruple mass spectrometer (Foster City, CA, USA).

LC-MS/MS method for CES1 activity assay

The hydrolytic metabolites enalaprilat, clopidogrel carboxylate, and LBQ657 were quantitated using the previously described assays with some modifications (Zhu et al.,

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2013; Shi et al., 2016b; Wang et al., 2016b). The analytes were isolated on a Shimadzu VP-ODS column (5 μm, 150 × 2.0 mm, Shimadzu, Japan) with mobile phase delivered at a constant flow rate (0.3 ml/min for enalaprilat, 0.25 ml/ min for clopidogrel carboxylate and 0.2 ml/min for LBQ657). The gradient conditions are summarized in the Supplementary Table S3. Column temperature was set at 50°C for enalaprilat and 40 °C for clopidogrel carboxylate and LBQ657. An injection volume of 10 μl was used for all analytes. Positive electrospray ionization mode was applied, and ions were monitored by multiple reaction monitoring (MRM) with the following m/z transitions: enalaprilat (349.0 > 206.0), clopidogrel carboxylate (308.0 > 197.9), LBQ657 (384.7 > 266.7), 5-hydroxy omeprazole (362.34 > 213.9), clopidogrel d4 carboxylic acid (312.1 > 202.0) and ritalinic acid (220.5 > 84.6).

LC-MS/MS method for CES1 protein quantification

To quantitate the relative CES1 protein expression levels in the transfected cells, an established targeted CES1 proteomics assay was adopted with some modifications (Wang et al., 2016a). In brief, six CES1 unique peptides (i.e. AISESGVALTSVLVK, FLSLDLQGDPR, TAMSLLWK, SYPLVC[CAM]IAK, ELIPEATEK, FWANFAR) and one GAPDH unique peptide (GALQNIIPASTGAAK), and the corresponding heavy isotope-labeled SILAC peptides were separated on a ZORBAX 300SB-C18 column (5 μM, 150 × 2.1 mm, Agilent Technologies, Santa Clara, CA, USA). The column temperature was set at 40 °C. Mobile phase was delivered at a constant flow rate of 0.2 ml/min with the gradient conditions listed on Supplementary Table S3. Under positive ionization mode, analytes were monitored at the transitions described previously (Wang et al., 2016a). The data were analyzed using the Skyline software (University of

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Washington, Seattle, WA, USA).

Data Analysis

Data are representative of three independent experiments and values are expressed as mean ± standard deviation (S.D.). Unpaired, two-tailed t-test was utilized to determine the differences of CES1 expression and activity between *CES1* variants and WT controls (GraphPad Prism software version 6.0; GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

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Results

Enzymatic activity of WT and mutant CES1 on enalapril, clopidogrel and sacubitril hydrolysis

A total of 21 stable CES1 expressing cell lines were established to evaluate the effect of the candidate *CES1* nsSNPs and haplotypes on CES1 activity utilizing the CES1 substrates enalapril, clopidogrel and sacubitril (Figure 1). All three CES1 substrates were efficiently hydrolyzed by WT CES1. No appreciable hydrolytic metabolites were formed after incubation of the three substrates with the S9 fractions from cells expressing the *CES1* nsSNPs L40Ter, G142E, G147C, Y170D and R171C, indicating that these five nsSNPs were loss-of-function variants for CES1. Among the five loss-of-function CES1 mutations, except for the L40Ter, all variants were located close to the CES1 active site. The Q169P (rs143718310), another nsSNP located near the CES1 active site, exhibited approximately 50% of CES1 activity on hydrolysis of the three substrates relative to the WT enzyme. Moreover, the CES1 SNP E220G, which has an MAF of 0.55% in South Asian and 3.1% in Korean populations, but is rare in other populations, significantly decreased CES1 hydrolytic activity on enalapril, clopidogrel, and sacubitril by 78.8% ± 2.6%, 82.3% ± 3.5% and 80.3% ± 3.0%, respectively. The variants A158V (MAF = 6.69% in South Asian) and R199H (MAF = 0.74% in African) showed significantly decreased activity on enalapril hydrolysis (53.4% ± 1.5% and 59.5% ± 2.0%, respectively, of the WT CES1). Interestingly, these two nsSNPs did not significantly affect the hydrolysis of clopidogrel and sacubitril. This substrate specific effect was also observed for the T290M (MAF = 1.24% in African, 2.86% in South Asian and 1.87% in Latino), which reduced the hydrolysis of clopidogrel by 50.7% ± 16.8% while having no

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significant effect on enalapril and sacubitril metabolism. In addition to the individual nsSNPs, the two common haplotypes D203E-A269S and S75N-D203E-A269S were also studied for their potential effect on CES1 catalytic activity. The *in vitro* incubation study indicated that none of the haplotypes significantly affect CES1 activity on hydrolyzing enalapril, clopidogrel and sacubitril.

CES1 protein and mRNA expression levels in WT and mutant *CES1* cell lines

CES1 mRNA and protein were abundantly expressed in the WT *CES1* transfected cells, with levels comparable to that in human livers (data not shown). No detectable CES1 mRNA and protein were found in the vector control cells. The nsSNP G147C decreased CES1 mRNA and protein expressions by 80.5% ± 0.7% and 86.7% ± 2.8%, respectively. The L40Ter expressing cells displayed normal CES1 mRNA expression levels, but null CES1 protein expression, which is likely due to the premature stop codon in exon 2 introduced by this variant. Interestingly, the CES1 protein levels of the Y170D and R171C variants were only 7.4% ± 2.8% and 5.9 ± 3.6% of WT CES1, respectively, whereas mRNA expressions were not significantly affected. Neither the haplotype S75N-D203E-A269S nor D203E-A269S affected the CES1 protein or mRNA expressions in the transfected cells.

***CES1* nsSNPs S75N, D203E, A269S had no effect on CES1 protein expression and activity in human livers**

CES1 mRNA extracted from a total of 104 individual human liver samples was reversely transcribed to cDNA, and subsequently sequenced for *CES1* nsSNPs. Twelve subjects were found to be the S75N heterozygotes and ten subjects were heterozygous for the D203E and A269S. It is noted that the D203E and A269S are in complete linkage

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disequilibrium (LD) with each other in our samples; nine out of the ten D203E and A269S carriers were also heterozygous for the S75N variant (i.e. S75N-D203E-A269S haplotype). No other candidate *CES1* nsSNPs were found in the liver samples, which is likely due to that 94 out of the 104 subjects were Caucasians, and the other candidate *CES1* nsSNPs are rare in Caucasians. In addition, the known loss-of-function *CES1* nsSNP G143E and several rare nsSNPs which are not on our candidate nsSNPs list were identified in the human liver samples, including I49V (rs3826193), L97I (rs571416840), A93V (rs202111709) and A156T (rs187158640). Table 2 provides a summary of the genotypes, CES1 protein expression, CES1 activity on enalapril metabolism, and the CES1 activity normalized by protein expression of all CES1 nsSNPs carriers from the 104 human liver samples. Consistent with the results from the transfected cells, neither CES1 protein expression nor activity was affected by the S75N and the haplotype S75N-D203E-A269S (Figure 3). The effect of the G143E on enalapril metabolism in human livers was described in a recently published study (Wang et al., 2016b). Statistical analysis was not performed for other variants due to the small samples size of the carriers.

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Discussion

CES1, the most abundant hepatic enzyme in humans (Achour et al., 2017), catalyzes the hydrolysis of many clinically important drugs (Shi et al., 2006; Zhu et al., 2008; Zhu et al., 2013; Shi et al., 2016a; Shi et al., 2016b; Shi et al., 2016c; Wang et al., 2016b; Stage et al., 2017). Considerable interindividual variability in CES1 expression and activity has been consistently demonstrated by our laboratory and others (Zhu et al., 2008; Laizure et al., 2013; Zhu et al., 2013; Rasmussen et al., 2015; Shi et al., 2016a; Wang et al., 2016b; Oh et al., 2017; Stage et al., 2017). Varied CES1 function may lead to variability in the pharmacokinetics and pharmacodynamics of drugs metabolized by CES1. *CES1* genetic variation has been established as a significant contributor to CES1 variability (Zhu et al., 2008; Zhu et al., 2013; Rasmussen et al., 2015; Shi et al., 2016a; Shi et al., 2016b; Shi et al., 2016c; Wang et al., 2016b; Stage et al., 2017). Among thousands of variants identified within the *CES1* gene, nsSNPs are of particular interest because, relative to other types of genetic variants, nsSNPs are more likely to be functionally significant due to associated changes in amino acid sequences. The discovery of the loss-of-function variant G143E has exemplified the functional significance and clinical implications of *CES1* nsSNPs (Zhu et al., 2008). However, only a very few *CES1* nsSNPs have been studied so far (Zhu et al., 2008; Shi et al., 2016c; Stage et al., 2017), leaving a large portion of CES1 variability unexplained. Several *in silico* programs such as SIFT and Polyphen2 were developed to predict functional consequences of nsSNPs. However, our previous study demonstrated that the *in silico* analysis was not predictive of the effect of *CES1* nsSNPs on enzyme function (Zhu et al., 2013). In the present study, we conducted a comprehensive *in vitro* functional study to determine the impact of *CES1* nsSNPs on

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CES1 expression and activity using *CES1* transfected cell lines and individual human liver tissues.

The MAFs of *CES1* nsSNPs vary markedly among different ethnic groups (**Table 1**), however, the majority of previous *CES1* pharmacogenomics studies have focused on common variants in Caucasians. The present study included *CES1* nsSNPs with an MAF greater than 0.5% in a given population to make the assessment applicable to all ethnic groups. Five *CES1* nsSNPs (i.e. L40Ter, E220G, A158V, R199H and T290M) were found to be functionally significant. The L40Ter (MAF=0.55% in African) was a loss-of-function variant for the hydrolysis of enalapril, clopidogrel, and sacubitril (**Figure 1**). This variant resulted in a premature stop codon in exon2, abolishing the production of mature CES1 protein, though the *CES1* mRNA level in the L40Ter transfected cells was comparable to the WT control (**Figure 2**). The E220G showed a significant decreasing effect on the hydrolysis of all three tested CES1 substrates. Consistent with our *in vitro* data, the E220G was predicted *in silico* to be deleterious to CES1 activity as it resides near the active site (Nzabonimpa et al., 2016). Given that CES1 protein and mRNA levels were unaltered in the E220G expressing cells (**Figure 2**), the effect of E220G on CES1 activity was likely due to the disruption of CES1-substrate bindings. The MAF of this variant was 0.55% in East Asian according to the dbSNP database; however, a recent study reported that this variant is very common in Koreans (MAF=3.1%) (Oh et al., 2017). This study also revealed that the E220G was associated with a greater systemic exposure to oseltamivir in healthy subjects who received a single dose of oseltamivir (75 mg). This clinical observation is in agreement with our *in vitro* study, indicating that the E220G reduces CES1 activity.

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It is interesting that the effects of the *CES1* nsSNPs A158V, R199H and T290M on CES1 activity are substrate dependent **(Figure 1)**. Enalapril hydrolysis was reduced to 46.6% ± 1.5% and 40.5% ± 2.0% of the WT enzyme by the A158V and R199H, respectively, whereas the hydrolysis of clopidogrel and sacubitril was not affected by the variants. Additionally, the T290M decreased clopidogrel hydrolysis by 50.7% ± 16.8%, while having no effect on enalapril and sacubitril metabolism **(Figure 1)**. Given the high frequencies of A158V in South Asians (MAF=6.69%) and R199H in Africans (MAF=0.74%), the A158V and R199H could be clinically significant for optimizing enalapril therapy in South Asians and Africans, respectively. The T290M is relatively prevalent in African, South Asian and Latino with an MAF of 1.24%, 2.86% and 1.87%, respectively. It should be noted that over 85% of ingested clopidogrel is metabolized by CES1, and the *CES1* nsSNP G143E has shown significant effects on clopidogrel activation as well as its therapeutic outcomes (Lewis et al., 2013; Zhu et al., 2013; Tarkiainen et al., 2015a). Therefore, the T290M could be a significant contributor to the interindividual variation in clopidogrel therapy in relevant populations. It should be noted that the differential effect of *CES1* genetic polymorphisms on different CES1 substrates has been observed in previous studies. For example, the G143E was a loss-of-function variant for the metabolism of methylphenidate, clopidogrel, enalapril, and dabigatran, but a decreased function variant for oseltamivir metabolism (Zhu et al., 2008; Zhu and Markowitz, 2009; Shi et al., 2016b; Wang et al., 2016b). Similar to the G143E, the A158V, R199H and T290M diminished CES1 activity without altering the mRNA or protein expressions (**Figure 1**-**3**), indicating these variants might directly affect the enzyme-substrate interaction. Given that chemical structures differ significantly among enalapril,

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clopidogrel, and sacubitril, we speculate that the involvement of amino acid residues in catalyzing the cleavage of the ester bonds of these compounds may vary, which may have resulted in the observed substrate dependent effect of the three nsSNPs.

A recent healthy volunteer study reported that the area under the curve (AUC) of d-methylphenidate plasma concentrations was increased by 68% in the A269S carriers compared to non-carriers after subjects were administered a single dose of 10 mg methylphenidate (Lyauk et al., 2016). However, our *in vitro* studies of transfected cells and human livers showed that the A269S was not associated with CES1 expression or activity. Given the fact that the A269S is in complete LD (D'=1, R^2=1) with the D203E and in high LD with the S75N (D' = 0.664, R^2=0.399), we established cell lines that stably expressed the haplotypes D203E-A269S and S75N-D203E-A269S, and evaluated the impact of the haplotypes on CES1 expression and the metabolism of the CES1 substrates clopidogrel, enalapril, and sacubitril. We also genotyped a large set of human liver tissues for the CES1 nsSNPs and haplotypes and determined the CES1 expression and activity on enalapril hydrolysis. As shown in **Figure 1**-**3**, neither the D203E-A269S nor the S75N-D203E-A269S exhibited a significant effect on CES1 protein expression or activity in the transfected cells and human liver tissues.

Eight *CES1* nsSNPs including the G142E, G143E, G147E, Q169P, Y170D, R171C, G173D, and E220G are located within a 5Å distance from the CES1 active site according to analysis of the CES1 three-dimensional structure (Nzabonimpa et al., 2016). One of the variants, G143E, has shown a profound impact on CES1 activity, and consequently impaired the metabolism of several CES1 substrate drugs (Zhu et al., 2008; Nemoda et al., 2009; Zhu et al., 2009b; Zhu and Markowitz, 2009; Lewis et al., 2013; Tarkiainen et

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al., 2015a; Tarkiainen et al., 2015b; Shi et al., 2016b; Wang et al., 2016a). In the present study, stable cell lines transfected with CES1 variants were developed to assess the functions of the rest of the seven *CES1* nsSNPs located in close proximity to the CES1 active site. As expected, all seven nsSNPs affected CES1 activity to a certain extent though effect magnitude and substrate specificity varied among the nsSNPs (**Figure 1**).

CES1 protein and mRNA expressions are highly correlated among the transfected cell lines with the exception of the L40T, Y170D, and R171C variants (**Supplemental figure S1**). The three variants markedly impaired CES1 protein expression without affecting mRNA levels (**Figure 2**). As expected, the correlation between CES1 activity and protein expression was significantly higher than that between the activity and mRNA expression for the tested variants (**Supplemental figure S2**). Furthermore, a number of nsSNPs, such as the G142E and E220G, significantly attenuated CES1 activity while imposing no effect on CES1 protein and mRNA expressions.

In summary, this study is the first comprehensive functional assessment of *CES1* nonsynonymous variants and haplotypes, and has revealed a number of nsSNPs that exhibit significant effects on the metabolism of CES1 substrates. Clinical investigations are warranted to determine the influence of the identified functional nsSNPs on the pharmacokinetics and pharmacodynamics of medications metabolized by CES1. It is noted that the functional nsSNPs identified from the present study may only explain a small portion of CES1 variability based on the frequencies of the variants. Thus, further investigations are needed to elucidate genetic and non-genetic elements regulating this important drug-metabolizing enzyme.

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Authorship Contributions

Participated in research design: Wang, Shi, Wu, Bleske, Zhu.

Conducted experiments: Wang, Shi, Rida.

Performed data analysis: Wang, Shi, Zhu.

Wrote or contributed to the writing of the manuscript: Wang, Shi, Rida, Wu, Bleske, Zhu.

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Footnotes

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Figure legends:

Figure 1: Effect of *CES1* nsSNPs and haplotypes on hydrolysis of enalapril (A), clopidogrel (B), and sacubitril (C) in *CES1* transfected cell lines. Enalapril (500μM), clopidogrel (100 μM), and sacubitril (200 μM) were incubated with s9 fractions prepared from cells stably transfected with WT *CES1* and candidate variants (18 nsSNPs and two haplotypes: D203E-A269S and S75N-D203E-A269S). Hydrolysis rates of enalapril, clopidogrel, and sacubitril were determined by measuring the formation of the respective hydrolytic metabolites enalaprilat, clopidogrel carboxylic acid, and LBQ465. Vector transfected cells were used as a blank control. Data are expressed as means ± S.D. (n = 3). *P<0.05, **P<0.001 (CES1 variants vs WT CES1).

Figure 2: CES1 protein (A) and mRNA (B) expressions in the *CES1* nsSNPs and haplotypes transfected cells. Relative CES1 protein and mRNA levels of the CES1 variants compared to WT CES1 were determined using LC-MS/MS and qRT-PCR assays, respectively. WT CES1 and vector transfected cells were included as the positive and negative controls, respectively. Data are presented as means ± S.D. (n = 3). *P<0.05, **P<0.001 (CES1 variants vs WT CES1).

Figure 3: Hepatic CES1 activity on enalapril activation (A) and absolute CES1 expression (B) among different CES1 genotypes (i.e. S75N (n=3), D203E-A269S (n=1), S75N-D203E-A269S (n=9), and WT (n=33)). The WT control contains 33 subjects who do not carry any nsSNPs. Statistical analysis was not performed for the D203E-A269S haplotype due to the very limited carrier number.

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Table 1. MAFs of *CES1* nsSNPs and haplotypes

	rs numbers	Protein residue changes	Nucleotide changes	MAF						
				African	European (FINN)	European (Non-FINN)	East Asian	South Asian	Latino	Other
nsSNPs	rs151291296	L40Ter	T227G	0.51%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	rs2307240	S75N	G332A	4.30%	3.83%	5.37%	4.68%	7.44%	2.06%	5.29%
	rs121912777	G142E	G533A	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	rs146456965	G147C	G547T	0.03%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%
	rs202121317	A158V	C581T	0.04%	0.00%	0.00%	0.03%	6.69%	0.04%	0.11%
	rs147694791	T167S	C608G	5.48%	0.00%	0.01%	0.00%	0.01%	0.32%	0.00%
	rs143718310	Q169P	A614C	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	rs148947808	Y170D	T616G	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	rs201065375	R171C	C619T	0.01%	0.00%	0.02%	0.00%	0.00%	0.05%	0.00%
	rs4784575	G173D	G626A	0.01%	0.00%	0.00%	0.39%	0.02%	0.12%	0.00%
	rs60054861	R186P	G665C	12.39%	0.02%	0.24%	0.01%	0.02%	0.69%	1.23%
	rs2307243	R199H	G704A	0.74%	0.00%	0.03%	0.00%	0.00%	0.03%	0.00%
	rs2307227	D203E	C717A	8.30%	0.71%	3.16%	1.18%	4.33%	1.70%	3.53%
	rs200707504	E220G	A767G	0.00%	0.00%	0.00%	0.55%	0.00%	0.00%	0.00%
	rs115629050	A269S	G913T	8.25%	0.71%	3.15%	1.24%	4.31%	1.71%	3.52%
	rs114119971	H284Q	C960G	0.18%	0.27%	0.71%	0.00%	0.06%	0.26%	0.44%
	rs202001817	T290M	C977T	0.24%	0.03%	0.19%	0.02%	0.21%	0.09%	0.11%
	rs576295379	N340K	T1128G	0.00%	0.00%	0.01%	0.00%	0.55%	0.01%	0.11%
Haplotypes	D203E-A269S			5.22%	0.00%	0.00%	0.00%	0.00%	0.00%	NA
	S75N-D203E-A269S			3.56%	1.01%	4.46%	1.49%	4.29%	2.02%	NA

***** Data were derived from the Exome Aggregation Consortium (ExAC) browser http://exac.broadinstitute.org/

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Table 2. CES1 genotypes, CES1 protein expression, CES1 activity on enalapril metabolism, and the activity normalized by CES1 protein expression in human livers carrying *CES1* nsSNPs.

Subject number	S75N	D203E	A269S	G143E	I49V	L97I	A93V	A156T	Enalapril Hydrolysis Rate (pmol/ min/ mg protein)	CES1 protein expressions (pmol/ mg protein)	Normalized CES1 Activity (pmol/min/pmol CES1)
L2	S/N	D/D	A/A	G/G	I/I	L/L	A/A	A/A	40.3	104.17	0.39
L16	S/N	D/D	A/A	G/G	I/I	L/L	A/A	A/A	45.3	84.45	0.54
L40	S/N	D/D	A/A	G/G	I/I	L/L	A/A	A/A	93.4	170.44	0.55
L8	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	62.4	184.06	0.34
L31	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	12.6	104.39	0.12
L45	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	41.1	118.41	0.35
L60	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	64.4	194.12	0.33
L61	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	50.3	141.49	0.36
L72	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	153.7	266.06	0.58
L90	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	36.3	102.01	0.36
L95	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	87.8	215.71	0.41
L102	S/N	D/E	A/S	G/G	I/I	L/L	A/A	A/A	83.7	104.85	0.80
L99	S/S	D/E	A/S	G/G	I/I	L/L	A/A	A/A	20.2	69.99	0.29
L41	S/S	D/D	A/A	G/E	I/I	L/L	A/A	A/A	15.9	236.16	0.07
L73	S/S	D/D	A/A	G/E	I/I	L/L	A/A	A/T	22.4	96.33	0.23
L94	S/S	D/D	A/A	G/E	I/I	L/L	A/A	A/A	28.4	156.11	0.18
L103	S/S	D/D	A/A	G/E	I/I	L/L	A/A	A/A	19.9	163.28	0.12
L104	S/S	D/D	A/A	G/E	I/I	L/L	A/A	A/A	17.6	273.32	0.06
L3	S/S	D/D	A/A	G/G	I/V	L/L	A/A	A/A	0.0	155.26	0.00
L5	S/S	D/D	A/A	G/G	I/V	L/I	A/A	A/A	26.4	102.37	0.26
L89	S/S	D/D	A/A	G/G	I/I	L/L	A/V	A/A	183.6	435.68	0.42

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Figure 1

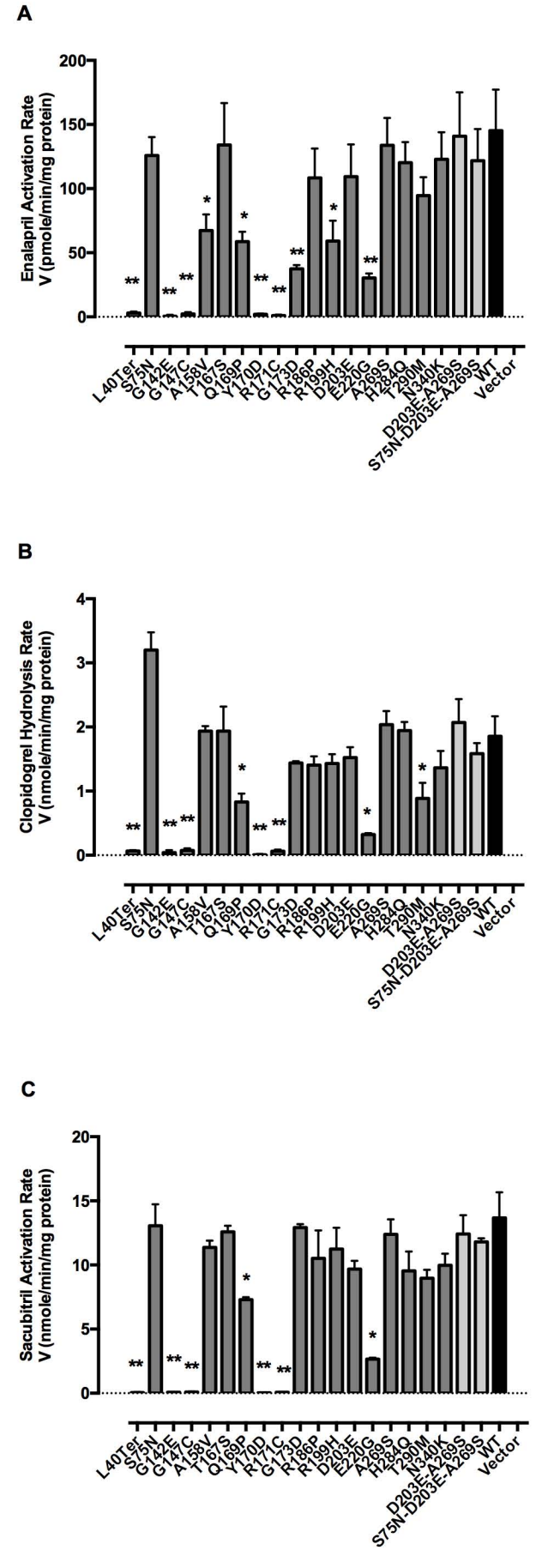
A
Enalapril Activation Rate
V (pmole/min/mg protein)
200
150
100
50
0
L40Ter
S75N
G142E
G147C
A158V
T167S
Q169P
Y170D
R171C
G173D
R186P
R199H
D203E
E220G
A269S
H284Q
T290M
N340K
D203E-A269S
S75N-D203E-A269S
WT
Vector
B
Clopidogrel Hydrolysis Rate
V (nmole/min/mg protein)
4
3
2
1
0
C
Sacubitril Activation Rate
V (nmole/min/mg protein)
20
15
10
5
0

Figure 2

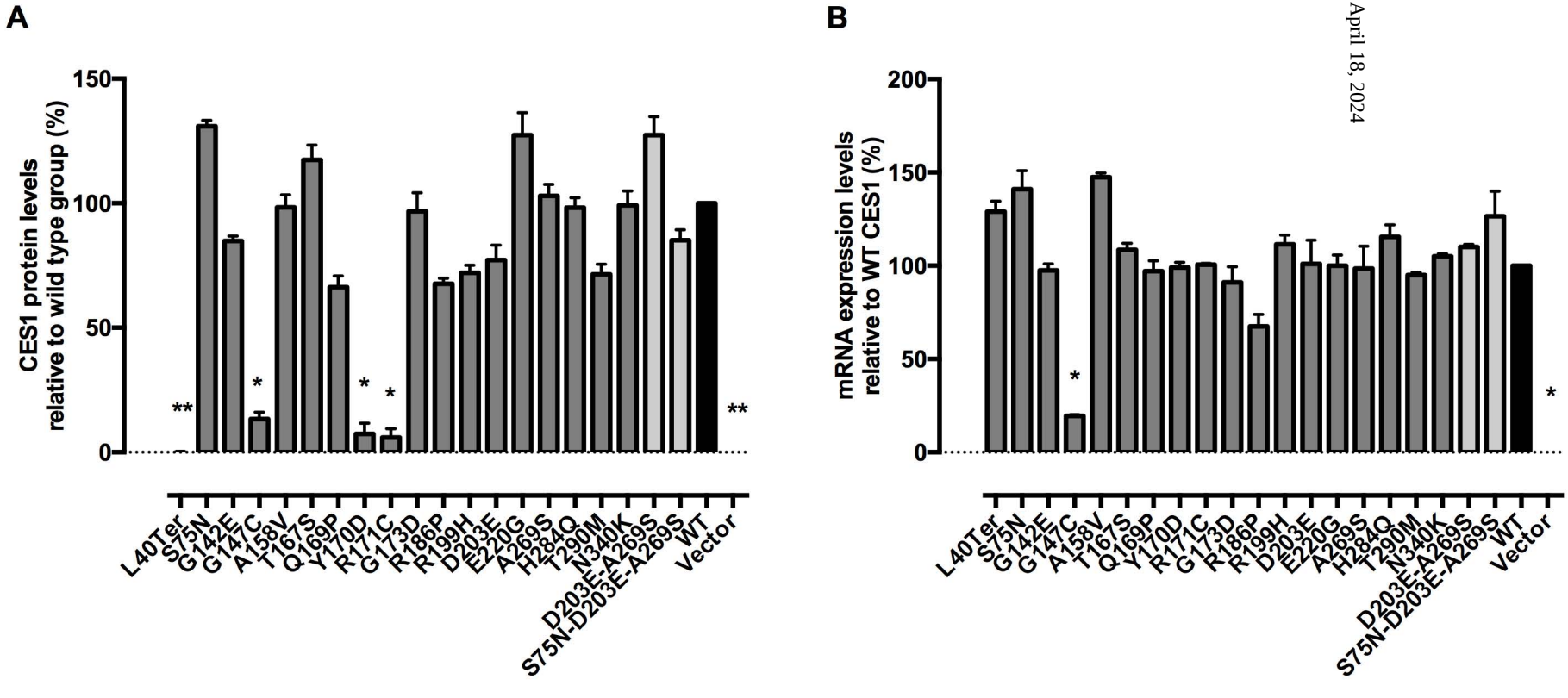

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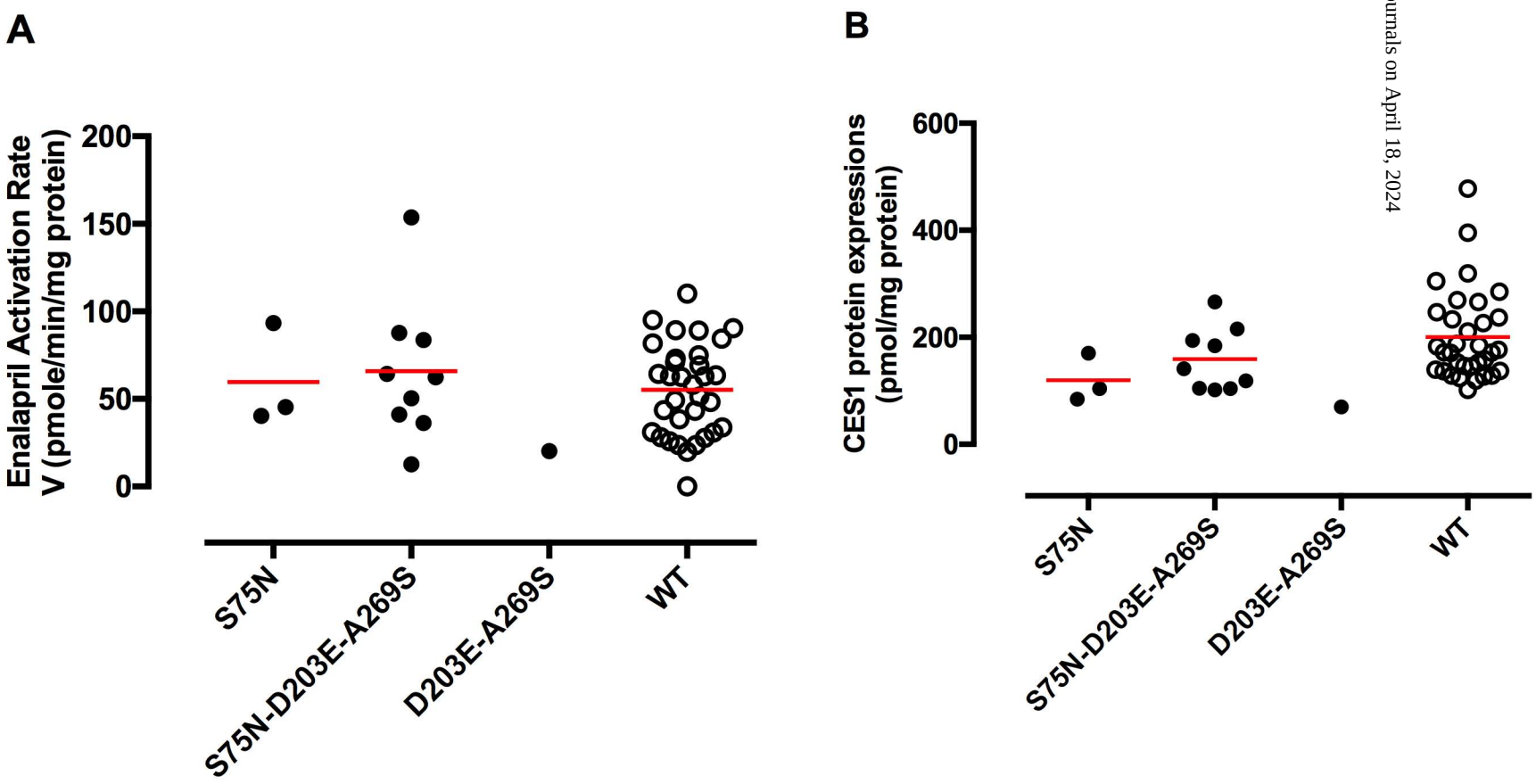
Figure 3



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Supplemental Materials

Title:
A comprehensive functional assessment of carboxylesterase 1 nonsynonymous polymorphisms

Authors:

Xinwen Wang, Nada Rida, Jian Shi, Audrey H. Wu, Barry E. Bleske, and Hao-Jie Zhu

Department of Clinical Pharmacy (X.W., N.R., J.S., H.-J.Z.), and Cardiovascular Center (A.H.W.), University of Michigan, Ann Arbor, Michigan; and Department of Pharmacy Practice and Administrative Sciences, University of New Mexico, Albuquerque, New Mexico (B.E.B.)

Journal Title: Drug Metabolism and Disposition

Supplementary materials for "Comprehensive functional assessment of CES1 nonsynonymous polymorphisms"

Table S1. Sequences of the primers used in this study

		Forward Primer	Reverse Primer
Site Directed Mutagenesis	L40Ter	5'-TGTGCAAATCCTTCTCAGCTGACGAACTTCCCCA-3'	5'-TGGGGAAGTTCGTCAGCTGAGAAGGATTTGCACA-3'
	S75N	5'-TGGCATTCTTCACAAAGTTCCATGGTTCTGCAGGC-3'	5'-GCCTGCAGAACCATGGAACTTTGTGAAGAATGCCA-3'
	G142E	5'-CACCATCAGCCCCTCTCCGTGGATCCA-3'	5'-TGGATCCACGGAGAGGGGCTGATGGTG-3'
	G147C	5'-GTTGATGCCGCACACACCATCAGCCCC-3'	5'-GGGGCTGATGGTGTGTGCGGCATCAAC-3'
	A158V	5'-CGTTTTCATGGGCAACAAGGGCCAGCCCA-3'	5'-TGGGCTGGCCCTTGTTGCCCATGAAAACG-3'
	T167S	5'-CAGGCGATATTGAATGCTCACCACCACCACGTT-3'	5'-AACGTGGTGGTGGTGAGCATTCAATATCGCCTG-3'
	Q169P	5'-GATGCCCAGGCGATATGGAATGGTCACCACCAC-3'	5'-GTGGTGGTGACCATTCCATATCGCCTGGGCATC-3'
	Y170D	5'-GCCCAGGCGATCTTGAATGGTCACCACCACCA-3'	5'-TGGTGGTGGTGACCATTCAAGATCGCCTGGGC-3'
	R171C	5'-GATGCCCAGGCAATATTGAATGGTCACCACCAC-3'	5'-GTGGTGGTGACCATTCAATATTGCCTGGGCATC-3'
	G173D	5'-TGAAGAATCCCCAGATGTCCAGGCGATATTGAATG-3'	5'-CATTCAATATCGCCTGGACATCTGGGGATTCTTCA-3'
	R186P	5'-ACCCCAGTTCCCCGGGCTGTGTTCATC-3'	5'-GATGAACACAGCCCGGGGAACTGGGGT-3'
	R199H	5'-CCTGGACCCAGTGCAGGGCAGCC-3'	5'-GGCTGCCCTGCACTGGGTCCAGG-3'
	D203E	5'-GCGCTGGGTCCAGGAAAACATTGCCAGCTTT-3'	5'-AAAGCTGGCAATGTTTTCCTGGACCCAGCGC-3'
	E220G	5'-CTCCTCCCGCTGACCCTCCAAAGATGGTC-3'	5'-GACCATCTTTGGAGGGTCAGCGGGAGGAG-3'
	A269S	5'-CCTTGGCTGAGCAAATTTCTATCACTGCTGGGTG-3'	5'-CACCCAGCAGTGATAGAAATTTGCTCAGCCAAGG-3'
	H284Q	5'-GTCGCAGGCACTGAACCATGACAGCAGAGG-3'	5'-CCTCTGCTGTCATGGTTCAGTGCCTGCGAC-3'
	T290M	5'-GAGCTCCTCTTCCATCTTCTGTCGCAGGCA-3'	5'-TGCCTGCGACAGAAGATGGAAGAGGAGCTC-3'
	N340K	5'-GGGGACAGTGTGGAACTTCCTTTCAGCTTGAAGCT-3'	5'-AGCTTCAAGCTGAAAGGAAGTTCCACACTGTCCCC-3'
Nested PCR	Outer P	5'-GGACAGCACAGTCCCTCTGA-3'	5'-ACTTGCTCCTTCATGGCCTC-3'
	Inner P	5'-GGACAGCACAGTCCCTCTGA-3'	5'-CCAGACAATTCCCCAGCCAT-3'
cDNA sequencing	cDNA P-1	5'-TGTGGCTCCGTGCCTTTATC-3'	5'-CACCACCAGACAATTCCCCA-3'
	cDNA P-2	5'-TGTCGCCCTTCCACGATG-3'	5'-CTTTCGCAAAAGACCCCAGT-3'
	cDNA P-3	5'-GATGTGGCTCCGTGCCTTTA-3'	5'-CACCACGTTTTCATGGGCAG-3'
	cDNA P-4	5'-CCCTCTGAACTGCACAGAGAC-3'	5'-TGGTCAAGTCAGCAGGAGTG-3'

Table S2. Thermocycling conditions for PCR

	A		B			C	
Step	Temperature	time	Temperature	time	Step	Temperature	time
Initial Denaturation	95 ℃	30s	95 ℃	30s	Initial Denaturation	50°C	2min
						95°C	10min
30 Cycles	95 ℃	30s	95 ℃	30s	40 Cycles	95°C	15s
	60℃	45s	72 ℃	40s		60°C	1min
	68 ℃	2min10s	68 ℃	2min10s		95°C	15s
Final Extension	68 ℃	5min	68 ℃	5min			
Hold	4 ℃	∞	4 ℃	∞			

A: the PCR conditions for the amplification of outer region of CES1 cDNA (nested PCR-1st outer);
B: the PCR conditions for the amplification of inner region of CES1 cDNA (nested PCR-2nd inner)
C: the real time PCR conditions for CES1 mRNA quantification

Table S3. The gradient conditions of HPLC-MS/MS analysis

Enalapril			Clopidogrel			Sacubitril			CES1 unique peptides		
Time (min)	A (%)	B (%)	Time (min)	A (%)	C (%)	Time (min)	A (%)	C (%)	Time (min)	D (%)	E (%)
0	80	20	0	52	48	0	70	35	1	95	5
1	80	20	22	end	end	7	70	90	20	63	37
5	10	90				8	5	90	25	0	100
7	10	90				9	5	35	35	0	100
8	80	20				12	end	end	36	95	5
10	end	end							40	end	end

Mobile Phase:
A: water containing 2 mM ammonium acetate and 0.2% formic acid
B: methanol containing 2 mM ammonium acetate and 0.2% formic acid
C: acetonitril with 0.2% formic acid
D: water containing 0.1% formic acid
E: acetonitril with 0.1% formic acid

Figure S1: Correlation between CES1 mRNA and protein expressions in different CES1 variants and WT CES1 transfected cell lines. The CES1 nsSNPs exhibiting significant impact on protein but not mRNA expressions are labeled. Data are the means from three independent experiments.

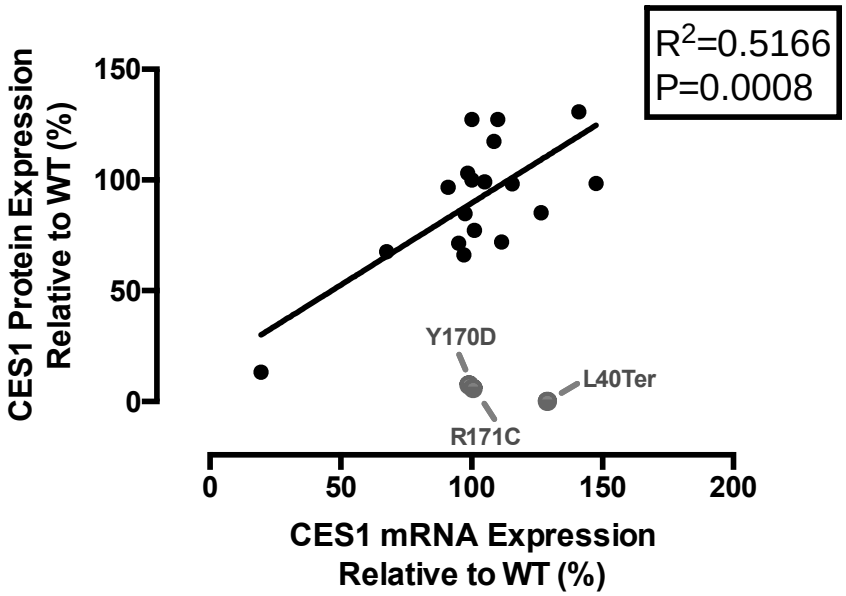
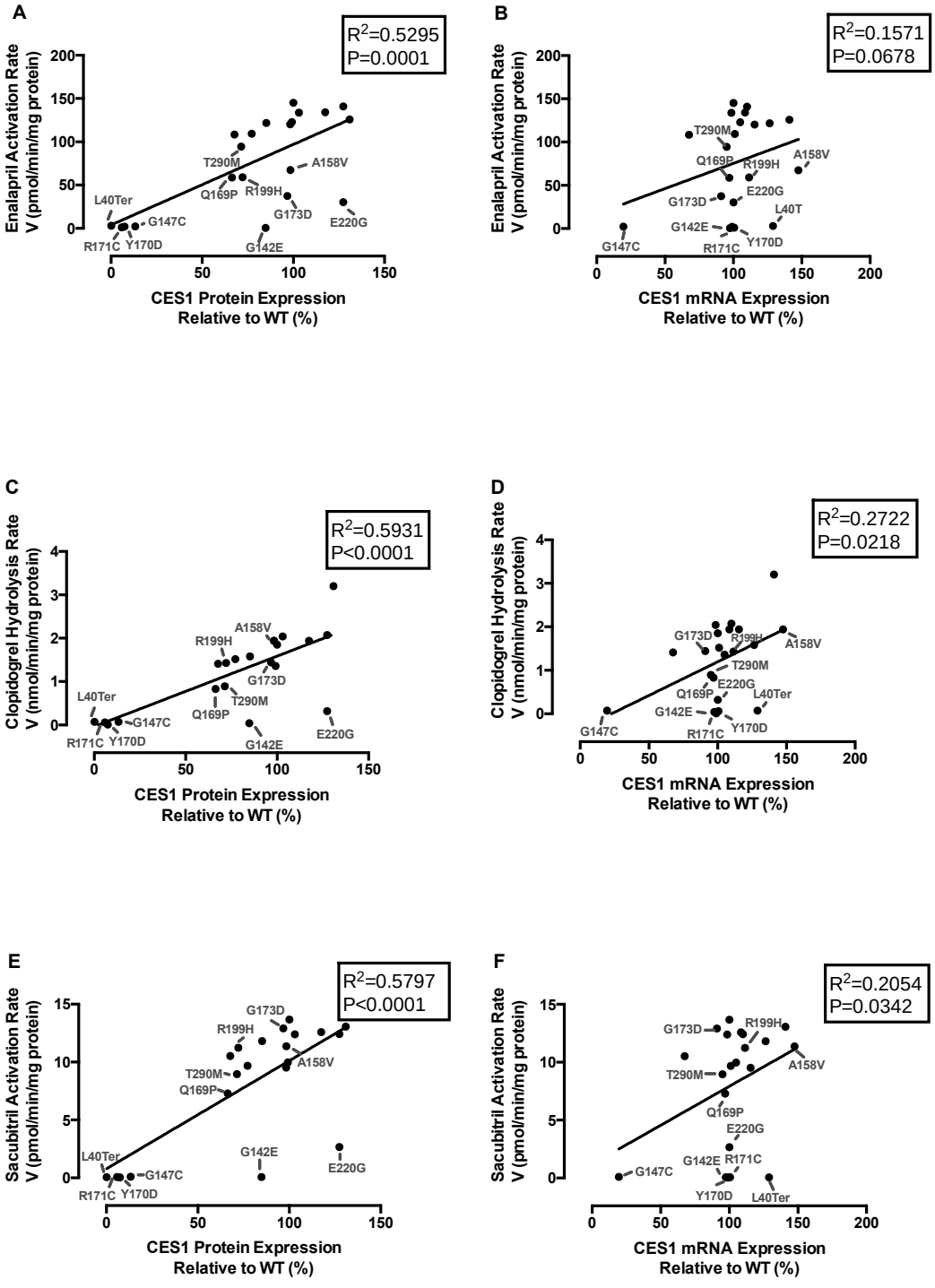
Figure S2. Correlation between CES1 protein, mRNA expressions and CES1 activities on enalapril, clopidogrel and sacubitril hydrolysis. (A) enalapril vs protein; (B) enalapril vs mRNA; (C) clopidogrel vs protein; (D) clopidogrel vs mRNA; (E) sacubitril vs protein; (F) sacubitril vs mRNA. CES1 nsSNPs showing significant impact on CES1 activity on a CES1 substrate are labeled. Data are the means from three independent experiments.