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Genome-Wide Analysis of Clopidogrel Active Metabolite Levels Identifies Novel Variants that Influence Antiplatelet Response

Joshua D. Backman¹, Jeffrey R. O'Connell¹, Keith Tanner¹, Cody J. Peer², William D. Figg², Shawn D. Spencer³, Braxton D. Mitchell^{1,4}, Alan R. Shuldiner^{1,4}, Laura M. Yerges-Armstrong¹, Richard B. Horenstein¹, and Joshua P. Lewis¹

¹Division of Endocrinology, Diabetes and Nutrition, and Program for Personalized and Genomic Medicine, University of Maryland School of Medicine, Baltimore, MD

²Clinical Pharmacology Program, National Cancer Institute, Bethesda, MD

³Applied and Developmental Research, SAIC-Frederick Inc., National Cancer Institute, Frederick, MD

⁴Geriatric Research and Education Clinical Center, Veterans Administration Medical Center, Baltimore, MD

Abstract

Clopidogrel is one of the most commonly used therapeutics for secondary prevention of cardiovascular events in patients with acute coronary syndromes. However, substantial inter-individual variation in clopidogrel response has been documented, resulting in suboptimal therapy and an increased risk of recurrent events for some patients. In this investigation, we conducted the first genome-wide association study of circulating clopidogrel active metabolite levels in 513 healthy participants in order to directly measure clopidogrel pharmacokinetics. We observed that the *CYP2C19* locus was the strongest genetic determinant of active metabolite formation ($P=9.5\times 10^{-15}$). In addition, we identified novel genome-wide significant variants on chromosomes 3p25 (rs187941554, $P=3.3\times 10^{-11}$) and 17q11 (rs80343429, $P=1.3\times 10^{-8}$), as well as 6 additional loci that showed suggestive evidence of association ($P < 1.0\times 10^{-6}$). Four of these loci demonstrated nominal associations with on-clopidogrel ADP-stimulated platelet aggregation ($P < 0.05$). Evaluation of clopidogrel active metabolite concentration may help identify novel genetic determinants of clopidogrel response, which has implications for the development of novel therapeutics and improved anti-platelet treatment for at-risk patients in the future.

Keywords

pharmacogenomics; precision medicine; clopidogrel; pharmacokinetics; platelet aggregation; genome-wide association study; CYP2C19

Clopidogrel is a thienopyridine prodrug frequently prescribed to reduce atherothrombotic events in patients with acute coronary syndromes. However, up to 40% of patients do not

Conflicts of Interest

All other authors have no conflict of interest to declare.

receive adequate benefit from clopidogrel therapy [1, 2]. Furthermore, up to 73% of the inter-individual variation in clopidogrel response is heritable [3]; yet, aside from well-described variants such as *CYP2C19**2, few polymorphisms have been linked with clopidogrel response, suggesting that most of the genetic variation that leads to variability in drug efficacy remains unidentified [3–7].

Most investigations to date have measured platelet reactivity to assess clopidogrel response. While evaluation of circulating levels of the clopidogrel active metabolite is a more direct measure of clopidogrel metabolism, it has been underutilized due to the instability of the clopidogrel active metabolite, which requires rapid derivatization for accurate assessment [8]. Herein, we performed a genome-wide association study (GWAS) of circulating clopidogrel active metabolite levels in 513 healthy subjects of the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study after 8 days of clopidogrel (300 mg loading dose followed by 75 mg/day for 7 days). Furthermore, we assessed the influence of identified variants on adenosine diphosphate (ADP)-stimulated platelet aggregation post-clopidogrel administration.

Participants

Amish individuals from Lancaster, Pennsylvania were recruited into the PAPI Study between 2006 and 2012 [3, 9]. Participants were over age 20, generally healthy, and agreed to discontinue the use of medications, supplements, and vitamins at least one week prior to study initiation. Baseline platelet aggregation measurements were recorded after overnight fast, after which participants received a 300 mg oral loading dose of clopidogrel followed by 75 mg/day for 7 days (Supplemental Digital Content, Figure S1). Follow-up platelet aggregation and clopidogrel metabolite measurements were obtained 1 hour following the last clopidogrel dose. Exclusion criteria and additional information regarding recruitment and design of the PAPI Study have been previously described [3, 9]. The protocol was approved by the University of Maryland, Baltimore Institutional Review Board. All subjects provided written informed consent.

Genotyping

Genotyping was performed with Affymetrix 500k and 6.0 genome-wide arrays (Affymetrix Inc., Santa Clara, California). Variants were called using Birdseed V2 and BRLMM, and imputed using the 1000 Genomes Project reference panel (Phase I version 3, March 2012) with Impute2 [10] and ShapeIt [11]. Polymorphisms with an imputation quality score < 0.5 and a minor allele frequency < 1% were excluded from analysis.

Clopidogrel metabolite quantification

Blood samples were collected within 1 hour after the last clopidogrel dose into EDTA tubes containing 2mmol/l (E)-2-bromo-3'-methoxyacetophenone (MPB; Sigma Aldrich, St Louis, Missouri) to derivatize the clopidogrel active metabolite. Plasma levels of clopidogrel MPB-derivatized active metabolite and parent drug were assessed using an ultra-high performance

liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay as previously described [5, 12].

Platelet aggregation

Platelet-rich plasma was isolated from blood samples drawn into tubes containing 3.2% sodium citrate (Becton-Dickinson, Franklin Lakes, New Jersey), and platelet counts were adjusted to 200,000 platelets/ μ l with platelet-poor plasma (PPP). Platelet function was assessed using a PAP8E aggregometer (Bio/Data Corporation, Horsham, Pennsylvania) after stimulation with ADP (20 μ mol/L, Chronolog Corp., Havertown, Pennsylvania) and was expressed as the maximal percentage change in light transmittance using PPP as a referent.

Statistical analysis

Association analyses were conducted with a variance component method using Mixed Model Analysis for Pedigrees (MMA) (<http://edn.som.umaryland.edu/mmap/>), [3, 13–15]. Analyses of clopidogrel active metabolite were performed under an additive model adjusted for age, sex, and relatedness. Analyses of on-clopidogrel ADP-mediated platelet aggregation were performed under an additive model and adjusted for age, sex, relatedness, and baseline platelet aggregation. Clopidogrel prodrug concentration was assessed for the top GWAS signals and conditional analyses were conducted with adjustment for the index SNP at each locus. A genome-wide significance threshold of $P = 5 \times 10^{-8}$ was used for GWAS analysis and $P < 0.05$ was used to establish significance of ADP-stimulated platelet aggregation analyses.

We tested 7,884,700 SNPs for association with clopidogrel active metabolite concentration in PAPI Study subjects (Figure 1, Supplemental Digital Content, Figure S2). Characteristics of these individuals are listed in Table 1. A locus on chromosome 10 near the *CYP2C9-CYP2C18-CYP2C19* gene cluster was significantly associated with active metabolite concentration (rs137891020, $P = 9.5 \times 10^{-15}$, Supplemental Digital Content, Figure S3). After adjustment for the *CYP2C19**2 variant, association of rs137891020 was markedly attenuated ($P = 9.1 \times 10^{-4}$), and no other variant in this region showed suggestive evidence of association ($P \geq 1.0 \times 10^{-6}$). In addition to *CYP2C19**2, novel variants on chromosomes 3p25 (rs187941554, $P = 3.3 \times 10^{-11}$, Supplemental Digital Content, Figure S4) and 17q11 (rs80343429, $P = 1.3 \times 10^{-8}$, Supplemental Digital Content, Figure S5) were significantly associated with clopidogrel active metabolite concentration. Another 6 independent loci exhibited suggestive evidence of association ($P \geq 1.0 \times 10^{-6}$, Table 2). The full list of SNPs with significant ($P \leq 5.0 \times 10^{-8}$) or suggestive ($P \geq 1.0 \times 10^{-6}$) evidence of association with active metabolite concentration can be found in Supplemental Digital Content, Table S1. Conditional analyses adjusted for the most significantly associated SNP at each locus fully accounted for the associations observed at all loci (Supplemental Digital Content, Table S2). We extended our findings by examining whether polymorphisms that influence active metabolite levels also impacted on-clopidogrel ADP-stimulated platelet aggregation. Indeed, nominal associations ($P < 0.05$) were observed between on-clopidogrel platelet aggregation and 4 regions identified in the active metabolite GWAS (Table 2). No associated SNPs were

significantly associated with clopidogrel prodrug levels and adjustment for prodrug levels did not appreciably change our results (data not shown).

We report the first GWAS of clopidogrel active metabolite concentration, a direct measure of clopidogrel pharmacokinetics. Clopidogrel is among the most widely used antiplatelet agents available; however, genetic background results in substantial variation in drug response [3]. To date, most studies have utilized ADP-based platelet function tests, a pharmacodynamic measure, in order to examine clopidogrel response. However, apart from *CYP2C19*2*, few actionable variants have been identified. We hypothesized that the genome-wide analysis of clopidogrel active metabolite concentration would facilitate the identification of novel pharmacokinetic variants that may remain unidentified in genetic investigations focused solely on pharmacodynamic measures.

The active metabolite of clopidogrel is highly unstable [16] and must be derivatized to be measured accurately [12]. Through genome-wide genotyping and assessment of clopidogrel active metabolite in a large cohort, we successfully identified novel loci on chromosomes 3p25 and 17q11 that are significantly correlated with formation of the clopidogrel active metabolite, as well as an additional 6 loci that may warrant further investigation. Consistent with our initial results, 4 of the 8 loci that influence formation of the clopidogrel active metabolite also impact on-clopidogrel ADP-stimulated platelet aggregation. Importantly, none of these variants were associated with ADP-mediated platelet aggregation at a genome-wide level and would not have been identified in traditional genome-wide investigations that solely utilize platelet function testing (Supplemental Digital Content, Figures S6 and S7). In addition, while we observed strong evidence of association between *CYP2C19*2* and formation of the clopidogrel active metabolite, no other variant in this or other cytochrome P450 genes was correlated with active metabolite levels after adjustment for *CYP2C19*2* and correction for multiple testing. It should be noted that our investigation was not properly powered to identify previously reported loss-of-function variants that are exceedingly rare in European-derived populations (e.g. *CYP2C19*3*); however, these data do provide initial evidence of little to no impact of other more common variants in *CYP2C19* (e.g. *CYP2C19*17*) or other cytochrome P450 genes on active metabolite formation.

Several variants identified in this study are in close proximity to genes with potential roles in clopidogrel metabolism. For instance, a cluster of SNPs on chromosome 3p25 are near *ATP2B2*, which codes for a plasma membrane calcium transporter [17] that is expressed in the liver [18] and was previously implicated in clopidogrel response [19]. On chromosome 1p25, the most strongly associated variant is located within Ring Finger Nuclease 2 (*RNF2*), which encodes a polycomb group protein that interacts with P-glycoprotein, a key enzyme for the intestinal absorption of clopidogrel [20]. Moreover, prior investigations have shown that RNF2 protein levels are negatively correlated with P-glycoprotein expression [20].

We acknowledge some limitations of this investigation. Clopidogrel active metabolite is not routinely measured, and no suitable replication cohort was available. This study is also potentially limited by the methodology used to quantify the clopidogrel active metabolite. The derivatization process with MPB does not differentiate between the racemic H4 (active) and H3 (inactive) stereoisomer of clopidogrel metabolites, potentially leading to some

measurement error. While high concordance between H3 and H4 has been noted previously [21], which may minimize the import of this potential limitation, the use of novel methodologies to isolate the H4 isomer, such as the method developed by Hua and colleagues [22], may improve variant detection in future studies. Additionally, all work presented herein was conducted in the Amish of Lancaster, PA, which could influence translatability of findings to non-Amish populations. However, this has not been the case in the past [3, 13–15].

While the identification of polymorphisms such as *CYP2C19**2 has informed clinicians in prescribing the most effective antiplatelet regimen and has improved outcomes in a percentage of cardiovascular disease patients, there is still significant ‘missing’ heritability that leaves many patients at an unnecessarily high risk of recurrent ischemic events. In this study, we aimed to identify novel genetic variants that influence clopidogrel active metabolite formation. Indeed, a number of novel variants with potential effects on clopidogrel efficacy were identified. While verification in an independent population is needed, this study demonstrates the utility of directly assessing clopidogrel metabolites. Furthermore, such investigations have implications for improved next-generation antiplatelet drug discovery through identification of variants that negatively influence drug efficacy thereby facilitating the development of novel drugs whose metabolism/efficacy are not dependent on proteins that harbor these mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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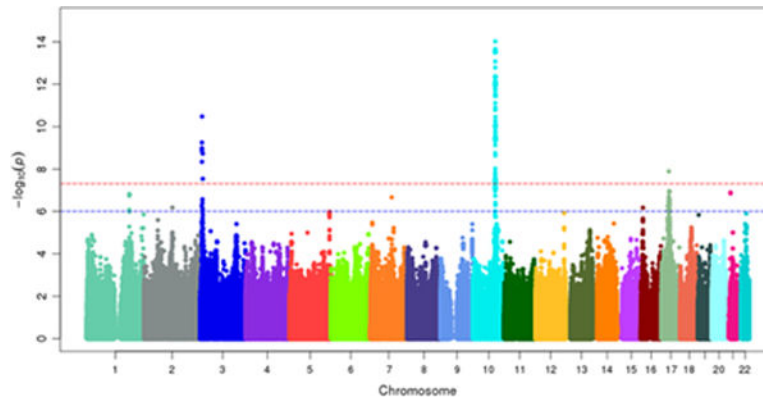


Figure 1. Manhattan plot of circulating clopidogrel active metabolite concentration as measured by HPLC MS/MS after clopidogrel administration (300 mg loading dose followed by 75 mg/day for 7 days). N=513. Red line indicates $P = 5.0 \times 10^{-8}$. Blue line indicates $P = 1.0 \times 10^{-6}$.

Table 1

Characteristics of PAPI Study Participants

Characteristics (units)	Total (N = 513)	Females (N = 256)	Males (N = 257)
Age (years)	44.9 ± 13.2	45.6 ± 13.6	44.2 ± 12.7
BMI (kg/m ²)	27.1 ± 4.7	28.2 ± 5.4	26.0 ± 3.6
Platelet Count (thousands)	239.7 ± 48.2	245.7 ± 51.3	234.1 ± 44.3
SBP (mm Hg)	116.8 ± 12.3	117.0 ± 13.1	116.6 ± 11.4
DBP (mm Hg)	70.2 ± 7.1	69.6 ± 7.0	70.8 ± 7.2
Cholesterol (mg/dl)	208.9 ± 46.8	210.0 ± 49.4	207.6 ± 43.5
LDL (mg/dl)	135.9 ± 42.7	132.6 ± 44.7	138.6 ± 40.2
HDL (mg/dl)	58.7 ± 15.2	62.4 ± 15.5	55.6 ± 14.5
Triglycerides (mg/dl)	71.4 ± 40.2	74.9 ± 42.1	67.3 ± 37.3
Diabetes (%)	0.4%	0.4%	0.4%

Values are listed as mean ± standard deviation or percent. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL; high-density lipoprotein; LDL, low-density lipoprotein; PAPI, Pharmacogenomics of Anti-Platelet Intervention; SBP, systolic blood pressure.

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Novel genomic regions with significant ($P < 5.0 \times 10^{-8}$) or suggestive ($P < 1.0 \times 10^{-6}$) associations with clopidogrel active metabolite concentration

Table 2

Index SNP	Chr:Pos	Minor Allele	MAF	Gene	Active Metabolite			On-Clopidogrel Platelet Aggregation		
					β	P-value	β	P-value	β	P-value
rs187941554	3:8987233	A	0.01	RAD18	25.2 ± 3.7	3.3×10^{-11}	-3.8 ± 4.8		0.42	
rs80343429	17:31556681	A	0.05	ASIC2	8.1 ± 1.4	1.3×10^{-8}	-3.5 ± 1.7		0.04	
rs79172967	21:18571112	T	0.02	N/A	15.5 ± 2.9	1.3×10^{-7}	-2.2 ± 3.7		0.55	
rs72392086	1:185033816	D	0.04	RNF2	7.6 ± 1.4	1.6×10^{-7}	-5.0 ± 1.8		5.3×10^{-3}	
rs73407739	7:92491283	T	0.01	LOC101927497	16.8 ± 3.2	2.2×10^{-7}	-11.9 ± 3.9		2.2×10^{-3}	
rs181524103	2:123209400	G	0.03	N/A	12.0 ± 2.4	6.6×10^{-7}	-3.1 ± 3.0		0.31	
rs138852022	16:9155981	G	0.01	N/A	17.0 ± 3.4	6.7×10^{-7}	-6.9 ± 4.1		0.09	
rs6892003	5:174191465	A	0.29	N/A	3.3 ± 0.7	1.0×10^{-6}	-2.2 ± 0.9		9.7×10^{-3}	

Betas are listed as mean ± standard deviation or percent. Abbreviations: MAF, minor allele frequency; Chr:Pos, chromosome and position; P, p-value; N/A, not applicable; D, deletion