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EXAMINE THE SERUM ACTIVE METABOLITE FINDS NEW VARIANTS THAT EFFECTS CLOPIDOGREL PHARMACOKINETICS

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Abstract :- Dual antiplatelet therapy (DAPT) with clopidogrel and aspirin is the most commonly used regimen for the secondary prevention of ischemic events in patients with acute coronary syndromes. However, substantial inter-individual variation in response to clopidogrel has been documented, resulting in sub-optimal therapy in some patients and an increased risk of recurrent events. Previous studies indicate that a significant portion of this variation is heritable and a number of genetic variants such as CYP2C19*2 have been found to influence clopidogrel response. However, a substantial amount of the genetic heritability remains unaccounted for. In this study, we conducted the first genome-wide association study of circulating clopidogrel active metabolite levels in 513 healthy participants of the Pharmacogenomics of Antiplatelet Intervention (PAPI) study in order to more directly measure clopidogrel pharmacokinetics. Consistent with previous investigations, we observed that the CYP2C19 locus was the strongest genetic determinant of clopidogrel active metabolite formation ($P = 9.5x10^{-15}$). In addition, we identified novel genome-wide significant signals on chromosome 3 (rs187941554, $P = 3.3x10^{-11}$), chromosome 17 (rs80343429, $P = 1.3x10^{-8}$), and chromosome 19 (rs142890248), as well as 6 additional loci that showed suggestive evidence of association ($P \leq 1x10^{-6}$).While further investigation is warranted to validate the findings in this study, the analysis of non- traditional measures of clopidogrel response, such as clopidogrel active metabolite concentration, holds promise for further elucidating the clopidogrel metabolic pathway, contributing to improved anti-platelet drug development, and ultimately leading to better patient care in the future.

Keywords: Dual antiplatelet therapy, acute coronary syndromes, clopidogrel pharmacokinetics

Introduction

Coronary artery disease, the most common form of cardiovascular disease, results in approximately 7.4 million deaths worldwide each year[16]. In order to prevent recurrent cardiovascular events, individuals with advanced cardiovascular disease are frequently prescribed dual anti-platelet therapy, most commonly with clopidogrel and aspirin.

Clopidogrel is a thienopyridine prodrug that must be activated *in* vivo in order to exert its anti-platelet function. After absorption in the intestine, the clopidogrel prodrug is transported to the liver where it becomes biologically active through a two-step conversion involving several cytochrome P450 enzymes, most notably CYP2C19. The clopidogrel active metabolite is then transported to the bloodstream, where it reduces platelet reactivity by irreversibly binding the adenosine diphosphate (ADP) receptor,P2YR12,onthesurfaceofcirculatingplatelets.While clopidogrelislargelyeffectiveat reducing platelet aggregation and recurrent ischemic events, up to 40% of patients do not receive adequate benefit from traditional clopidogrel doses. Furthermore, heritability estimates suggest that up to 73% of the inter-individual variation in clopidogrel response, as measured by *ex vivo* platelet reactivity, is heritable. However, aside from notable variants such as *CYP2C19**2, few polymorphisms have been clearly linked with clopidogrel response, suggesting that most of the genetic variation that leads to inter-individual variability in clopidogrel efficacy remain sunidentified.

Most studies to date have utilized pharmacodynamic measures such as *ex* vivo platelet reactivity or thromboglobulin levels to assess clopidogrel response. However, despite strong scientific interest and multiple published investigations, elucidation of pharmacokinetic factors that influence clopidogrel metabolism remains incomplete. Inthis study, we aimed to identify novel genetic variants that impact clopidogrel metabolism by performing the first genome-wide association study of circulating clopidogrel active metabolite concentration. While evaluation of clopidogrel active metabolite, as opposed to *ex vivo* platelet function tests, is a more direct measure of clopidogrel metabolism, it has remained underutilized, likely due to the instability of the clopidogrel active metabolite and intermediate metabolite (2-oxo-clopidogrel), which require rapid derivatization for accurate assessment. Herein, we measured circulating levels of clopidogrel prodrug and active metabolite in 513 healthy subjects after 8 days of clopidogrel administration (a single 300 mg loading dose followed by 75 mg/d for 7 days). Furthermore, adenosine diphosphate (ADP)-induced platelet aggregation was measured pre-and post-clopidogrel treatment. In order to extend our findings, polymorphisms that significantly influenced clopidogrel active metabolite levels were assessed in 1,400 clopidogrel-treated patients of the International Clopidogrel PharmacogenomicsConsortium(ICPC)inordertoevaluatethe effectofthesevariantson ADP-induced platelet aggregation in patients with severe cardiovascular burden as well as to gain insights regarding their impact on clinicalendpoints.

Methods

Participant Characteristics:

Participants were over age 20, relatively healthy and agreed to discontinue the use of all prescription medications, supplements, and vitamins at least one week prior to the start of the study. Medical and family history, anthropometry, physical examinations, and blood samples were all taken after an overnight fast (Table 4.1). Exclusion criteria for this study included: blood pressure > 160/95 mmHg, currently pregnant or nursing, platelet count > 500,000/µl or < 75,000/µl, hematocrit < 32%, TSH < 0.05 or > 5.5 mU/L, AST or ALT > 2 times the upper normal limit, creatinine > 2.0 mg/dl, surgery in the past 6 months, history of gastrointestinal bleeding, allergy to clopidogrel or aspirin, inability to safely withdraw from medication use, or any other coexisting malignancy that might interfere with the study. After baseline platelet aggregation measurements were recorded, participants were given a 300-mg oral loading dose of clopidogrel followed by 75 mg per day for 7 days. Follow-up platelet aggregation and clopidogrel metabolite measurements were obtained 1 hour following the last dose of clopidogrel. Additional information regarding recruitment and design of the PAPI Study has been previously described.

SNP Selection

Correlated SNP sets for loci strongly associated with clopidogrel active metabolite concentration were developed by assessing linkage disequilibrium between the index SNP and all SNPs with a p-value for association less than or equal to 1×10^{-6}. SNPs with D' >= 0.9 with the index SNP in each locus were included in the SNP set.

Multiple testing correction for follow-up of top hits in the International Clopidogrel Pharmacogenomics Consortium (ICPC) was determined by the number of correlated SNP sets.

Genotyping:

Genotyping was performed on 2,734 ICPC DNA samples with the Illumina (Illumina, Inc. San Diego, CA USA) Human Omni Express Exome v1.2 Chip, yielding 586,379 SNPs with minor allele frequency greater than 0.05. The total genotyping call rate was 99.96%. Additionally, uncalled genotypes for ICPC were imputed using the 1000 genomes "Cosmopolitan" reference panel (Phase I version 3, released March 2012) with Impute2 and ShapeIt. This yielded 9,702,644 SNPs with a minor allele frequency greater than or equal to 1% and an imputation quality score of at least 0.5.

Statistical Analysis:

Analyses of platelet aggregation and clinical endpoints were restricted to European samples of the ICPC. Linear

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regression analyses were used to determine the relationship between genotype and platelet aggregation. Models are adjusted for age, sex, and first four principal components. ADP-mediated platelet aggregation was measured at each respective study site and then normalized and standardized for analysis with Plink[121]. Principal components for the subjects included in analyses were calculated with GCTA (http://cnsgenomics.com/software/gcta/pca.html)[122]. Logistic regressions todetermine the relationship between genotypes and clinical events using age, sex, and the first four principal components as covariates are also completed in Plink.

Results

Participant Characteristics:

In total, 513 subjects (257 males and 256 females) participated in this investigation. All participants were generally healthy, with low incidences of hypertension and diabetes, although BMI and lipid levels were slightly elevated (Table 1)[12]. Overall, approximately 10% of participants were active smokers; however, this was driven entirely by men, who smoke at rates comparable with the outbred population[124], while women, keeping with cultural norms, typically do not smoke[12]. No anthropometric measures were significantly correlated with clopidogrel active metabolite concentration with the exception of BMI and platelet count ($P = 9.6 \times 10^{-3}$ and 3.4×10^{-2}, respectively). On-clopidogrel ADP-mediated platelet aggregation was significantly associated with gender, age, BMI, platelet count, cholesterol levels and triglycerides (6.5×10^{-3}, 1.0×10^{-5}, 3.1×10^{-3}, 1.6×10^{-4}, 1.8×10^{-2}, and 1.5×10^{-2}, respectively).

Table 1: participant characteristics

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 (mmHg)	116.8 ± 12.3	117.0 ± 13.1	116.6 ± 11.4
DBP (mmHg)	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%
Current Smoker (%)	10.3%	0.0%	20.5%

Values are listed as mean ± standard deviation or percent

**Association with on-clopidogrel ADP-stimulated platelet aggregation P < 0.05 in full cohort*

†Association with clopidogrel active metabolite P < 0.05 in full cohort

Heritability and Correlation of clopidogrel-related traits

In concordance with previous estimates, the residual heritability of on-clopidogrel ADP-mediated platelet aggregation was estimated to be 75% after adjustment for age, sex, baseline platelet aggregation, and family relatedness ($P = 2.3 \times 10^{-13}$)[7]. Residual heritability of serum clopidogrel active metabolite concentration after adjustment for age, sex, and relatedness was estimated at 26% ($P = 3.1 \times 10^{-3}$). Clopidogrel prodrug levels were not heritable in this population (0%, P = 1.0).

As expected, clopidogrel active metabolite concentration was significantly correlated with on-clopidogrel ADP-mediated platelet aggregation ($r^2 = 0.12$, $P = 2.2 \times 10^{-16}$). However, clopidogrel prodrug levels were not correlated with on-clopidogrelADP-mediated platelet aggregation ($r^2 = 0.001$, P = 0.40) and only modestly correlated with clopidogrel active metabolite concentration ($r^2 = 0.01$, P = 0.01).

GWAS of Serum Clopidogrel Active Metabolite Concentration

Using a genome-wide approach, we tested for association between 7,884,700 SNPs and clopidogrel active metabolite concentration in 513 PAPI Study subjects while simultaneously adjusting for age, sex, and relatedness (Figure 1). A locus on chromosome 10 near the CYP2C9-CYP2C18-CYP2C19 gene cluster was significantly associated with clopidogrel active metabolite concentration (rs137891020, $P = 9.5 \times 10^{-15}$). After adjustment for the known CYP2C19*2 variant, no variants in this regionreached genome-wide significance (Top remaining signal was rs137891020, $P = 9.1 \times 10^{-4}$). In addition to CYP2C19*2, novel variants significantly impacted clopidogrel active metabolite concentration on

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chromosome 3p25 (strongest signal was rs187941554, P=3.3 x 10^{-11}) (Figure 2) and chromosome 17q11 (strongest signal was rs80343429, P=1.3 x 10^{-8}) (Figure 3). Another 6 independent loci exhibited suggestive evidence of association (P ≤ 1.0 x 10^{-6}) (Table 2). Conditional analyses that adjusted for the most significantly associated SNP at each locus fully accounted for the associations observed at all loci (Table 3). In addition, all index variants that exceeded genome-wide significance or showed suggestive evidence of association (P ≤ 1.0 x 10^{-6}) were tested to see if they impacted on-clopidogrel ADP-mediated platelet aggregation. While no evidence of association was observed between on-clopidogrel platelet aggregation and the chromosome 3p25 locus, rs80343429 on chromosome 17q11 as well as 3 other suggestive loci (rs72392086, rs73407739, and rs6892003 on chromosomes 1q25, 7q21, and 5q32, respectively) reached a nominal level of significance (P < 0.05) (Table 2).To verify the significant signals identified through the GWAS of clopidogrel active metabolite, highly associated imputed variants on chromosome 3 (rs111985173, a proxy for rs187941554) and chromosome 17 (rs80434329) were Taqman genotyped in the full cohort of 513. Concordance between imputation and Taqman genotyping was greater than 98% for both SNPs. Accordingly, the association with active metabolite was still quite strong after direct genotyping, with a P = 5.2 x 10^{-7} for rs111985173 and P = 1.4 x 10^{-7} for rs80434329.

GWAS of On-Clopidogrel ADP-Mediated Aggregation

A GWAS of ex vivo ADP-mediated platelet aggregation in the same 513 PAPI Study subjects again identified CYP2C19*2 as the strongest determinant of high on- treatment platelet reactivity in the Old Order (chr10:96609093, P = 1.3x10^{-17}) (Figure 4). After adjustment for CYP2C19*2, no variants on chromosome 10q24 reached genome-wide significance (Top remaining signal was chr10:96609093, P = 6.4 x 10^{-6}). Furthermore, no other loci were significantly associated with on-clopidogrel ADP- mediated platelet aggregation after correction for multiple testing.

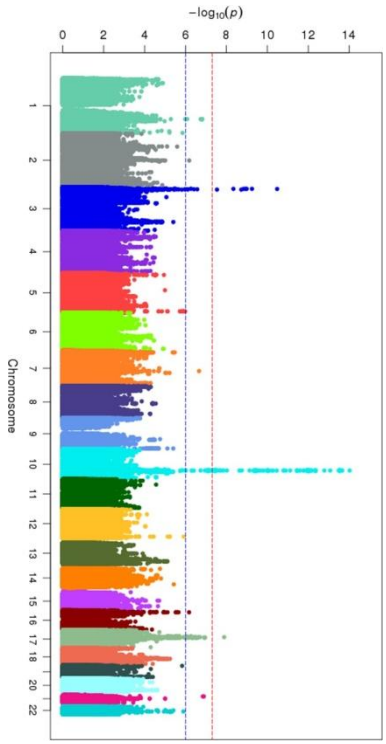

Figure 1. Manhattan plot of serum clopidogrel active metabolite concentration as measured by HPLC MS/MS after 7-day 75-mg/day clopidogrel intervention. N=513. Red line indicates P < 5 x 10^{-8}. Blue line indicates P < 1 x 10^{-6}.

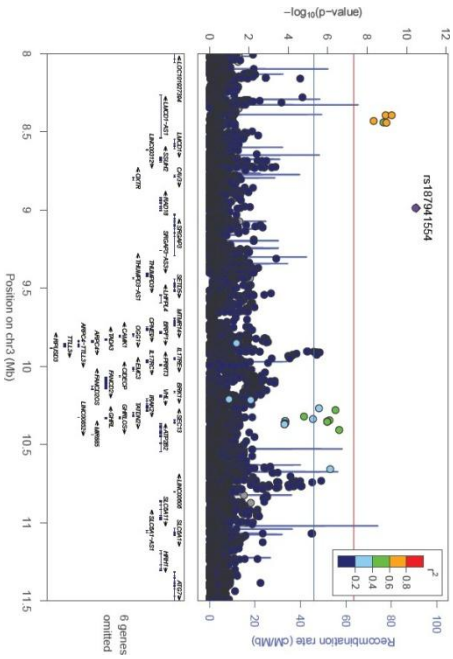

Figure 2. LocusZoom plot of the chromosome 3 locus (8MB-11.5MB) identified in the association analysis of clopidogrel active metabolite concentration in the PAPI Study. N=513. Red line indicates P < 5 x 10^{-8}. Blue line indicates P < 1 x 10^{-6}.

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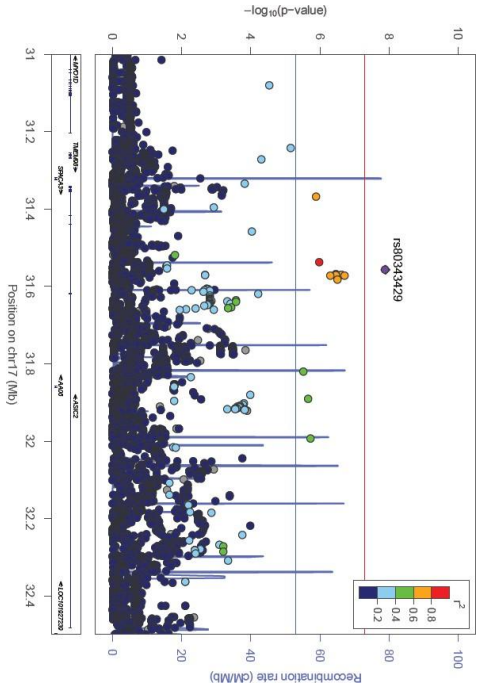

Figure 3. LocusZoom plot of the chromosome 17 locus (31MB-32.5MB) identified in the association analysis of clopidogrel active metabolite concentration in the PAPI Study. N=513. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

Table 2. Regions in genome with suggestive associations ($P \leq 1.0 \times 10^{-6}$) with clopidogrel active metabolite concentration. Abbreviations: MAF, minor allele frequency; Chr:Pos, chromosome and position; P, p-value.

SNP	Chr:Pos	MAF	Gene	Beta—Active Metabolite	P—Active Metabolite	Beta—On-Clopidogrel Platelet Aggregation	P—On-clopidogrel Platelet Aggregation
rs111985173	3:8441942	0.01	LMCD1-AS1	16.8±2.7	1.00×10^{-6}	-0.9 ± 3.4	8.00×10^{-1}
rs80343429	17:31556681	0.05	ASIC2	8.1±1.4	1.30×10^{-8}	-3.5 ± 1.7	4.30×10^{-2}
rs72392086	1:185033816	0.04	RNF2	7.6±1.4	1.60×10^{-7}	-5.0 ± 1.8	5.32×10^{-3}
rs79172967	21:18571112	0.02		15.5±2.9	1.30×10^{-7}	-2.2 ± 3.7	5.50×10^{-1}
rs73407739	7:92491283	0.01	LOC101927497	16.8±3.2	2.20×10^{-7}	-11.9 ± 3.9	2.23×10^{-3}
rs181524103	2:122209400	0.03		12.0±2.4	6.60×10^{-7}	-3.1 ± 3.0	3.10×10^{-1}
rs138852022	16:9155981	0.01		17.0±3.4	6.70×10^{-7}	-6.9 ± 4.1	9.30×10^{-2}
rs6892003	5:174191465	0.29		-3.3±0.7	1.00×10^{-6}	2.2 ± 0.9	9.67×10^{-3}

Table 3. Top remaining SNP in each locus associated with clopidogrel active metabolite in the PAPI Study after conditioning on the original index SNP. Abbreviations: MAF, minor allele frequency; Chr:Pos, chromosome and position; P, p-

SNP	Chr:Pos	MAF	Gene	Beta—Active	P—Active Metabolite
rs6766130	3:11625202	0.08	VGLL4	-5.31±1.2	8.96×10^{-4}
rs116990978	17:32869119	0.03		5.58±1.8	2.17×10^{-3}
rs2823968	21:18050085	0.39		-2.23±0.7	1.62×10^{-3}
rs145982435	1:185352761	0.02		6.38±2.5	9.89×10^{-3}
rs73219929	7:92990291	0.01	CCDC132	14.6±4.3	6.44×10^{-4}
rs296072	2:123650738	0.4		1.85±0.6	3.94×10^{-3}
rs35614103	16:9155981	0.06		5.3±1.7	1.83×10^{-3}
rs1422909	5:174679840	0.04		-5.91±2.3	1.04×10^{-2}

Discussion

In this study we conducted the first genome-wide analysis of circulating clopidogrel active metabolite concentration, a direct measure of clopidogrel pharmacokinetics, to identify novel genetic variants that influence clopidogrel metabolism. Clopidogrel, a prodrug that must be metabolized *in vivo* in order to be biologically active, is among the most widely distributed and efficacious anti-platelet agents available, and the contribution of genetic variation to patient response, estimated at 73% by *ex vivo* platelet aggregation [7], has been highly studied for more than a decade. To date, the majority of studies have utilized platelet function tests that are based on *ex vivo* ADP-mediated platelet aggregation, a pharmacodynamic measure, in order to examine clopidogrel response. Interestingly however, the majority of variants that have been identified through platelet function testing influence clopidogrel pharmacokinetics. For instance, *ABCB1* C3435T, a missense mutation in a gene integral in the intestinal absorption of the clopidogrel prodrug, results in reduced clopidogrel active metabolite levels in carriers, and therefore reduced effectiveness of clopidogrel treatment [111,112]. Carriers of *CES1* G143E, a mutation in the major protein responsible for the deactivation and clearance of clopidogrel and its metabolites, have elevated levels of the active metabolite, and therefore increased anti-platelet response [91]. The hallmark mutation for clopidogrel efficacy, *CYP2C19**2, a

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nonsynonymous SNP in the major cytochrome P450 enzyme responsible for clopidogrel bioactivation, has repeatedly been linked to decreased clopidogrel active metabolite formation, increased on-treatment platelet aggregation, and altered clinical events rates in patients with cardiovascular disease, prompting the FDA to update the clopidogrel label to inform clinicians to consider alternative treatment for genetically susceptible patients. However, apart from these polymorphisms, few variants have been identified that are consistently linked to clopidogrel efficacy. Therefore, based on current heritability estimates, a large proportion of the genetic variants that impact clopidogrel response remain unknown.

Through combining genome-wide genotyping and assessment of clopidogrel active metabolite in a relatively large sample of subjects, we successfully identified novel loci on chromosomes 3p25 and 17q11 that are significantly correlated with clopidogrel metabolism as well as several other loci that may warrant further investigation. Consistent with our hypothesis, 5 of the 9 loci strongly associated with clopidogrel active metabolite exhibit, at minimum, a trending association with on- clopidogrel platelet aggregation. However, none of these variants were associated with ADP-mediated platelet aggregation at a genome-wide level and would not have been identified in traditional investigations that solely utilize platelet function testing.

Furthermore, none of the pharmacokinetic variants identified in this study were correlated with differing starting concentrations of the clopidogrel parent drug. Gene-level testing using SKAT again identified the chromosome 17q11 locus as a modifier of clopidogrelactive metabolite levels. Additionally, we utilized a bivariate approach with canonical correlation analysis, which has been demonstrated to increase analytical power in the genetic analysis of pleiotropic or correlated traits[131]. The results of this analysis lendedfurther support for the chromosomes 3p25 and 17q11 loci identified by clopidogrel active metabolite GWAS.

In addition,pharmacogeneticvariantssignificantlyinfluencec lopidogrelefficacy.However, while the identification of polymorphisms such as *CYP2C19**2 has informed clinicians to alter treatment plans and improved outcomes in a percentage of cardiovascular disease patients, there is still large 'missing' heritability that undoubtedly leaves a number of patientsatunnecessarilyhighriskofrecurrentischemicevent s.Inthisstudy,weaimedto identify novel genetic variants that significantly influenced formation of the clopidogrel active metabolite. Indeed, a number of novel variants with potential effects on clopidogrel efficacy were identified. While these results still need further verification in an independent population, this study demonstrates the utility of directly assessing clopidogrel metabolites and has implications for improved next-generation drug design and improved anti-platelet treatment for at risk patients in the future.

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