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Targeted deep resequencing of *ALOX5* and *ALOX5AP* in patients with diabetes and association of rare variants with leukotriene pathways

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Abstract. The aim of the present study was to investigate a possible association between the accumulation of rare coding variants in the genes for arachidonate 5-lipoxygenase (*ALOX5*) and ALOX5-activating protein (*ALOX5AP*), and corresponding production of leukotrienes (LTs) in patients with type 2 diabetes mellitus (T2DM) receiving acetylsalicylic therapy. Twenty exons and corresponding introns of the selected genes were resequenced in 303 DNA samples from patients with T2DM using pooled polymerase chain reaction amplification and next-generation sequencing, using an Illumina HiSeq 2000 sequencing system. The observed non-synonymous variants were further confirmed by individual genotyping of DNA samples comprising of all individuals from the original discovery pools. The association between the investigated phenotypes was based on LTB_4 and LTE_4 concentrations, and the accumulation of rare missense variants (genetic burden) in investigated genes was evaluated using statistical collapsing tests. A total of 10 exonic variants were identified for each resequenced gene, including 5 missense and 5 synonymous variants. The rare missense variants did not exhibit statistically significant differences in the accumulation pattern between the patients with low and high LTs concentrations. As the present study only included patients with T2DM, it is unclear whether the absence of observed association between the accumulation of rare missense variants in investigated genes and LT production is associated with diabetic populations only or may also be applied to other populations.

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Introduction

Acetylsalicylic acid (ASA) is an effective inhibitor of platelet thromboxane A_2 (TxA_2) synthesis by cyclooxygenase-1 (COX-1). Furthermore, ASA is able to cause alternative processing of arachidonic acid (AA) via the 5-lipoxygenase (ALOX5) pathway, resulting in increased production of proinflammatory leukotrienes (LTs) (1,2). The synthesis of LTs in the vasculature depends strongly on leukocyte recruitment and activation, in addition to cell-cell interaction between leukocytes and vascular cells in the inflamed areas. In leukocytes, ALOX5 and ALOX5-activating protein (ALOX5AP), initialize the biosynthesis of LTs from AA (2-6). Previous studies indicate that a number of genetic variants in LT pathway genes may modulate LT production, metabolism and response (7-17). In addition, genetic linkage and association studies, in addition to gene expression studies, have indicated an association between the ALOX5/ALOX5AP pathway and cardiovascular disease (7-17).

Previous association studies investigating the *ALOX5* and *ALOX5AP* genes have been limited by a limited number of common polymorphisms [minor allele frequency (MAF), >5%] in the coding sequences of the two genes, in particular non-synonymous variants. The only known relatively common non-synonymous variant is rs2228065 in *ALOX5* (MAF, ~0.03

in Caucasian populations) and the other variants are either rare or even private (7-9,11,12,14-18). This situation has lead to the use of a number of common polymorphisms in the untranslated sequences of these genes as the potential proxies for the coding parts (14-17). The majority of these studies (including one from our laboratory) have produced no significant evidence of an association between investigated polymorphisms in the *ALOX5* and *ALOX5AP* genes and the 5-LOX pathway, when investigated using measurement of different LT products, including LTB_4 and LTE_4 (7-9,11,12,14-18). Therefore, the aim of the present study was to determine whether there is association between the accumulation of rare coding variants in *ALOX5* and *ALOX5AP* and LT and the production of its associated metabolites. This study employed a next generation sequencing (NGS) approach in order to re-sequence the *ALOX5* and *ALOX5AP* genes in the pools of 303 patients with type 2 diabetes mellitus (T2DM) treated with low doses of ASA, followed by individual genotyping of all non-synonymous variants observed in pools. Using this approach we investigated the association between rare variants in the coding sequences (CDS) of *ALOX5* and *ALOX5AP* (variants accumulation or genomic burden) and differences in the urine LTB_4 and LTE_4 concentrations in the investigated patients.

Materials and methods

Patient population and study design. The ethics committee of the Medical University of Warsaw (Warsaw, Poland) approved the study protocol and the informed consent form. The study was conducted in accordance with the current version of the Declaration of Helsinki (18) at the time when the study was designed, and informed written consent was obtained. The genotyping component of the study was reviewed and approved by the Institutional Review Board of Penn State Hershey Medical Center (Hershey, PA, USA). Patients with T2DM participating in a multi-center, prospective, randomized, and open-label *AVOCADO* (*Aspirin Vs/Or Clopidogrel in Aspirin-resistant Diabetics inflammation Outcomes*) study were recruited consecutively from the outpatient clinic of the Central Teaching Hospital of the Medical University of Warsaw (Table I) (19). The full characterization of the study population, including the inclusion and exclusion criteria were published previously (20,21). DNA samples were collected from 303 Caucasian subjects with T2DM that had been administered ASA tablets (75 mg per day) for ≥3 months at the time of enrollment for primary or secondary prevention of myocardial infarction (MI). No clopidogrel or antiplatelet drugs other than ASA were used in any of the investigated patients. All patients had received oral antidiabetic agents and/or insulin for at ≥6 months, and diet-controlled diabetic patients were not included. Exclusion criteria for the enrollment included: Bleeding diathesis; history of gastrointestinal bleeding; platelet count, <150,000/mm^3; hemoglobin concentration, <10 g/dl; hematocrit <30%; end-stage chronic renal disease requiring dialysis; anticoagulants (i.e., low-molecular-weight heparin, warfarin or acenocoumarol) or alternative antiplatelet therapy other than ASA (i.e., ticlopidine, clopidogrel, prasugrel, or dipyridamole); self-reported use of nonprescription non-steroidal anti-inflammatory drugs or drugs containing ASA within 10 days of enrollment; recent history (≤12 months) of MI, unstable angina, coronary angioplasty or coronary artery bypass grafting and major surgical procedure within the previous 8 weeks.

Table I. Demographic and clinical characteristics of the study patients (n=303).

Parameter	Value
Demographics	
Age, years	67.6±8.7
Gender, female	135 (47.5)
BMI, kg/m^2	31.19±12.0
SBP, mmHg	142.3±18.9
DBP, mmHg	80.5±11.3
Dyslipidemia, n (%)	234 (82.4)
Hypertension, n (%)	262 (92.3)
CAD, n (%)	162 (57.0)
Prior MI, n (%)	87 (30.6)
Prior stroke, n (%)	23 (8.1)
Heart failure, n (%)	107 (37.7)
History of smoking, n (%)	160 (56.3)
Current smoker, n (%)	28 (9.8)
Concurrent medication, n (%)	
Oral hypoglycemics	243 (85.6)
Insulin	93 (32.7)
Beta-blockers	205 (72.2)
ACE inhibitors	185 (65.1)
Statins	206 (72.6)
Biochemical and hematological parameters	
HGB, g/dL	13.8±1.3
HCT, %	41.3±4.5
WBC, $10^3/mm^3$	7.1±2.2
PLT, $10^3/mm^3$	227.8±58.3
MPV, fL	9.9±1.2
eGFR, ml/min/1.73	70.8±20.9
HbA1c, %	7.0±1.3
hsCRP, mg/l	4.1±5.6
S-TXB2, ng/ml	0.153 (0.142)
LTB_4, pg/ml	102.585 (51.26)
LTE_4, pg/mg creatine	808.915 (82.637)

Continuous data expressed as the mean ± standard deviation, discrete data as n (%) and S-TXB2, LTB_4 and LTE_4 expressed as median (interquartile range). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CAD, coronary artery disease; MI, myocardial infarction; ACE, angiotensin-converting enzyme; HGB, hemoglobin; HCT, hematocrit; WBC, white blood cells; PLT, platelet count; MPV, mean platelet volume; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; hsCRP, high-sensitivity C-reactive protein; CEPI-CT, collagene/epinephrine closure time (sec); CADP-CT, collagen/adenosine diphosphate closure time (sec); IQR, interquartile range; S-TXB_2, serum thromboxane B_2; LTB_4, leukotriene B_4; LTE_4, leukotriene E_4.

Biochemical analysis. Regular laboratory testing was performed at the laboratory of the Central Teaching Hospital,

Medical University of Warsaw using standard techniques to determine complete blood cell and platelet counts, fasting glycaemia, glycosylated hemoglobin (HbA1c), lipid profile, C-reactive protein and serum creatinine concentrations. In addition, serum thromboxane B2 level (S-TXB2) was measured using a Thromboxane B2 enzyme-linked immunosorbent assay (ELISA) kit (cat. no. 501020) according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA). Samples with results outside the standard curve were re-assayed using appropriate dilutions. An optimal compliance was confirmed by S-TXB2 levels <7.2 ng/ml in all patients, as described previously (21). The first morning following night rest, urine specimen was collected and brought in by the patient within 2 h of collection. The samples were collected in tubes containing indomethacin (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80˚C for further analysis (19). Urinary LTE_4 and LTB_4 levels were detected using ELISA kits, according to the manufacturer's instructions (ELISA kit; cat. nos. 501060 and 520111, respectively; Cayman Chemical Co.) following extraction and purification using solid phrase extraction (C18) columns (Waters Corporation, Milford, MA, USA). Data were normalized against urinary creatinine concentrations (20).

NGS resequencing of DNA samples. Genomic DNA (gDNA) was extracted from venous ethylenediaminetetraacetic acid (EDTA) antecubital vein-whole blood sample using a FujiFilm Life Sciences membrane ultrafiltration method (AutoGen, Holliston, MA, USA) (19-21), according to the manufacturer's recommendations. The selected number of gDNA samples were pooled in an equimolar manner and verified according to the previously described method from our laboratory (19). Pooled gDNA samples were submitted to Otogenetics Corporation (Norcross, GA, USA) for target capture and sequencing. Briefly, gDNA was subjected to agarose gel electrophoresis and optical density ratio tests (22) to confirm the purity and concentration prior to fragmentation using a Covaris NGS method (Covaris S220 System; Covaris, Inc., Woburn, MA, USA). Fragments were tested for size distribution and concentration using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and NanoDrop (ND-2000; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Libraries were constructed using qualified fragmented gDNA using NEBNext DNA Library Prep Reagent Set for Illumina (cat. no. E6000; New England Biolabs, Ipswich, MA, USA) and the resulting libraries were subjected to enrichment of the two selected genes using custom probes targeting selected loci as previously described (19). The resultant libraries were tested for enrichment of selected genes by quantitative polymerase chain reaction (qPCR) according to the manufacturer's instructions. The thermal cycling conditions were as follows: Initial denaturation for 30 sec at 98˚C, denaturation for 10 sec at 98˚C, annealing/extension for 75 sec at 65˚C and final extension for 5 min at 65˚C. The samples were also tested for size distribution and concentration by an Agilent 2100 Bioanalyzer. The samples were then sequenced using an Illumina HiSeq 2000 sequencing system (Illumina, Inc., San Diego, CA, USA) which generated paired-end reads of 100 nucleotides each.

Sequencing data analysis and identification of variants. The original source sequencing data files (from Illumina 1.8+ pipeline in FASTQ format; http://support.illumina.com/sequencing/sequencing_software/casava.html) were analyzed using the web-based Galaxy (main.g2.bx.psu.edu) and DNAnexus (www.dnanexus.com) platforms. For the Galaxy platform, the original pair-end sequencer FASTQ data files were converted using the FASTQ Groomer algorithm into the Sanger format (23). Subsequently, the short sequences (queries) in the FASTQ files were aligned against a sequence of the human reference genome (Human Feb. 2009-GCRCh37/hg19; http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/) using the Galaxy Burrows-Wheeler transform routine. This resulted in the generation of the sorted Sequence Alignment/Map (SAM) format files for each pooled sample, which were transformed into the indexed BAM files (24,25). Variant detection in each BAM file was performed using the Galaxy FreeBayes Bayesian genetic variant detector (github.com/ekg/freebayes#readme) (26).

In addition, each of the pooled sample FASTQ sequence files was independently analyzed using the DNAnexus platform in the Nucleotide-Level Variation analysis mode. This platform, although similar to the Burrows-Wheeler transform routine used in Galaxy, employed a different algorithm for variant detection; GATK (https://www.broadinstitute.org/gatk/). Our approach applied GATK for base quality score recalibration, indel realignment, duplicate removal, and performed single-nucleotide polymorphism (SNP) and insertion/deletion (INDEL) discovery and genotyping across all pooled samples simultaneously using standard hard filtering parameters or variant quality score recalibration, according to GATK best practice recommendations (27-29). The final variants output files from the Galaxy and DNAnexus platforms were annotated using a web interface to the ANNOVAR software (wANNOVAR; http://wannovar.usc.edu/). ANNOVAR is a rapid and efficient tool for annotating the functional consequences of genetic variation from high-throughput sequencing data. Functional prediction and annotation of all potential non-synonymous single-nucleotide variants (nsSNVs) in the analyzed genes was performed using the dbNSFP database, version 2.7 (19,30,31).

Individual SNP genotyping. Genotyping for selected markers in individual DNA samples was performed at the Boston Children's Hospital (Boston, MA, USA) using a custom Sequenom iPLEX assay in conjunction with a MassARRAY platform (Sequenom Inc., La Jolla, CA, USA). Panels of SNP markers were designed using Sequenom Assay Design software, version 3.2 (Sequenom, Inc.) (20,21,32).

Analysis of burden association signal due to rare variants. In order to identify associations between a trait and the number of genetic markers containing rare variants, a number of previously described statistical pooling methods were used, including genetic burden or accumulation tests, collapsing tests or aggregating tests (33). The specific testing systems used included the score test (from logistic regression model) (SCORE) (34,35), replication based test (RBT) (36), adaptation score test (ASCORE) (37,38), sequential score sum test (SEQSUM) (39) and t-test = Hotelling's T^2 test

Table II. Details of resequenced genes in the study patients.

Gene	Length (bp)	Chromosome location	Exons (n)
ALOX5	71,934	Chr10: 45, 869, 629-45, 941, 563	14
ALOX5AP	50,950	Chr13: 31, 287, 615-31, 338, 565	6

ALOX5, arachidonic acid 5-lipoxygenase; ALOX5AP, arachidonic acid 5-lipoxygenase activating protein.

(TTEST) (40). All tests were performed using the R software package AssotesteR (https://cran.r-project.org/web/packages/AssotesteR/index.html). For primary analysis, the burden tests were restricted to only rare missense variants both described and not described in dbSNP or the 1000 Genomes Project (released, June 2011) under the hypothesis that truly rare mutations are more likely to be pathogenic. All tested variants were verified by individual genotyping using the Sequenom MassARRAY platform, and the number of analyzed alleles were obtained from the verification stage of the study. In this analysis, only rare missense variants with MAF <5% variants were included. Statistical significance was assessed using 100,000 case-control permutations, with exception of *t*-test, which used asymptotic P-value.

Table III. Summary of variants observed in all resequenced genes.

Variant	*ALOX5*	*ALOX5AP*
Non-synonymous	2/2	1/0
Synonymous	2/2	1/0
Introns	187/66	79/12
Untranslated 3' or 5'	8/0	0/0

Data are presented as: Number of known variants/number of newly identified variants. ALOX5, arachidonic acid 5-lipoxygenase; ALOX5AP, arachidonic acid 5-lipoxygenase activating protein.

Results

NGS resequencing and analysis of pooled genomic regions. The total output from the Illumina sequencer for the 12 pooled samples included 11,281,876 reads (100 bp each) for the total of 1.1 GB. This sequencing yielded a large quantity of high-quality data for each pool, capturing 99% of the amplified target region of ~123 kb (Table II). For the variants discovery in pool samples, only bases with a FRED quality scale of at least Q30 for a given position were accepted. The median final read depth for the analyzed coding sequences was ≥ x230 per combined pool (23-30 diploid genomes in each pool), indicating that each base position was interrogated ≥4 times for each allele. This level of coverage was sufficient to provide 97% sensitivity for the detection of a single alternate allele in pools (41).

The total number of observed single variant polymorphisms was 352 over a 2-gene loci span (data not shown). In summary, 342 variants were located outside of CDS sequences of the interrogated genes (i.e. in introns and untranslated sequences) and 10 were located within the CDS sequences. These CDS variants included 5 synonymous variants and 5 non-synonymous variants. All observed non-synonymous variants had a MAF of <5%. Compared with available databases (dbSNP, version 141 and the 1000 Genome Project), 2 non-synonymous variants were reported before, and 3 were new (i.e. unreported in available databases).

To further increase the sensitivity and predictive power of the study 2 combined pools (n=160 patients in each) were analyzed with the contrasting urine LTB_4 and LTE_4 concentrations in order to identify potential associations with the various observed platelet pathway phenotypes.

Further characterization of all non-synonymous variants, new and previously described, included interrogation using the full list of functional prediction algorithms in the dbNSFP database (version 2.7, September 12, 2014) (42,43). This analysis revealed that the majority of new variants and of known, rare missense have potential significance for the protein function, defined as potentially damaging, not tolerated or highly conserved in a number of the functional prediction algorithms.

Verification of the rare missense variants by individual genotyping. Individual genotyping was performed in a total of 303 ASA-treated T2DM patients and treated with ASA comprising the initial discovery group as previously described (19). The results of this genotyping, presented as observed MAF for all interrogated alleles, for the 5 rare missense variants identified in the discovery phase are presented in Tables III and VI.

Accumulation of non-synonymous rare variants in diabetic individuals with various LT concentrations. The contrasting groups for LTE_4 and LTB_4 were separately created by dividing all individually genotyped subjects into two equal groups (for each assay), one with the LTE_4 (or LTB_4) levels above median and another with LT levels below the median. When combined, the non-synonymous variants for the two investigated genes did not show any nominal burden signal of association (P>0.05) for the group differences based on LTE_4 or LTB_4 (data not shown). In these genes, all rare non-synonymous variants in the ALOX5 and ALOX5AP genes were included in the tests. The absence of burden signal in the investigated genes corresponded with an accumulation of non-synonymous rare variants.

In addition, the absence of burden signal of association in these two genes could not be attributed to a bias in the sequencing coverage between case and control pools, as all ten variants included in the burden tests showed coverage in each pool.

Table IV. Summary of variants observed in the coding sequences of *ALOX5* and *ALOX5AP* in the study patients from the pooled discovery group (n=303).

Position	dbSNP	Function	Base switch	AA switch	MAF	SIFT prediction	PolyPhen prediction
ALOX5							
45891311	rs144260980	Non-syn	G/T	A/S	0.002	T	D
45920506	rs2228065	Non-syn	G/A	E/K	0.004	T	B
45936008	New	Non-syn	G/A	R/Q	0.002	T	B
45938974	New	Non-syn	G/A	R/H	0.002	T	N
45869748	rs4987105	Syn	C/T	-	0.11	N/A	N/A
34939617	rs2229136	Syn	A/G	-	0.05	N/A	N/A
45891370	New	Syn	G/A	-	0.005	N/A	N/A
45907708	New	Syn	T/C	-	0.002	N/A	N/A
ALOX5AP							
31318223	rs146973182	Non-syn	G/C	E/Q	0.002	T	D
31318249	rs41351946	Syn	C/T	-	0.008	N/A	N/A

All variants were SNPs. SIFT is an algorithm for prediction whether an amino acid substitution affects protein function. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences. PolyPhen is a tool which predicts the impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. ALOX5, arachidonic acid 5-lipoxygenase; ALOX5AP, arachidonic acid 5-lipoxygenase activating protein; dbSNP, Single Nucleotide Polymorphism Database; AA, amino acid; MAF, minor allele frequency; SIFT, Sorting Intolerant from Tolerant; PolyPhen, Polymorphism Phenotyping; Functional predictions for SIFT and PolyPhen: D, damaging; T, tolerated; Non-syn, non-synonymous; Syn, synonymous; G, guanine; T, thymine; A, adenine; C, cytosine; A, alanine; S, serine; E, glutamic acid; K, lysine; R, arginine; Q, glutamine; H, histidine.

Discussion

In previous epidemiological correlation studies, common genetic variations, predominantly in the untranslated regions, in ALOX genes have been associated with an increased risk of colorectal cancer, stroke, bronchial asthma and atherosclerosis (2,7-17). To the best of our knowledge, the present study is the first to conduct a complete resequencing of the two LT pathway genes *ALOX5* and *ALOX5AP* in patients with diabetes and receiving ASA therapy, and its association with LT metabolite production. The next-generation resequencing of pools of individuals, as performed in the present study, is an established approach that provides genetic polymorphism data at a considerably lower cost compared with individual sequencing (44). In addition, NGS studies, as applied to platelet function, provided significant results indicating that potentially damaging variants in platelet genes have low individual frequencies, but are collectively abundant among the population (45). Another previous study reported that potentially damaging variants are present in pedigrees with inherited platelet function disorders and may contribute to complex laboratory phenotypes (46).

The genetic variants involved in the function of *ALOX5* and *ALOX5AP* were described previously with mixed results regarding their involvement in the regulation of LT metabolism or vascularity, including platelet functions (7-17). However, the majority of previous studies have investigated the common polymorphic variants in the *ALOX5* and *ALOX5AP* genes, as neither gene exhibits any significant common variants in their coding sequences. The results of a previous study by the present authors was unable to confirm whether the selected 14 common intronic variants in the *ALOX5AP* gene within the LT metabolism pathway contribute to platelet reactivity in a diabetic population treated with ASA (20).

By contrast, certain previous studies have indicated that the *ALOX5AP* SG13S114 polymorphism is associated with susceptibility to ischemic stroke in Chinese population (47,48). *ALOX5AP* encodes a protein which, in addition to 5-lipoxygenase, is required for LT synthesis and localizes to the plasma membrane. Inhibitors of its function impede the translocation of 5-lipoxygenase from the cytoplasm to the cell membrane and inhibit 5-lipoxygenase activation. Despite its length (~51 kb, 6 exons) only a relatively limited number of variants (~1,097) are currently included in the various databases, including 20 rare missense or nonsense variants and 5 splice variants. The remainder of the variants are located in the untranslated or intronic regions of the gene. The present study did not identify any new variants, in addition to the previously known rare 1 non-synonymous and one 1 synonymous variants, in either coding sequences or splice variant locations of the *ALOX5AP* gene. However, 10 new intronic variants were identified, with currently unidentified significance for gene function.

ALOX5 contains comparatively more known non-synonymous variants compared with *ALOX5AP*. The 1000 Genome database describes 69 non-synonymous coding variations for *ALOX5*; however, only the *ALOX5* Glu254Lys (rs2228065) variant has a global frequency of >1%, and is therefore classified as common. This variant was detected in the present study, in addition to other known rare non-synonymous

variant (rs144260980). In a previous paper describing *in vitro* functional consequences of the non-synonymous variants in *ALOX5* gene, Horn *et al* indicated that the majority of analyzed proteins translated from known rare variants are localized on the enzyme surface, suggesting that they may only exert minor effects on enzyme functionality (49). By contrast, genetic variations, which affect functional important amino acid residues or lead to truncated enzyme variations (nonsense mutations) are usually rare, with a global allele frequency of <0.1%. These data indicate an evolutionary pressure on the coding regions of the lipoxygenase genes, preventing the accumulation of loss-of-function variations in the human population.

The present study included patients with T2DM exclusively, therefore it is unclear whether the observed lack of effect is associated with the diabetic status of the study population or if the rare variant accumulation may become evident in other populations, such as patients with coronary heart disease or non-diabetics. However, in general, the observation of only a limited number of rare nonsense rare variants in the present study of 303 patients reduces the plausibility of their involvement alone in the function of the LT pathway, as numerous coding variants spread across the investigation group would be required in order to assume that the genetic burden differed between the groups selected on the different concentrations of LT metabolites.

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References

1. DiChiara J, Bliden KP, Tantry US, Hamed MS, Antonino MJ, Suarez TA, Bailon O, Singla A and Gurbel PA: The effect of aspirin dosing on platelet function in diabetic and nondiabetic patients: An analysis from the aspirin-induced platelet effect (ASPECT) study. Diabetes 56: 3014-3019, 2007.
2. Martel-Pelletier J, Lajeunesse D, Reboul P and Pelletier JP: Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann Rheum Dis 62: 501-509, 2003.
3. Peters-Golden M and Henderson WR Jr: Leukotrienes. N Engl J Med 357: 1841-1854, 2007.
4. Carlos TM and Harlan JM: Leukocyte-endothelial adhesion molecules. Blood 84: 2068-2101, 1994.
5. Sala A, Rossoni G, Berti F, Buccellati C, Bonazzi A, Maclouf J and Folco G: Monoclonal anti-CD18 antibody prevents transcellular biosynthesis of cysteinyl leukotrienes in vitro and in vivo and protects against leukotriene-dependent increase in coronary vascular resistance and myocardial stiffness. Circulation 101: 1436-1440, 2000.
6. Rossi A, Cuzzocrea S and Sautebin L: Involvement of leukotriene pathway in the pathogenesis of ischemia-reperfusion injury and septic and non-septic shock. Curr Vasc Pharmacol 7: 185-197, 2009.
7. Zhang K, Wang YY, Liu QJ, Wang H, Liu FF, Ma ZY, Gong YQ and Li L: Two single nucleotide polymorphisms in ALOX15 are associated with risk of coronary artery disease in a Chinese Han population. Heart Vessels 25: 368-373, 2010.
8. Zhao J, He Z, Ma S and Li L: Association of ALOX15 gene polymorphism with ischemic stroke in northern Chinese Han population. J Mol Neurosci 47: 458-464, 2012.
9. Helgadottir A, Gretarsdottir S, St Clair D, Manolescu A, Cheung J, Thorleifsson G, Pasdar A, Grant SF, Whalley LJ, Hakonarson H, *et al*: Association between the gene encoding 5-lipoxygenase-activating protein and stroke replicated in a Scottish population. Am J Hum Genet 76: 505-509, 2005.
10. Helgadottir A, Manolescu A, Helgason A, Thorleifsson G, Thorsteinsdottir U, Gudbjartsson DF, Gretarsdottir S, Magnusson KP, Gudmundsson G, Hicks A, *et al*: A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. Nat Genet 38: 68-74, 2006.
11. Linsel-Nitschke P, Gotz A, Medack A, König IR, Bruse P, Lieb W, Mayer B, Stark K, Hengstenberg C, Fischer M, *et al*: Genetic variation in the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) is associated with myocardial infarction in the German population. Clin Sci (Lond) 115: 309-315, 2008.
12. Hartiala J, Li D, Conti DV, Vikman S, Patel Y, Tang WH, Brennan ML, Newman JW, Stephensen CB, Armstrong P, *et al*: Genetic contribution of the leukotriene pathway to coronary artery disease. Hum Genet 129: 617-627, 2011.
13. Werz O and Steinhilber D: Therapeutic options for 5-lipoxygenase inhibitors. Pharmacol Ther 112: 701-718, 2006.
14. Camacho M, Martinez-Perez A, Buil A, Siguero L, Alcolea S, López S, Fontcuberta J, Souto JC, Vila L and Soria JM: Genetic determinants of 5-lipoxygenase pathway in a Spanish population and their relationship with cardiovascular risk. Atherosclerosis 224: 129-135, 2012.
15. Burdon KP, Rudock ME, Lehtinen AB, Langefeld CD, Bowden DW, Register TC, Liu Y, Freedman BI, Carr JJ, Hedrick CC and Rich SS: Human lipoxygenase pathway gene variation and association with markers of subclinical atherosclerosis in the diabetes heart study. Mediators Inflamm 2010: 170153, 2010.
16. Crosslin DR, Shah SH, Nelson SC, Haynes CS, Connelly JJ, Gadson S, Goldschmidt-Clermont PJ, Vance JM, Rose J, Granger CB, *et al*: Genetic effects in the leukotriene biosynthesis pathway and association with atherosclerosis. Hum Genet 125: 217-229, 2009.
17. Girelli D, Martinelli N, Trabetti E, Olivieri O, Cavallari U, Malerba G, Busti F, Friso S, Pizzolo F, Pignatti PF and Corrocher R: ALOX5AP gene variants and risk of coronary artery disease: An angiography-based study. Eur J Hum Genet 15: 959-966, 2007.
18. World Medical Association: WMA Declaration of Helsinki: Ethical principles for medical research involving human subjects. http://www.wma.net/en/30publications/10policies/b3/. Accessed April 28, 2016.
19. Postula M, Janicki PK, Eyileten C, Rosiak M, Kaplon-Cieslicka A, Sugino S, Wilimski R, Kosior DA, Opolski G, Filipiak KJ and Mirowska-Guzel D. Next-generation re-sequencing of genes involved in increased platelet reactivity in diabetic patients on acetylsalicylic acid. Platelets 24: 1-8, 2015.
20. Rosiak M, Postula M, Kaplon-Cieslicka A, Kondracka A, Trzepla E, Zaremba M, Filipiak KJ, Kosior DA, Czlonkowski A, Opolski G and Janicki PK: Lack of effect of common single nucleotide polymorphisms in leukotriene pathway genes on platelet reactivity in patients with diabetes. Mol Med Rep 8: 853-860, 2013.
21. Postula M, Kaplon-Cieslicka A, Rosiak M, Kondracka A, Serafin A, Filipiak KJ, Czlonkowski A, Opolski G and Janicki PK: Genetic determinants of platelet reactivity during acetylsalicylic acid therapy in diabetic patients: Evaluation of 27 polymorphisms within candidate genes. J Thromb Haemost 9: 2291-2301, 2011.
22. Glasel JA: Validity of nucleic acid purities monitored by A260/A280 absorbance ratios. Biotechniques 18: 62-63, 1995.
23. Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J and Nekrutenko A; Galaxy Team: Manipulation of FASTQ data with Galaxy. Bioinformatics 26: 1783-1785, 2010.
24. Li H and Durbin R: Fast and accurate short read alignment with burrows-wheeler transform. Bioinformatics 25: 1754-1760, 2009.
25. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G and Durbin R; 1000 Genome Project Data Processing Subgroup: The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078-2079, 2009.
26. Garrison E and Marth G: Haplotype-based variant detection from short-read sequencing. 2012 Available from: http://arxiv.org/pdf/1207.3907.pdf.

27. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M and DePristo MA: The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20: 1297-1303, 2010.
28. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, *et al*: A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43: 491-498, 2011.
29. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, *et al*: From FastQ data to high confidence variant calls: The genome analysis toolkit best practices pipeline. Curr Protoc Bioinformatics 11: 11.10.1-11.10.33, 2013.
30. Chang X and Wang K: wANNOVAR: Annotating genetic variants for personal genomes via the web. J Med Genet 49: 433-436, 2012.
31. Wang K, Li M and Hakonarson H: ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38: e164, 2010.
32. Janicki PK, Vealey R, Liu J, Escajeda J, Postula M and Welker K: Genome-wide association study using pooled DNA to identify candidate markers mediating susceptibility to postoperative nausea and vomiting. Anesthesiology 115: 54-64, 2011.
33. Bansal V, Libiger O, Torkamani A and Schork NJ: Statistical analysis strategies for association studies involving rare variants. Nat Rev Genet 11: 773-785, 2010.
34. Clayton D, Chapman J and Cooper J: Use of unphased multilocus genotype data in indirect association studies. Genet Epidemiol 27: 415-428, 2004.
35. Chapman J and Whittaker J: Analysis of multiple SNPs in a candidate gene or region. Genet Epidemiol 32: 560-566, 2008.
36. Liu X, Jian X and Boerwinkle E: dbNSFP: A lightweight database of human non-synonymous SNPs and their functional predictions. Hum Mutat 32: 894-899, 2011.
37. Han F and Pan W: A data-adaptive sum test for disease association with multiple common or rare variants. Hum Hered 70: 42-54, 2010.
38. Pan W, Kim J, Zhang Y, Shen X and Wei P: A powerful and adaptive association test for rare variants. Genetics 197: 1081-1095, 2014.
39. Basu S and Pan W: Comparison of statistical tests for disease association with rare variants. Genet Epidemiol 35: 606-619, 2011.
40. Xiong M, Zhao J and Boerwinkle E: Generalized T2 test for genome association studies. Am J Hum Genet 70: 1257-1268, 2002.
41. Pihlstrom L, Rengmark A, Bjornaråa KA and Toft M: Effective variant detection by targeted deep sequencing of DNA pools: An example from Parkinson's disease. Ann Hum Genet 78: 243-252, 2014.
42. Liu X, Jian X and Boerwinkle E: dbNSFP v2.0: A database of human non-synonymous SNVs and their functional predictions and annotations. Hum Mutat 34: E2393-E2402, 2013.
43. Liu X, Jian X and Boerwinkle E: dbNSFP: A lightweight database of human nonsynonymous SNPs and their functional predictions. Hum Mutat 32: 894-899, 2011.
44. Schlötterer C, Tobler R, Kofler R and Nolte V: Sequencing pools of individuals-mining genome-wide polymorphism data without big funding. Nat Rev Genet 15: 749-763, 2014.
45. Jones ML, Norman JE, Morgan NV, Mundell SJ, Lordkipanidzé M, Lowe GC, Daly ME, Simpson MA, Drake S, Watson SP, *et al*: Diversity and impact of rare variants in genes encoding the platelet G protein-coupled receptors. Thromb Haemost 113: 826-837, 2015.
46. Leo VC, Morgan NV, Bem D, Jones ML, Lowe GC, Lordkipanidzé M, Drake S, Simpson MA, Gissen P, Mumford A, *et al*: Use of next generation sequencing and candidate gene analysis to identify underlying defects in patients with inherited platelet function disorders. J Thromb Haemost 13: 643-650, 2015.
47. Wang G, Liu R and Zhang J: The arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene SG13S114 polymorphism and ischemic stroke in Chinese population: A meta-analysis. Gene 533: 461-468, 2014.
48. Yi XY, Zhou Q, Lin J, Chi LF and Chi WZ: Interaction between ALOX5AP-SG13S114A/T and COX-2-765G/C increases susceptibility to cerebral infarction in a Chinese population. Genet Mol Res 12: 1660-1669, 2013.
49. Horn T, Reddy Kakularam K, Anton M, Richter C, Reddanna P and Kuhn H: Functional characterization of genetic enzyme variations in human lipoxygenases. Redox Biol 1: 566-577, 2013.