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Aspirin Exposure Reveals Novel Genes Associated With Platelet Function and Cardiovascular Events

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Objectives The aim of this study was to develop ribonucleic acid (RNA) profiles that could serve as novel biomarkers for the response to aspirin.

Background Aspirin reduces death and myocardial infarction (MI), suggesting that aspirin interacts with biological pathways that may underlie these events.

Methods Aspirin was administered, followed by whole-blood RNA microarray profiling, in a discovery cohort of healthy volunteers (HV1) ($n = 50$) and 2 validation cohorts of healthy volunteers (HV2) ($n = 53$) and outpatient cardiology patients (OPC) ($n = 25$). Platelet function was assessed using the platelet function score (PFS) in HV1 and HV2 and the VerifyNow Aspirin Test (Accumetrics, Inc., San Diego, California) in OPC. Bayesian sparse factor analysis identified sets of coexpressed transcripts, which were examined for associations with PFS in HV1 and validated in HV2 and OPC. Proteomic analysis confirmed the association of validated transcripts in platelet proteins. Validated gene sets were tested for association with death or MI in 2 patient cohorts ($n = 587$ total) from RNA samples collected at cardiac catheterization.

Results A set of 60 coexpressed genes named the "aspirin response signature" (ARS) was associated with PFS in HV1 ($r = -0.31$, $p = 0.03$), HV2 ($r = -0.34$, Bonferroni $p = 0.03$), and OPC ($p = 0.046$). Corresponding proteins for the 17 ARS genes were identified in the platelet proteome, of which 6 were associated with PFS. The ARS was associated with death or MI in both patient cohorts (odds ratio: 1.2 [$p = 0.01$]; hazard ratio: 1.5 [$p = 0.001$]), independent of cardiovascular risk factors. Compared with traditional risk factors, reclassification (net reclassification index = 31% to 37%, $p \leq 0.0002$) was improved by including the ARS or 1 of its genes, *ITGA2B*.

Conclusions RNA profiles of platelet-specific genes are novel biomarkers for identifying patients who do not respond adequately to aspirin and who are at risk for death or MI. (J Am Coll Cardiol 2013;62:1267–76) © 2013 by the American College of Cardiology Foundation

The identification of novel biomarkers for patients at risk for coronary artery disease (CAD) mortality, primarily because of platelet-mediated cardiovascular events such as

See page 1277

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Abbreviations and Acronyms

ARS = aspirin response signature
CAD = coronary artery disease
CATHGEN = Catheterization Genetics
CI = confidence interval
DUMC = Duke University Medical Center
HV1 = healthy volunteer discovery cohort
HV2 = healthy volunteer validation cohort
MI = myocardial infarction
MPV = mean platelet volume
OPC = outpatient cardiology cohort
OR = odds ratio
PCR = polymerase chain reaction
PFS = platelet function score
RNA = ribonucleic acid
RT-PCR = real-time polymerase chain reaction

myocardial infarction (MI), is a priority for reducing the burden of cardiovascular disease. Although genomewide surveys of genomic variation and gene expression can identify loci associated with CAD (1–3), few can serve as biomarkers for cardiovascular events (4).

Aspirin is prescribed for the prevention of cardiovascular events, suggesting that aspirin interacts with biological pathways that may underlie these events. Platelet function assays are a surrogate biomarker for the effects of aspirin and are associated with cardiovascular events (5). However, platelet function testing is not widely available, primarily because of technical complexity. By contrast, whole-blood ribonucleic acid (RNA) profiling using polymerase chain reaction (PCR)–based assays is currently a widely available diagnostic testing platform (6,7). Therefore, we hypothesized that aspirin could be used as a probe in conjunction with whole-blood RNA profiling to elucidate novel biomarkers for platelet function in response to aspirin and for cardiovascular outcomes.

Methods

Platelet function outcomes in healthy volunteer cohorts at Duke University Medical Center. We previously described (8) the healthy volunteer discovery cohort (HV1) and the healthy volunteer validation cohort (HV2) (Online Fig. 1) and the platelet function score (PFS), a composite metric of the following platelet function assays: PFA-100 (collagen/epinephrine; Siemens Healthcare, Erlangen, Germany) closure time and the areas under the optical aggregometry curve induced by adenosine diphosphate (10, 5, and 1 μmol/l), epinephrine (10, 1, and 0.5 μmol/l), and collagen (5 and 2 mg/ml). We measured the PFS and mean platelet volume (MPV) in HV1 (n = 50) after 2 weeks of dosing with 325 mg/day non–enteric-coated, immediate-release aspirin and HV2 (n = 53) after 4 weeks of dosing with 325 mg/day aspirin. In both cohorts, whole-blood RNA was collected into PAXgene Blood RNA tubes (Becton Dickinson and Company, Franklin Lakes, New Jersey) before and after aspirin exposure and stored at −80°C until microarray profiling. Platelet count was measured in platelet-rich plasma in HV1.

Because 3 subjects in HV2 had participated in HV1, they were dropped from HV2, leaving 50 unique HV2 subjects. The Duke University Medical Center (DUMC) institutional review board approved the study protocols.

Platelet function outcomes in patients at risk for cardiovascular events at George Washington University. We previously described (9) the outpatient cardiology cohort (OPC) (Online Fig. 1), treated with 81 mg/day aspirin and assessed using the VerifyNow Aspirin Test (Accumetrics, Inc., San Diego, California) and whole-blood RNA microarray analysis.

Clinical outcomes in DUMC patients. CATHETERIZATION GENETICS BIOREPOSITORY. The Catheterization Genetics (CATHGEN) biorepository has banked, whole-blood RNA in PAXgene tubes from DUMC patients from the time of cardiac catheterization, baseline medical history, and follow-up for all-cause death and MI (10,11). Two cohorts had available microarray data (Online Fig. 2): in the observational cohort, 224 sequential samples were selected for RNA analysis, of which 191 had sufficient RNA for microarray analysis, and the case-control cohort consisted of participants who had experienced death or MI (n = 250) after their index catheterization and age-matched, sex-matched, and race-matched controls (n = 250) who were free of death or MI for >2 years after cardiac catheterization (12). Four hundred forty-seven had sufficient RNA for microarray analysis; 44 overlapped with the observational cohort and were dropped, leaving 403 subjects for analysis.

Follow-up for death and MI was ascertained in both cohorts in October 2011; the median follow-up duration was 3.8 years. Patients with incomplete follow-up were censored at the time of last contact. Patients who had histories of cardiac transplantation at the time of catheterization (n = 5), died within 7 days (n = 1), or failed quality control (n = 1) were excluded. The remaining datasets left 190 samples in the observational cohort (48 death or MI events) and 397 (202 death or MI events) in the case-control cohort.

RNA extraction, labeling, microarray hybridization, quality control, and normalization. See the Online Appendix for full details. Two microarray platforms were used: the Affymetrix U133A2 array (HV1, before aspirin; Affymetrix, Santa Clara, California) and the U133 Plus 2.0 array (all others). The robust multichip average method was used for normalization.

Real-time PCR. See the Online Appendix for full details. Forty-five transcripts were selected for verification in the original RNA samples on the basis of 2 criteria: 1) the strength of correlation of the probe set with PFS; and 2) the strength of membership between the probe set and the set of coexpressed genes of interest.

Platelet purification, protein sample preparation, and proteomics analysis by liquid chromatography–mass spectrometry/mass spectrometry. See the Online Appendix for full details.

Statistical analysis. The raw and normalized microarray data are available in the Gene Expression Omnibus for the OPC cohort (GSE38511). The data for the HV1, HV2, and CATHGEN cohorts are available through the Database of Genotypes and Phenotypes (phs000548.v1.p1 and phs000551.v1.p1). Unless stated otherwise, all tests were 2