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Aspirin insensitive thrombophilia: Transcript profiling of blood identifies platelet abnormalities and HLA restriction

Payam Fallahi[a,c], **Richard Katz**[a,c], **Ian Toma**[a,b], **Ranyang Li**[a,b], **Jonathan Reiner**[a,c], **Kiersten VanHouten**[a,b], **Larry Carpio**[a,b], **Lorraine Marshall**[a,c], **Yi Lian**[a,b], **Sujata Bupp**[a,b], **Sidney W. Fu**[a,b,e], **Frederick Rickles**[a,d], **David Leitenberg**[e], **Yinglei Lai**[f], **Babette B. Weksler**[g], **Frederik Rebling**[a,b], **Zhaoqing Yang**[a,b], and **Timothy A. McCaffrey**[a,b,e,*]

[a] Department of Medicine, The George Washington University Medical Center, Washington DC, USA

[b] Division of Genomic Medicine, The George Washington University Medical Center, Washington DC, USA

[c] Division of Cardiology, The George Washington University Medical Center, Washington DC, USA

[d] Division of Hematology/Oncology, The George Washington University Medical Center, Washington DC, USA

[e] Department of Microbiology, Immunology, and Tropical Medicine, The George Washington University Medical Center, Washington DC, USA

[f] Department of Statistics, Biostatistics Center, The George Washington University Medical Center, Washington DC, USA

[g] The Department of Medicine, Division of Hematology/Oncology, The Weill/Cornell University Medical College, USA

Abstract

Aspirin is the most widely used antiplatelet agent because it is safe, efficient, and inexpensive. However, a significant subset of patients does not exhibit a full inhibition of platelet aggregation, termed ‘aspirin resistance’ (AR). Several major studies have observed that AR patients have a 4-fold increased risk of myocardial infarction (MI), stroke, and other thrombotic events. Arachidonic acid-stimulated whole blood aggregation was tested in 132 adults at risk for ischemic events, and identified an inadequate response to aspirin therapy in 9 patients (6.8%). Expression profiling of blood RNA by microarray was used to generate new hypotheses about the etiology of AR. Among the differentially expressed genes, there were decreases in several known platelet transcripts,

* Corresponding author at: The George Washington Medical Center, Department of Medicine, Division of Genomic Medicine, 2300 I Street NW, Ross Hall 443, Washington, D.C. 20037, USA. Tel.: +1 202 994 8919; fax: +1 202 994 8924. mcc@gwu.edu (T.A. McCaffrey).

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including clusterin (CLU), glycoproteins IIb/IIIa (ITGA2B/3), lipocalin (LCN2), lactoferrin (LTF), and the thrombopoetin receptor (MPL), but with increased mRNA for the T-cell Th1 chemokine CXCL10. There was a strong association of AR with expression of HLA-DRB4 and HLA-DQA1. Similar HLA changes have been linked to autoimmune disorders, particularly antiphospholipid syndrome (APS), in which autoantibodies to phospholipid/protein complexes can trigger platelet activation. Consistent with APS, AR patients exhibited a 30% reduction in platelet counts. Follow-up testing for autoimmune antibodies observed only borderline titers in AR patients. Overall, these results suggest that AR may be related to changes in platelet gene expression creating a hyperreactive platelet, despite antiplatelet therapy. Future studies will focus on determining the protein levels of these differential transcripts in platelets, and the possible involvement of HLA restriction as a contributing factor.

Keywords

Aspirin; Coagulation; Genetics; Platelets; Thrombosis; Transcript profiling

1. Introduction

Aspirin is the most widely prescribed anti-platelet drug worldwide. According to a recent collaborative meta-analysis of 287 randomized trials of anti-platelet therapy, comprising more than 200,000 patients, aspirin reduced the risk of stroke, myocardial infarction or death by approximately 22% in patients with pre-existing cardiovascular disease (Baigent, 2002). In patients with acute coronary syndrome (ACS) or a coronary intervention, anti-platelet therapy including aspirin has been shown to improve outcome and decrease mortality (Yusuf et al., 2001).

The primary molecular target for aspirin in platelets is cyclooxygenase (COX), which converts arachidonic acid (AA) to thromboxane A2 (TxA_2), a potent platelet agonist and vasoconstrictor. By acetylating COX1 at serine 529, aspirin results in steric inhibition of AA binding, thus irreversibly preventing its metabolism to TxA_2. Because platelets lack nuclear DNA, irreversible inhibition of COX1 lasts for the lifespan of platelets (~10 days).

While life-saving in many cases, aspirin is not equally effective for all patients. Despite aspirin therapy, up to 20% of patients experience recurrent cardiovascular events after stent placement (Gurbel et al., 2005), raising the concept of clinical “aspirin resistance” (AR). However, patients with AR are probably not pharmacologically ‘resistant’, rather, they exhibit an insufficient antiplatelet response to aspirin (Sweeny et al., 2009). About 90% of AR subjects are also poor responders to clopidogrel, suggesting that AR may reflect a general hyperreactivity of the platelets. Further, AR subjects exhibit elevated platelet aggregation to submaximal concentrations of ADP and arachidonic acid (AA) even in the absence of aspirin treatment (Guthikonda et al., 2008), which in part, has led others to conclude that AR may reflect a subclinical thrombophilia, which has led to the evolution of the term “high on-aspirin platelet responsiveness” (HAPR) (Linden et al., 2012).

Numerous studies have investigated the relationship between aspirin responsiveness and cardiovascular outcomes. In a recent meta-analysis, the rate of lab-defined AR ranged from

5% to 65% (mean 27%), with a pooled odds ratio (OR) of 3.8 for all cardiovascular events (Snoep et al., 2007). Using the VerifyNow whole blood aggregation test, Chu and colleagues recently reported an AR incidence of 9.6% and a markedly increased risk (OR = 10) of adverse events in AR patients in the 6 months after presenting with ACS (Chu et al., 2010). Likewise, an incomplete response to aspirin, and other antiplatelet agents, is considered an important factor in the proper management of patients with drug-eluting stents (Del Castillo-Carnevali et al., 2012).

A number of hypotheses have been offered to explain AR, including pharmacodynamic (Mattiello et al., 2011), thromboxane-independent platelet activation (Weber et al., 2002), NSAID interference (Catella-Lawson et al., 2001), genetic variations (Su et al., 2007), and noncompliance with aspirin therapy (Biondi-Zoccai et al., 2006), but there is no consensus explanation for AR. The present studies were undertaken to determine whether changes in gene expression in blood cells might help to generate new hypotheses about its etiology. Whole blood gene expression was profiled by microarray in patients with and without AR. Differentially expressed genes included striking changes in HLA expression, and potentially important changes in a small set of platelet transcripts with known relevance to platelet aggregation.

2. Materials and methods

2.1. Study subjects

The study protocol was approved by the Institutional Review Board of The George Washington University, and all subjects gave informed consent. Of 180 subjects screened in the course of routine medical exams, 132 subjects met inclusion/exclusion criteria (Supplementary Table 1), and were assessed for the presence of AR. The subjects began a 7–10 day regimen of non-enteric coated aspirin (81 mg/day) taken prior to breakfast. In the morning of last day of aspirin treatment, thus typically 2–4 h after aspirin ingestion, blood was drawn in 3.2% sodium citrate tubes (Greiner) for platelet function testing, in PAXgene Blood RNA tubes (BD) for genome-wide RNA profiling, and in BD Vacutainer K2 tubes for complete blood counts with differentials. Urine was collected in BD Vacutainer urine C&S preservative for 11-dehydro-thromboxane B_2 and creatinine measurement.

2.2. Identification of AR subjects—platelet function testing

Platelet function tests were done within 2 h after blood collection using VerifyNow Aspirin (Accumetrics). This system is based on optical detection of platelet aggregation to AA and fibrinogen-coated beads in whole blood. Patients with aspirin reaction units (ARU) ≥550 (>2 S.D. above mean ARU) are defined as aspirin resistant (Coleman, 2004). High normal (HN) response was defined as 500–549 ARU, reflecting essentially 1 SD above the mean (Fig. 1). One AR RNA sample was lost due to technical failure, and the remaining 26 subjects selected for microarray analysis were divided into three groups: aspirin resistant (AR, n = 8, >550 ARU), high normal (HN, n = 9, ARU 500–549) and aspirin sensitive (AS, n = 9, ARU < 500).

2.3. Urinary thromboxane

Urinary 11-dehydro-thromboxane B_2 measurement ($dTxB_2$) is an inactive metabolite of thromboxane A_2 and detection of this metabolite in urine indicates systemic TxA_2 generation. $dTxB_2$ concentrations were measured using an enzyme-linked immunoassay kit (AspirinWorks, Corgenix, Broomfield, CO). Urinary $dTxB_2$ concentrations were normalized against urinary creatinine concentrations to correct for the dilution of urine.

2.4. Whole blood total RNA purification

Total RNA was purified from whole blood using PAXgene Blood RNA kit (PreAnalytiX), including an on-column DNAse treatment. RNA samples were further purified using the RNAeasy Mini kit (Qiagen Sciences). RNA concentration was quantitated by absorbance at 260 nm (Nanodrop, Thermo Scientific) and the RNA integrity was tested by capillary electrophoresis on an Agilent 2100 Bioanalyzer.

2.5. Microarray expression profiling

Purified RNA (100 ng) was reverse transcribed with SuperScript III using random hexamers and the cDNA was amplified with Ovation RNA Amplification System V2 kit (NuGEN Technologies). The cDNA was purified with DNA Clean & Concentrator-25 (Zymo Research) and 3.75 μg of cDNA was fragmented and labeled with FL-Ovation cDNA Biotin Module V2 kit (NuGEN Technologies) and hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array for 18 h. The arrays were washed and then fluorescence was quantitated on an Affymetrix GeneChip Scanner 3000 7G.

2.6. Microarray data analysis

The microarray probeset levels were converted to transcript levels using RMA summarization in GeneSpring GX 10 (Agilent Technologies). For identifying a small set of diagnostically useful transcripts, the 54,000 total transcripts were analyzed by an unpaired *t*-test (p-value cut-off ≤0.05), and further filtered for a fold-change of >1.5 between groups (AS + HN = 18 subjects, vs. 8 AR). To identify potentially causally-related transcripts, only the AS and AR groups were compared by a combined *t*-test/fold change analysis. The differentially expressed gene (DEG) list was further analyzed with Ingenuity Pathway Analysis, and gene ontologies analyzed with DAVID (Huang da et al., 2009).

2.7. Autoimmune parameters

A subset of subjects (5 AR, 4 AS) was reconsented for a second whole blood aggregometry and further laboratory testing for anti-phospholipid antibodies. Subjects were administered a single oral dose of 325 mg aspirin and, 1 h later, blood was collected for VerifyNow testing, and anti-platelet antibodies (Quest Diagnostics): anti-phosphatidylserine antibodies (IgG and IgM), anti-prothrombin antibodies (IgG and IgM), anti-β2 glycoprotein (IgG and IgM), and anti-cardiolipin antibodies (IgG and IgM). The presence of lupus anticoagulant was tested using the activated partial thromboplastin time-lupus anticoagulant (PTT-LA) and dilute Russell's viper venom time (DRVVT) assays. HLA-DR and -DQ genotyping was performed on buccal DNA (Oragene) by PCR and hybridization with sequence-specific oligonucleotide (SSO) probes (Quest Diagnostics).

3. Results

3.1. Clinical characteristics of aspirin resistant (AR) subjects

From a series of typical outpatient cardiology subjects, screening for AR using VerifyNow whole blood aggregation identified 9 of 132 subjects as AR using a threshold of >550 aspirin response units (ARU) of residual aggregation after 1 week on 81 mg/day aspirin (AR = 6.8%). This incidence of AR agrees with the reported incidence (6.5%) in 200 patients presenting with apparent ACS at the Cleveland Clinic emergency room (Glauser et al., 2010). The clinical characteristics of the 3 groups of aspirin-sensitive, high normal, and aspirin-insensitive subjects, are shown in Table 1. The only differences of note are a significantly lower platelet count in the AR subjects (179 K/μl) versus sensitive patients (254 K/μl), and a non-significant reduction in lymphocyte count in AR subjects. Platelet volume, sometimes used as a surrogate index of platelet turnover, did not vary significantly between groups. Other clinical variables did not vary significantly between groups.

3.2. Pharmacologic effect of ASA

Despite the significantly higher residual platelet reactivity in the AR patients, there was no significant difference in urinary $dTxB_2$ among the 3 groups, though there was a trend toward higher urinary $dTxB_2$ in the AR group (16%). No patients were AR by residual urinary $dTxB_2$ levels, which is defined as $dTxB_2$ >1500 pg/mg creatinine (Table 1). Further, urinary $dTxB_2$ correlated only modestly with platelet function testing (r = 0.21) suggesting that a) combined with verification of aspirin ingestion, non-compliance with aspirin is not a probable explanation of AR in this study, and b) aspirin is exerting the intended COX inhibitory action in the AR subjects, but their platelets remain reactive to stimulation. The platelet response to aspirin, measured by the VerifyNow whole blood aggregometry, showed a mean residual aggregation of 453.9 ARU, with a standard deviation of 52.1, and had a distinctly non-normal distribution, which was bimodal with positive skew (skewness = 0.8, Fig. 1).

3.3. Identification of differentially expressed genes (DEGs) for diagnostic use

Using PaxGene blood RNA stabilization and the U133 + 2 GeneChip, 54,000 transcripts were quantified and filtered for transcripts which were differentially expressed in AR compared to AS + HN subjects. With a minimum difference of 1.5-fold change in expression, and a p value of <0.001, there were 124 differentially expressed transcripts (Supplementary Table 2). These 124 transcripts were used to train a Support Vector Machine (SVM) machine-learning algorithm to classify subjects into sensitive or resistant groups. A leave-one-out cross-validation strategy indicated that the SVM classification was robust, and predicted the aspirin response with 100% sensitivity and specificity. This SVM algorithm will need to be retested in an independent, prospective validation cohort. The mRNA levels of the 124 gene set are shown graphically in Fig. 2, as applied to all subjects divided into the aspirin-sensitive (n = 18) versus aspirin-insensitive (n = 8) groups.

3.4. Analysis of DEGs to discover underlying causes of AR

To identify differences between the most sensitive and resistant patients, the high normal (HN) group was omitted and statistical testing was performed to identify DEGs. As shown in Fig. 3, the majority of transcripts are unchanged, but there are striking changes in some transcripts. For functional analysis, transcripts with >1.5-fold change at a $p < 0.05$ were filtered, yielding 447 transcripts, of which 280 had a useful gene annotation. An impartial analysis of gene ontologies using DAVID (Huang da et al., 2009), demonstrated that the 10 most significant functional annotations pertained to coronary stent thrombosis, platelet alpha granules, and extracellular matrix/receptor interactions, as shown in Table 2. The functional analysis was used to compose a select gene list with emphasis on thrombosis-related transcripts, as shown in Table 3.

3.4.1. HLA genotype and aspirin sensitivity—By far the largest magnitude changes were in multiple probesets for HLA-DQA1 (–14-fold in AR) and HLA-DRB4 (+15-fold in AR), which are members of MHC Class II. HLA-DQ and DR expression was validated in 16 subjects by quantitative RT-PCR with similar results (not shown). For closer inspection of HLA status, the 26 subjects were broken into 3 groups of sensitivity: aspirin sensitive (ARU < 500), high normal sensitivity (500–550 ARU), and fully aspirin resistant (>550 ARU). The changes in HLA expression shown in Table 3 are very abbreviated, and the U133 + 2 array actually has >60 probesets for HLA transcripts. All 3 major HLA-DQA alleles were present on the array and usage of more than 1 DQA allele was strongly protective against AR (Fig. 4). Heterozygosity for DQA alleles, such as expressing from DQA1 and DQA5, was present in 9/9 AS, but was infrequent (2/9) in HN and AR (1/8) (chi-square, $p = 0.003$). Likewise, the presence of the DRB4 allele was associated with an increased risk of AR: DRB4+ in AS (0/9), HN (5/9), and AR (5/8) was significantly different ($p = 0.01$). The combined DQA1 heterozygosity and DRB4− status was highly associated with sensitivity: AS (9/9), HN (0/9), and AR (0/8) ($p = 0.0004$). Standardized HLA genotyping of a subset of patients confirmed that the RNA expression analysis was predictably linked to the presence of the MHC Class II alleles.

3.4.2. Glycoproteins IIb/IIIa—Platelet glycoprotein (GP) IIb (ITGA2B) and GPIIIa (ITGB3) are well-defined platelet fibrinogen receptors, whose mRNA transcripts were decreased in the AR patients by 1.7 and 1.9-fold, respectively. While this reduction may partially reflect the reduced platelet count, the nearly 2-fold reduction is far larger than the 30% reduction in platelet count, and platelets would represent only a portion of total IIb/IIIa mRNA, suggesting that the IIb/IIIa transcripts would be reduced on a per platelet basis. In addition to changes in IIb/IIIa, a pathway analysis revealed potentially important changes in IIb/IIIa interacting proteins (Fig. 5), such as neutrophil elastase (ELANE), which is decreased almost 2-fold in AR blood. Neutrophil elastase has been shown to activate glycoprotein IIb (Si-Tahar et al., 1997) and cleave GPIIIa without destroying their fibrinogen binding activities (Bykowska et al., 1990). Overall, however, the combined reductions in IIb/IIIa and elastase should make platelets less reactive, and thus might reflect a selection for low IIb/IIIa platelets, or an inverse relationship between IIb/IIIa mRNA and protein levels. As human platelets age ex vivo, translation of platelet mRNAs causes the surface expression of IIb/IIIa to increase 2–4 folds while the mRNA decreases with a half-life of about 2.9 days

(Thon and Devine, 2007). Thus, the reduced IIb/IIIa mRNA levels may reflect older platelets, or accelerated turnover of platelets that have higher IIb/IIIa mRNA levels.

3.4.3. Glycoprotein Ibα and thromboxane A2 receptor—Prior genotyping studies identified SNPs in the platelet glycoprotein Ib α chain (GP1BA) and thromboxane A2 receptor (TBXA2R) associated with aspirin resistance (Fujiwara et al., 2007) and platelet activation (Fontana et al., 2006). Other TBXA2R SNPs have been associated with aspirin-intolerant urticaria (Palikhe et al., 2011). In the present data, TBXA2R was decreased 1.6-fold in AR, with an intermediate value in the HN subjects.

Furthermore, when a microarray analysis of primary antiphospholipid syndrome (PAPS) (Bernales et al., 2008) was compared to the current AR dataset, using an automated database LOLA (Cahan et al., 2005), the only common transcript was GP1BA, which was decreased in both conditions. The mRNA reductions in TBXA2R and GP1BA would seem to be inconsistent with elevated platelet reactivity, but, similar to IIb/IIIa, it is possible that a) mRNA levels are reduced due to increased translation, and thus, elevated protein levels, or b) it reflects selection for platelets with reduced mRNA as a compensatory response.

3.4.4. Lactotransferrin—Of particular interest, lactotransferrin (LTF, lactoferrin) mRNA was reduced by 2.4-fold in the AR patient's blood. LTF is a known neutrophil activation marker, which interacts with elastases (Fig. 4), and is an autoantigen with a potential role in autoimmune pancreatitis and liver disease (Hayakawa et al., 2009). LTF, and peptide fragments of it (Levy-Toledano et al., 1995), bind to a platelet receptor and inhibit platelet aggregation (Leveugle et al., 1993). A potential mechanism of reduced lactoferrin mRNA would be the well known effect of promoter methylation on lactoferrin transcription (Zhang et al., 2011).

3.4.5. MPL, thrombopoietin receptor—Another known platelet transcript, the thrombopoietin receptor (MPL), was decreased 1.8-fold in AR patients. Thrombopoietin (TPO), via the MPL receptor, is a key factor in megakaryocyte maturation and differentiation, as well as in platelet production by megakaryocytes (Yu and Cantor, 2012). Further, TPO acts directly on platelet MPL receptors to increase activation in response to external stimuli, presumably by an ADP-dependent pathway (Kubota et al., 1996). In essential thrombocythemia, TPO levels are normal or elevated, but platelet MPL protein and mRNA are markedly reduced (Horikawa et al., 1997). Similarly, in patients with unstable angina, serum TPO levels are elevated, but platelet MPL receptor is decreased (Lupia et al., 2006).

3.4.6. Pleckstrin—While many transcripts were decreased, pleckstrin mRNA was elevated 1.6-fold in AR patient blood. Pleckstrin can constitute up to 1% of total protein in platelets, monocytes, macrophages, lymphocytes, and granulocytes, and is a major substrate for protein kinase C. Platelets from mice with a null mutation in pleckstrin have severe defects in exocytosis of granules and PMA-induced aggregation (Lian et al., 2009). Upon platelet activation, pleckstrin comigrates with serum deprivation response protein (SDRP) to the platelet surface, where both proteins are phosphorylated by PKC, and the complex exerts important effects on platelet adhesion and aggregation (Baig et al., 2009).

3.4.7. CXCL10—Another important transcript in the blood of AR patients is the chemokine ligand CXCL10, which was elevated 2-fold. Known to be associated with the T helper 1 (Th1) subset of lymphocytes, CXCL10 has been previously associated with immune thrombocytopenia (ITP) (Gu et al., 2010), and other autoimmune disorders such as multiple sclerosis (Michalowska-Wender et al., 2006), and lupus (Bauer et al., 2009). Plasma levels of CXCL10 were elevated in patients with active ITP compared to controls, and decreased during remission of symptoms (Gu et al., 2010). Interestingly, in those studies, lower peripheral platelet counts correlated with higher CXCL10 levels (Gu et al., 2010).

3.4.8. Platelet transcripts—The consistent reduction in several known platelet-enriched transcripts, such as CLEC2B, CLU, GP1BA, ITGA2B, ITGA3, LCN2, PLEK, and TBXA2R, suggested that there might be a general reduction in platelet transcripts. The platelet transcriptome has been measured by isolation of purified platelets prior to RNA isolation, followed by microarray and SAGE analysis (Gnatenko et al., 2003). With the exception of the transcripts noted above, there was not a detectable shift in expression of more than 50 other major platelet transcripts (not shown), suggesting that there is a relatively selective decrease in a subset of transcripts in the platelets.

3.5. Antiplatelet antibodies in AR patients

Three factors suggested the potential involvement of autoimmune antiplatelet antibodies in the AR patients: 1) the clustering of the AR patients in a particular HLA subgroup, 2) the significant reduction in platelet counts in the AR patients, and 3) the 2-fold elevation in CXCL10 mRNA, which has previously been associated with immune thrombocytopenia (Gu et al., 2010). Thus, the AR patients (n = 5) and AS patients (n = 4) that were available for reconsent underwent a second whole blood aggregation test and a standard laboratory analysis for antiplatelet antibodies. While there were some changes in the platelet aggregation after aspirin treatment, overall the AR appeared to be a stable trait (r = 0.33), despite an average inter-test interval of 28 months. Some of the variability in apparent aspirin response could be due to different treatment protocols (1 week of 85 mg, vs. 1 h of 325 mg). Of the 5 AR patients available for retesting, none showed unequivocal evidence of antiplatelet antibodies against the major platelet autoantigens. However, 3 patients (1 AR, 1 HN, 1 AS) showed borderline or equivocal titers for antiplatelet antibodies or lupus anticoagulant.

4. Discussion

Aspirin is a widely used and life-saving cardiovascular therapeutic, due its low cost, generally tolerable side effects, and high efficacy in the management of prothrombotic states. Aspirin resistance, however, is a complex disorder, with clinical, biochemical, and genetic components (Fitzgerald and Pirmohamed, 2011). Thus, the present studies had 2 goals: 1) to identify a gene signature in peripheral blood that would allow identification of AR prior to, and independent of aspirin therapy, and 2) to generate new hypotheses about the etiology of AR. The first goal was met by identifying a 124 gene signature that correlates with AR, which will be tested in an independent prospective trial. Further, two specific hypotheses can be envisioned and tested on the basis of the present results: 1) a seronegative

antiphospholipid syndrome, 2) a platelet production defect, resulting in platelets with an abnormal repertoire of mRNA transcripts, as shown schematically in Fig. 6.

A potentially important clue to the etiology of AR is the HLA genotype of the patients. Particular HLA genotypes are often found in disorders that have an autoimmune component, such as diabetes, rheumatoid arthritis, lupus, antiphospholipid syndrome, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. In primary APS, or APS secondary to lupus, the HLA-DRB4 allele is over-represented (Kapitany et al., 2009). An extensive literature indicates that the HLA haplotype is strongly associated with the development of antiphospholipid antibodies, yielding odds ratios in the 4–8 range (Bertolaccini et al., 2000; Caliz et al., 2001). It is interesting to speculate that HLA genotype partially explains the heritable component of the antiplatelet response to aspirin (Shen et al., 2009).

APS patients share the common laboratory finding of antibodies reactive against phospholipid-rich proteins, such as β2-glycoprotein I (β2GPI) or prothrombin (McIntyre et al., 2003b). In a series of 775 normal blood donors, a surprisingly high 8.1% were identified with one or more anti-phospholipid antibodies (McIntyre et al., 2003a), which is consistent with the 6.8% rate of AR observed in the present study. Like AR, APS and antiphospholipid antibodies are more common with advancing age and female gender (Colucci et al., 1992). Consistent with the present findings, many APS patients exhibit an inadequate, or ‘resistant’, antiplatelet response to aspirin (Joseph et al., 1998) and APS patients have an increased prevalence of mild, asymptomatic thrombocytopenia (Uthman et al., 2008), which is consistent with the mild reduction in platelet counts (30%) observed in the present study. This might be suggestive of increased platelet turnover, which has recently been suggested as a possible cause of the reduced efficacy of aspirin in some patients (Rocca and Petrucci, 2012). While many of the features of AR in the present study are consistent with an autoimmune basis, autoantibodies to the major platelet antigens were not detected, except in 2–3 borderline cases. However, the antibodies screened represent only a subset of the potential autoimmune antibodies that have been documented, and thus, patients with phenotypic indications of APS, but lacking detectable antibodies, are referred to as “seronegative APS” (Sanmarco, 2009).

Alternatively, it is possible that changes in megakaryocyte function produces platelets that possess a prothrombotic phenotype, or with an increased ability to translationally regenerate COX1 protein from pre-existing transcripts in the platelet, or have accelerated translation of GP IIb/IIIa, leading to higher protein, but lower transcript levels. There are clear changes in a subset of transcripts that are known platelet transcripts, such as clusterin, lactoferrin and glycoproteins IIB/IIA. On the one hand, these might be non-specific changes that merely reflect a change in the turnover rate of the platelets, but on the other hand, they might reflect more specific changes in the ‘transcript set’ of the parent megakaryocytes. Potentially, the changes in platelet gene expression could be related to some prior transient infectious or autoimmune event, which produced a sustained alteration in the megakaryocyte population. Future studies can employ the present results to determine whether this subset of transcripts is especially sensitive to platelet maturation or turnover, or whether there are underlying changes in the megakaryocyte transcript profile in AR patients. Combined, the present

results are the first to describe specific transcriptional changes associated with AR, thereby informing future mechanistic studies of this serious subclinical thrombophilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	arachidonic acid
aPLs	antiphospholipid antibodies
APS	antiplatelet syndrome
AR	aspirin resistance
ARU	aspirin reaction units
CAD	coronary artery disease
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8
CLEC7A	C-type lectin domain family 7A
CLU	clusterin
COL1	collagen I
COL4	collagen IV
COL5A3	collagen 5 α3
COX	cyclooxygenase
CTTN	cortactin
CXCL5	chemokine (C-X-C motif) ligand 5
DEG	differentially expressed genes
dTxB2	11-dihydro-thromboxane B2
ELANE	neutrophil elastase

FAK	focal adhesion kinase
FBLN5	fibulin 5
FL	femtoliter
GP1BA	glycoprotein 1b, α chain
HLA	human leucocyte antigen
ITP	immune thrombocytopenia purpura
ITGA2B	integrin α 2B, platelet glycoprotein 2B
ITGB3	integrin beta 3, platelet glycoprotein IIIa
LCN2	lipocalin 2
LFT	lactoferrin, lactotransferrin
MI	myocardial infarction
MMP	matrix metalloproteinase
MPL	myeloproliferative leukemia virus oncogene, thrombopoietin receptor
MYO10	myosin X
NCOA7	nuclear receptor coactivator 7
OLR1	oxidized low density lipoprotein receptor 1
PAPS	primary antiphospholipid syndrome
PDGF	platelet-derived growth factor
PSAT1	phosphoserine aminotransferase 1
SDPR	serum deprivation response protein
TBXA2R	thromboxane A2 receptor
TPO	thrombopoietin
TxA2	thromboxane A2
THBS1	thrombospondins 1
TM4SF1	transmembrane 4 L six family member 1

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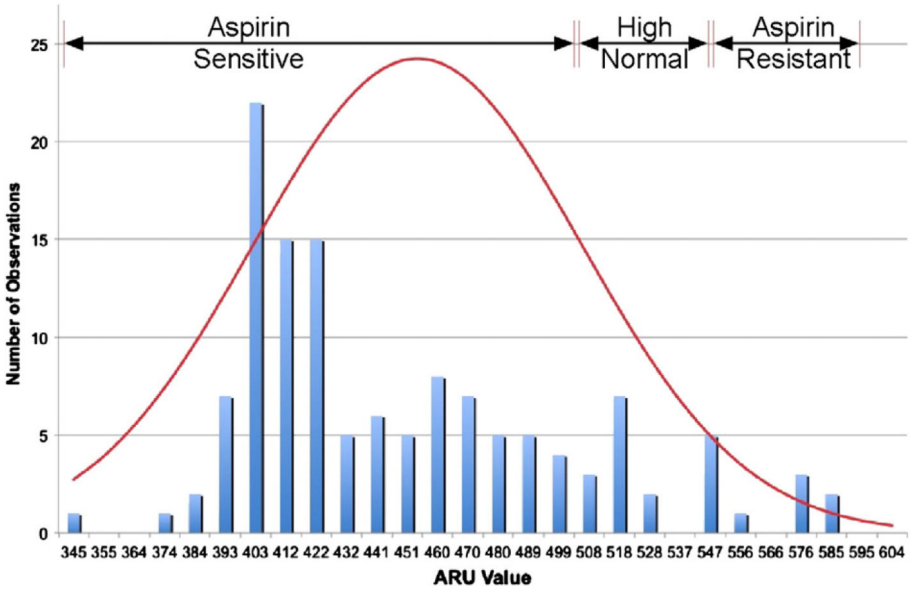

Fig. 1.

Distribution of aspirin resistance scores in patient samples. Platelet function was evaluated on a cardiology outpatient population (n = 132), after 1 week on low dose aspirin (81 mg/day). The residual platelet aggregation was measured by VerifyNow ASA using AA-stimulated whole blood aggregometry. The rate of aggregation is reported as aspirin response units (ARU) with higher values indicating increased aggregation despite aspirin treatment. ARUs above 550 are defined as aspirin resistant (AR). Blue bars indicate the frequency of ARUs within the bin range reported on X-axis. Red line indicates a normal distribution based on the mean (453.9 ARU) and standard deviation (52.1) of the sample.

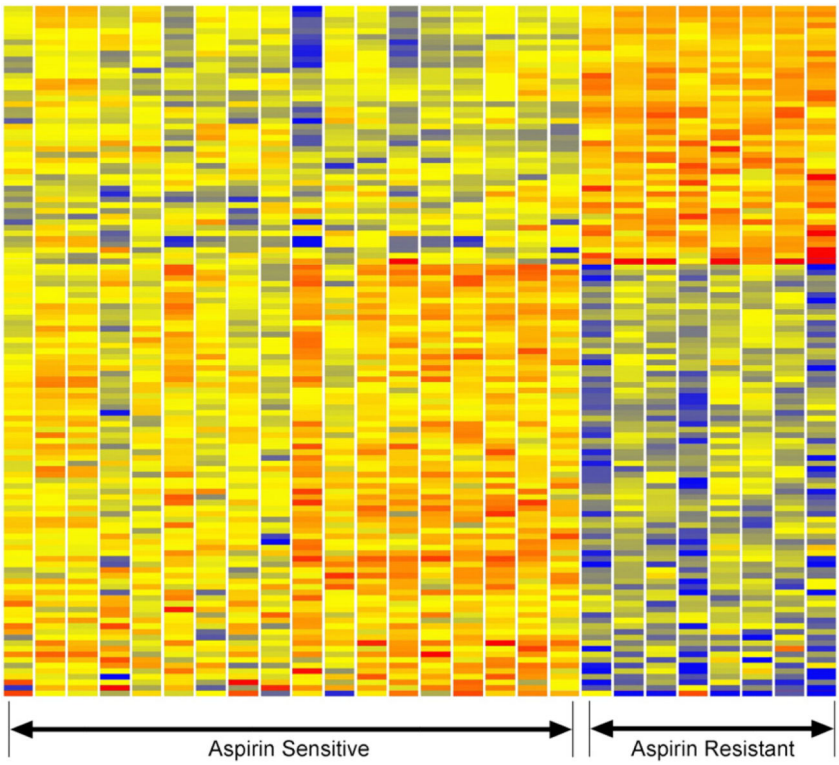

Fig. 2.

Heatmap of transcripts used for Support Vector Machine classification of aspirin sensitive versus resistant patients. Microarray analysis was conducted on whole blood RNA from subjects with varying antiplatelet responses to aspirin. DEGs were identified by a combined fold-change (>1.5 folds) and t test cut-off ($p < 0.001$) between sensitive (n = 18) and resistant (n = 8) patients. Transcripts are shown in rows and transcript names are shown in Supplementary Table 2. Red indicates elevated expression and blue indicates decreased expression relative to the mean expression for each transcript.

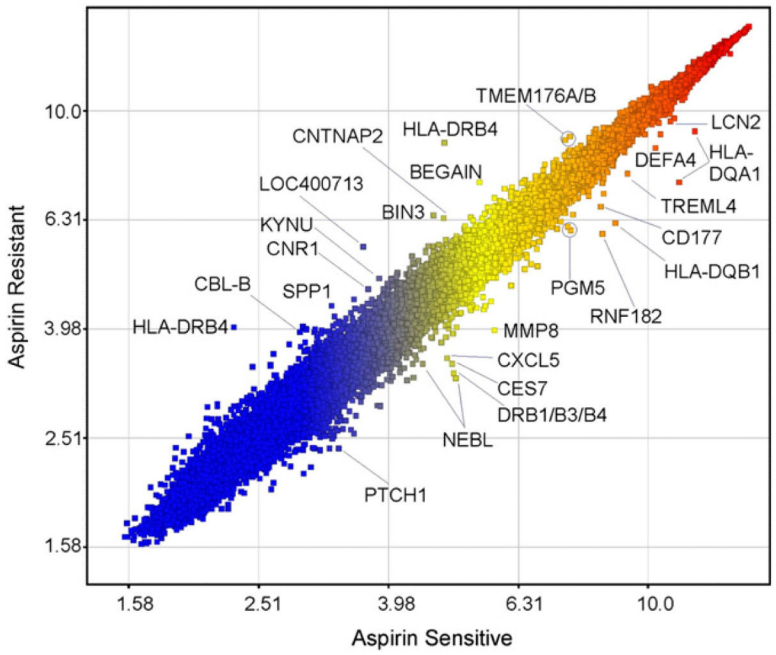

Fig. 3.

Scatter plot of transcripts in aspirin-sensitive versus aspirin-resistant groups. Gene expression levels were compared between aspirin-resistant (Y-axis) and aspirin-sensitive patients (X-axis) with absolute expression plotted on a log2 scale color-coded from low expression (below median expression, blue), to high expression (red). Each point reflects one transcript of the 54,000 transcripts quantitated on the Affymetrix U133 + 2 microarray. Transcripts deviating either up or down in aspirin-resistant patients, and with potential relevance to thrombotic pathways, are marked with their gene symbols, and more detailed levels shown in Table 3.

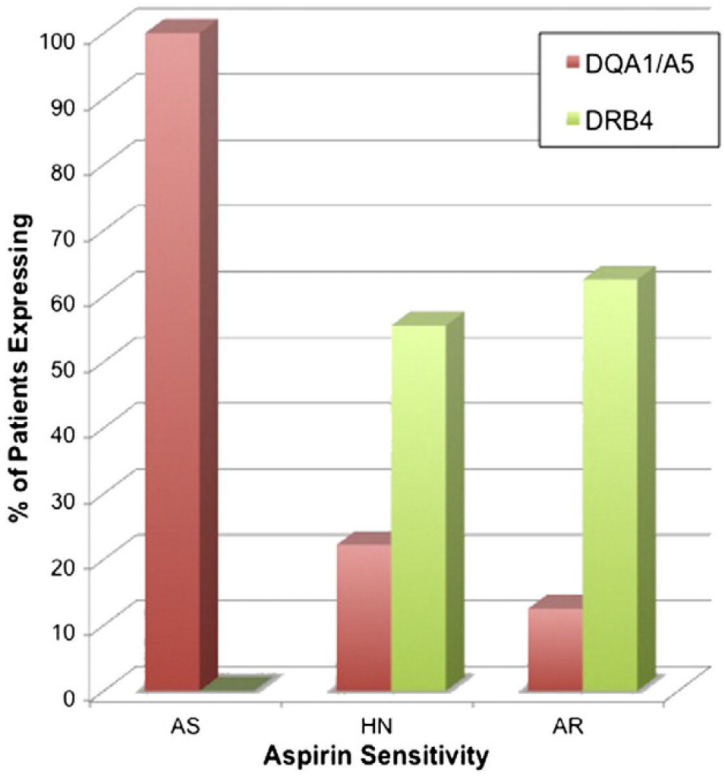

Fig. 4.

Expression from HLA-DR and HLA-DQ alleles in patients with varying sensitivity to the antiplatelet effect of aspirin. The expression levels of HLA-DR and HLA-DQ alleles shown in Table 3 were analyzed with respect to which alleles were expressed by the patients as a function of their response to the antiplatelet effect of aspirin. All of the sensitive (AS) subjects were expressing from DQA1 and DQA5 alleles, but none (0%) were expressing from DRB4 alleles, while the high normal (HN) or resistant (AR) had a significantly higher percentage using DRB4 alleles and much lower percentage using both DQA1 and DQA5 alleles, tending to use just one or the other.

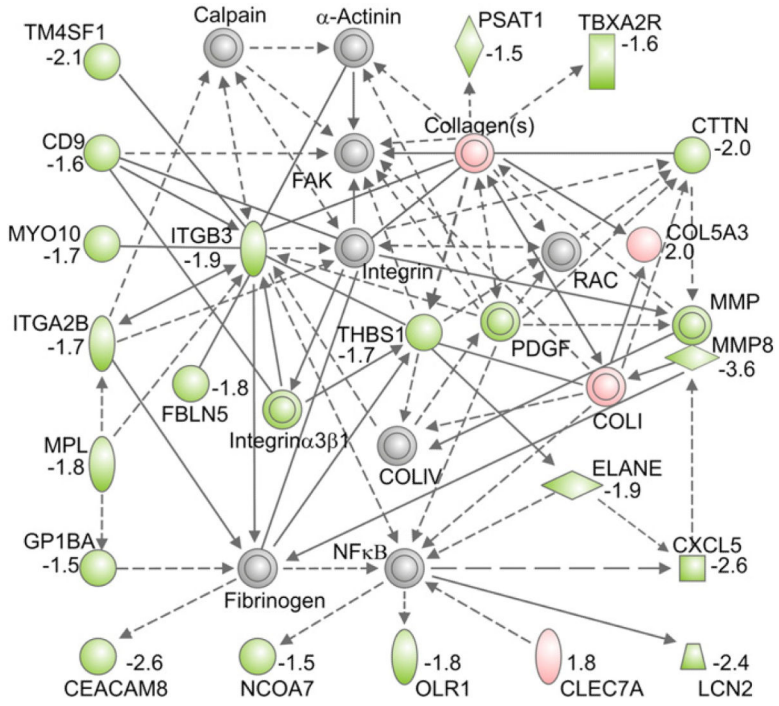

Fig. 5.

Pathway analysis of transcripts modulated in blood associated with aspirin resistance. The differentially expressed genes (Table 3, and Supplementary Table 2) from patients with aspirin resistance were analyzed by an automated comparison to a database of known gene–gene interactions (Ingenuity Pathway Analysis). A high scoring pathway of platelet adhesion is illustrated using transcript abbreviations and linear connections defining known relationships. The core of the pathway emanates from a reduction in glycoproteins IIb (ITGA2B) and IIIa (ITGB3), which are the principal platelet fibrinogen receptors. Neutrophil elastase (ELANE), which is decreased in AR, has an activating effect upon IIb/IIIa. Transcripts elevated in AR are shown in red, decreased transcripts are shown in green.

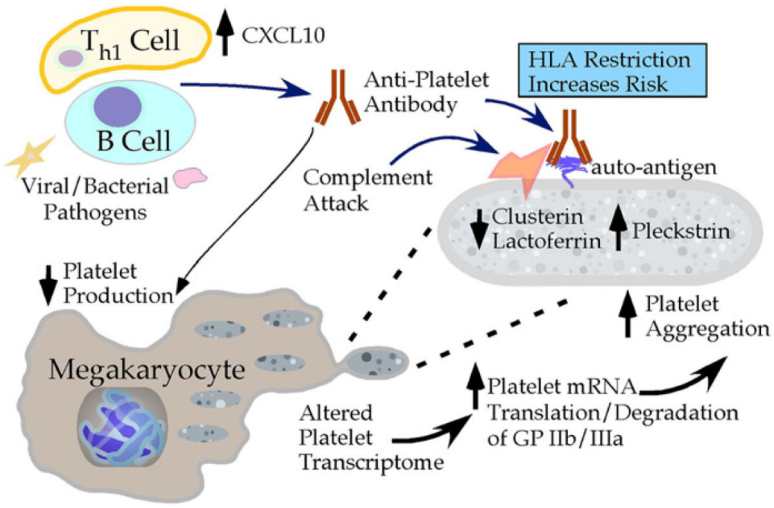

Fig. 6.

Schematic model of two potential etiologies of aspirin resistance. Using primary antiplatelet syndrome (PAPS) as a model, the general molecular and cellular relationships of the proposed etiology of AR is shown in the upper left. It is commonly believed that autoimmune syndromes such as PAPS or lupus, have an infectious trigger, in which a viral or bacterial antigen is processed through the immunoproteasome in antigen-presenting cells, and presented on HLA to generate a cross-reactive B-cell antibody clone. Certain HLA combinations are more likely to generate auto-reactive antibodies, in this case, reacting against platelet antigens, which leads to platelet activation, possibly via chronic complement reactivity. Alternatively, as shown in the lower right, the primary defect may reside in megakaryocytes, whereby an infectious or inflammatory event may affect the megakaryocyte population and lead to production of platelets with an aberrant repertoire of mRNA, as observed in the present transcript profiling, thereby leading to elevated susceptibility to aggregation.

Table 1

Clinical characteristics of subjects.

		Sensitive		Resistant	p-Value
		Low normal	High normal		
ARU range		**384–481**	**511–548**	**551–590**	
Platelet function	ARU	423.0	527.0	568.0	
Age	Years	62.2	63.7	58.1	0.45
Gender	%Male	56.0	56.0	63.0	NS
WBC	T/μl	6.2	6.7	5.9	0.61
RBC	M/μl	4.5	4.2	4.3	0.48
Hematocrit	%	38.8	39.0	38.7	0.98
MCV	FL	85.9	91.7	91.0	0.22
Platelets	T/μl	254.0	219.4	179.0	*0.03
Platelet volume	FL	8.5	8.9	8.8	0.36
Neutrophils	/μl	3756.9	3806.0	3825.5	0.99
Lymphocytes	/μl	1838.0	2035.7	1453.0	0.06
Mono	/μl	446.9	608.8	480.5	0.11
Creatinine (urine)	mg/dl	167.2	161.8	124.5	0.77
TxB2 (urine)	pg/mg	710.8	767.9	824.4	0.58

* indicates $p<0.05$.

Table 2

Gene ontology analysis of AR-related transcripts.

Functional annotation	Count	Count %	p-Value
Coronary artery stent thrombosis	4	1.10	3.07E–05
Cytoplasmic vesicle part	13	3.58	1.99E–04
Alternative splicing	165	45.45	2.70E–04
Platelet alpha granule	7	1.93	5.83E–04
ECM-receptor interaction	8	2.20	9.50E–04
Myocardial infarct; restenosis	3	0.83	1.18E–03
Blood transfusion complications	3	0.83	1.18E–03
Myocardial infarction; stroke, ischemic	3	0.83	1.18E–03
Thrombus formation, arterial	3	0.83	1.18E–03

Table 3

Selected Transcripts Differentially Expressed in AR Patient Blood.

Fold change	Aspirin sensitive	High normal	Aspirin resistant	Gene symbol	Gene title	Entrez Gene ID
3.8	–1.40	–0.28	0.52	BEGAIN	Brain-enriched guanylate kinase-associated	57596
1.5	–0.23	0.06	0.36	CAPN12	Calpain 12	147968
2.0	–0.32	0.09	0.71	CBLB	Cas-Br-M	868
–3.5	0.81	–0.61	–0.97	CD177	CD177 molecule	57126
–1.6	0.36	0.23	–0.30	CD9	CD9 molecule, tetraspanin	928
1.6	–0.31	0.10	0.33	CLEC2B	C-type lectin domain family 2, member B	9976
–1.6	0.28	0.07	–0.40	CLU	Clusterin	1191
2.0	–0.50	0.32	0.53	CNR1	Cannabinoid receptor 1 (brain)	1268
–2.0	0.53	–0.12	–0.49	CTTN	Cortactin	2017
2.0	–0.30	0.10	0.72	CXCL10	Chemokine (C-X-C motif) ligand 10	3627
–2.6	0.40	0.67	–0.97	CXCL5	Chemokine (C-X-C motif) ligand 5	6374
–1.9	0.56	0.05	–0.34	ELANE	Elastase, neutrophil expressed	1991
–1.5	0.42	0.00	–0.18	GP1BA	Glycoprotein Ib (platelet), alpha polypeptide	2811
–13.5	0.11	–3.25	–3.65	HLA-DQA1	MHC class II, DQ alpha 1	3117
14.7	–0.17	3.04	3.71	HLA-DRB4	MHC class II, DR beta 4	3126
1.6	–0.13	–0.05	0.53	HNMT	Histamine N-methyltransferase	3176
–1.7	0.35	0.06	–0.43	ITGA2B	Integrin, alpha 2b-platelet glycoprotein IIb	
–1.9	0.14	0.20	–0.79	ITGB3	Integrin, beta 3-platelet glycoprotein IIIa	3690
–2.4	0.93	–0.50	–0.32	LCN2	Lipocalin 2	3934
–2.4	0.88	–0.71	–0.40	LTF	Lactotransferrin, lactoferrin	4057
–3.6	0.85	–0.27	–0.98	MMP8	Matrix metallopeptidase 8, collagenase	4317
–1.8	0.33	0.13	–0.55	MPL	Thrombopoetin receptor	4352
1.6	–0.51	0.19	0.18	PLEK	Pleckstrin	5341
–1.6	0.25	–0.09	–0.41	TBXA2R	Thromboxane a2 receptor	6915
–1.7	0.54	0.08	–0.20	THBS1	Thrombospondin 1	7057
2.0	–0.59	0.09	0.40	XRCC4	X-ray repair complementing	7518