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Characterization of the platelet transcriptome by RNA sequencing in patients with acute myocardial infarction

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Abstract

Transcripts in platelets are largely produced in precursor megakaryocytes but remain physiologically-active as platelets translate RNAs and regulate protein/RNA levels. Recent studies using transcriptome sequencing (RNA-seq) characterized the platelet transcriptome in limited numbers of non-diseased individuals. Here, we expand upon these RNA-seq studies by completing RNA-seq in platelets from 32 patients with acute myocardial infarction (MI). Our goals were to characterize the platelet transcriptome using a population of patients with acute MI and relate gene expression to platelet aggregation measures and ST-segment elevation MI (STEMI) (n=16) versus non-STEMI (NSTEMI) (n=16) subtypes. Similar to other studies, we detected 9,565 expressed transcripts, including several known platelet-enriched markers (e.g., *PPBP*, *OST4*). Our RNA-seq data strongly correlated with independently ascertained platelet expression data and showed enrichment for platelet-related pathways (e.g., wound response, hemostasis, and platelet activation), as well as actin-related and post-transcriptional processes. Several transcripts displayed suggestively higher (*FBXL4*, *ECHDC3, KCNE1, TAOK2*, *AURKB*, *ERG*, and *FKBP5*) and lower (*MIAT, PVRL3*and *PZP*) expression in STEMI platelets compared to NSTEMI. We also identified transcripts correlated with platelet aggregation to TRAP (*ATP6V1G2*, *SLC2A3*), collagen (*CEACAM1*, *ITGA2*), and ADP (*PDGFB*, *PDGFC*, *ST3GAL6*). Our study adds to current platelet gene expression resources by providing transcriptome-wide analyses in platelets isolated from patients with acute MI. In concert with prior studies, we identify various genes for further study in regards to platelet function and acute MI. Future platelet RNA-seq studies

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Declaration of Interests

The authors report no declarations of interest.

examining more diverse sets of healthy and diseased samples will add to our understanding of platelet thrombotic and non-thrombotic functions.

Keywords

gene expression; RNA-seq; myocardial infarction; platelet aggregation

Introduction

Platelets are anucleate cells that do not actively transcribe the nuclear genome. For years, it was believed that RNA-related processes were irrelevant to platelet biology. However, studies have shown that platelets contain RNAs (including messenger RNA [mRNA] and non-coding RNAs [ncRNAs]), perform translation, and regulate transcript/protein expression [1,2]. Platelet RNAs are produced in precursor megakaryocytes and variation in transcript levels may reflect changes in megakaryocyte transcriptional activity. Platelets translate RNA into proteins and regulate translation as well as modify, sequester, and degrade RNAs. Changes in RNA expression in the megakaryocyte or platelet can impact platelet physiology. Further insight into platelet RNA expression patterns may provide mechanistic insight into platelet functions.

The first studies of the platelet transcriptome used microarray-based assays [3,4]. However, there are limitations to the use of microarrays in characterizing RNA expression, particularly in the case of platelets. Microarrays typically require higher expression thresholds to detect transcripts, a challenge due to the relatively low amounts of RNA in platelets [5]. Additionally, microarrays require the design and construction of chips. While covering most coding regions, contemporary microarrays incompletely capture the transcriptome and can miss many ncRNAs as well as alternatively spliced transcripts. Transcriptome sequencing (RNA-seq) can detect lower expressed genes and offers less biased, more complete detection of expressed RNAs.

Recently, a limited number of studies have used RNA-seq to profile the platelet transcriptome [6-9]. In these studies, platelets from a relatively limited number of healthy individuals, ranging from 2 to 10 subjects, were examined using RNA-seq as reviewed by Schubert et al. [10]. By detecting more lowly expressed transcripts in a less biased manner, these RNA-seq studies have built upon microarray studies in characterizing the expression profiles of platelets from healthy subjects. However, platelets become activated during thrombosis and play a role in cardiovascular diseases and events [11,12]. Differences in platelet transcript levels may reveal causal or responsive mechanisms that contribute to platelet reactivity as well as to the onset and/or prognosis of various cardiovascular traits and events. Specifically, platelet activation and atherothrombosis contribute to myocardial infarction (MI), a major cause of morbidity and mortality worldwide [11-14]. The platelet transcriptome has only been characterized by RNA-seq in healthy subjects and not in subjects with MI. The characterization of the platelet transcriptome in patients with MI may provide insights into causal or responsive platelet factors relevant to MI events.

MIs are clinically heterogeneous events. Current American Heart Association and American College of Cardiology guidelines recognize two forms of MI: ST segment elevation myocardial infarction (STEMI) and non-ST segment elevation myocardial infarction (NSTEMI) [15]. These two types of MI differ significantly with respect to pathogenesis, prognosis, and treatment [16]. High on-treatment platelet reactivity is linked to worse outcomes for both STEMI and NSTEMI patients undergoing procedures [17]. Platelet reactivity and anti-platelet resistance is suggested to be higher in STEMI patients than NSTEMI patients [18-20]. These differences in platelet reactivity and disease progression may result from gene expression differences between STEMI and NSTEMI cases. Previous studies in whole blood identified several differentially expressed transcripts between STEMI and NSTEMI cases including *KCNE1*, *ECHDC3*, and *MIAT* [21,22]. Another study identified 54 differentially expressed transcripts in platelets between STEMI and stable coronary artery disease (CAD) patients, with *CD69* and *S100A9* being the strongest candidates [23]. Identification of transcripts that differ between these clinically distinct MI subtypes may be important in ascertaining different casual or responsive pathways and could lead to the development of specifically tailored treatments for NSTEMI and STEMI events.

Here, we present a RNA-seq study of platelets from 32 patients presenting with acute MI, 16 STEMI and 16 NSTEMI. Our transcriptome-based study allows us to discover new expression differences between STEMI and NSTEMI patients and to validate prior gene transcripts thought to differ between the two MI subtypes in an unbiased manner. The overall goals of this study were (1) to perform RNA-seq of isolated peripheral blood platelets from MI patients to characterize their expression profiles, (2) to use RNA-seq to identify differentially expressed genes related to STEMI/NSTEMI status, and (3) to relate platelet aggregation to several agonists to STEMI/NSTEMI status and gene expression.

Methods

Participants and Blood Samples

We enrolled 32 participants presenting with acute MI for urgent cardiac catheterization in our study. The cohort was comprised of 16 patients with STEMI and 16 with NSTEMI, all of whom were referred to the University of Massachusetts Medical Center's cardiac catheterization laboratory for urgent left heart catheterization and coronary angiography. Participants included 22 men and 10 women with mean age of 65.6 years and body mass index (BMI) of 27.6 kg/m^2. The participants had a moderate burden of comorbid cardiovascular diseases and were taking several medications (Table I). Study approval was granted by the Institutional Review Board at the University of Massachusetts Medical Center (IRB Docket #14125), and all participants provided informed consent to study protocols. Two arterial blood samples were collected from each participant at the beginning and end of their cardiac catheterization procedure. Samples were collected with 8 mL CPT tubes (Becton Dickinson, Franklin Lakes, NJ) for RNA-seq and 8.5 mL blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) for platelet aggregation measures, both with sodium citrate (3.8%).

Platelet/RNA Isolations and RNA-sequencing

For RNA-seq analyses, the CPT tubes were centrifuged at 1700 × g for 25 minutes at room temperature. After centrifugation, mononuclear cells and platelets were collected and centrifuged at 288 × g. To obtain platelets, the supernatant was pelleted by centrifugation, and lysed with RLT solution (Qiagen, Germantown, MD). The resulting platelet lysate was kept at −80 °C for RNA isolation in preparation for sequencing [24]. Leukocyte contamination in platelet solution was < 1/50,000. Previous platelet isolations with this protocol have demonstrated relative purity of platelet cell populations by cell count, flow cytometry, and gene expression [24].

For RNA-seq samples, total RNA was purified from Trizol-treated samples and treated with DNase. The quality of resulting RNA was assessed by Bioanalyzer assay. DNA-free total RNA was used as input for library construction using Ovation RNA Seq v.2 kits (NuGen, Inc., San Carlos, CA). STEMI and NSTEMI samples were batched in two groups of 16 to balance case status, age, and gender across batches. After the final amplification step, libraries were size selected between 250 to 450 base pairs. Barcoded RNA-seq libraries were mixed and subjected to 75 base pair paired-end sequencing on an Illumina HiSeq-2000 machine, with each sample having the equivalent of approximately one dedicated flow cell. Sequencing data were demultiplexed and converted to FASTQ format. Paired-end reads were aligned to RefSeq using TopHat2, and RNA reads-per-kilobase-per million mapped (RPKM) was calculated with RSeQC v2.3.9. We instituted a minimum RPKM threshold of 0.3 for all downstream analyses of the RNA-seq data to ensure that expression measurements reflected actual transcript levels. RNA sequencing data from the platelet samples and subjects' clinical characteristics have been submitted to GEO (Accession Number: GSE65705).

Comparisons to Existing Platelet Expression Data

In order to determine whether our newly generated platelet transcriptome data were similar to other studies, we used two independent platelet gene expression datasets as comparison sets. The first set of data comes from quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses of 80 candidate genes in 2,245 participants from the Framingham Heart Study (FHS). Details on the experiments are presented elsewhere [24]. Spearman correlations between threshold cycle (Ct) in the FHS qRT-PCR and RPKM values in the present RNA-seq study were calculated in SAS v9.3 using PROC CORR. The second dataset is publically available RNA-seq analysis of platelets from 10 individuals, 5 of European-American and 5 of African-American descent [8]. Ensembl identifiers from Londin et al. (2014) were converted to RefSeq identifiers to allow for direct comparison to our data. Spearman correlations between β-actin normalized RPKM values from Londin et al. (2014) and RPKM values from the present study were also calculated in SAS v9.3 using PROC CORR.

Platelet Aggregation

In addition to RNA-seq analyses, a second blood sample was taken to assess platelet aggregation to various agonists. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 × g for 20 min, and the supernatant, representing PRP, was separated and used for

platelet aggregation experiments. Washed platelets for aggregation measures were obtained from PRP as described previously [24]. Briefly, PRP was diluted 1:1 with platelet wash buffer (10 mM sodium citrate, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 1% dextrose, pH 7.4), with prostaglandin E1 (1:10,000; Calbiochem, San Diego, CA), and centrifuged at 740 × g for 20 min. The resulting platelet pellet was diluted in HEPES buffer (140 mM NaCl, 6 mM KCl, 2.4 mM $MgSO_4.7H_2O$, 1.7 mM Na_2HPO_4, 6 mM Hepes, 0.35% BSA, 0.1% dextrose, pH 7.4) to reach platelet count 2×10^8 platelets/mL. Platelet counts were determined in a Coulter Counter (Coulter ACT Series Analyzer, Coulter Inc., Miami, FL).

Platelet aggregation was determined by measuring the change in the optical density (light transmittance) of stirred (1,200 rpm) PRP or washed platelets after addition of 2.5 μM human thrombin receptor activating peptide (TRAP; Phoenix Pharmaceuticals, Belmont, CA), 10 μg/mL collagen or 10 μM ADP (collagen and ADP; Chrono-log Corporation, Havertown, PA) as agonists. PRP was used for ADP, while washed platelets were used for TRAP, collagen, and ADP. Platelets aggregation was monitored for 10 min at 37°C by a PAP-4 platelet aggregometer (Bio/Data Corporation, Horsham, PA) as previously described [25].

Statistical Analyses

To determine mean expression levels, we averaged RPKM across the 32 MI platelet samples after imposing a minimal expression cutoff of RPKM≥0.3. Pathway analyses were completed using Gene Ontology (GO), KEGG, and Pathway Commons on WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) [26]. Partek Genomic Suites v6.6 (St. Louis, MO) was used to perform principal component analysis (PCA) of expression data and correlation of gene expression with platelet aggregation. Two methods were used to identify differentially expressed transcripts between NSTEMI and STEMI cases. Both corrected for batch, gender, age, and BMI. First, we performed ANOVA comparing RPKM expression between NSTEMI and STEMI cases in Partek Genomic Suites v6.6. Second, we used EdgeR in Bioconductor v3.1 with raw reads as input [27]. As all findings were suggestive in nature, we provide results from both analyses. All other correlations were completed using PROC CORR in SAS v9.3. Differences in platelet aggregation measures between NSTEMI/STEMI subtypes, uncorrected and corrected for gender, age, and BMI, were performed using PROC GLM in SAS v9.3.

Results

Overall Platelet Transcriptome

Across the 32 MI platelet samples, there was an average of 191 million mapped reads per sample with an average unique mapping rate of 72.1%. Using a cutoff of RPKM ≥ 0.3 averaged across the 32 samples, we found 9,565 expressed transcripts, similar to previous platelet RNA-seq studies [6-9]. There were no substantial batch, gender, age, body mass index (BMI), or time interval effects on general gene expression levels, as indicated by principal component analysis (PCA) (Supplemental Figure 1). Both STEMI and NSTEMI groups had a moderate burden of cardiovascular disease comorbidity and similar medication

use profiles, including anti-platelet aggregation agents (Plavix and ticagrelor), aspirin, and heparin/Lovenox (Table I).

Similar to other platelet RNA-seq studies, expression of mitochondrial transcripts was high (Supplemental Table 1) [9]. In fact, the two most highly expressed transcripts were from the mitochondrial genome, *MT-RNR2* and *MT-RNR1*. Highly expressed genes were similar in STEMI and NSTEMI platelets (Table II, Supplemental Table 1). Several noted platelet RNA markers, including *PPBP* and *OST4*, were detected in both groups (Table II). We performed pathway analyses of genes expressed in the 32 platelet samples at two expression thresholds: logRPKM ≥ 3 and logRPKM ≥ 2 (Supplemental Tables 2-5, Supplemental Figures 2-3). At the higher gene expression threshold of logRPKM ≥ 3, similar pathways were enriched, including wound response, hemostasis, and platelet activation (Supplement Tables 2-3, Supplemental Figure 2). At the lower threshold of log RPKM ≥ 2, we observed enrichment of additional pathways, including actin binding proteins, translational related processes, GTPase activity, and nonsense mediated decay (Supplemental Tables 4-5, Supplemental Figure 3).

Before further examining our transcriptome results, we compared our newly generated data with extant platelet expression data. Previously, our group performed qRT-PCR on candidate genes in 2,245 platelet samples from the Framingham Heart Study (FHS), which are population samples unrelated to the MI samples described here. 80 transcripts tested by qRT-PCR were detectable both in the RNA-seq MI samples and in at least 10% of FHS samples (Supplemental Table 6). Recently, tightly controlled experiments by the Sequencing Control (SEQC)/MAQC-III have verified high rates of concordance between RNA-seq and qRT-PCR experiments [28]. Overall relative expression levels, as measured by threshold cycle (Ct) and RPKM values, respectively, were highly correlated (Spearman correlation = −0.83) between FHS qRT-PCR and the non-FHS MI RNA-seq samples, despite different demographic makeup and ascertainment strategies. This negative correlation is expected as lower Ct and higher RPKM values are indicative of higher expression levels in qRT-PCR and RNA-seq experiments, respectively. Additionally, we obtained publically-available transcriptome data from the largest prior platelet RNA-seq study in 10 healthy individuals (5 European-American and 5 African-American) [8]. Expression data were highly correlated (Spearman correlation = 0.63), further increasing the confidence in the platelet-specificity and fidelity of our data (Supplemental Table 7).

Comparing NSTEMI and STEMI Platelet Transcriptomes

Platelet expression patterns of STEMI and NSTEMI cases were highly correlated (Supplemental Tables 8-11). Each subtype showed comparable correlations of gene expression between and within STEMI/NSTEMI groups (average Spearman correlation = 0.76; range = 0.68-0.83) (Supplemental Tables 8-11). PCA showed no noticeable clustering of STEMI/NSTEMI cases status by gene expression, further indicating that there are no categorical differences in gene expression between STEMI and NSTEMI subtypes (Supplemental Figure 6).

Although we found no evidence for systematic transcriptome-wide differences between STEMI and NSTEMI cases, individual differentially expressed genes may reveal insight into

underlying causal and responsive mechanisms. We identified numerous candidate genes differentially expressed in platelets between STEMI and NSTEMI cases using two different methods (Table III, Supplemental Tables 12-13). These included genes with increased expression in STEMI platelets (*NDUFAF4*, *AURKB*, *RAI14*, *ERG*, *GAMT*, *FBXL4*, *SLC4A8*, *FKBP5*, *FTSJ3*, *ELL3*, and *NPR3*), as well those with increased expression in NSTEMI platelets (*PVRL3*, *WDR62*, *PVRIG2P*, *MYL4*, *TMEM60, PCYT2*, and *PZP*) (Table III). Statistically, these differences are suggestive and do not pass a strict Bonferroni correction for multiple testing. However, we do provide support for the increased expression of *KCNE1* (p=0.00793) and *ECHDC3* (p=0.0141) as well as decreased expression of *MIAT* (p=0.0449) in STEMI platelets compared to NSTEMI, as previously reported in whole blood, as well as increased expression of *FKBP5* (p=0.000694) in platelets from STEMI patients (Table IIIb, Table IV) [21-23]. Our observation of increased *MYL4* expression in NSTEMI compared to STEMI platelets is in the opposite direction as previously reported [21]. Pathway analyses of suggestively differentially expressed genes ($p \leq 0.05$) showed enrichment for metabolic and mitochondrial-related pathways, including the synthesis of metabolic enzymes (panthothenate and CoA) and metabolism of nucleotide sugars, amino acids, and glycerophospholipids (Supplemental Tables 14-17). Additionally, multiple signaling pathways including Afr6, S1P, and transmembrane small molecule transport as well as Beta1 integrin interactions were enriched (Supplemental Tables 14-17).

Platelet Aggregation

In addition to examining differences in gene expression, we also determined whether there were platelet aggregation differences between STEMI and NSTEMI samples using three agonists. In uncorrected analyses, STEMI samples showed a trend toward increased aggregation to TRAP in washed platelets (Student's T-test p=0.11) and ADP in PRP (Student's T-test p=0.33) compared to NSTEMI samples (Figure 1a). When corrected for gender, age, and BMI, there were trends toward decreased platelet aggregation to collagen in washed platelets (p=0.07) and increased aggregation to ADP in PRP (p=0.10) in STEMI cases compared to NSTEMI cases (Figure 1b). To further examine this trend of platelet aggregation differences between STEMI and NSTEMI, we looked at the correlation of our candidate differentially expressed genes ($p<0.05$, Supplemental Table 12) with platelet aggregation to collagen and ADP in PRP. Generally, transcripts expressed more highly in STEMI cases were correlated with decreased aggregation to collagen (e.g., *RDH13*, *PZP*, and *KCNE1*) and increased aggregation to ADP in PRP (e.g., *NPR3*), with the inverse relationship also largely observed (Supplemental Tables 18-19).

As platelet aggregation differences between STEMI and NSTEMI were only trends, we further examined the correlation between gene expression and platelet aggregation transcriptome-wide. None of the correlations pass strict Bonferonni correction for multiple testing, but several intriguing candidates emerged (Table V, Supplemental Tables 20-23). Some of the strongest correlations included expression of *ATP6V1G2* (TRAP), *KCNE1* (TRAP), and *SLC2A11* (ADP) with increased aggregation, as well as *CEACAM1* (collagen), *TERC* (collagen), *PDGFB* (ADP), and *CCNG1* (ADP) with decreased platelet aggregation (Table V). There were several other biologically notable correlations including *SLC2A3* (TRAP), *TAZ* (TRAP), *KLF1* (collagen), *ITGA2* (collagen), *ST3GAL6* (ADP),

PDGFB (ADP), *PDGFC* (ADP), and *RHOA* (ADP) (Supplemental Tables 20-23). These gene expression and platelet aggregation correlations indicate possible genes and pathways that influence platelet reactivity to different agonists.

Discussion

In this study, we aimed to characterize the transcriptome of platelets in 32 patients with acute MI by RNA-seq and to determine whether gene expression could be related to NSTEMI/STEMI subtype and platelet aggregation measures. Our platelet transcriptome data add to the growing platelet transcriptomic literature, including evidence for moderate to high expression of hemostatic, cytoskeletal, mitochondrial, and post-transcriptional regulatory genes and pathways. Our study augments these resources by characterizing gene expression in platelets from MI patients, not healthy individuals. There were no categorical gene expression differences between platelets isolated from STEMI and NSTEMI patients. However, we did identify candidate genes and pathways suggestively differentially expressed. These expression differences may indicate differences in causative or responsive factors in disease etiology and prognosis, and may relate to platelet aggregation differences observed between STEMI and NSTEMI cases. Additionally, we identified biologically intriguing correlations between gene expression and platelet aggregation to TRAP, collagen, and ADP. Together, these results highlight several candidate genes and pathways that warrant further investigation in relation to acute MI and platelet thrombotic functions.

Transcriptome-wide analyses of platelets have been limited in number due to the low levels of active transcription in mature platelet cells and low RNA copy number. However, the active role RNAs play in platelet functions, as well as technical improvements, have allowed for a less biased assessment of the platelet transcriptome. Recent RNA-seq studies have put forth a “baseline” expression pattern of platelets from healthy individuals with sample sizes up to 10 individuals [6-10]. Here, we build upon these studies by performing RNA-seq on 32 platelet samples. Our study further demonstrates the use of RNA-seq in examining expression in platelets, detecting many more transcripts than microarray-based studies [3,4,29,30]. Similar to other RNA-seq studies, we detected a wide range of expressed genes, including mitochondrial, hemostatic, cytoskeletal, and post-transcriptional regulatory factors [6-10]. These findings underscore the known importance of hemostatic and cytoskeletal factors to platelet functions. Intriguingly, the enriched expression of post-transcriptional regulatory factors in our samples further buttresses likely roles for RNA and protein expression regulation in platelet function. As nuclear transcription can only be directly regulated in the precursor megakaryocyte, platelets can only modulate expression at the RNA and protein level. This expression regulation in platelets may occur through mechanisms including ncRNAs (e.g., microRNA [miRNA] and long ncRNA [lncRNA]), non-sense mediated decay, and 5’/3’-UTR mediated regulation [10,31,32]. Additional studies are needed to tease apart more of the mechanistic and functional implications of RNA/protein regulation in platelets and how they contribute to human health and disease.

A novel aspect of our platelet RNA-seq investigation is the examination of the platelet transcriptome in relation to disease status. Here, we present the first RNA-seq characterization of platelets isolated from MI patients and then examined transcriptional

differences between two MI subtypes, STEMI and NSTEMI. There are marked clinical differences in the pathogenesis, prognosis, and treatment of these two MI subtypes [16]. However, little is known regarding the roles platelets may play in differences between STEMI and NSTEMI subtypes. Previous studies examining expression differences between STEMI and NSTEMI cases in whole blood showed that *ECHDC3* and *KCNE1* are differentially higher expressed and *MIAT* lower expressed, respectively [21,22]. We provide support for these expression differences with the same direction of effect in platelets. *MIAT* and *KCNE1* are particularly intriguing due to their past genetic associations with MI and long QT syndrome, respectively [33,34]. In light of these previous associations, our results for *MIAT* and *KCNE1* in STEMI/NSTEMI cases suggest that these transcripts may influence MI pathophysiology and/or prognosis, although further experimentation is needed to determine temporal and causal relationships. Another study used a microarray approach in platelets from STEMI and stable CAD patients to identify expression differences [23]. Although our comparison groups are different, both studies show evidence for increased platelet expression of *FKBP5* in STEMI patients. The results of both studies suggest that *FKBP5*, an immunophillin protein involved in immunoregulation and stress response, contributes to the vasculature's response to acute STEMI events.

Additionally, we identified several new candidates differentially expressed in STEMI and NSTEMI cases (Table III). Some of these top differentially expressed genes are biologically intriguing. *AURKB* is a microtubule associated protein that influences megakaryocyte ploidy, possibly through its function in endomitosis [35,36]. Changes in *AURKB* expression and function may reflect differences in platelet production in response to the pathophysiology contributing to NSTEMI and STEMI events. Similarly, the microtubule related protein *TAOK2* has high sequence homology with *TAOK1*, which was associated with mean platelet volume and ADP-induced platelet reactivity through GWAS [37,38]. Another intriguing candidate is *ERG*, which is essential for platelet adhesion and important in the maturation of megakaryocytes [39]. Increased expression of *ERG* in STEMI platelets may reflect increased platelet production and function following more acutely severe disease states and events. Several of the associated genes, including *GAMT*, *PCYT2*, and *FBXL4*, are also highlighted through our analyses implicating metabolic pathways. The increased severity of STEMI events, compared to NSTEMI events, may increase the local energy needs and require hemostatic activation of metabolic and signaling pathways [16].

In addition to examining differential gene expression, we also compared platelet aggregation between STEMI and NSTEMI cases. Here, we observed a trend of increased aggregation to ADP and decreased aggregation to collagen in platelets from STEMI compared to NSTEMI patients. Although only suggestive in nature, differences in platelet activation suggest a role of platelets and hemostatic factors in STEMI/NSTEMI pathogenesis and prognosis. Increased platelet aggregation to ADP following STEMI has been reported in the literature and hypothesized to be an indicator of poorer outcomes [40-42]. However, these differences in platelet aggregation between STEMI and NSTEMI cases should be independently validated in larger samples and could also be influenced by differential exposure to environmental factors including medications.

Nonetheless, we explored whether there was a relationship between the most differentially expressed genes and ADP and collagen platelet aggregation. Largely, RNAs with increased expression in STEMI platelets were correlated with increased aggregation to ADP and decreased aggregation to collagen. Of note was the positive correlation of PZP expression, which was reduced in STEMI cases, with increased aggregation to collagen. *PZP* lies in close proximity to and interacts with alpha-2 macroglobulin (*A2M*), an inhibitor of coagulation. PZP inhibits human tissue kallikrein and type IV collagenases MMP2 and MMP9 [43]. Inhibition of platelet metalloproteinases prevents clearance of GP6 and retains collagen reactivity, evoking a model consistent with our observations: ↑PZP > ↓MMP > ↑GP6 > ↑collagen reactivity [44,45]. In concert with evidence from prior *Mmp2−/−* results, lower PZP expression and loss of MMP2 inhibition in human STEMI platelets could promote pro-thrombotic outcomes [46]. Conversely, *NPR3* expression was increased in STEMI cases and was correlated with increased aggregation to ADP. Recent endothelial-specific mouse knock studies of *Npr3* support roles in the regulation of blood pressure and platelet reactivity [47]. Future mediation analyses with larger samples may reveal stronger statistical relationships between expression of these specific genes, platelet reactivity, and MI subtype.

As prior RNA-seq studies were unable to examine the relationships between expression levels and platelet aggregation, we sought to identify transcripts that were correlated with platelet aggregation measures on a transcriptome-wide scale. Generally, there was not a large degree of overlap among the transcriptional correlates of the three agonist responses, suggesting the genes involved in platelet aggregation to the different agonists are relatively distinct. While our results are correlative, several intriguing candidates did emerge. The H^+, ATPase subunit *ATP6V1G2* showed the strongest positive correlation (i.e, increased expression correlated with increased aggregation) with TRAP platelet aggregation. *ATP6V1G2* is not the first vacuolar-type proton pump subunit associated with platelet related traits as shown by previous work on *ATP6V0D2*, *ATP6V1F*, and *ATP6V1B2* [38,48-50]. Another interesting transcriptional correlate with TRAP platelet aggregation was the positive correlation of *SLC2A3* expression (also known as *GLUT3*), which has been shown to increase glucose transport during thrombin activation of platelets [51]. The strongest correlation seen with platelet aggregation to collagen was a negative relationship with *CEACAM1*. In mice, both Ceacam1 and Ceacam2 are important *in vivo* regulators of thrombus formation via collagen-related signaling, with *CEACAM1* being the closest human homolog for both based on BLAT analysis [52,53]. Ceacam1 is also expressed in rat platelets, with increased accessibility to antibodies upon collagen or ADP stimulation [54]. The negative regulation of GP6-collagen interactions by both Ceacam1 and Ceacam2 in mice is consistent with the negative correlations between platelet reactivity and *CEACAM1* expression in human platelets [52,53]. To our knowledge, our study is only the second report of an association between CEACAM1 and aggregation in human platelets [53]. Also associated with increased platelet aggregation to collagen was expression of *ITGA2*, a collagen receptor that contributes to platelet adhesion and may contribute to collagen-mediated vessel occlusion [55,56]. Several intriguing genes' expressions, including *PDGFB*, *PDGFC*, and *RHOA*, were correlated with platelet aggregation to ADP. PDGFB and PDGFC are members of the PDGF family of proteins important in wound healing, platelet biology,

and angiogenesis [57,58]. The inhibition of platelet aggregation by PDGF is mediated through PDGFA receptors, though both PDGFB and PDGFC have been shown to heterodimerize with PDGFA and to interact with PDGFA receptors [57-59]. *RHOA*, a small GTPase involved in cytoskeletal dynamics, is essential to platelet activation and resulting platelet shape change as well as the secretion of alpha and dense granules [60]. Our study further supports the role of these genes in platelet function and suggests that their expression levels as they may vary in platelets across clinical or temporal contexts may modify platelet functions. However, a deeper understanding of these specific relationships requires further replication and direct experimentation using *in vitro* and *in vivo* models.

Our study had several limitations. The sample size of this study (16 NSTEMI and 16 STEMI) may hinder our ability to detect small transcription differences between groups. Future studies should aim to increase the sample size of expression and platelet aggregation studies and to replicate findings. There also a difference in the time interval between MI event and sample collection among NSTEMI and STEMI cases. PCA of time interval revealed no noticeable clustering of expression patterns. Additionally, sample preparation and isolation can influence expression detection. Leukocyte depletion protocols were not performed on our washed platelet samples as completed in other platelet RNA-seq studies [6-10], leading to the possibility of detecting trace to moderate amounts of leukocyte-derived transcripts. However, we observed strong correlations, similar total number of expressed genes, and concordance of the most highly expressed transcripts between our data and extant platelet expression data that completed leukocyte depletion protocols indicating our sample reflects a relatively pure platelet population [6-9]. Likewise, prior gene expression studies utilizing the same washed platelet protocols demonstrated low leukocyte contamination levels as assessed by flow cytometry, cell counting, and targeted expression measurements [24]. Nonetheless, there may be leukocyte contamination in our expression data that could influence our findings. We do detect expression of white blood cell markers *CD45* (annotated as *PTPRC*) and *CD14* in our dataset. These markers are also detected in other platelet RNA-seq investigations, although at lower levels likely as a result of their platelet isolation methods [6,8]. This indicates there is likely higher leukocyte contamination in the current study than the other reported platelet RNA-seq studies [6-10]. Currently, there is no consensus on platelet isolation protocols for RNA-seq experiments, differential isolation protocol effects on the characteristics of measured platelet cell populations, and the best approach for interpreting possible leukocyte contaminants, particularly in the use of white blood cell surface markers in evaluating platelet purity.

Here, we present platelet transcriptome data of platelet samples from 32 individuals, further demonstrating the utility of RNA-seq in examining platelet biology, particularly in human studies. Platelet RNA expression can lead to new insights into the normal hemostatic and thrombotic functions of platelets as well as how platelets may contribute to disease onset, response, and prognosis. We identified candidate genes whose expression was related to platelet aggregation and STEMI/NSTEMI status. These candidates include genes previously implicated in platelet biology and/or MI and new genes whose relationship with hemostasis and thrombosis warrants further investigation. The therapeutic implications and use of gene expression as preventative biomarkers, particularly of identified candidates, also should be further evaluated in clinically and cohort ascertained subjects. Future transcriptome analyses

of platelets in both healthy and affected individuals are likely to reveal novel insights into the mechanisms underlying both normal function and disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

mRNA	messenger RNA
ncRNA	non-coding RNA
RNA-seq	transcriptome sequencing
MI	myocardial infarction
STEMI	ST-segment elevation myocardial infarction
NSTEMI	non-ST-segment elevation myocardial infarction
CAD	coronary artery disease
BMI	body mass index
	platelet-rich plasma
RPKM	reads-per-kilobase-per million mapped
qRT-PCR	quantitative reverse transcription polymerase chain reaction
FHS	Framingham Heart Study
GO	gene ontology
PCA	principal component analysis
Ct	threshold cycle
miRNA	micro RNA
lncRNA	long non-coding RNA
SNP	single nucleotide polymorphism
GWAS	genome-wide association study

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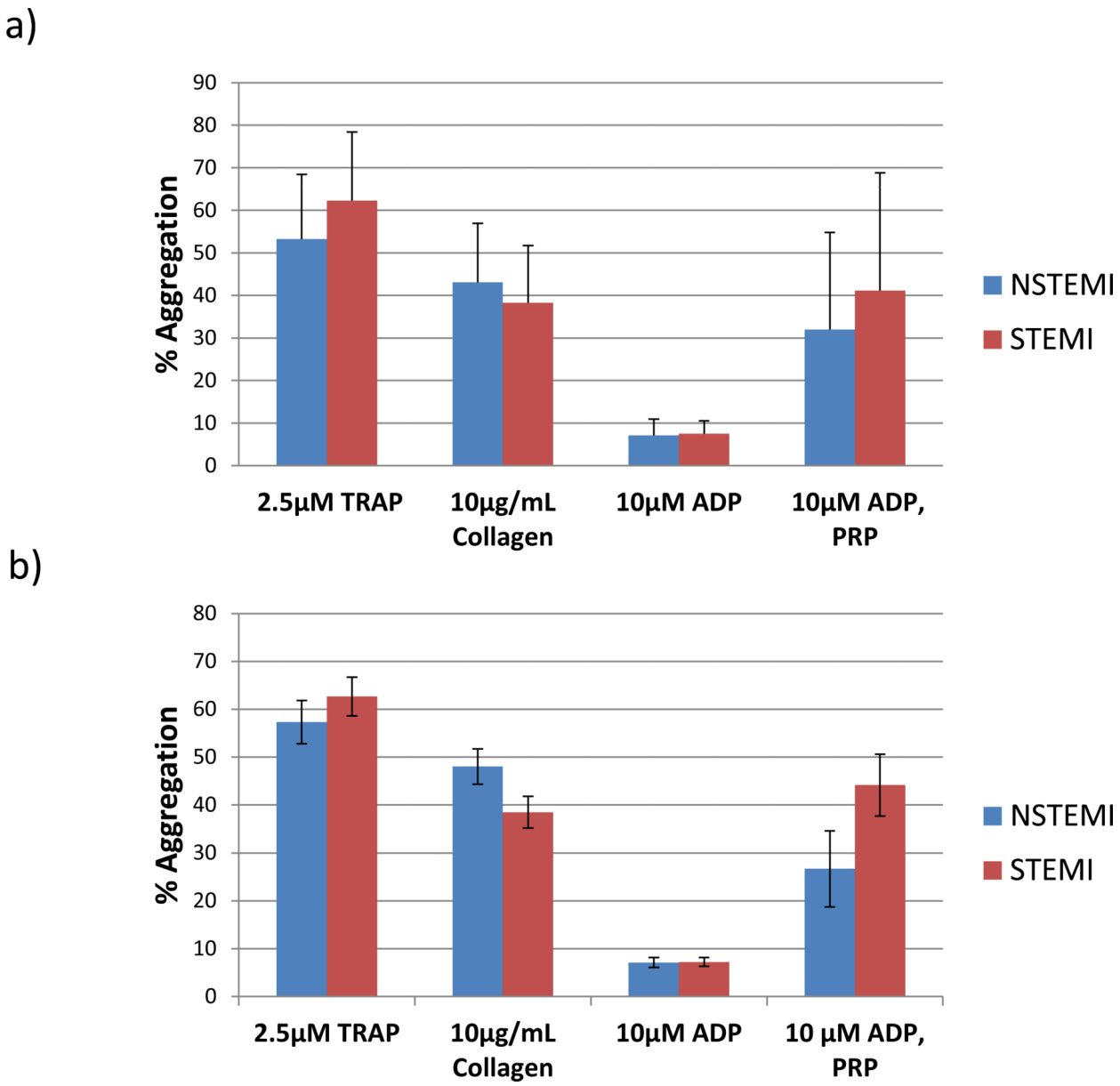

Figure 1.

Comparison of platelet reactivity measures between NSTEMI and STEMI samples **(a)** uncorrected and **(b)** corrected for age, gender, and body mass index (BMI). In the uncorrected models, STEMI samples showed a trend toward increased reactivity to TRAP in washed platelets (Student's T-test p=0.11) and ADP in PRP (Student's T-test p=0.33) compared to NSTEMI. In the corrected models, there were trends toward decreased platelet reactivity to collagen in washed platelets (p=0.0695) and increased reactivity to ADP in platelet rich plasma (p=0.1019) in STEMI cases compared to NSTEMI cases.

Table I

Subject characteristics, medical history, and medication status.

	NSTEMI n=16	STEMI n=16	
Demographics	**Mean ± SD**	**Mean ± SD**	**p-value**
Gender	13 male, 3 female	9 male, 7 female	0.15
Age (years)	68.9 ± 14.9	62.8 ± 11.6	0.20
Body Mass Index (kg/m^2)	29.1 ± 5.1	26.1 ± 5.8	0.13

Past Medical History	**N (%)**	**N (%)**	**p-value**
Diabetes Mellitus	8 (50.0)	6 (37.5)	0.49
Hypertension	15 (93.8)	11 (68.8)	0.09
Dyslipidemia	13 (81.3)	12 (75.0)	0.68
Coronary Artery Disease	5 (31.3)	2 (12.5)	0.22
History of Cardiac Catheterization	5 (31.3)	1 (6.3)	0.09
Cardiomyopathy	2 (12.5)	1 (6.3)	0.55
Family History Coronary Artery Disease	6 (37.5)	9 (56.3)	0.30
History of Smoking	9 (56.3)	11 (68.8)	0.48
Currently Smoking	6 (37.5)	8 (50.0)	0.49

Labs	**Mean ± SD**	**Mean ± SD**	**p-value**
White Blood Cell (thousands/mm^3)	8.9 ± 2.9	11.4 ± 3.9	0.055
Hemoglobin (g/dL)	13.4 ± 2.1	13.9 ± 2.1	0.48
Hematocrit (%)	38.9 ± 6.5	41.6 ± 5.8	0.22
Platelets (thousands/mm^3)	232 ± 77.7	249.3 ± 113.7	0.62

Vital Signs	**Mean ± SD**	**Mean ± SD**	**p-value**
Heart Rate (bpm)	81.8 ± 21.8	75.9 ± 17.8	0.42
Systolic Blood Pressure (mmHg)	150.7 ± 27.5	140.9 ± 23.9	0.31
Diastolic Blood Pressure (mmHg)	81.9 ± 19.6	84.4 ± 16.8	0.71

Medications	**N (%)**	**N (%)**	**p-value**
Acetylsalicylic Acid	16 (100.0)	15 (93.8)	0.33
Plavix*	11 (68.8)	4 (25.0)	0.03
Glycoprotein IIb/IIIa Inhibitors*	1 (6.3)	2 (12.5)	0.55
Ticagrelor	2 (12.5)	11 (68.8)	0.006
Heparin/Lovenox	16 (100.0)	16 (100.0)	1

* $p<0.05$

Table II

Top expressed non-mitochondrial transcripts in RPKM averaged across 32 platelet MI samples and STEMI/NSTEMI subgroups after imposing a cutoff of RPKM ≥ 0.3

RefSeqID	Name	logRPKM MI	logRPKM STEMI	logRPKM NSTEMI
NM_002704	PPBP*	4.44	4.44	4.44
NM_001190702	MTRNR2L8	4.30	4.32	4.29
NM_001134693	OST4*	4.21	4.23	4.18
NM_001101	ACTB	4.08	4.07	4.09
NM_000146	FTL	3.79	3.77	3.80
NR_003259	GNAS	3.78	3.77	3.78
NM_031286	SH3BGRL3	3.74	3.72	3.76
NM_001077489	GNAS	3.72	3.72	3.73
NM_080426	GNAS	3.72	3.72	3.73
NM_000516	GNAS	3.71	3.71	3.72
NM_001077488	GNAS	3.71	3.71	3.72
NM_021109	TMSB4X	3.63	3.64	3.62

Abbreviations: RPKM, reads per kilobase per million mapped reads; MI, myocardial infarction; STEMI, ST-segment elevation MI; NSTEM, non-STEMI

*Reported platelet RNA markers.

Table III

Top unique differentially expressed transcripts between platelets from STEMI and NSTEMI cases using (a) ANOVA and (b) edgeR *

a)

RefSeq ID	Transcript	STEMI RPKM	NSTEMI RPKM	p-value	Fold Change	Up/Down in STEMI
NM_014165	NDUFAF4	3.54	2.35	0.000392	2.03	Up
NM_015480	PVRL3	0.69	1.37	0.000417	8.37	Down
NM_000156	GAMT	2.38	1.42	0.000890	5.25	Up
NM_012160	FBXL4	22.60	11.75	0.000907	1.98	Up
NM_001039960	SLC4A8	0.64	0.43	0.000974	1.34	Up
NM_017647	FTSJ3	4.19	2.03	0.00153	2.50	Up
NM_001204376	NPR3	0.62	0.66	0.00158	4.13	Up
NR_103728	PVRIG2P	3.23	9.40	0.00178	5.19	Down
NM_032936	TMEM60	15.12	24.26	0.00178	2.27	Down
NM_018335	ZNF839	1.82	0.95	0.00187	2.04	Up
NM_001256435	PCYT2	0.59	1.19	0.00192	2.88	Down
NM_001252043	TAOK2	2.18	1.12	0.00196	2.19	Up
NM_002864	PZP	1.29	2.24	0.00213	3.38	Down
NM_024886	C10orf95	1.38	2.48	0.00242	35.30	Down
NM_015480	SERPINF1	0.69	1.37	0.00275	3.95	Down

b)

RefSeq ID	Transcript	STEMI RPKM	NSTEMI RPKM	p-value	Fold Change	Up/Down in STEMI
NM_004217	AURKB	1.89	1.47	0.000259	8.63	Up
NM_001145522	RAI14	2.72	1.07	0.000273	7.99	Up
NM_001243432	ERG	3.63	1.77	0.000455	5.97	Up
NM_017752	TBC1D8B	2.78	0.80	0.000591	3.67	Up
NM_001725	BPI	10.15	2.03	0.000601	7.51	Up
NM_004117	FKBP5	144.60	50.48	0.000694	1.22	Up
NM_012160	FBXL4	22.60	11.75	0.00127	1.20	Up
NM_203416	CD163	29.46	8.99	0.00132	1.88	Up
NM_173636	WDR62	0.92	3.13	0.00135	5.38	Down
NM_001128147	NF1	5.01	2.75	0.00234	2.05	Up
NR_027051	THAP7-AS1	0.41	1.88	0.00251	4.44	Down
NM_002476	MYL4	38.64	58.80	0.00268	8.72	Down
NM_001134745	LRRTM4	N/A	1.33	0.00317	6.34	Down
NM_153321	PMP22	3.39	2.06	0.00322	5.58	Up
NM_025165	ELL3	3.27	0.96	0.00414	3.28	Up

* Full differentially expressed transcripts ($p \leq 0.01$) are presented in Supplement Tables 12-13

Table IV

Validation of prior associated transcripts (KCNE1, ECHDC3, MIAT) as being differentially expressed in platelets in STEMI and NSTEMI cases.

RefSeq ID	Transcript	p-value	Fold Change	Up/Down in STEMI
NM_001127670	KCNE1	0.00793*	1.65	Up
NM_001270404	KCNE1	0.0395*	1.48	Up
NM_001127668	KCNE1	0.155	1.34	Up
NM_001270405	KCNE1	0.155	1.34	Up
NM_001127669	KCNE1	0.180	1.36	Up
NM_001270402	KCNE1	0.189	1.26	Up
NM_000219	KCNE1	0.215	1.25	Up
NM_001270403	KCNE1	0.227	1.28	Up
NM_024693	ECHDC3	0.0141*	5.03	Up
NR_033321	MIAT	0.0450*	2.02	Down
NR_033319	MIAT	0.0451*	2.02	Down
NR_033320	MIAT	0.0633	2.06	Down
NR_003491	MIAT	0.0636	2.05	Down

*$p<0.05$

Table V

Transcripts correlation (Spearman correlation p≤0.001) with platelet aggregation to multiple agonists: (i) TRAP, (ii) Collagen, (iii) ADP in whole blood, and (iv) ADP in platelet rich plasma. N reflects the number of samples with both platelet aggregation data and gene expression for each gene with RPKM ≥ 0.3. No positive correlations were observed for collagen reactivity with p≤0.001. All such correlations with p≤0.01 for each agonist are given in Supplemental Tables 20-23, respectively.

(i)

TRAP

Positively Correlated					**Negatively Correlated**				
RefSeqID	**Gene**	**Corr**	**p-value**	**N**	**RefSeqID**	**Gene**	**Corr**	**p-value**	**N**
NM_001204078	*ATP6V1G2*	0.91	4.63E-05	12	NR_027083	*AFMID*	−0.96	4.54E-04	7
NM_001127670	*KCNE1*	0.89	5.42E-04	10	NM_025261	*LY6G6C*	−0.73	5.28E-04	18
NM_001145248	*FAM157A*	0.66	5.43E-04	23	NM_001128160	*UBP1*	−0.56	7.77E-04	32
NR_046723	*SGOL1-AS1*	0.76	6.70E-04	16	NM_001144900	*MIEF2*	−0.88	8.14E-04	10
					NM_031954	*KCTD10*	−0.56	9.63E-04	32
					NM_014517	*UBP1*	−0.55	9.86E-04	32

(ii)

Collagen

RefSeqID	**Gene**	**Corr**	**p-value**	**N**
NM_001184813	*CEACAM1*	−0.78	3.66E-04	16
NM_001184815	*CEACAM1*	−0.77	4.51E-04	16
NM_001184816	*CEACAM1*	−0.77	4.69E-04	16
NM_000696	*ALDH9A1*	−0.58	4.76E-04	32
NM_148672	*CCL28*	−0.82	6.50E-04	13
NR_038278	*LINC00665*	−0.88	8.14E-04	10
NR_001566	*TERC*	−0.81	8.39E-04	13
NR_027489	*MTHFSD*	−0.69	8.40E-04	20
NM_001177479	*HDX*	−0.85	8.70E-04	11
NM_144657	*HDX*	−0.85	8.70E-04	11

(iii)

ADP in whole blood

Positively Correlated					Negatively Correlated				
RefSeqID	**Gene**	**Corr**	**p-value**	**N**	**RefSeqID**	**Gene**	**Corr**	**p-value**	**N**
NM_001024939	*SLC2A11*	0.71	1.05E-04	24	NM_001191015	*GSTO2*	−0.99	3.09E-04	6
NM_030807	*SLC2A11*	0.67	2.87E-04	25	NM_152470	*RNF165*	−0.96	4.75E-04	7
NM_001024938	*SLC2A11*	0.65	3.85E-04	25	NM_001256758	*RNF165*	−0.96	4.75E-04	7
NM_001039888	*ANKRD34A*	0.96	4.75E-04	7	NM_152361	*EID2B*	−0.87	9.46E-04	10
NM_032139	*ANKRD27*	0.58	6.17E-04	31					
NM_031491	*RBP5*	0.95	8.05E-04	7					

(iv)

ADP in platelet rich plasma

Positively Correlated					Negatively Correlated				
RefSeqID	**Gene**	**Corr**	**p-value**	**N**	**RefSeqID**	**Gene**	**Corr**	**p-value**	**N**
NM_001080449	*DNA2*	0.67	2.01E-04	26	NM_004060	*CCNG1*	−0.63	1.81E-04	30
NR_102264	*DNA2*	0.66	2.39E-04	26	NM_199246	*CCNG1*	−0.63	1.90E-04	30
NM_030809	*CSRNP2*	0.63	5.50E-04	26	NM_024840	*ZNF613*	−0.84	3.24E-04	13
NR_045072	*CSRNP2*	0.63	5.50E-04	26	NM_173517	*VKORC1L1*	−0.61	3.36E-04	30
NM_001270519	*LATS1*	0.59	5.85E-04	30	NM_001031721	*ZNF613*	−0.83	4.96E-04	13
NM_001171931	*CDH23*	0.77	8.77E-04	15	NM_002608	*PDGFB*	−0.62	7.88E-04	26
NM_005688	*ABCC5*	0.63	9.80E-04	24					