A Genome-wide Association Study Identifies Three Loci Associated with Mean Platelet Volume

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Mean platelet volume (MPV) is increased in myocardial and cerebral infarction and is an independent and strong predictor for postevent morbidity and mortality. We conducted a genome-wide association study (GWAS), the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) F3 500K study, and found MPV to be strongly associated with three common single-nucleotide polymorphisms (SNPs): rs7961894 located within intron 3 of *WDR66* on chromosome 12q24.31, rs12485738 upstream of the *ARHGEF3* on chromosome 3p13-p21, and rs2138852 located upstream of *TAOK1* on chromosome 17q11.2. We replicated all three SNPs in another GWAS from the UK and in two population-based samples from Germany. In a combined analysis including 10,048 subjects, the SNPs had p values of 7.24×10^{-48} for rs7961894, 3.81×10^{-27} for rs12485738, and 7.19×10^{-28} for rs2138852. These three quantitative trait loci together accounted for 4%–5% of the variance in MPV. In-depth sequence analysis of *WDR66* in 382 samples from the extremes revealed 20 new variants and a haplotype with three coding SNPs and one SNP at the transcription start site associated with MPV ($p = 6.8 \times 10^{-5}$). In addition, expression analysis indicated a direct correlation of *WDR66* transcripts and MPV. These findings may not only enhance our understanding of platelet activation and function, but may also provide a focus for several novel research avenues.

Platelets are anucleate blood cells and play an important role in atherogenesis and atherothrombosis, two key processes underlying cardiovascular disease.[1,2] MPV is increased in myocardial (MIM 608446, MIM 608557) and cerebral (MIM 601367, MIM 606799) infarction and is an independent and strong predictor for postevent morbidity and mortality.[3,4] Platelets are formed from polyploid bone marrow precursor cells, the megakaryocytes, through a process of proplatelet formation. The volume of platelets is tightly regulated but the precise molecular machinery that controls it is only partially understood and involves outside-in signals emanating from extracellular matrix proteins and growth factors.[5]

There is ample evidence that the blood cell indices under which is also MPV have a high level of heritability. In twin studies, heritability estimates for hemoglobin levels and the counts of white blood cells and platelets ranged from 0.37 to 0.89.[6] Studies in baboons and rodents confirmed these findings and found (not surprisingly) that also the volumes of red cells and platelets are under genetic control.[7]

We conducted a genome-wide association study (GWAS) in individuals sampled from the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) F3 500K study population. The study population for the GWAS was recruited from the MONICA S3 survey, a population-based sample from the general population living in the region of Augsburg, Southern Germany, which was carried out in 1994/95. The standardized examinations applied in this survey including 4856 participants aged 25 to 74 years (response 75%) have been described in detail elsewhere.[8,9] In a follow-up examination of S3 in 2004/05 (KORA F3), 3006 subjects participated. For KORA F3 500K we selected 1644 subjects of these participants then aged 35 to 79 years, including 1606 individuals with MPV values available. Genotyping was performed with the Affymetrix Gene Chip Human Mapping 500K Array Set as described in Döring et al.[10] In brief, on SNP level from a total of 500,568 SNPs, we excluded for the purpose of this analysis all SNPs on chromosome X, leaving 490,032 autosomal SNPs for the GWA screening step. The X chromosome SNPs were excluded from the analysis because the X chromosome has to be treated differently from the autosomes (note that the Affymetrix Chip used does not assay the Y chromosome). Because most loci on the X chromosome are subject to X chromosome inactivation, it can not be predicted which allele is active. Furthermore, because there is only one copy of X in males, sample sizes and accordingly

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power are different from the autosomes. From the 490,032 autosomal SNPs, 335,152 (68.39%) SNPs passed all quality control criteria and were selected for the subsequent association analyses. Criteria leading to exclusion were genotyping efficiency <95% (N = 49,325) and minor allele frequency (MAF) <5% (N = 101,323). An exact Fisher test has been used to detect deviations from Hardy-Weinberg equilibrium, and we excluded all SNPs with p values below 10^{-5} (N = 4,232) after passing the other criteria.[10]

We used three independent samples for replication. The first was a GWAS sample from the UK National Blood Services collection of Common Controls (UKBS-CC) typed with the same Affymetrix Chip. Details of genotyping and quality criteria are given in the original study.[11] In brief, the UKBS-CC collection is an anonymized collection of DNA samples from 3100 healthy blood donors. The collection has been established by the three British blood services of England, Scotland, and Wales as part of the Wellcome Trust Case Control Consortium (WTCCC) study.[11] Data from 1203 English individuals of panel 1 (UKBS-CC1) with available genotypes were used in this study, because no MPV data were available for the Scottish and Welsh samples.

The second replication cohort was recruited from the KORA S4 survey, an independent population-based sample from the general population living in the region of Augsburg, Southern Germany, conducted in 1999/2001. The standardized examinations applied in the survey (4261 participants, response 67%) have been described in detail elsewhere.[8,10] Genotyping of SNPs was performed with the iPLEX (Sequenom, San Diego, CA) method by means of matrix-assisted laser desorption ionization-time of flight mass spectrometry method (MALDI-TOF MS, Mass Array, Sequenom) according to the manufacturer's instructions. Details of genotyping and quality criteria are given elsewhere.[10]

The third replication sample, the Study of Health in Pomerania (SHIP), is a cross-sectional population-based health survey conducted between 1997 and 2001 in West Pomerania, a region in the northeastern part of Germany. The detailed objectives and the study design have been published elsewhere.[12] The final SHIP population comprising 4310 participants (response 68.8%) was invited to attend a 5-year follow-up examination, termed SHIP1, which was conducted between 2002 and 2006 (3300 participants; response 76.6%). For replication analysis, the SHIP1 population was included. The SNPs were genotyped with custom-made 5′ nuclease allelic discrimination (Taqman) assays (AppliedBiosystems, Foster City, CA). Quality control included the independent replication of 3% of genotypes and the inclusion of 2% negative controls on all DNA sample plates.

In all samples, MPV was measured on fresh venous EDTA blood with an automatic analyzer (Coulter STKS in KORA F3, KORA S4, and UKBS-CC1 and Sysmex SE-9000 analyzer in SHIP; reference MPV values were 7.8–11.0 fl in KORA F3, KORA S4, and UKBS-CC1 and 9.0–12.5 fl in SHIP).

A description of the GWA study population and the replication samples is given in Table S1 available online.

In all studies, informed consent was obtained from participants and the studies were approved by the local ethical committees.

We used additive genetic models assuming a trend per copy of the minor allele to test the association of MPV values and genotypes. MPV values were natural log transformed before analysis to approximate the normal distribution. All models were adjusted for age and gender, and additionally for collection center within the UK sample. We used linear regression algorithms as implemented in the statistical analysis packages R (KORA F3 500K), PLINK[13] (KORA F3 500K, UKBS-CC1), and SAS version 9.1 (KORA S4, SHIP). Imputation of genotypes in KORA F3 500K used to fine-map the replicated regions in Figures 1B–1D was performed with the software MACH based on HapMap II. Meta-analysis statistics were obtained with a weighted z-statistics method, where weights were proportional to the square root of the number of individuals examined in each sample and selected such that the squared weights sum was 1. Calculations were implemented in the METAL package. Combined betas and SEs were calculated with Inverse Variance meta-analysis, together with Cochran's Q and I^2 with R scripts.

To select significant SNPs in the genome-wide screening and in the replication studies, we used conservative Bonferroni thresholds that corresponded to an uncorrected significance level of 0.05. The associated quantile-quantile plot in Figure S1 shows good agreement with the null distribution.

The GWAS identified several genomic locations as potentially associated with MPV (Figure 1A). Of the 335,152 SNPs tested by regression analysis, 10 representing 8 distinct genetic regions reached p values below 10^{-5} (Table 1; Tables S2 and S3). One SNP rs7961894 ($p = 2.09 \times 10^{-11}$; Table 1; Figure 1B), located within intron 3 of the *WDR66* (WD repeat domain 66) gene at 12q24.31, reached genome-wide significance with a Bonferroni corrected significance level of 1.5×10^{-7}. The 10 SNPs were taken forward to replicate them in the UKBS-CC1 GWAS sample, and at the same time 8 SNPs (representing 8 different loci) were taken forward for replication in the KORA S4 Study. One of those SNPs could not be replicated in KORA S4 because of problems with the assay design (Table S3). The SNPs, which were successfully replicated in both studies, were rs7961894 in *WDR66*, rs12485738 on 30 and 56 kb distance from the transcription start sites of two short isoforms of the *ARHGEF3* gene at 3p13-p21 (Rho guanine nucleotide exchange factor 3) (MIM 612115), and rs2138852 upstream of the *TAOK1* gene at 17q11.2 (TAO Kinase 1; Figures 1B–1D; Table 1) (MIM 610266). None of the other tested SNPs reached significance in the UKBS-CC1 or KORA S4 sample given a corrected significance of 0.005 (Table S3). Finally, only the three loci that have been successfully replicated in both studies were taken forward to additional replication in the SHIP study where these SNPs again showed a significant association with MPV values (Table 1).

In further analysis in the GWA population, it was examined whether the three lead SNPs are associated with other

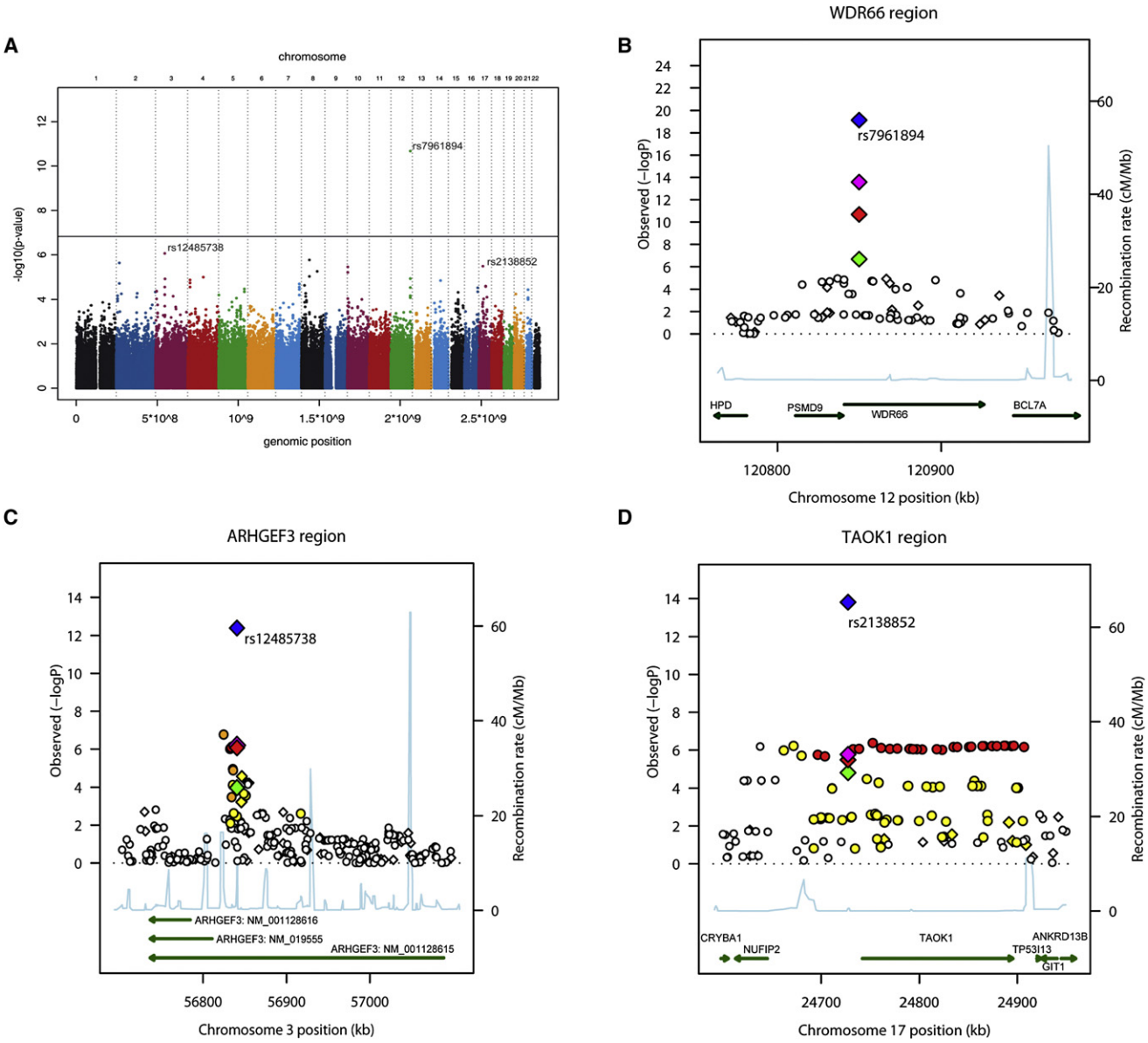

Figure 1. Summary of Genome-wide Association and Replication Results

(A) Genome-wide association study for log-transformed MPV on a population-based sample of 1606 individuals from the KORA F3 500K study. The *x* axis represents the genomic position (in Gb) of 335,152 SNPs; the *y* axis shows −log10(P). The horizontal line indicates the threshold for genome-wide significance at 1.5×10^{-7}. After correcting for multiple testing, we found that one SNP on chromosome 12 attained genome-wide statistical significance.

(B–D) p value plots showing the association signals in the region of *WDR66* on chromosome 12 (B), *ARHGEF3* on chromosome 3 (C), and *TAOK1* on chromosome 17 (D). −log10 p values are plotted as a function of genomic position (NCBI Build 36). Large diamonds indicate the p value for the lead SNP in KORA F3 500K (red), KORA S4 (blue), UKBS-CC1 (green), and SHIP (magenta). Proxies are indicated with diamonds for genotyped SNPs and circles for imputed SNPs of smaller size, with colors determined from their pairwise r^2 values from KORA F3 500K. Red diamonds indicate high LD with the lead SNP ($r^2 > 0.8$), orange diamonds indicate moderate LD with the lead SNP ($0.5 < r^2 < 0.8$), yellow indicates markers in weak LD with the lead SNP ($0.2 < r^2 < 0.5$), and white indicates no LD with the lead SNP ($r^2 < 0.2$). Recombination rate estimates (HapMap Phase II) are given in light blue, Refseq genes (NCBI) are displayed by green bars.

traits, such as white blood cell count, red blood cell count, mean corpuscular volume, hematocrit, and hemoglobin. None of the lead SNPs showed a significant association ($p < 0.05$) with any of these traits (data not shown).

In the combined sample of 10,048 individuals, the SNP rs7961894 reached a p value of 7.24×10^{-48} (effect per minor allele copy = 0.032 per log fl, CI 0.028–0.037), the SNP rs12485738 a p value of 3.81×10^{-27} (effect per minor allele copy = 0.015 per log fl, CI 0.012–0.017), and the third SNP (rs2138852) a combined p value of 7.19×10^{-28} (effect per minor allele copy = −0.015 per log fl, CI −0.018–−0.013).

The reference values were about 15% higher in SHIP than in the other studies, which is best explained by the different

Table 1. Association between Mean Platelet Volume and Three Lead SNPs in the GWAS and Three Replication Cohorts

	Chromosome	Position	Minor Allele	Major Allele	Genotyping Efficiency	p Value HWE	N (MAF in %)	Estimate (SE) (fl)	p Value	Variance Explained
rs12485738	3	56840816								
KORA 500K F3			A	G	98.6	0.706	1,584 (36.03)	0.019 (0.0038)	8.57×10^{-7}	1.52%
UKBS-CC1					99.9	0.449	1,219 (36.30)	0.017 (0.0043)	5.61×10^{-5}	1.27%
KORA S4					94.8	2.2×10^{-16}[a]	4,137 (30.14)	0.015 (0.0022)	4.02×10^{-13}	1.11%
SHIP					96.2	0.2922	3,024 (36.97)	0.012 (0.0024)	6.31×10^{-7}	0.87%
Combined[b]							9,964	0.015 (0.0014)	3.81×10^{-27}	
rs7961894	12	120849966								
KORA 500K F3			A	G	99.7	0.013	1,602 (11.92)	0.040 (0.0059)	2.09×10^{-11}	2.77%
UKBS-CC1					100.0	0.685	1,220 (11.32)	0.033 (0.0063)	3.04×10^{-7}	1.90%
KORA S4					97.9	0.937	4,070 (11.18)	0.034 (0.0037)	7.26×10^{-20}	2.04%
SHIP					98.2	0.3628	3,142 (11.14)	0.028 (0.0036)	2.61×10^{-14}	1.84%
Combined[b]							10,034	0.032 (0.0022)	7.24×10^{-48}[c]	
rs2138852	17	24727475								
KORA 500K F3			C	T	99.9	1.000	1,605 (49.33)	−0.017 (0.0037)	3.31×10^{-6}	1.34%
UKBS-CC1					99.5	0.307	1,220 (47.80)	−0.018 (0.0041)	1.62×10^{-5}	1.38%
KORA S4					99.8	0.5329	4,139 (47.17)	−0.018 (0.0023)	1.57×10^{-14}	1.42%
SHIP					96.2	0.1123	3,084 (48.21)	−0.011 (0.0024)	1.70×10^{-6}	0.74%
Combined[b]							10,048	−0.015 (0.0014)	7.19×10^{-28}	

Effect sizes (estimates and SE) are given for each copy of the minor allele and are expressed as natural logarithm of MPV.

[a] Violation of HWE equilibrium, also after regenotyping.

[b] No study heterogeneity (I2 range 0–43, p values > 0.05).

[c] The p value excluding the KORA S4 sample ($n = 5964$) is 1.087×10^{-29}.

analysis platforms with the Coulter-method (KORA, UKBS-CC1) or light scatter analysis (Sysmex SE-9000, SHIP). However, this fact may be negligible for the analysis, provided that the values are not differentially variable over the range. An internal comparison of the methods carried out in the SHIP project resulted in the regression equation Y (fl Sysmex SE-9000) = 1.000*X (fl Coulter-method) + 1.850, indicating that all values are shifted by the constant value of 1.850 upwards. We carried out an analysis corrected with MPV values for SHIP and found rather higher effect estimates for all three SNPs. We decided to use the conservative uncorrected values resulting in a slight underestimation of the effects.

Because the lead SNP in *WDR66* reached the best p value and accounted for about 2.0% of the MPV variance, we decided to analyze the coding sequence of *WDR66* in more detail (Tables S4 and S5). High-resolution melting analysis was used as mutation scanning technology to analyze the coding region of *WDR66*. *WDR66* exons were PCR amplified with intronic primers with ~5 ng genomic DNA with a final denaturation step at 94°C for 1 min (0.25 units Thermo-Start Taq DNA polymerase [Abgene], 1× LCGreen Plus [BIOKE], 0.25 μM of each primer; Table S5). High-resolution melting analysis was performed on a LightScanner instrument (Idaho Technology). In the presence of the saturating double-stranded DNA-binding dye, amplicons were slowly heated from 77°C until fully denatured (96°C) while the fluorescence was monitored. Melting curves were analyzed by LightScanner software (Idaho Technology), with normalized, temperature-shifted curves displayed as difference plots (−dF/dT). Detected samples with altered melting curves compared with the average of multiple wild-types were directly sequenced with a BigDye Cycle sequencing kit (Applied Biosystems).

We analyzed the sequence of all 21 coding exons and the 5′ UTR in 382 samples selected from the high and low extremes of the MPV distribution in 4000 individuals (KORA S4). We found variants or variation in 4 of the 9 coding SNPs, which were already annotated in dbSNP. None of these showed an association with MPV, but the A allele of the lead SNP rs7961894 was overrepresented in the high-MPV group ($p = 1.3 \times 10^{-6}$, Fisher's exact test for allele distribution, Figure 2; more detailed information in Table S4). In addition, we detected 10 nonsynonymous SNPs, one nonsense and five synonymous variants, a 15 bp and an 18 bp insertion, one 3′ UTR SNP and one SNP (C → T) a single bp upstream of the UCSC annotated 5′ end of the *WDR66* transcript (see Table S4). The latter variant (ss107795092) with a minor allele frequency (MAF) of 3.6% falls within a conserved region (LOD = 24, phastCons program) and is significantly overrepresented in the low-MPV group ($p = 6.8 \times 10^{-5}$). This variant is linked ($r^2 > 0.9$, see Table S6) with three other newly discovered coding SNPs (ss107795081-3, p.C304C, p.V307I, and p.R417Q) and they define—in the background of the G allele of the lead SNP rs7961894—a rare haplotype (MAF 2.5%). This haplotype may contribute to the significant association of rs7961894 with MPV, but the strongest association was found for the lead SNP followed by ss107795092 alone.

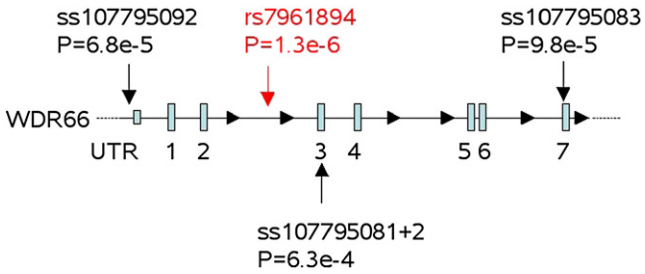

Figure 2. Localization of MPV-Associated SNPs within the 5′ Part of the *WDR66* Gene

The p values given are based on Fisher's exact test in 382 samples from the most extreme (high and low) MPV distribution in KORA S4.

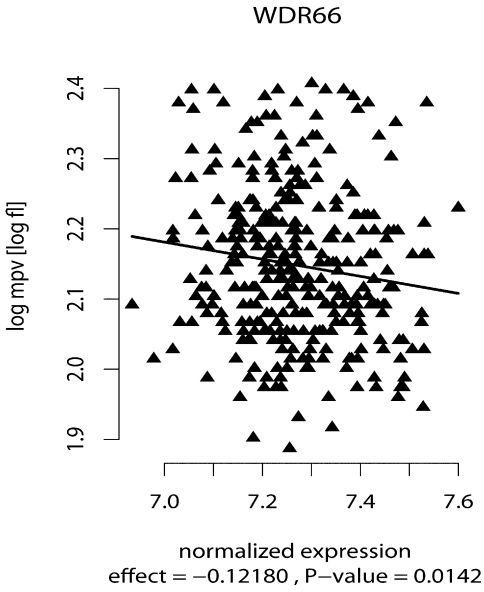

Figure 3. Expression Analysis of *WDR66* and Association with Log MPV

WDR66 expression was analyzed via whole-blood genome-wide transcription profiling in a subgroup of 323 KORA F3 samples with Illumina Human-6 v2 Expression BeadChip (probe ID 2630343).

The strong correlation of the SNP prompted us to investigate the transcript levels of *WDR66* in a randomly selected subgroup of 323 KORA F3 samples with whole-genome expression profiles available. Gene-expression analysis was performed with the Illumina Human-6 v2 Expression BeadChip as described in Döring et al.[10] In brief, blood samples were collected under fasting conditions in PAXgene (TM) Blood RNA tubes (PreAnalytiX) and RNA extraction was performed with the PAXgene Blood RNA Kit (QIAGEN). RNA was reverse transcribed and biotin-UTP labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion). The raw data were exported from the Illumina "Beadstudio" Software to R, converted into logarithmic scores, and normalized.[10] We observed no association between intronic lead SNP rs7961894 and WDR66 transcript level, but a significant association of the levels of the *WDR66* transcript with MPV ($p = 0.01$, Figure 3) via the linear regression model. In addition, we looked at correlation between gene expression and genotypes for the other two lead SNPs and found no significant association. Based on the small samples size for the expression studies, the analysis has a limited power. However, the lacking association between the intronic SNP and WDR expression argues against a direct effect on WDR66 expression. On the other side, the correlation of WDR66 expression with MPV supports the hypothesis that WDR66 is involved in the determination of MPV.

In summary, we identified three loci associated with MPV, a quantitative trait that is increasingly recognized as being associated with the post-MI event risk of major complications. These three loci accounted for about 5% of the variance in MPV values in the normal population. All three genes are plausible biological candidates that could modify the process of platelet formation. The process of proplatelet formation is critically dependent on reorganization of cytoskeletal components and localized apoptosis seems to play an important role.[5,14] WD-repeat proteins are present in all eukaryotes but not in prokaryotes. It is hypothesized that they are involved in the regulation of cellular functions ranging from signal transduction and transcription regulation to cell-cycle control and apoptosis.[15] Our expression experiment indicates a direct correlation of *WDR66* transcript level and MPV. Previous studies have shown that *ARHGEF3* (*XPLN*), which encodes the rho guanine-nucleotide exchange factor 3 (RhoGEF3), is expressed in the brain, skeletal muscle, heart, kidney, and platelets as well as macrophage and neuronal cell tissues.[16] RhoGEFs activate RhoGTPases, which play an important role in many cellular processes such as regulation of cell morphology, cell aggregation, cytoskeletal rearrangements, and transcriptional activation.[17]

TAOK1, which is expressed in a wide variety of different tissues that include brain, heart, lung, testis, skeletal muscle, placenta, thymus, prostate, and spleen, encodes the TAO kinase 1 peptide (hTAOK1 also known as MARKK or PSK2) a microtubule affinity-regulating kinase that has been identified recently as an important regulator of mitotic progression, required for both chromosome congression and checkpoint-induced anaphase delay.[18] TAOK1 activates c-Jun N-terminal kinase (JNK) and induces apoptotic morphological changes that include cell contraction, membrane blebbing, and apoptotic body formation.[19]

In conclusion, to our knowledge we identified the first three quantitative trait loci associated with MPV in the general population. Identification of primary genetic determinants of MPV may not only enhance our understanding of platelet activation and function, but may also provide a focus for several novel research avenues.

Supplemental Data

Supplemental Data include one figure and six tables and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Genome browser, http://genome.ucsc.edu/
Markov Chain Haplotyping Package, http://www.sph.umich.edu/csg/abecasis/mach/
METAL Package, http://www.sph.umich.edu/csg/abecasis/Metal.index.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
The R project for Statistical Computing, http://www.r-project.org/
Sequenom, http://www.sequenom.com
SNP database, http://www.ncbi.nlm.nih.gov/SNP/

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