Genome-wide association analysis of red blood cell traits in African Americans: the COGENT Network


1Division of Epidemiology and Biostatistics, Mel and Enid Zuckerman College of Public Health, University of Arizona, Tucson, AZ 85724, USA, 2Department of Statistics and Department of Genetics, Stanford University, Stanford, CA 94305, USA, 3GeneSTAR Research Program, Division of General Internal Medicine, Johns Hopkins School of Medicine, Baltimore, MD 21287, USA, 4Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98195, USA, 5Molecular Genetics Section, Laboratory of Neurogenetics, National Institute on Aging, Bethesda, MD 20892, USA, 6Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA, 7Center for Applied Genomics, Abramson Research Center and Division of Human Genetics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA, 8The Charles Bronfman Institute for Personalized Medicine, The Genetics of Obesity and Related Metabolic Traits Program, 9The Charles Bronfman Institute for Personalized Medicine, 10Department of Genetics and Genomic Sciences, Institute of Genomics and Multiscale Biology, 11Division of Psychiatric Genomics, Department of Psychiatry and 12Department of Child Health and Development, The Genetics of Obesity and Related Metabolic Traits Program, Mount Sinai School of Medicine, New York, NY 10029, USA, 13Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, USA, 14Department of Medicine and 15Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA, 16Program in Medical and Population Genetics, Broad Institute, Cambridge, MA, USA, 17Department of Epidemiology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands, 18Division of Rheumatology, Immunology, and Allergy and 19Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA, 20Medical and Population Genetics Program, Broad Institute, Cambridge, MA 02142, USA, 21Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan, 22Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA, 23Laboratory of Neurogenetics and 24Department of Genetics, University of North Carolina, Chapel Hill, NC 27599, USA, 25Health Disparities Research Section, National Institute on Aging, National Institutes of Health, Bethesda, MD 20225, USA, 26Division of Cardiovascular Sciences, National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD, USA, 27Laboratory of Personality and Cognition, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA, 28Department of Medicine, University of California, San Francisco, CA 94143, USA, 29Division of Cardiology, University of Michigan Health System, Ann Arbor, MI 48109, USA, 30Department of Genetics, Harvard Medical School, Cambridge, MA, USA.
INTRODUCTION

Laboratory red blood cell (RBC) measurements are clinically important, heritable and differ among ethnic groups. To identify genetic variants that contribute to RBC phenotypes in African Americans (AAs), we conducted a genome-wide association study in up to ~16,500 AAs. The alpha-globin locus on chromosome 16pter [lead SNP rs13335629 in ITFG3 gene; \( P < 1 \times 10^{-13} \) for hemoglobin (Hgb), RBC count, mean corpuscular volume (MCV), MCH and MCHC] and the G6PD locus on Xq28 [lead SNP rs1050828; \( P < 1 \times 10^{-13} \) for Hgb, hematocrit (Hct), MCV, RBC count and red cell distribution width (RDW)] were each associated with multiple RBC traits. At the alpha-globin region, both the common African 3.7 kb deletion and common single nucleotide polymorphisms (SNPs) appear to contribute independently to RBC phenotypes among AAs. In the 2p21 region, we identified a novel variant of PRKCE distinctly associated with Hct in AAs. In a genome-wide admixture mapping scan, local European ancestry at the 6p22 region containing HFE and LRRRC16A was associated with higher Hgb. LRRRC16A has been previously associated with the platelet count and mean platelet volume in AAs, but not with Hgb. Finally, we extended to AAs the findings of association of erythrocyte traits with several loci previously reported in Europeans and/or Asians, including CD164 and HBS1L-MYB. In summary, this large-scale genome-wide analysis in AAs has extended the importance of several RBC-associated genetic loci to AAs and identified allelic heterogeneity and pleiotropy at several previously known genetic loci associated with blood cell traits in AAs.

RESULTS

Descriptive analysis

Since not all RBC traits were available in every COGENT cohort, the numbers of individuals available for meta-analysis varied by each RBC trait (Supplementary Material, Table S1). Only Hgb (\( n = 16,485 \)) and Hct (\( n = 16,496 \)) were available in all cohorts. MCHC (\( n = 12,152 \)), MCV (\( n = 6,438 \)), RBC count (4,818), MCH (\( n = 4,066 \)) and RDW (\( n = 3,811 \)) were available in subsets of participating cohorts. There were varying degrees of pairwise correlation between RBC traits (Supplementary Material, Table S2). Pearson’s correlation coefficients were highest (>0.95) between Hgb and Hct, and between MCV and MCH and were lowest between the RDW and RBC count (0.03).

GWAS of RBC traits in COGENT AAs

The GWA results for each RBC trait are summarized by Manhattan (Fig. 1) and quantile-quantile (Supplementary Material, Fig. S1) plots. The meta-analysis inflation factors were all near unity (0.998–1.005), suggesting that confounders and other technical artifacts were well-controlled. In total, seven independent genomic loci met the experiment-wide significance threshold (\( P < 1 \times 10^{-8} \)) for one or more RBC traits (Table 1 and Supplementary Material, Table S3). Three loci (1p31.1, 13q31.2, 16p13.3centromeric) have not been previously associated with RBC traits, whereas four loci (2p21,
6q21, 16p31.3 telomeric, and Xq28) have been associated with at least one such trait in populations of European, Japanese or African descent.

Previously reported RBC loci

The two top Xq28 single nucleotide polymorphisms (SNPs) (rs762516, rs1050828) for Hgb, Hct, MCV and RBC count are located in the G6PD gene. rs1050828 encodes the G6PD amino acid substitution Val68Met that results in the G6PD A allele known to cause G6PD deficiency (MIM #305900). The G6PD A-variant has been previously associated with lower Hct, Hgb and RBC count, and with higher MCV in AAs (9). Here, we additionally report that the G6PD A allele is associated with the lower RDW. Given the extent of the association signal at Xq28, we repeated the Hgb and Hct association analyses in women from Women Health Initiative (WHI), the largest AA cohort (n = 8304) for Hgb and conditioning the lead SNPs, rs1050828. After adjusting for rs1050828, the strength of association with Hgb for the remaining SNPs on Xq28 was greatly attenuated and no longer significant (data not shown).

The index SNP on 16p13, which encompasses the α-globin (HBA2-HBA1) locus, was rs13335629 within an intron of ITFG3. rs13335629 met the genome-wide significance threshold for association with lower Hgb, MCH, MCHC and MCV and also with a higher RBC count. The rs13335629 variant was also nominally associated with lower Hct (β = −0.215 ± 0.056; P = 1.33E−04) and higher RDW (β = 0.0053 ± 0.0021; P = 0.01). Lo et al. previously reported a common rs1211375 variant within the 16p13 region associated with lower Hgb, MCH and MCV in AAs, and that these associations were not present in Caucasians (9). Our index SNP rs13335629 is in moderate linkage disequilibrium (LD) with rs1211375 (r² = 0.33 in HapMap YRI).

Three intronic variants of the protein kinase C (PKC)-epsilon gene PRKCE on 2p21 were associated with lower

Figure 1. Manhattan plots of GWAS analysis for RBC traits (A) Hct; (B) Hgb; (C) MCHC; (D) MCH; (E) MCV; (F) RBC count and (G) RDW. The dashed horizontal red line indicates P = 1 × 10⁻⁸. The dashed horizontal blue line indicates P = 5 × 10⁻⁸.
Table 1. Results of genome-wide significant SNPs for RBC traits in COGENT AA

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chromosome</th>
<th>Number of SNPs with $P &lt; 5 \times 10^{-8}$</th>
<th>Top SNP in the region</th>
<th>Position Hg18</th>
<th>Candidate genes</th>
<th>Minor/major allele</th>
<th>MAF</th>
<th>Effect size (SE)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT</td>
<td>Xq28</td>
<td>6</td>
<td>rs762516</td>
<td>153 417 857</td>
<td>G6PD, TKTL1, MECP2, MPP1</td>
<td>T/C</td>
<td>0.148</td>
<td>–0.452 (0.055)</td>
<td>2.17E + 16</td>
</tr>
<tr>
<td>HCT</td>
<td>2p21</td>
<td>3</td>
<td>rs13008603</td>
<td>46 209 352</td>
<td>PRKCE, ITFG3, LUCL7, NPRL3, POLR3K</td>
<td>A/C</td>
<td>0.163</td>
<td>–0.277 (0.047)</td>
<td>4.09E + 09</td>
</tr>
<tr>
<td>HCT</td>
<td>16p13.3</td>
<td>11</td>
<td>rs13335629</td>
<td>250 381</td>
<td>MCV, MCH, MCHC, RDW</td>
<td>A/G</td>
<td>0.120</td>
<td>–0.190 (0.019)</td>
<td>2.63E + 23</td>
</tr>
<tr>
<td>HGB</td>
<td>Xq28</td>
<td>9</td>
<td>rs762516</td>
<td>153 417 857</td>
<td>G6PD, TKTL1, MECP2, MPP1</td>
<td>T/C</td>
<td>0.146</td>
<td>–0.1614 (0.0186)</td>
<td>3.73E + 18</td>
</tr>
<tr>
<td>MCH</td>
<td>16p13.3</td>
<td>38</td>
<td>rs13335629</td>
<td>250 381</td>
<td>ITFG3, LUCL7, NPRL3, POLR3K</td>
<td>A/G</td>
<td>0.117</td>
<td>–0.3298 (0.0227)</td>
<td>8.66E + 48</td>
</tr>
<tr>
<td>MCH</td>
<td>16p13.3</td>
<td>28</td>
<td>rs13339636</td>
<td>238 589</td>
<td>ITFG3, LUCL7, NPRL3, POLR3K</td>
<td>G/A</td>
<td>0.132</td>
<td>–0.6847 (0.0559)</td>
<td>1.87E + 34</td>
</tr>
<tr>
<td>MCH</td>
<td>1q31.2</td>
<td>4</td>
<td>rs9559892</td>
<td>88 166 665</td>
<td>–</td>
<td>A/C</td>
<td>0.253</td>
<td>–0.2589 (0.0444)</td>
<td>5.46E + 09</td>
</tr>
<tr>
<td>MCH</td>
<td>16p13.3</td>
<td>1</td>
<td>rs7192051</td>
<td>4482 118</td>
<td>HMOX2</td>
<td>G/T</td>
<td>0.360</td>
<td>–0.2396 (0.0411)</td>
<td>5.70E + 09</td>
</tr>
<tr>
<td>MCH</td>
<td>6q21</td>
<td>10</td>
<td>rs9386791</td>
<td>109 715 190</td>
<td>CD164</td>
<td>C/T</td>
<td>0.416</td>
<td>–0.2294 (0.0401)</td>
<td>1.20E + 08</td>
</tr>
<tr>
<td>MCV</td>
<td>16p13.3</td>
<td>21</td>
<td>rs13335629</td>
<td>250 381</td>
<td>ITFG3, LUCL7, NPRL3, POLR3K</td>
<td>A/G</td>
<td>0.134</td>
<td>–0.648 (0.0669)</td>
<td>3.61E + 22</td>
</tr>
<tr>
<td>MCV</td>
<td>Xq28</td>
<td>6</td>
<td>rs762516</td>
<td>153 417 857</td>
<td>G6PD, FAM3A, F8, MMP1</td>
<td>T/C</td>
<td>0.137</td>
<td>1.5768 (0.2083)</td>
<td>3.76E + 14</td>
</tr>
<tr>
<td>MCV</td>
<td>16p13.3</td>
<td>12</td>
<td>rs13335629</td>
<td>250 381</td>
<td>HMOX2</td>
<td>G/T</td>
<td>0.365</td>
<td>–0.259 (0.0475)</td>
<td>4.83E + 08</td>
</tr>
<tr>
<td>RBC</td>
<td>Xq28</td>
<td>9</td>
<td>rs1050828</td>
<td>153 417 411</td>
<td>G6PD, F8, MPP1, MECP2, CTAG2</td>
<td>A/G</td>
<td>0.120</td>
<td>0.1699 (0.0169)</td>
<td>7.48E + 24</td>
</tr>
<tr>
<td>RDW</td>
<td>Xq28</td>
<td>1</td>
<td>rs1050828</td>
<td>153 417 411</td>
<td>G6PD</td>
<td>T/C</td>
<td>0.116</td>
<td>–0.326 (0.0048)</td>
<td>1.70E + 11</td>
</tr>
<tr>
<td>RDW</td>
<td>1p31.1</td>
<td>1</td>
<td>rs10493739</td>
<td>83 698 745</td>
<td>–</td>
<td>T/C</td>
<td>0.334</td>
<td>0.0128 (0.0023)</td>
<td>3.02E + 08</td>
</tr>
</tbody>
</table>

*Novel loci.

Hct. The index SNP rs13008603 was also nominally associated with a lower RBC count ($P = 0.044 \pm 0.013; P = 4.69E-04$), but not with other RBC traits ($P > 0.05$ for Hgb, MCV, MCH, MCHC, RDW). The three Hct-associated PRKCE variants are in strong LD (pairwise $r^2 > 0.7$). Another intronic variant of PRKCE (rs10495928) was previously associated with Hgb and Hct in Europeans (8) and with the RBC count in Japanese (6), but showed no evidence of association in AAs ($P = 0.50$ and 0.71 for Hct and Hgb, respectively). In European and African HapMap populations, there is no evidence of LD between rs10495928 and any of the three Hct-associated variants observed in COGENT AA. These results strongly suggest ethnicity-specific allelic heterogeneity for RBC traits at the PRKCE locus.

At 6q21, a haplotype comprised of 10 SNPs (lead SNP = rs9386791) was associated with a lower MCH, and nominally with a lower MCV ($P = 1.09E-05$), Hgb ($P = 0.007$), Hct ($P = 0.03$), MCHC ($P = 0.02$), RBC count ($P = 0.01$). These variants are located ~50 kb upstream of CD164, which encodes a mucin-like molecule expressed by human CD34(+ ) hematopoietic progenitor cells that regulate erythropoiesis. Other variants of the CD164 5' flanking region have been associated with RBC, MCH and MCV in Japanese (rs11966072) (6) and with MCV in Europeans (rs9374080) (8). In HapMap CEU, rs9374080 is in LD with our AA index SNP rs9386791 ($r^2 = 0.87$).

Newly discovered RBC loci

Of the three novel loci associated with RBC traits, rs10493739 at 1p31.1 (associated with RDW) and rs9559892 at 1q31.2 (associated with MCH) are both located in regions devoid of known genes. TILL7 is the closest gene to rs10493739 (400 kb away) and encodes a tubulin polyglutamylase, which modifies beta-tubulin (11). There are no known genes within 500 kb on either side of rs9559892. The lead SNP at the third locus, rs7192051 is located within the second intron of the heme oxygenase-2 gene (HMOX2) and was associated with lower MCH and MCV. Heme oxygenase 2, the protein product of HMOX2, degrades heme and is important in erythropoiesis (12). Although HMOX2 is located ~4 Mb centromic to the alpha-globin locus, it is not in LD with the previously identified 16p13 association signals (maximum $r^2 = 0.004$ with rs13335629).

We attempted to validate two of our three novel RBC loci discovered in COGENT in two independent population-based samples: ~7700 AA youths ages 8–21 years from CHOP and 2010 AA adults from the Mount Sinai eMERGE study. There was no evidence of replication of rs9559892 with MCH, nor of rs7192051 with MCV or MCH (Supplementary Material, Table S4) in the validation sample. It was not possible to pursue replication of rs10493739 in CHOP and eMERGE because this SNP was not genotyped and it could not be imputed in the available replication samples. In over 20 000
Europeans from the CHARGE consortium and 14,000 Japanese from RIKEN, there was no evidence of association of rs9559892 with MCH. Similarly, there was no evidence of association of rs7192051 with MCV or MCH in CHARGE Europeans (Supplementary Material, Table S4).

Admixture mapping analysis of Hgb, Hct and MCHC traits in WHI AA

As a complementary approach to identifying variants associated with RBC traits in AAs that occur at disparate frequencies in ancestral African versus European populations, we performed admixture mapping for Hgb, Hct and MCHC in WHI, the largest cohort comprising COGENT. For MCHC, there was one genome-wide significant association signal at the p-term of 16 containing the alpha-globin locus (Supplementary Material, Fig. S2). Local African ancestry in this region was associated with lower MCHC. The admixture association peak is at rs7203694 ($P = 2.78 \times 10^{-6}$) located within RAB40C, and the genome-wide significant region spans $0-0.78$ Mb (build 36). There were no genome-wide significant admixture associations for Hct (data not shown). For Hgb, a 2 Mb region on chromosome 6p22.2–6p22.1 (25.2–27.1 Mb, build 36) reached genome-wide significance, with increased European ancestry associated with higher Hgb levels (Fig. 2A). The Hgb admixture signal appears to be comprised of two peaks (Fig. 2B). Underlying the centromeric peak is $HFE$, the hemochromatosis protein-coding gene, which regulates iron absorption by modulating the interaction of the transferrin receptor with transferrin. Two known $HFE$ mutations, C282Y (rs1800562) and H63D (rs1799945) cause hereditary hemochromatosis, an autosomal recessive iron storage disorder (13). Among individuals of European descent, the frequencies of C282Y and H63D are 3.23 and 16.6%, respectively. Both the mutations are essentially absent in the HapMap YRI populations, and therefore the frequency in AAs is low and is the result of European admixture. C282Y was directly typed in WHI SHARE and other COGENT cohorts (total $N = 15,584$); the genotype association test yielded an Hgb association $P = 0.0003$ in WHI alone and $4.3 \times 10^{-6}$ in COGENT overall (minor allele frequency = 0.015; $\beta = 0.239 \pm 0.052$). H63D was not directly typed; however, it is tagged by rs129128 ($r^2 = 1.0$ in CEU), which was associated with Hgb levels in WHI ($P = 0.008$) but not when all COGENT cohorts were analyzed together ($P = 0.07$).

After adjusting for genotypes at C282Y and the H63D proxy rs129128 in WHI, the admixture $P$ value for chromosome 6p22 was attenuated, but remained significant (from $3.28 \times 10^{-7}$ to $1.12 \times 10^{-5}$), suggesting the existence of additional variants in this region that contribute to interpopulation differences in Hgb levels. Located within the telomeric peak of the admixture signal (Fig. 2B) are a number of additional variants that have $F_{st} > 0.3$, including several near $LRRC16A$, which has been associated with both serum transferrin levels in whites and the platelet count in AAs from COGENT (14). The most strongly associated $LRRC16A$ variant rs9356970 is located $\sim$25 kb upstream of the $S'$ flanking region (MAF = 0.09; $\beta = 0.118 \pm 0.028$; $P = 2.7 \times 10^{-5}$). According to the HapMap, the minor allele is present in 30% of European chromosomes, but only 2.5% of YRI chromosomes. In a regression model simultaneously adjusting for $LRRC16A$ rs9356970 in addition to $HFE$ C282Y and rs129128, the association signal for local African ancestry at 6p22 was further attenuated, but remained nominally associated with lower Hgb ($\beta = -0.058 \pm 0.029$; $P = 0.045$). Together, these results suggest that several European-derived alleles in the 6p22 region, including those of $HFE$ and $LRRC16A$, may contribute to higher Hgb levels observed in populations of European descent compared with AAs.

CNV analysis and assessment of allelic heterogeneity at 16p13 alpha-globin region

Given the genetic complexity of the alpha-globin locus on chromosome 16p13, including the presence of a common 3.7 kb alpha-thalassemia deletion in AAs (15), and the extent and magnitude of the observed GWAS signal for RBC traits at 16p13, we assessed structural variation at the 16p13 alpha-globin locus using data from 1000 genomes. First, we confirmed the presence of a common deletion (−α3.7) among African Americans (AAs) and West Africans that removes one alpha-globin gene copy ($HBA2$)
(Supplementary Material, Fig. S3). Using pooled sequence data from 16 samples (9 YRI, 5 LWK, 1 ASW, 1 CLM) that appear to be homozygous for $-\alpha^{3.7}$ deletion, we further localized the breakpoints, which appear to be bounded by $\sim 300$ bp of nearly identical sequence located within the 5$^\prime$ flanking regions of HBA1 and HBA2 (Supplementary Material, Fig. S4). Second, we identified a rare deletion spanning HBM through HBQ1, in three Han Chinese individuals, and several other possible (but uncertain) rare copy number variations (CNVs) including one duplication (Supplementary Material, Fig. S5) and a very rare deletion that deletes a known regulatory element MCS-R1 (16) (Supplementary Material, Fig. S6).

Among all typed and imputed SNPs in the WHI dataset, the strongest correlation with alpha37 in 63 YRI samples from 1000 Genomes was observed in the region of ITFG3. This includes rs13335629 (r-squared = 0.6), which is also the top Hgb- and MCHC-associated SNP in COGENT AA. We repeated the association analyses in WHI ($n = 8304$) for Hgb and MCHC conditioning on rs13335629. The top SNP for Hgb in the conditional analysis was POLR3K rs798693 ($P = 8.7 \times 10^{-6}$). For MCHC, rs2541612 in NPRL3 remained genome-wide significantly associated with lower MCHC ($P = 1.14 \times 10^{-9}$). When both ITFG3 rs13335629 and NPRL3 rs2541612 were included as covariates in the conditional analysis, the SNP most strongly associated with lower MCHC was LUC7L rs1211375 ($P = 1.50 \times 10^{-6}$). Taken together, the 1000 Genomes CNV analyses and the results of conditional regression analyses for Hgb and MCHC suggest that while some of the red cell GWAS association signal may be due to the common African alpha37 deletion, there appears to be independent signals coming from other structural variants and/or SNP(s) in the region.

Cross-ethnic transferability of previously reported RBC to COGENT AA

We assessed whether 72 SNPs previously associated with RBC traits in European or Japanese populations are associated with RBC traits in COGENT AA (Supplementary Material, Table S5). Using the conservative Bonferroni multiple comparison corrected significance threshold ($P < 0.0001$), we validated four associations. In addition to the association of $HFE$ rs1800562 with Hgb, these include ITFG3 rs1122794 (previously associated with MCH in Europeans) with higher MCHC ($P = 1.5 \times 10^{-8}$), MCH ($P = 7.2 \times 10^{-5}$) and MCV ($P = 7.1 \times 10^{-5}$) in AAs; ITFG3 rs7189020 (previously associated with MCV in Europeans) with higher MCHC ($P = 1.0 \times 10^{-5}$) and MCV ($P = 1.5 \times 10^{-5}$) in AAs; and HBS1L-MYB rs7775698 (previously associated with HCT, MCH, MCHC, MCV and RBC count in Japanese) with a lower RBC count ($P = 3.3 \times 10^{-5}$) in AAs.

RBC-associated genetic variants and anemia in AA women

To identify genetic variants associated with anemia, defined dichotomously as Hgb < 12 g/dl, we performed a GWA scan in 8304 AA women 50–79 years old from WHI. Two loci, Xq28 and 16p13, met the threshold of genome-wide significance. The G6PD rs1050828 A-variant was associated with a 1.49-fold (95% CI: 1.33–1.67) increased risk of anemia ($P = 3.3 \times 10^{-12}$). The index SNP at 16p13 (rs1088638) is located $\sim 20$ kb 3’ to POLR3K, and was associated with a 1.42-fold (95% CI: 1.26–1.60) increased risk of anemia ($P = 1.2 \times 10^{-8}$). We also constructed a composite RBC genetic risk score (GRS) by summing genotyped or imputed allele dosage at the 15 SNPs associated with at least one RBC trait in AA through the GWA scan, admixture mapping scan, conditional analyses or cross-ethnic transferability analyses described above. The GRS ranged from 5 to 19, with a median of 12. When modeled as a quantitative trait, the GRS was strongly associated with anemia ($P = 3.5 \times 10^{-18}$), explaining 1.4% of the anemia phenotypic variance, or 2.2% of the variance in Hgb concentration. When WHI participants were grouped into four GRS categories, those in the highest GRS category had a 1.95-fold increased risk of anemia (95% CI: 1.56–2.42) compared with those in the lowest GRS category ($P = 3.6 \times 10^{-9}$).

DISCUSSION

In this first reported GWAS meta-analysis of RBC traits in AAs, we report genome-wide associations for four loci (G6PD on Xq28, alpha-globin locus on 16pter, PRKCE on 2p21 and CD164 on 6q21). We also validated the association in AAs of variants in genes such as HFE and HBS1L-MYB, which have previously been associated with RBC traits in other ethnicities. At the alpha-globin locus, there appears to be allelic heterogeneity (particularly for MCHC), with both copy number variants and SNPs having apparent independent effects. At PRKCE, the variants associated with lower Hct in our AA sample appear to be distinct from another set of PRKCE variants that have been associated with Hgb, Hct and RBC count in Europeans and Japanese.

Hemizygous males and in some instances female carriers of the X-linked G6PD A- allele are predisposed to acute episodes of drug- or infection-induced hemolytic anemia. Under basal conditions, however, the G6PD A-allele is not generally thought to be associated with RBC abnormalities, and hemizygous G6PD A-individuals have been reported to have normal baseline red cell survival in the absence of oxidant stress (17). Nonetheless, the association of low RDW with G6PD deficiency may be due to low grade hemolysis resulting in an increase in the MCV with rightward shift of the overall distribution of RBC volume without change in the shape of the distribution (18). The G6PD A-variant is in LD with other nearby genetic variants that plausibly could influence Hgb or RBC morphology. TKTL1 encodes a transketolase enzyme that links the pentose phosphate pathway with anaerobic glycolysis, which constitutes the two major metabolic pathways for glucose utilization in human erythrocytes. MPP1 encodes the red cell membrane protein p55, a scaffolding protein that anchors the actin cytoskeleton to the plasma membrane by forming a ternary complex with protein 4.1R and glycophorin C (19).

Aside from genes involved in Hgb synthesis or metabolism, other genetic loci such as CD164 and PRKCE may be associated with RBC traits through effects on erythropoiesis. CD164 (endolyn) is an adhesive receptor present on early hematopoietic progenitors and maturing erythroid cells that...
regulates the adhesion of CD34+ cells to bone marrow stroma and affects migration and proliferation of hematopoietic stem cells and progenitor cells (20,21). The upstream region harboring the RBC trait-associated variants contains an erythroblastic leukemia cell line (K562)-specific cluster of histone modifications and ENCODE transcription factor ChIP-seq binding sites including those for GATA-2 and C-Jun. PRKCE encodes an isofrom of PKC, PKC epsilon, which is expressed in hematopoietic progenitor cells in a lineage- and stage-specific manner and appears to influence erythroid and megakaryocytic progenitor proliferation and differentiation by modulating the response of hematopoietic precursors to a tumor necrosis factor-related apoptosis-inducing ligand (22–24).

Though the finding was not validated in independent AA samples, one of our novel genome-wide significant associations in our discovery cohorts was the association of MCH and MCV with HMOX2, which encodes heme oxygenase-2, a constitutively expressed enzyme with a major role in heme catabolism. Heme induces expression of globin genes in erythocyte progenitor cells and thus plays an important role in erythropoiesis (12,25,26). The lead SNP in this region, rs7192051, is within 5 kb of predicted HMOX2 regulatory elements such as transcription factor binding sites, DNase sites and histone modification sites (27). Therefore, further study of this variant in larger, independent samples of AA may be warranted.

Our findings have potential clinical implications. Although previous studies have explored the role of common genetic variation in the regulation of these RBC phenotypes in populations of European and Asian descent (6,7,10), no systematic genetic association studies of these traits have been reported in African-ancestry populations. This is particularly important, as there are marked differences in these RBC indices among ethnic groups, and anemia is more prevalent in populations of African descent (28). While it appears that some of the phenotypic variations for RBC and other hematologic traits are controlled by genetic variation shared across ethnic groups (29), other RBC loci are relatively unique to Africans. Rare variants, which are not well captured by GWASs, and undetected common variants of more modest effect may account for additional genetic variance. Discovery and validation of these and additional genetic variants associated with RBC traits in other ethnic populations are likely to uncover new mechanisms and pathways that affect hematopoiesis and RBC turnover, offering insights that may inform further research into red cell biology. Indeed, recent reports have shown that genetic loci uncovered through an unbiased genome-wide study in human populations, together with follow-up functional studies incorporating gene expression, bioinformatic analyses and insights from mouse models and gene knockdown experiments, can greatly contribute to our understanding of the biological mechanisms underlying RBC production (30,31).

MATERIALS AND METHODS

Primary subjects and data collection

We performed GWA analysis of RBC traits in over 16,000 AAs from seven population-based cohorts that comprise the Continental Origins and Genetic Epidemiology Network (COGENT). The characteristics of each cohort were described in previous publications (14,29). Fasting blood samples were drawn and analyzed for RBC traits at designated clinical laboratories using an automated electronic cell counter. These counters directly measure Hgb concentration (in grams per deciliter), RBC count (in millions per microliter) and MCV, the average size of the RBC in femtoliters. Electronic cell counters calculate MCH, MCHC, Hct and RDW. Hct is the percentage of blood by volume that is occupied by RBC and is calculated by dividing the RBC count in millions/microliter by the MCV in femtoliters. MCH is the average amount of Hgb inside an RBC expressed in picograms and is calculated by dividing the Hgb concentration by the RBC count in billions per microliter, then multiplying by 10. The MCHC is the average concentration of Hgb in RBCs and is calculated by dividing Hgb in grams per deciliter by Hct. The RDW is a measure of the variance in RBC size and is calculated by dividing the standard deviation of RBC volume by the MCV and multiplying by 100.

All participants self-reported their race/ethnicity. Additional clinical information was collected by self-report and clinical examination. Participants provided written informed consent as approved by local Human Subjects Committees. Study participants who were pregnant or had a diagnosis of cancer or AIDS at the time of blood count were excluded.

Replication subjects and data collection

For validation of novel, genome-wide significant associations identified in the COGENT discovery sample, we performed association analyses in two independent population-based samples: ~7700 AA youths ages 8–21 years from Children’s Hospital of Philadelphia (CHOP) and 2010 AA adults from the Mount Sinai electronic Medical Records and Genomics (eMERGE) study. We also attempted to replicate novel loci in two other ethnic populations: 14,088 Japanese from RIKEN and up to 30,000 European Americans from CHARGE. Details of each validation cohort are provided under Supplementary Material.

Genotyping and quality-control

Genomic DNA was extracted from peripheral blood leukocytes and genotyping was performed on the Affymetrix 6.0 array or Illumina Omni or 1 M platforms within each cohort using methods described previously (14,29). DNA samples with a genome-wide genotyping success rate of <90% or sex discordance were excluded, as were genetic ancestry outliers (identified by cluster analysis using principal components analysis or multi-dimensional scaling). SNPs with a genotyping success rate of <95% or MAF <1%, monomorphic SNPs and SNPs that map to several genomic loci were removed from the analyses. Participants and SNPs passing basic quality control thresholds were imputed to >2.2 million autosomal SNPs based on HapMap2 haplotype data using a 1:1 mixture of Europeans (CEU) and Africans (YRI) as the reference panel. Details of the genotype imputation procedure have been described previously (14,29). Prior to discovery meta-analyses, SNPs were excluded if imputation quality metrics
coefficients by the square-root of the study-specific standard errors (SEs) of the regression coefficients and standard errors from study-level regression results for each SNP, to derive pooled estimates. Study-level results were corrected for genomic inflation factors ($\lambda$) by multiplying the standard errors (SEs) of the regression coefficients by the square-root of the study-specific $\lambda$. Meta-analyses were implemented in the METAL software.

For each phenotype, meta-analysis was conducted using inverse-variance weighted fixed-effects models to combine $\beta$ coefficients and standard errors from study-level regression results for each SNP, to derive pooled estimates. Study-level results were corrected for genomic inflation factors ($\lambda$) by multiplying the standard errors (SEs) of the regression coefficients by the square-root of the study-specific $\lambda$. Meta-analyses were implemented in the METAL software. Between-study heterogeneity of results was assessed by Cochran’s $Q$ statistic and the $I^2$ inconsistency metric. A threshold of $\alpha = 1 \times 10^{-8}$ was used to declare genome-wide statistical significance. This statistical threshold accounts for the greater nucleotide diversity and lower LD in African descent populations combined with testing of multiple, correlated RBC traits (31,33). We carried out replication testing of ‘suggestive’ SNPs selected on the basis of a more liberal significance threshold in our primary AA discovery GWAS ($P < 5 \times 10^{-8}$).

To assess the potential existence of multiple, independent variants influencing a trait at the same locus (allelic heterogeneity), regression analyses were repeated in the largest sample (WHI, $n = 8095$), conditional on the most strongly associated (index) SNP in that region. We also assessed the transferability to AAs of SNPs previously associated with RBC traits in populations of European or Japanese ancestry by assessing association with RBC traits in the COGENT discovery meta-analyses. For validation, we considered consistency of direction of effect, and assessed statistical significance using a simple Bonferroni adjustment for the total number of SNPs assessed, using a two-sided hypothesis test.

Local ancestry estimation and admixture mapping in WHI

For each AA individual in the WHI sample, locus-specific ancestry was estimated using an extension of the model described by Tang et al. (34). We used phased haplotype data from HapMap3 CEU and YRI individuals as reference panels. An admixture mapping analysis was performed in WHI to test for association between Hgb levels and ancestry at each genomic location (local ancestry), while adjusting for the first 10 principal components, regions of recruitment, clinical trial, age and age-squared. The critical value for genome-wide significance level of admixture mapping is substantially lower than the genotype test due to the extensive correlation in local ancestry between adjacent markers that result from the recent admixture in AAs. We therefore adopted an empirically determined genome-wide significance threshold of $P < 7.1 \times 10^{-6}$, which corresponds to a Bonferroni correction of $\sim 7000$ independent tests (35).

Copy number variation (CNV) analysis using 1000 genomes data

We used the 1000 Genomes sequencing data to investigate CNVs at the chromosome 16 p31 alpha-globin locus, studying 946 African-ancestry samples at roughly 4x sequencing coverage. As a result of noise in depth-based genotyping at this locus (due to low-pass sequencing, high %GC and potential overlapping variants), some of our analyses were confined to the 76 YRI samples, which have higher sequence coverage in 1000 Genomes data and more complete genotyping (call rate 84% at 95% CI).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGMENTS

The authors wish to acknowledge the support of the National Heart, Lung and Blood Institute and the contributions of the involved research institutions, study investigators, field staff and study participants of Atherosclerosis Risk in Communities (ARIC), Coronary Artery Risk in Young Adults (CARDIA), Jackson Heart Study (JHS) and Broad Institute in creating the Candidate-gene Association Resource for biomedical research (CARe; http://public.nhlbi.nih.gov/GeneticsGenomics/home/care.aspx).

The authors also wish to thank the investigators, staff and participants of GeneSTAR, Health ABC, Healthy Aging in Neighborhoods of Diversity across the Life Span Study (HANDLS) and Women Health Initiative (WHI) for their important contributions. A listing of WHI investigators can be found at http://www.whiscience.org/publications/WHI_investigators_shortlist.pdf.

We thank all the children who donated blood samples for genetic research purpose. The CHOP study was funded by the Institute Development Funds to the Center for Applied Genomics at the Children’s Hospital of Philadelphia and an Adele S. and Daniel S. Kubert Estate gift to the Center for Applied Genomics.

The Mount Sinai IPM Biobank Program is supported by The Andrea and Charles Bronfman Philanthropies.

The authors acknowledge the essential role of the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium in development and support of this manuscript. CHARGE members include the Rotterdam Study (RS), Framingham Heart Study (FHS), Cardiovascular Health Study (CHS), the NHLBI’s Atherosclerosis Risk in Communities (ARIC) Study and the NIA’s Iceland Age,
Gene/Environment Susceptibility (AGES) Study. The collaboration of studies such as the Health Aging and Body Composition Study (Health ABC), the Baltimore Longitudinal Study of Aging (BLSA), the Invecchiare in Chianti Study (InChianti), and the Heart and Vascular Health Study (HVH) also played a vital role.

The following parent studies contributed study data, ancillary study data and DNA samples through the Broad Institute (N01-HC-65226) to create this genotype/phenotype data base for wide dissemination to the biomedical research community:

Atherosclerosis Risk in Communities (ARIC): University of North Carolina at Chapel Hill (N01-HC-55015), Baylor Medical College (N01-HC-55016), University of Mississippi Medical Center (N01-HC-55021), University of Minnesota (N01-HC-55019), Johns Hopkins University (N01-HC-55020), University of Texas, Houston (N01-HC-55017), University of North Carolina (N01-HC-55018). Other NIH support contributing to the GWAS in ARIC are: R01HL087641, R01HL59367, R01HL86694, U01HG004402 and HHSN268200625226C.

Coronary Artery Risk in Young Adults (CARDIA): University of Alabama at Birmingham (N01-HC-48047), University of Minnesota (N01-HC-48048), Northwestern University (N01-HC-48049), Kaiser Foundation Research Institute (N01-HC-48050), University of Alabama at Birmingham (N01-HC-95095), Tufts-New England Medical Center (N01-HC-45204), Wake Forest University (N01-HC-45205), Harbor-UCLA Research and Education Institute (N01-HC-05187), University of California, Irvine (N01-HC-45134, N01-HC-95100).

Jackson Heart Study (JHS): Jackson State University (N01-HC-95170), University of Mississippi (N01-HC-95171), Tougaloo College (N01-HC-95172).

Healthy Aging in Neighborhoods of Diversity across the Life Span Study (HANDLS): this research was supported by the Intramural Research Program of the NIH, National Institute on Aging and the National Center on Minority Health and Health Disparities (intramural project Z01-AG000513 and human subjects protocol 2009-149). Data analyses for the HANDLS study utilized the high-performance computational capabilities of the Biowulf Linux cluster at the National Institutes of Health, Bethesda, MD, USA (http://biowulf.nih.gov).

Health ABC: this research was supported by NIA contracts N01AG62101, N01AG62103 and N01AG62106. The GWAS was funded by NIA grant 1R01AG032008-01A1 to Wake Forest University Health Sciences and genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University, contract number HHSN268200782096C. This research was supported in part by the Intramural Research Program of the NIH, National Institute on Aging.

GeneSTAR: this research was supported by the National Heart, Lung and Blood Institute (NHBLI) through the PROGENI (U01 HL72518) and STAMPEED (R01 HL087698-01) consortia. Additional support was provided by grants from the NIH/National Institute of Nursing Research (R01 NR08153), and the NIH/National Center for Research Resources (M01-RR000052) to the Johns Hopkins General Clinical Research Center.

WHI: the WHI program is funded by the National Heart, Lung and Blood Institute, National Institutes of Health, US Department of Health and Human Services through contracts N01WH22110, 24152, 32100–2, 32105–6, 32108–9, 32111–13, 32115, 32118–32119, 32122, 42107–26, 42129–32 and 44221.

AGES: The Age, Gene/Environment Susceptibility Reykjavik Study is funded by NIH contract N01-AG-12100, the NIA Intramural Research Program, Hjartavernd (the Icelandic Heart Association) and the Althingi (the Icelandic Parliament).

Framingham: the National Heart, Lung and Blood Institute’s Framingham Heart Study is a joint project of the National Institutes of Health and Boston University School of Medicine and was supported by the National Heart, Lung, and Blood Institute’s Framingham Heart Study (contract No. N01-HC-25195) and its contract with Affymetrix, Inc. for genotyping services (contract No. N02-HL-6-4278). Analyses reflect the efforts and resource development from the Framingham Heart Study investigators participating in the SNP Health Association Resource (SHArE) project. A portion of this research was conducted using the Linux Cluster for Genetic Analysis (LinGa-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center.

InChianti: the InChianti Study was supported as a “targeted project” (ICS 110.1RS97.71) by the Italian Ministry of Health, by the US National Institute on Aging (Contracts N01-AH-91643, N01-AH-821336, 263 MD 9164 13 and 263 MD 821336) and in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health, USA.

Rotterdam: Rotterdam Study GWAS database of the Rotterdam Study was funded through the Netherlands Organization of Scientific Research NWO (no. 175.010.2005.011, 911.03.012) and the Research Institute for Diseases in the Elderly (RIDE). This study was supported by the Netherlands Genomics Initiative (NGI)/NWO project number 050 060 810 (Netherlands Consortium for Healthy Aging). We thank Dr Michael Moorhouse, Pascal Arp, Mila Jhamai, Marijn Verkerk and Sander Bervoets for their help in creating the genetic database. We thank the laboratory technicians Jeannette M Vergeer—Drop, Bernadette H M van Ast—Copier, Andy A L J van Oosterhout, Sue Ellen Mauricia, Andrea J M Vermeij—Verdoold, Els Halmeijejer—van der Plas, Debby M S Lont and Hasna Kariouh for their help in phenotype assessment. The Rotterdam Study is supported by the Erasmus Medical Center and Erasmus University, Rotterdam; the Netherlands organization for scientific research (NWO), the Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Netherlands Heart Foundation, the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports, the European Commission (DG XII) and the Municipality of Rotterdam.

RIKEN: we would like to thank all the staff of the Laboratory for Statistical Analysis at RIKEN for their technical assistance. The BioBank Japan Project was supported by Ministry of Education, Culture, Sports, Science and Technology, Japan.
FUNDING

Additional support for this work was provided by NIH (R01 HL71862-06 and ARRA N000949304 to A.P.R.). Some of the results of this paper were obtained by using the program package S.A.G.E., which is supported by a US Public Health Service Resource Grant (RR03655) from the National Center for Research Resources. Additional support came from the National Cancer Institute (grant R25CA094880 to U.M.S.).

REFERENCES