Genomic characterization of the RH locus detects complex and novel structural variation in multi-ethnic cohorts

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Abstract

Purpose—Rh antigens can provoke severe alloimmune reactions, particularly in high-risk transfusion contexts such as, sickle cell disease. Rh antigens are encoded by the paralogs, RHD and RHCE, located in one of the most complex genetic loci. Our goal was to characterize RH genetic variation in multi-ethnic cohorts, with the focus on detecting RH structural variation (SV).

Methods—We customized analytical methods to estimate paralog-specific copy number from next generation sequencing (NGS) data. We applied these methods to clinically-characterized samples, including four WHO genotyping references and 1135 Asian and Native American blood donors. Subsequently, we surveyed 1715 African American samples from the Jackson Heart Study.
Results—Most samples in each dataset exhibited SV. SV detection enabled prediction of the immunogenic RhD and RhC antigens in concordance (>99%) with serological phenotyping. RhC antigen expression was associated with exon 2 hybrid alleles (RHCE*CE-D(2)-CE). Clinically-relevant exon 4-7 hybrid alleles (RHD*D-CE(4-7)-D) and exon 9 hybrid alleles (RHCE*CE-D(9)-CE) were prevalent in African Americans.

Conclusions—This study shows custom NGS methods can accurately detect RHSV, and that SV is important to inform prediction of relevant RH alleles. Additionally, this study provides the first large NGS survey of RH alleles in African Americans.

Keywords
RH; structural variation; hybrid allele; blood group; next generation sequencing

Introduction
Blood group systems are inherited entities with direct clinical importance in transfusion and transplantation medicine. Blood group antigens are expressed on the surface of red blood cells (RBCs); most are glycoproteins with specificity determined by their oligosaccharide or amino acid sequence. The genes that encode nearly all blood group systems are known and several exhibit substantial genetic complexity and population-specific heterogeneity.

The Rh blood group system contains highly immunogenic antigens and commonly exhibits complex genetic variation including structural variation (SV). It is comprised of >50 different antigens, including the polymorphic RhD (D) and RhCE (C, c, E and e) antigens. This antigenic diversity stems from genetic variation in two homologous paralogs, RHD and RHCE, which lie in close proximity at the RH locus. At present, RHD and RHCE encode >280 reported alleles (haplotypes) which include RHD gene deletions and RHD-RHCE hybrids. This level of complexity poses clinical challenges and can provoke significant rates of Rh allosensitization. In one study up to 45% of chronically transfused African American patients with sickle cell disease (SCD) experienced allosensitization, primarily due to undetected variation in the Rh blood group system. High rates of Rh alloimmunization persist even when patients receive transfusions from serologically-matched African American donors, demonstrating the need for higher resolution Rh blood group information.

Serology is the mainstay of clinical RBC typing, including Rh. However, serology has known limitations which can be overcome with molecular testing. In clinical laboratories, DNA-based prediction is typically performed using genotyping platforms (e.g. SNP arrays), Sanger sequencing, and variant-specific methods (e.g. PCR-SSP, RFLP). These can be used to characterize patients with unexpected alloantibodies, patients at risk for allosensitization or recently transfused patients. DNA-based methods are also used to identify alleles for which antisera are unavailable and to test for paternal zygosity of the D antigen for pregnancies at risk of hemolytic disease of the fetus and newborn. In addition, RBC genotyping methods can aid in discriminating Rh phenotypes which can produce indeterminate or conflicted serological results. Genotyping methods can discriminate RH partial alleles which lead to missing antigen epitopes and antibody formation when exposed
to the conventional antigen\textsuperscript{10}. Genetic methods can also discern weak \textit{RH} alleles which reduce the quantity of antigens on the surface of RBCs but maintain display of the same epitopes as conventional Rh antigens\textsuperscript{11}.

Currently, there is growing interest in applying next generation sequencing (NGS) to Rh antigen prediction\textsuperscript{12–16}. NGS can systematically survey for genetic variants, including SV, and is scalable for high-throughput screening. To date, efforts to detect \textit{RH} variation using NGS have shown success in detecting clinically-relevant variation but technical challenges have limited the interpretation of \textit{RH} variation and the detection of SV\textsuperscript{12–16}. Our primary goal was to develop an \textit{RH} genotyping method that addressed RH SV, including \textit{RHD-RHCE} hybrid alleles that alter Rh antigen expression. We customized paralog-specific SV analyses\textsuperscript{17} and first applied these methods to four WHO RBC genotyping reference samples and to 1135 clinically immunophenotyped and clinically genotyped samples from Asian and Native American blood donors\textsuperscript{18}. Subsequently, we applied our methods to survey \textit{RH} variation in 1715 unrelated African American samples from the Jackson Heart Study (JHS). This cohort was whole genome sequenced (WGS) by the NHLBI Trans-Omics for Precision Medicine (TOPMed) program and analyzed in this study to provide the first NGS survey of \textit{RH} alleles for this population.

Materials and methods

Samples

We purchased four WHO reference DNAs (RBC1, RBC4, RBC5, RBC12) from the National Institute for Biological Standards and Control. WHO references were clinically characterized and genotyped by a variety of methods\textsuperscript{19} but to our knowledge, not by NGS. These samples represent common European (RBC1, RBC4, RBC5) and African (RBC12) \textit{RH} alleles (Table 1) including alleles encoding D positive (D\textsuperscript{+}), D negative (D\textsuperscript{−}), and combinations of C, c, E, e antigens (Table 1)\textsuperscript{19}.

Asian and Native American samples (N=1168) were selected from a prior population study of blood donors\textsuperscript{18}. Blood samples were collected from consented volunteer donors by Bloodworks Northwest. All samples were previously clinically tested for D and C antigens by serology and for C, c, E and e genotype using a SNP array, HEA BeadChip\textsuperscript{TM} Kit (Bioarray Solutions Ltd., Immucor)\textsuperscript{18}. This sample set included 82 samples discrepant between C serology and SNP (N=16) or indeterminate on the SNP array (N=66).

African American samples (N=1715) were selected from JHS samples (phs000964) WGS by the NHLBI TOPMed program. The JHS is a community-based observational study in which individuals were recruited from the tri-county area surrounding Jackson, Mississippi, including a subset who participated in the Atherosclerosis Risk in Communities Study\textsuperscript{20}. The samples in this study were randomly selected from the maximum unrelated JHS sample set as identified using KING v1.4.0 (no individuals with a 1st- or 2nd-degree relationship).

Library preparation and next generation sequencing

DNA libraries from WHO and Asian and Native American samples were captured with a targeted panel designed to capture 41 blood group-relevant genes (1473Kb; Nimblegen,
Table S1). For RH, this panel captured 269Kb of continuous sequence including introns, exons, UTRs, and promoter regions. Library preparation followed a shotgun library construction method\textsuperscript{21} and were hybridized in multiplex (22–24 samples per reaction). Sequencing was performed on Illumina HiSeq 2500 machines using paired-end 100bp reads to a mean coverage of approximately 150x. In total, 1139 samples (1135 Asian and Native American and 4 WHO samples) passed sequencing quality thresholds. No samples were excluded based on performance at the RH locus.

JHS African American samples were WGS by the NHLBI TOPMed program. Library preparation for JHS samples similarly followed a shotgun library construction method\textsuperscript{21}. Sequencing was performed on Illumina HiSeq X machines using paired-end 150bp to a mean coverage of approximately 30x. Raw sequence data was aligned to the human reference genome (GRCh37) using BWA-MEM\textsuperscript{22}.

Detection of RH SV

SV in RHD and RHCE was identified using an adaptation of methods described previously\textsuperscript{17}. SV was identified by leveraging singly unique nucleotides (SUNs) within a repeat masked, pairwise sequence alignment of RHD and RHCE. SUNs were similarly identified in the Rhesus boxes flanking RHD\textsuperscript{3}. SUNs were used to anchor DNA sequence k-mers (k=70) which were screened for uniqueness against GRCh37 (BLAT v3.5, UCSC). K-mers were omitted if they contained >1 perfect match. Read depth was estimated for remaining k-mers using a mapping quality >= 40. Copy number was estimated by normalizing using sequencing depth and mean read depth for samples visually confirmed to have no SV. In total, 9189 k-mers for RHD and RHCE and 2054 k-mers in the Rhesus boxes informed SV analyses. K-mers were distributed across the RH locus except for RHCE exon 10. RHD exon 10 k-mers were identified in alignment of the Rhesus boxes. SV breakpoints were identified by change point analysis using the R changepoint package\textsuperscript{23}. SV impacting RH exons was prioritized.

Detection of RH SNVs and indels and RH allele identification

Single nucleotide variants (SNVs) and small insertions and deletions (indels) were genotyped using GATK HaplotypeCaller and haplotype phased using statistical methods (Beagle v4.1)\textsuperscript{24}. Functional annotation was incorporated using SeattleSeq Annotation (http://snp.gs.washington.edu/SeattleSeqAnnotation138/). All variants were annotated relative to the RefSeq transcripts, NM_016124.3 (RHD) and NM_020485.4 (RHCE). To identify RH alleles, SNVs, indels, and SVs were cross-referenced with alleles listed by the International Society of Blood Transfusions (ISBT) v2.0 110914, supplemented by information from Rhesusbase\textsuperscript{4}. For cross-referencing, cDNA coordinates associated with ISBT alleles were converted to GRCh37 coordinates. Chr1:25643553 (NM_016124.3:c.1136) and chr1:25747230 (NM_020485.4:c.48) are variant in GRCh37 relative to ISBT v2.0 110914. Novel variants were selected based on their absence in ISBT v2.0 110914 and prioritized as impactful based on variant function (e.g., predicted loss of function). Genotype quality (GQ) was assessed for novel and annotated ISBT SNVs and indels. Chr1:25643553 which encodes the primary variant of the DAU cluster (DAU0 allele), had variable GQ because it is present in a multiply-mapping region in exon 8. GQ was low when
Chr1:25643553 was variant relative to GRCh37 which contains the DAU0 variant (NM_016124.3:c.1136T). Low GQ resulted from low coverage of RHD exon 8 due to the misalignment of reads from this region to its highly homologous region in RHCE.

Quantitative multiplex PCR of short fluorescent fragments

To validate NGS-detected RSV, we performed quantitative multiplex PCR of short fluorescent fragments (QMPSF)\(^25\). Fluorescently-tagged primers were used to amplify WHO and 18 Asian and Native American samples (N=22) representative of RHD gene deletions, RHD-RHCE hybrid alleles or deletions/duplications, and to have no SV. QMPSF primers amplified gene-specific RHD and RHCE exons. F9 exon 7 and HFE exon 2 amplicons served as positive amplification markers and as normalization controls. QMPSF products were separated via capillary gel electrophoresis (ABI 3130xl, Applied Biosystems). Fluorescence peaks were analyzed using the R Fragman package\(^26\) and normalized using the maximum HFE peak height.

Combinatorial PCR and Sanger sequencing

To confirm RHCE*CE-D(2)-CE alleles (see Results) as hybrid alleles, we designed allele-specific long-range PCRs. Primer pairs were designed to target unique sequences between intron 1 - exon 2 and exon 2 - exon 3 (Table S2). PCRs were performed pairing RHD- and RHCE-specific primers in a combinatorial manner. PCRs consisted of 12.5 μL of Q5 Hot Start High-Fidelity Master Mix (NEB #M0494S), 0.5 μM of forward and reverse primers, and 50 ng DNA. Cycling conditions for intron 1 – exon 2 were: 98°C for 30 sec followed by 30 cycles of 98°C for 10 sec, 76°C for 30 sec, 72°C for 6 min, and 72°C for 2 min. Cycling conditions for exon 2 – exon 3 were identical except annealing and extension temperatures were 68°C for 30 sec and 72°C for 3 min, respectively. PCR was performed on 21 samples (including WHO samples). Two samples with PCR-confirmed RHCE*CE-D(2)-CE events were cloned into pMiniT vector (NEB PCR Cloning Kit). Insert-positive clones were Sanger sequenced with vector-specific and gene-agnostic primers (Table S3). Products were aligned against RHD and RHCE (GRCh37) using Geneious R8 software.

Results

NGS-based characterization of WHO reference samples

We used custom paralog-specific NGS analyses to detect SV at the RH locus. These analyses detected SV in all WHO reference samples. In RBC1 and RBC4, NGS analyses detected SV signals (Figure 1A and 1C) indicative of RHD-to-RHCE hybrid alleles (RHCE*CE-D(2)-CE), similar to alleles previously associated with the C+ phenotype\(^27,28\). Zygosity for this event was consistent with C and c phenotypes (Table 1)\(^19\). In RBC5, analyses detected a homozygous RHD deletion causal for its reported D− phenotype (Figure 1B, Table 1). In RBC12, analyses detected a hemizygous RHD deletion and SV indicative of an exon 9 hybrid allele (RHCE*CE-D(9)-CE) (Figure 1D). The latter event was not reported previously for RBC12\(^19\). Each SV event was validated by QMPSF (Figure 1). The one discrepancy between QMPSF and NGS analyses related to whether SV in RBC12 impacts exon 8 in addition to exon 9: QMPSF amplification is suggestive of exon 8 SV, but NGS-based breakpoints predicted exon 8 to be unaffected (Figure 1D). The homozygous RHD
deletion in RBC5 and RHCE*CE-D(2)-CE alleles predicted in RBC1 and RBC4 were additionally validated by allele-specific PCR (Figure S1). PCR confirmed RHCE*CE-D(2)-CE events to be hybrid alleles and not separate SV events.

RBC1, RBC4, and RBC12 also harbored SNVs indicative of previously characterized alleles (Table 1). In RBC1 and RBC4, we detected variants indicative of weak RHCE alleles (Table 1). RBC12 contained hemizygous RHD SNVs representative of an RHD null allele including a 37 bp insertion and the stop-gained variant casual for its D– phenotype (Table 1). RBC12 also harbored missense variants associated with the RHCE*01.20.02 allele and the V+VS+ phenotype, a known finding for RBC1219.

NGS-based characterization of clinically-characterized Asian and Native American samples

Paralog-specific analyses detected SV in 90% of Asian and Native American samples (Figure 2A, genotypes listed in Tables S4–S5). Note we do not provide representative allele frequencies for these populations because this sample set was selected in a non-random manner. The RHD deletion was detected in 373 samples (100 homozygotes and 273 hemizygotes, Figure 2A). The predicted mean length for this event was 70154 ± 1888bp and exhibited recombination signals between the flanking Rhesus boxes (similar to Figure 1B)3. RHCE*CE-D(2)-CE alleles were detected in 832 samples (388 homozygotes and 444 heterozygotes, Figure 2A). The mean length for this event was 4953 ± 238bp, with the most common variant being 4959bp in size (n=823) but other differently-sized RHCE*CE-D(2)-CE were detected and ranged in size from 1038bp to 7183bp. In 25 samples, we detected SV events impacting other RHD and RHCE exons, including RHD gene duplications and extensive RHD-RHCE hybrid alleles (see Figures 2A and 3). Three of these events are annotated in ISBT v2.0 110914: RHD*D-CE(4-7)-D (RHD*01N.07, Figure 3B), RHD*D-CE(3-9)-D (RHD*01N.04, Figure 3C) and RHD*D-CE(4-8)-D (RHD*01N.07). RHD*D-CE(4-7)-D and RHD*D-CE(4-8)-D share ISBT allele names because previous genotyping methods could not determine whether exon 8 was affected4.

Standard SNV/indel calling methods detected SNVs associated with established serological phenotypes (Table S6, Tables S4–S5). In RHD, SNVs indicative of 2 RHD null allele, 7 weak D and Del alleles, and 6 partial D alleles were detected (Table S6). Six samples with weak D and partial D alleles were predicted to inform D phenotype because of compound heterozygosity with RHD deletions. For example, one serologically D– sample harbored a splice site variant (RHD*DEL1) and was hemizygous for a RHD gene deletion. In RHCE, variants were indicative of 10 RHCE alleles (Table S6). Predicted loss-of-function variants not reported in ISBT included 1 splice site variant in RHD and 1 splice region variant in RHCE (Table S7).

QMPSF and allele-specific PCR for clinically-characterized Asian and Native American samples

Using QMPSF, we tested 18 samples which collectively represented a variety of SV events (Figure 3, Figures S2–S4). QMSP validated NGS-predicted events in all samples tested. As
above, the discrepancy between QMPSF and NGS-based analyses related to the size of SV in RHCE*CE-D(9)-CE and RHCE*CE-D(8-9)-CE alleles (Figures S3–S4).

Allele-specific PCRs further validated samples encompassing no SV, RHD deletions, and RHCE*CE-D(2)-CE events (N=17, Figure S5–S6). Cloning and sanger sequencing of two samples exhibiting the common RHCE*CE-D(2)-CE confirmed a RHCE intron 1 SNV identified by NGS analysis in the larger dataset (chr1:25736299, Figure S7). This SNV has not been previously reported and is positioned consistent with the RHCE-RHD intron 1 breakpoint. The RHCE*CE-D(2)-CE intron 2 breakpoint in these two samples was defined by a 109 bp insertion which has been previously reported28.

**Comparisons between NGS-based RH alleles with SNP array-based typing and D and C serology**

In Asian and Native American samples, NGS-based RH alleles were predicted blind to serology and SNP genotyping. NGS-genotype considered SNVs, indels, and SVs within each sample. Briefly, D− in this dataset was mainly predicted from homozygous loss of RHD. However, one D− sample was predicted to be DEL due to hemizygous loss of RHD and the presence of the RHD*DEL1 allele, a relevant distinction as DEL can provoke anti-D29. Another D− sample exhibited hemizygous loss of RHD and a deletion of RHD exon 3 (see example in Figure 3F), predicting a partial D phenotype. C and c antigens were predicted based on the presence of RHCE*CE-D(2)-CE alleles, while E and e genotypes were assigned using the ISBT annotated RHCE missense (NM_020485.4:c.676G>C).

Subsequent comparisons of NGS-genotype with serology showed agreement with the D antigen in 99.8% of samples and with the C+ antigen in 99.2% of samples. Comparisons with clinical SNP-genotype showed 99.9% agreement for prediction of E and e antigens. Direct comparison between all C SNP-array predictions and all C NGS-based predictions was not possible due to indeterminate SNP array results in 66 samples (see Methods). In samples that did have SNP-based c and C predictions, our results were 99.5% and 99.7% concordant, respectively. All 66 samples with indeterminate C SNP array calls were predicted by NGS in agreement with serology. Most C SNP indeterminate samples (59/66) were NGS-predicted to be C+; all 66 of these samples were 100% concordant between NGS and serology. Moreover, NGS resolved 9 of 16 samples that were discordant between C SNP array-based genotype and C serology.

**NGS-based characterization of African American samples**

RHSV was detected in 61% of African American samples (Figure 2B, genotypes listed in Tables S8–S9). RHD gene deletions were present in 579 samples (mean length = 70572 ± 3352bp) including 56 homozygotes and 523 hemizygotes (Figure 2B). RHCE*CE-D(2)-CE events were present in 406 samples (mean length = 5216 ± 796bp) including 33 homozygotes and 373 heterozygotes. We additionally detected hybrid alleles at relatively high prevalence including: RHD*D-CE(4-7)-D (RHD*01N.07) and RHCE*CE-D(9)-CE (Figure 2B).

SNVs identified in African American samples were indicative of several RHD null alleles, weak D alleles, partial D alleles and RHCE alleles (Table S10–11, Tables S8–S9). SNV-
based RH alleles with allele frequencies >1% are shown in Table 2, with previously reported SNP-array based allele frequencies\textsuperscript{30}. Note we detected DAU alleles in several samples (Table 2) but GQ for the primary variant was variable due to sequence homology. In African American samples, we also identified 5 predicted loss-of-function variants not reported in ISBT. In RH\textit{D}, this included 1 splice site variant and 2 frameshifts. In RH\textit{CE}, this included 1 splice site variant and 1 stop-gained variant (Table S12).

**Discussion**

In recent years, there has been growing interest in applying NGS to predict Rh antigens\textsuperscript{12–16}. This has been motivated, in part, by the high rates of Rh allosensitization in multiply-transfused patients, particularly, in African American patients with sickle cell disease\textsuperscript{31,32}. In this population, high rates of allosensitization persist even after patients have been matched by serology for D, C, c, E, e antigens and received racially-matched RBC transfusions\textsuperscript{5}. Evidence suggests this is primarily due to the presence of undetected RH variation in patients and donors\textsuperscript{5}, emphasizing the need to predict Rh antigens in a systematic and locus-informed manner.

To this end, studies have shown NGS is a viable approach for predicting RBC antigens\textsuperscript{12–16}. However, these studies have applied NGS on a limited scale, mostly to a small number of well-characterized individuals and have been largely insensitive in identifying complex SV, including RH\textit{D}-RH\textit{CE} hybrid alleles\textsuperscript{12–16}. Here, we show customized NGS-based methods can detect known and novel RH variation in two large cohorts comprised of individuals of Asian American, Native American, and African American descent.

This customized RH method leverages nucleotide differences between RH\textit{D} and RH\textit{CE} to exclude mapping artifacts associated with NGS short read data. This approach enabled SV detection in previously problematic regions including exons 1, 2, and 8\textsuperscript{12,13,15,16} by using information in flanking intronic sequences. Importantly, this approach performs robustly both in targeted capture and whole genome sequencing, indicating it is generalizable to datasets where NGS spans the RH locus. In addition, this approach provides the ability to detect RH SV at scale to measure allele frequencies in large genomic datasets.

We specifically detected RH\textit{CE}*CE-D(2)-CE hybrid alleles as prevalent across all datasets. Similar alleles were reported previously and associated with C+ expression, such as by Carrit et al. (1997)\textsuperscript{28}. However, at present there is lack of clarity as to whether these alleles are causal for C+ expression. Recent exome studies report exon 2 read depth signals associated with C+, which is indicative of SV\textsuperscript{15,16}; however, the majority of modern literature including RH\textit{CE} genotyping references report exon 1 and 2 RH\textit{CE} SNVs as causal for C+\textsuperscript{2}. In these large-scale analyses, the most common RH\textit{CE}*CE-D(2)-CE allele spanned ~5Kb and a subset of samples with RH\textit{CE}*CE-D(2)-CE were validated by multiple orthogonal methods. Sanger sequencing characterized the common RH\textit{CE}*CE-D(2)-CE intronic breakpoints, including an RH\textit{CE} 109bp “insertion” currently used in C genotyping as well as a previously undetected SNV at the RH\textit{CE}*CE-D(2)-CE breakpoint in RH\textit{CE} intron 1. Our analyses also show RH\textit{CE}*CE-D(2)-CE correctly predicted C serology in
99.2% of clinically-characterized samples, strongly supporting that \textit{RHCE*CE-D(2)-CE} is causal for C+ antigen expression.

We further identified multiple \textit{RH} hybrid alleles consistent with named ISBT alleles. We identified the clinically known \textit{RHD*01N.07 (RHD*D-CE(4-7)-D)} in both large cohorts and validated this NGS signature by QMSPF (Figure 3B). This allele was prevalent (2.5%) in African Americans, consistent with a recent study reporting this allele to occur in 2.9% of African American individuals and sickle cell disease patients\textsuperscript{30} and 10-fold higher than in European populations\textsuperscript{33}.

Our methods identified novel \textit{RH}SV alleles which impacted exons 8 and 9. This finding suggests previous genotyping efforts may have been hindered by sequence homology across these exons, a notion supported by our finding of \textit{RHCE*CE-D(9)-CE} allele in the well-characterized WHO reference, RBC12. Notably, \textit{RHCE*CE-D(9)-CE} was common (3.9%) in African American samples. In Asian and Native American samples, QMPSF validated \textit{RHCE*CE-D(9)-CE} alleles but also showed amplification of exon 8 in a subset of samples. QMPSF infers exon 8 copy number through amplification of nearby intronic sequences, leading us to hypothesize intrinsic variation associated with \textit{RHCE*CE-D(9)-CE} may have impacted this QMPSF result. Alternatively, our NGS-based methods could have excluded exon 8 as part of the SV due the breakpoint being in a region of high homology.

Although our analyses were focused on SV, we genotyped SNVs indicative of known ISBT alleles. Notably, in an Asian American sample we detected hemizygous loss of \textit{RHD} and an \textit{RHD} splice site variant causal for the DEL phenotype (\textit{RHD*DEL1}). This correlated with the D– phenotype reported in this blood donor, but this is a relevant finding as DEL is not null for D protein expression and can provoke D alloimmunization. This DEL allele has been reported as a common cause of D– in Asian populations\textsuperscript{29}; although, in this study of Asian Americans homozygous loss of \textit{RHD} was the primary cause of D–. We further found weak and partial \textit{RH} alleles known to be prevalent and clinically consequential in African populations (Table 2). Consistent with previous NGS work\textsuperscript{15}, we detected common \textit{RHD} SNVs in African Americans indicative of DAU alleles. The primary DAU00 SNV had variable genotype quality leading us (and others)\textsuperscript{15} to provide caution when interpreting DAU allele frequencies derived from NGS. The limitation we observed was low coverage in the absence of the DAU0 SNV due to increased sequence homology with \textit{RHCE}. Additional customization of NGS analyses, such as the use of an alternative mapping locus, should resolve this limitation. Separately, in RBC12, we detected SNVs indicative of \textit{RHD*04N.01} and \textit{RHCE*01.20.02}. In African Americans, we detected \textit{RHD*04N.01} at a frequency of ~3% (Table 2), consistent with allele frequencies reported by other studies in individuals of African descent\textsuperscript{30}. \textit{RHD*04N.01} co-occurred with hemizygous \textit{RHD} gene deletions predicting D– in 1.4% of African Americans, while 3.2% of African Americans were D– due to homozygous \textit{RHD} gene deletions.

In summary, our results show the ability of NGS-based methods to systematically identify \textit{RH}SV and detect known, complex and novel \textit{RH}SV. This represents the first scale study of \textit{RH} variation in Asian and Native Americans and the largest population survey of \textit{RH}SV in African Americans to date. We found complex SV to be common suggesting additional
clinically-relevant RH variation remains undiscovered. Altogether, this study shows locus-informed genomic approaches can detect RH alleles and characterize complex genetic variation in large and diverse datasets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structural variation detected in WHO reference samples
A, B, C and D panels show paralog-specific analyses (top) with corresponding QMPSF results (below) for RBC1, RBC5, RBC4 and RBC12, respectively. Each paralog-specific panel shows scale RHD (blue) and RHCE (red) gene schematics (top) and the location of single unique nucleotides within genic regions (black) and in Rhesus boxes (gray). Gray circles within panels represent normalized mean read depth for k-mers corresponding to SUNs. The dashed gray line denotes a copy number of 2; solid blue and red lines indicate inferred copy numbers over the RHD and RHCE genes, respectively. In QMPSF panels, QMPSF peak heights are fluorescence measurements normalized to the amplified exon for HFE. The F9 peak serves as an additional positive amplification control. Light yellow panels in QMPSF results for RBC5 (B) and RBC12 (D) highlight RHD whole gene deletions. (*) highlight amplicons with results indicative of structural variation. Note: In (D), QMPSF amplification of exon 8 is suggestive of structural variation, although exon 8 by NGS analyses is predicted to be unaffected.
Figure 2. Schematic summary of structural variation detected in 1135 Asian and Native American samples (A) and 1715 Jackson Heart Study, African American samples (B)

For (A), allele frequencies are not reported because this dataset was selected non-randomly from a prior study. In both (A) and (B), RHD and RHCE exons are depicted by black and gray boxes (respectively) and oriented 5′ to 3′ for simplification. Yellow boxes correspond to exons exhibiting duplication events. The RHD schematic summarizes structural variants detected in RHD with its corresponding ISBT allele name (if present) and the sample number (A) or its corresponding allele frequency estimate shown as a percent (B). The RHCE schematic summarizes structural variants detected in this gene with its corresponding ISBT allele name (if present) and the sample number (A) or its corresponding allele frequency estimate (B). For both RHD and RHCE, structural variation is ordered by Sample No. (A) or allele frequency estimate shown as a percent (B).
Figure 3. Selected structural variation detected in Asian and Native American samples
A, B, C, D, E, and F panels show paralog-specific analyses (top) and corresponding QMPSF results (below) for samples exhibiting: no structural variation (A), a RHD*CE*-D(4-7)-D (B), a RHD*CE*-D(3-9)-D and RHCE*CE*-D(2)-CE (C), a RHCE*CE*-D(2-9)-CE (D), a RHD duplication (E) and a RHD exon 3 deletion and RHCE*CE*-D(2)-CE (F), respectively.
Paralog-specific panels show scale RHD (blue) and RHCE (red) gene schematics (top) and the location of single unique nucleotides within genic regions (black) and in Rhesus boxes (gray). Gray circles within panels represent normalized mean read depth for 70-mers corresponding to single unique nucleotides. The dashed gray line denotes copy number of 2; solid blue and red lines indicate inferred copy numbers over the RHD and RHCE genes, respectively. In QMPSF panels, peak heights are fluorescence measurements normalized to the HFE control. The F9 peak is a positive amplification control. Light yellow within QMPSF panels highlight multiple affected exons. (*) highlight individual amplicons indicative of structural variation.
Table 1

Summary of NGS-predicted RH alleles, known serology and DNA variants in WHO reference samples

| Sample | Rh Serology | ISBT Alleles | NGS-predicted Antigens | NGS-based Alleles | Relevant Variation |
|--------|-------------|--------------|------------------------|------------------|--------------------|
| RBC1   | D+          | RHD*01; RHD*01 | D+                     | RHD*01; RHD*01   |                    |
|        | C+c+        | RHCE*C; RHCE*c | C+c+                  | RHCE*C; RHCE*c   | Het. RHCE*CE-D(2)-CE hybrid allele |
|        | E+e+        | RHCE*e; RHCE*e | E+e+                  | RHCE*03; RHCE*01.01 | Het. missense variant (c.676G>C); Het. missense variant (c.48G>C) |
| RBC4   | D+          | RHD*01; RHD*01 | D+                     | RHD*01; RHD*01   |                    |
|        | C+c−        | RHCE*C; RHCE*C | C+c−                  | RHCE*C; RHCE*c   | Hom. RHCE*CE-D(2)-CE allele |
|        | E−e+        | RHCE*e; RHCE*e | E−e+                  | RHCE*e; RHCE*01.01 | Het. missense variant (c.48G>C) |
| RBC5   | D−          | RHD*01N; RHD*01N | D−                     | RHD*01N; RHD*01N |                    |
|        | C−c+        | RHCE*c; RHCE*c | C−c+                  | RHCE*c; RHCE*c   |                    |
|        | E−e+        | RHCE*e; RHCE*e | E−e+                  | RHCE*e; RHCE*e   |                    |
| RBC12  | D−          | RHD*04N.01 (RHD*Γ) | D−                     | RHD*04N.01; RHD*01N | Hemi. variants (37 bp insertion, c.807T>G); Hemi. RHD deletion |
|        | C−c+, V+ VS+ | RHCE*c; RHCE*c | C−c+                  | RHCE*c; (RHCE*01.20.02) | Het. missense (c.48G>C); Het. missense (c.733C>G) |
|        | E−e+, V+ VS+ | RHCE*e; RHCE*e | E−e+                  | RHCE*e; (RHCE*01.20.02) | Het. missense (c.48G>C); Het. missense (c.733C>G) |

1. Rh Serology and ISBT alleles shown are those previously reported in Boyle et al. 2013 or ISBT v2.0 110914. ISBT allele names are adapted to avoid assuming phase between C, c and E, e indicative variants
2. NGS-based predicted antigen expression and NGS-based alleles are predicted based on detected variants shown in the ‘Relevant Variation’ column
3. cDNA positions are relative to NM_016124.3 for RHD and NM_020485.4 for RHCE
4. In RBC12, variants under “Relevant Variation” co-occurred with 4 other variants which define the RHD*04N.01
| Allele Name | Phenotype | Allele No. | Allele Frequency (%) | Previously Reported Frequency (%) |
|------------|-----------|-----------|----------------------|----------------------------------|
| RHD*04N.01 (RHD \(\Psi \)) | D null | 109 | 3.178 | 3.4 |
| RHD*[186G>T; 410C>T; 455A>C; 602C>G; 667T>G; 819G>A] | DIIIa | 40 | 1.166 | 1.4 |
| RHD*03.04 | DIII type 4 | 77 | 2.245 | 0.1 |
| RHD*09.03 | DAR | 49 | 1.429 | 1.6 |
| RHD*10.00 | DAU0 | 763 | 22.24 | 16.1 |
| RHD*10.03 | DAU3 | 66 | 1.924 | 1.9 |
| RHCE*01.01 | e weak | 1254 | 36.560 | 42.8 |
| RHCE*01.02 | partial e | 147 | 4.286 | 4.5 |
| RHCE*01.06 | partial eCEAG- | 41 | 1.195 | 1.6 |
| RHCE*01.07 | partial e partial e | 4753 | 13.790 | - |
| RHCE*01.20.03 | partial e partial c, V− VS+ | 473 | 3.469 | - |
| RHCE*01.20.02 | partial e partial c, V+ VS+ | 119 | 3.557 | - |
| RHCE*01.20.01 | partial e partial c, V− VS+ | 122 | 3.557 | - |

1. Allele names and phenotypes are as designated by ISBT v2.0 110914.
2. The number of alleles present and allele frequency in this dataset.
3. Allele frequencies in African American and SCD patients reported in Reid et al. 2014.
4. Novel RH allele relative to ISBT v2.0 110914. The “[ ]” and “;” follow HGVS conventions to denote variants were present on the same chromosome.
5. Genotype quality for the primary variant of the DAU cluster (NM_016124.3:c.1136C>T) was variable due to low coverage in the absence of DAU0 and high sequence homology between RHD and RHCE.

‡ Alleles were observed in Reid et al. 2014 but were reported jointly.