Check for updates

blood advances

Machine learning to optimize automated *RH* genotyping using whole-exome sequencing data

Ti-Cheng Chang,[1] Jing Yu,[2] Zhaoming Wang,[3] Jane S. Hankins,[4] Mitchell J. Weiss,[4] Gang Wu,[1] Connie M. Westhoff,[5] Stella T. Chou,[6] and Yan Zheng[2]

[1]Center for Applied Bioinformatics, [2]Department of Pathology, [3]Department of Epidemiology and Cancer Control, and [4]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN; [5]Laboratory of Immunohematology and Genomics, New York Blood Center Enterprises, New York, NY; and [6]Department of Pediatrics, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA

Key Points

- Machine learning optimized RHtyper for automated and accurate Rh blood group genotyping from WES data.

Rh phenotype matching reduces but does not eliminate alloimmunization in patients with sickle cell disease (SCD) due to *RH* genetic diversity that is not distinguishable by serological typing. *RH* genotype matching can potentially mitigate Rh alloimmunization but comprehensive and accessible genotyping methods are needed. We developed RHtyper as an automated algorithm to predict *RH* genotypes using whole-genome sequencing (WGS) data with high accuracy. Here, we adapted RHtyper for whole-exome sequencing (WES) data, which are more affordable but challenged by uneven sequencing coverage and exacerbated sequencing read misalignment, resulting in uncertain predictions for (1) *RHD* zygosity and hybrid alleles, (2) *RHCE*C* vs. *RHCE*c* alleles, (3) *RHD* c.1136C>T zygosity, and (4) *RHCE* c.48G>C zygosity. We optimized RHtyper to accurately predict *RHD* and *RHCE* genotypes using WES data by leveraging machine learning models and improved the concordance of WES with WGS predictions from 90.8% to 97.2% for *RHD* and 96.3% to 98.2% for *RHCE* among 396 patients in the Sickle Cell Clinical Research and Intervention Program. In a second validation cohort of 3030 cancer survivors (15.2% Black or African Americans) from the St. Jude Lifetime Cohort Study, the optimized RHtyper reached concordance rates between WES and WGS predications to 96.3% for *RHD* and 94.6% for *RHCE*. Machine learning improved the accuracy of *RH* predication using WES data. RHtyper has the potential, once implemented, to provide a precision medicine-based approach to facilitate *RH* genotype–matched transfusion and improve transfusion safety for patients with SCD. This study used data from clinical trials registered at ClinicalTrials.gov as #NCT02098863 and NCT00760656.

Introduction

Blood transfusion is an essential treatment for chronic anemia disorders, including sickle cell disease (SCD) and thalassemia. Exposure to donor red blood cell (RBC) antigens can lead to alloimmunization and increase the risk of hemolytic transfusion reactions with subsequent transfusions.[1] Prophylactic matching for Rh (C, E or C/c, E/e) and K antigens lowers the risk of alloimmunization in patients with

Submitted 13 September 2023; accepted 25 February 2024; prepublished online on *Blood Advances* First Edition 24 March 2024. https://doi.org/10.1182/bloodadvances.2023011660.

Whole-genome and whole-exome sequencing data are available at St. Jude Cloud (https://platform.stjude.cloud/data/cohorts) for the 396 patients with sickle cell disease from the Sickle Cell Clinical Research and Intervention Program study (accession number: SJC-DS-1006), and the 3030 cancer survivors from St. Jude Lifetime Cohort Study (accession number: SJC-DS-1002).[18] The *RH* genotypes of the patients are included in supplemental Tables 3 and 4. The source code and tutorial of RHtyper can be accessed via GitHub (https://github.com/disonchang/RHtyper).

The full-text version of this article contains a data supplement.

SCD and thalassemia but alloantibody formation against the Rh blood group remains a challenge because of the genetically diverse *RH* genes of Black patients and blood donors.[2,3] The Rh blood group consists of 5 major antigens, D, C, c, E, and e, and is encoded by the highly homologous *RHD* and *RHCE* genes.[4] *RHD* and *RHCE* genes of individuals of African descent exhibit high diversity with single nucleotide polymorphisms (SNPs), insertions/deletions (indels), and structural variants. Approximately 450 *RHD* and 190 *RHCE* alleles have been identified, and >50 Rh variant antigens have been described serologically. We found in our practice that 48% to 49% of patients with SCD and 41% of Black blood donors in the United States have an *RHD* or *RHCE* variant (excluding altered alleles of *RHD**10.00 or *RHD**DAU0 and *RHCE**01.01 or *RHCE**ce48C),[5,6] and 7% of D-positive patients with SCD have a partial D.[7] In Brazil, 15% of patients with SCD and 8% of African Brazilian blood donors have both variant *RHD* and *RHCE* alleles.[8] These variant *RH* alleles encode proteins associated with the loss of epitopes or the expression of neoepitopes. Individuals with variant *RH* alleles are at risk of alloimmunization when exposed to conventional or variant Rh antigens differing from their own. Because serological antigen typing cannot distinguish the presence of most variant Rh antigens,[2] *RH* genotyping and consideration of *RH* genotype matching can potentially improve the resource allocation of valuable Black blood donors and avoid Rh alloimmunization.

Next-generation sequencing (NGS) data, such as whole-genome sequencing (WGS) and whole-exome sequencing (WES), offer comprehensive evaluation of the genome and have been used for *RH* genotyping.[9-13] Genotyping *RH* using NGS data is challenging because *RHD* and *RHCE* are duplicated genes that share 97% sequence identity. Sequencing reads from highly homologous regions may map ambiguously, making it difficult to determine the true genomic origin of these reads. Therefore, analysis of NGS data from *RH* loci requires sophisticated bioinformatics tools that can differentiate between true genetic variants and sequencing artifacts. We previously developed RHtyper for automated and accurate detection of the complex *RH* genotypes of Black or African American individuals using WGS data.[6] RHtyper relies on a Bayesian likelihood-based framework to infer *RH* genotypes directly after short-read sequence alignment. Both sequence consistency at each SNP/indel and phase consistency across adjacent SNPs/indels are considered to improve prediction accuracy. RHtyper also incorporates coverage profiling to determine *RHD* zygosity and hybrid alleles and can further define potential breakpoints of the hybrid *RH* alleles using the Circular Binary Segmentation algorithm. In a validation cohort of 57 patients with SCD, RHtyper achieved 100% accuracy for *RHD* and 98.2% accuracy for *RHCE* when compared with *RH* genotypes verified by multiple molecular methods. Upon application to the Sickle Cell Clinical Research and Intervention Program (SCCRIP) study cohort, RHtyper achieved high concordance rates of 98.3% with C serological typing (n = 360 patients) and 99.54% with D serological typing (n = 219 patients).

WES is a focused and cost-effective strategy to identify exonic variations but has limitations we sought to overcome with machine learning. Sequencing coverage of WES is uneven because of variable sequencing read enrichment by capturing oligonucleotides at different locations, leading to inaccurate prediction for copy numbers of exons and SNPs. WES data lacks most intronic sequence markers essential for aligning sequencing reads, resulting in misalignments among highly homologous exons. Here, we adapted RHtyper for WES data by leveraging machine learning to address uneven coverage and sequence misalignments and improved WES-based *RH* genotyping substantially.

Methods

Patients

Existing WES and WGS data from 396 patients with SCD enrolled in the SCCRIP study and 3030 cancer survivors enrolled in St. Jude Lifetime Cohort Study (SJLIFE) at St. Jude Children's Research Hospital (SJCRH) were included in this study. Of the 396 patients with SCD, 56 had *RH* genotypes tested by the standard *RH* genotyping method, SNP-based and targeted molecular assays, and confirmed by Sanger sequencing and NGS as previously described[6] and in supplemental Methods. They were used to further verify the WES-predicted genotypes. The SCCRIP is a lifetime longitudinal cohort study of patients with SCD, in which clinical information is prospectively collected and biologic samples are banked, including blood for genomics and proteomics studies (NCT02098863).[14] The SJLIFE is a retrospective cohort study with prospective follow-up and ongoing accrual of oncology patients treated at SJCRH who were aged ≥18 years and ≥10 years after diagnosis from their malignancy (NCT00760656).[15]

WES, WGS, and serological typing

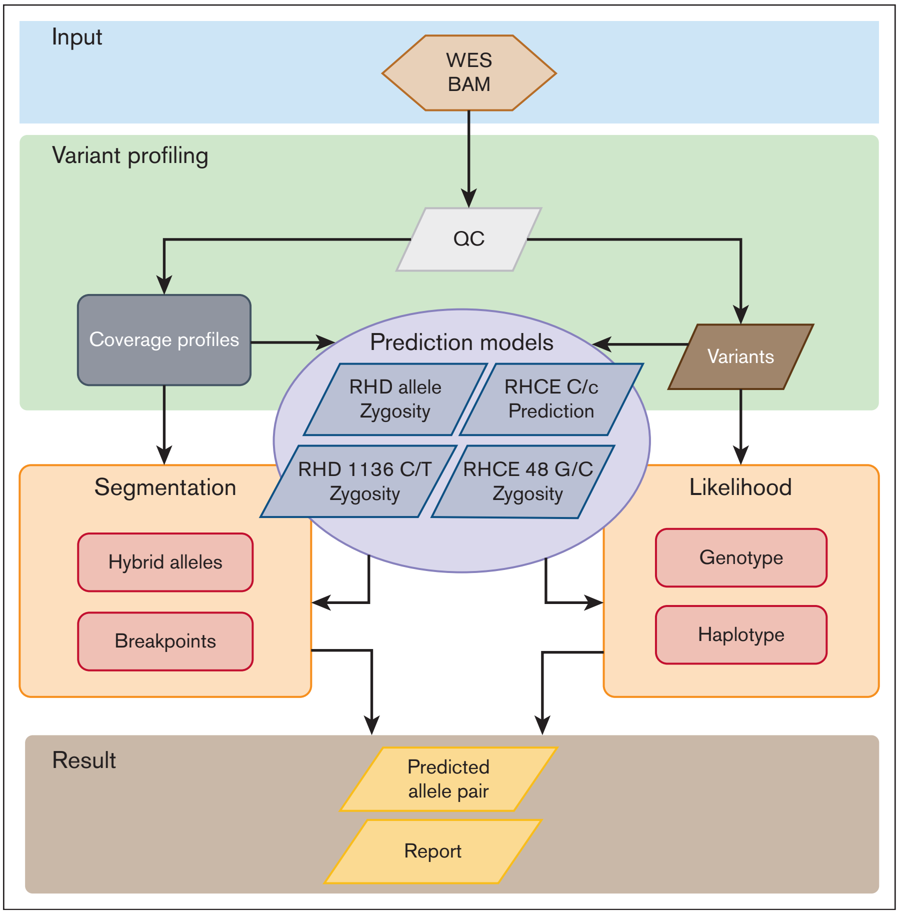
Genomic DNA was extracted from peripheral blood mononuclear cells using standard methods, and WGS and WES were performed at the HudsonAlpha Institute for Biotechnology and the SJCRH Hartwell Center for Bioinformatics and Biotechnology, as previously described.[14,16] The paired-end reads were aligned against the human genome (hg38) using the Burrows-Wheeler Aligner software package.[17] For patients in the SJLIFE cohort, serological typing of RhD only was performed.

Adjustment of RHtyper for WES data

The *RH* allele database was curated from the International Society of Blood Transfusion database and the now-retired National Center for Biotechnology Information-Blood Group Antigen Gene Mutation (BGMUT) database, as previously described.[6] The consolidated database included 419 *RHD* and 130 *RHCE* alleles annotated for genotype determination. Variants were determined according to conventional *RH* messenger RNA sequences (*RHD*, L08429; *RHCE*, DQ322275), which differ from the reference genomic sequence (hg38) by 2 SNPs in the coding region (conventional *RHD* sequence, c.1136T, reference genomic sequence, c.1136C; conventional *RHCE* sequence, c.48G, reference genomic sequence, c.48C).

The WES-based RHtyper algorithm was developed according to the WGS-based *RH* genotyping approach[6] with modification for WES data and by adding machine learning models to improve the prediction accuracy (Figure 1). Specifically, the WES-based RHtyper algorithm consists of 4 main steps: (1) variant profiling for SNPs/indels and coverage alterations; (2) predicting *RHD* zygosity and hybrid alleles, *RHD* c.1136C>T and *RHCE* c.48G>C, and the presence of *RHCE**C or *RHCE**c alleles using established machine learning models; (3) refining the hybrid allele and hybrid

Figure 1. Modification of RHtyper for WES data by adding machine learning. The WES-based RHtyper algorithm consists of 4 main steps: (1) variant profiling of SNPs/indels and coverage alterations. (2) Predicting *RHD* zygosity and hybrid alleles, *RHCE*C* and *RHCE*c,* and the zygosity of *RHD* c.1136C>T and *RHCE* c.48G>C using machine learning models. (3) Refining hybrid allele and breakpoint predictions using segmentation. (4) Generating likelihood scores using genotypes and phased haplotype likelihoods to rank candidate allele pairs. Finally, the candidate allele pair with the highest likelihood scores is considered as the predicted genotype. BAM, binary alignment map; QC, quality control.



breakpoint predictions using segmentation; and (4) generating likelihood scores using genotypes and phased haplotype likelihoods to rank candidate allele pairs. Finally, the candidate allele pair with the highest likelihood score was reported as the predicted genotype.

RH variant calling and coverage profiling. Variants were called via the Samtools pileup method[17] using WES reads that met predefined read criteria (base read quality ≥15, mapping read quality ≥10, and average read quality ≥15). Counts of A, T, G, and C nucleotides and indels were generated for each exonic position of *RHD/RHCE* genes. Exonic positions with variant allele-frequency >10% were classified as heterozygous sites. SNPs and indels were annotated subsequently with encoded amino acid changes. *RHD/RHCE* coverage profiling was performed as previously described, using WES data.[6]

Construction of machining learning models. The WGS-predicted genotypes served as control references.[6] Informative features were selected using the Boruta algorithm (10.18637/jss.v036.i11), based on per-base coverage and variant allele frequency. The selected features were then incorporated to construct XGBoost models for model learning, using 75% of the WES data from the SCCRIP study. The modified RHtyper was next validated using the remaining 25% of the data from the SCCRIP study as well as a second patient cohort, SJLIFE.

This study was approved by the SJCRH institutional review board, and all participants or guardians provided written informed consent.

Results

Uneven sequencing coverage of WES data

The average *RH* sequencing coverage for 396 patients with SCD in the SCCRIP cohort was 56.3× for WES compared with 35.7× for WGS. WES coverage demonstrated high regional unevenness, the normalized coverage per *RHD* exon ranged from −6.09 ± 5.09 to 0.21 ± 1.60 (mean ± standard deviation, "0" representing 2 copies), and the normalized coverage per *RHCE* exon ranged from −0.80 ± 0.78 to 1.01 ± 0.35 (Figure 2; supplemental Table 1). In contrast, the normalized WGS coverage fluctuated less, ranging from −3.58 ± 3.50 to −0.40 ± 0.85 per *RHD* exon, and from −0.70 ± 0.33 to 0.35 ± 0.41 per *RHCE* exon. Notably, *RHD* coverage varied more than *RHCE* regardless of sequencing method because *RHD* and *RHCE* have identical exon 8, and most sequencing reads from exon 8 align to *RHCE*, reducing *RHD* exon 8 coverage markedly. The unevenness of the WES coverage of *RH* genes affected the prediction of zygosity of alleles and SNPs.

Limitation of RHtyper using WES data

Because RHtyper was initially designed for WGS data analysis, we first modified the algorithm for WES data to not rely on intronic markers for identification. *RHCE*C* can be predicted using WGS

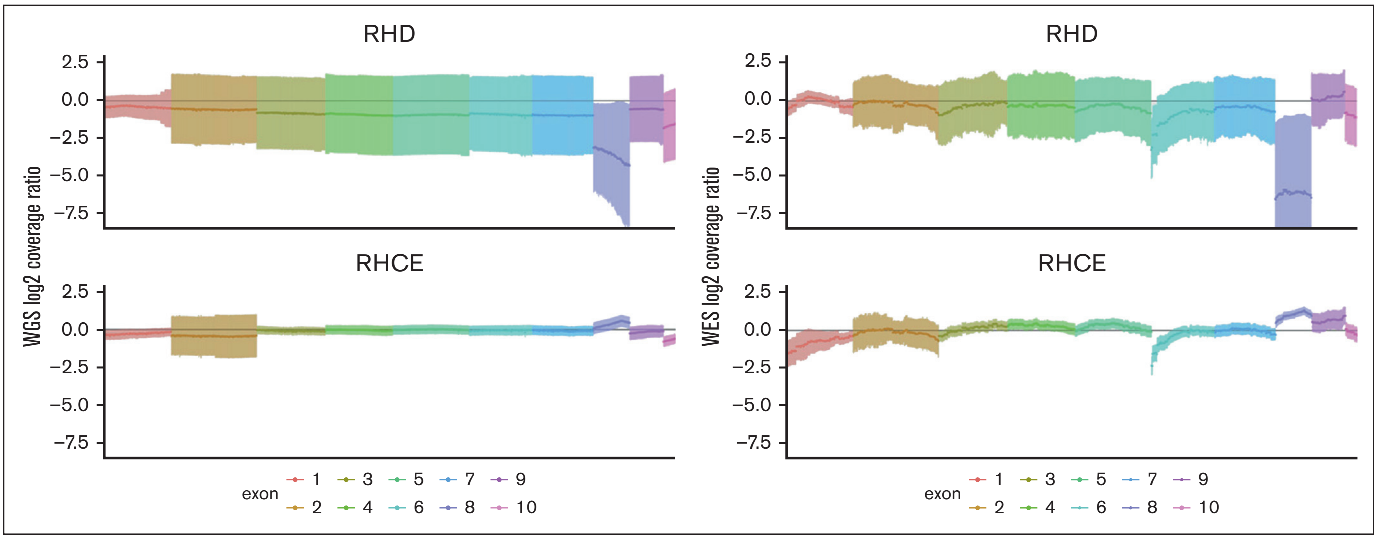


Figure 2. Uneven sequencing coverage of *RH* genes by WES compared with WGS. Sequencing coverage is normalized by log2 transformation of the ratio between each exon and the average coverage of the sample. A value of "0" represents 2 copies. The exons are differentiated by color. Lines represent the mean sequencing coverage. Shadows represent the standard deviation.

data with high confidence using a 109-base pair insertion in the *RHCE*C* intron 2. Because this intronic region is not covered by WES, *RHCE*C* was instead identified by increased coverage of *RHD* exon 2, because *RHCE*C* and *RHD* exon 2 are identical, and the reads from *RHCE*C* typically align with *RHD*.[9] Notably, WES data cannot be used to identify alleles with only intronic variations.

We next determined *RH* genotypes using WES data for 396 patients with SCD from the SCCRIP cohort, all of whom were Black or African American. The concordance rates between WES and previously reported WGS predictions[6] were 90.3% (715/792 alleles) for *RHD* and 96.3% (763/792 alleles) for *RHCE* (Figure 3A). Problematic determinations included (1) *RHD* zygosity and hybrid alleles, (2) *RHCE*C* vs *RHCE*c* alleles, (3) *RHD* c.1136C>T zygosity, and (4) *RHCE* c.48G>C zygosity. *RHD* zygosity, hybrid alleles, and *RHCE*C* were predicted based on sequencing coverage of the whole gene (ie, *RHD* zygosity) or certain exons of the gene (ie, *RHCE* exon 4 to 7 for *RHD*03N.01* or *RHD*DIIIa-CEVS(4-7)-D*, *RHD* exon 2 for *RHCE*C*), which was less accurate with WES data due to the fluctuated sequencing coverage. *RHD* c.1136C>T (p. Thr379Met), located in exon 8, is the most common missense *RHD* SNP in patients with SCD and is the characteristic SNP that defines the *RHD DAU* cluster.[6,19] Because the reference genomic sequence of *RHD* represents *RHD*10.00 or RHD*DAU0* with c.1136T, and the conventional *RHD* shares exon 8 with *RHCE* with c.1136C, almost all sequence reads from conventional *RHD* align with *RHCE*, resulting in reduced coverage of *RHD* exon 8. To circumvent the skewed coverage of exon 8, *RHD* c.1136 C>T zygosity was determined for the WGS data by dividing the reads containing the SNP by genome-wide average read coverage rather than position-specific read coverage. However, this approach was no longer reliable with WES data, given the highly variable exome-wide sequencing coverage. *RHCE* c.48G>C (p. Trp16Cys) resides in exon 1 and is the most common missense *RHCE* SNP found in patients with SCD.[6] In addition, *RHCE*01.01* or *RHCE*ce48C* is as common as the conventional *RHCE*01* or *RHCE*ce* allele in Black individuals.[5,6] The sequences of conventional *RHD* and *RHCE* exon 1 are highly homologous, differing only by 1 nucleotide: c.48C for *RHD* and c.48G for *RHCE*. Without the paired-mate sequencing reads that cover the surrounding introns to differentiate *RHD* from *RHCE*, sequence reads with *RHCE* c.48G>C frequently misaligned to *RHD*, resulting in an erroneous G/C fraction and subsequent incorrect allele prediction.

Modification and validation of RHtyper for WES data

Given the low concordance between WES and WGS predictions, we sought to improve RHtyper by incorporating machine learning specific to the problematic alleles and SNPs. The SCCRIP cohort was used for training and validating the machine learning models because (1) all patients in the SCCRIP cohort were of African descent with highly diverse *RHD* and *RHCE* genes[6] and (2) despite Rh phenotype–matched blood transfusion, patients with SCD are still at high risk for Rh alloimmunization because of the genetic diversity of *RH* genes and will likely benefit the most from receiving *RH* genotype–matched blood transfusion.[2,5,20] A total of 1547 informative features for *RHD* zygosity and hybrid alleles, 255 for *RHCE*C* vs *RHCE*c* allele differentiation, 240 for *RHD* c.1136C>T, and 253 for *RHCE* c.48G>C zygosity, were selected to build machine learning models (supplemental Figure 1). The *RH* genotypes predicted using the WGS data were used as reference genotypes because of their high accuracy.[6] We randomly selected 75% of the WES data from the SCCRIP cohort for model training, and the remaining 25% for validation. Machine learning improved the concordance rates between WES and WGS predictions to 98.0% for *RHD* zygosity and hybrid alleles, 97.0% for *RHCE*C* vs. *RHCE*c* alleles, 97.0% for *RHCE* c.48G>C zygosity, and 96.0% for *RHD* c.1136C>T zygosity. The overall concordance rates for the SCCRIP cohort were 97.2% (770/792 alleles) for *RHD* and 98.2% (778/792 alleles) for *RHCE* (Figure 3B; supplemental Tables 2-3). The remaining discrepancies were due to, with substantially fewer numbers though, *RHD* zygosity and hybrid alleles (6 alleles 0.8% of total 792 *RHD* alleles), *RHCE*C* vs *RHCE*c* (8

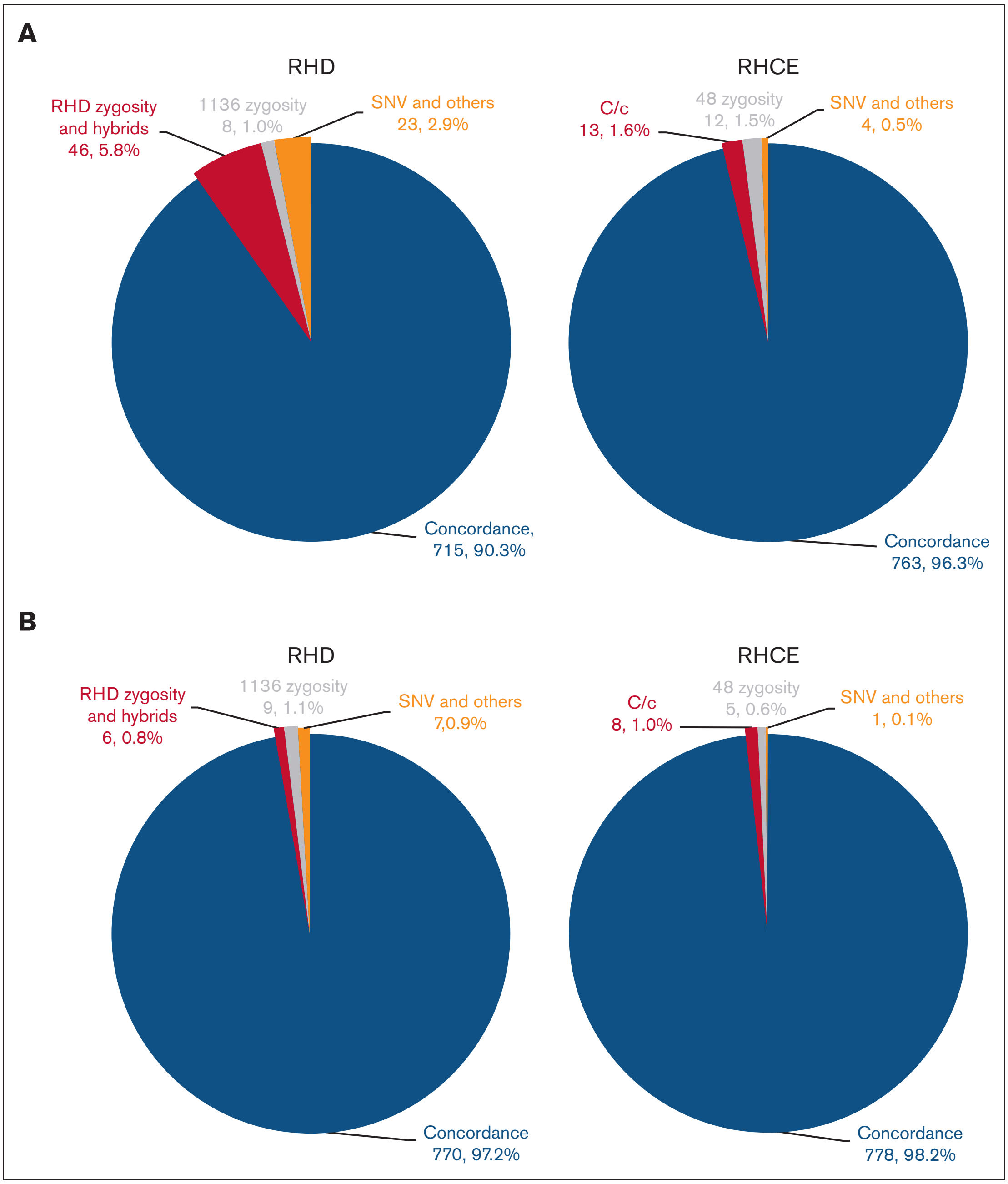


Figure 3. Concordance between WES and WGS predictions for the SCCRIP study. For the 396 patients with SCD enrolled in the SCCRIP study, machine learning increased the overall concordance rates between WES- and WGS-predicted *RH* genotypes from 90.3% for *RHD* and 96.3% for *RHCE* (A) to 97.2% for *RHD*, 98.2% for *RHCE* (B). The number and percentage of concordant alleles and various types of discordant alleles are shown in parentheses.

alleles, 1% of total 792 *RHCE* alleles; 6 *RHCE*C* misidentified as *RHCE*c*, 0.8% of total *RHCE* alleles; 2 *RHCE*c* as *RHCE*C*, 0.2% of total *RHCE* alleles), *RHCE* c.48G>C zygosity (5 alleles, 0.6%), *RHD* c.1136C>T zygosity (9 alleles, 1.1%), and other SNP discrepancies (*RHD*, 7 alleles, 0.9%; *RHCE*, 1 allele, 0.1%).

In the SCCRIP cohort, the *RH* genotypes of 56 patients were also determined by standard *RH* genotyping methods of *RH* SNP array and targeted molecular assays, verified by Sanger sequencing or a second independent NGS as described previously[6] and in supplemental Methods. Compared with the known genotypes, the modified RHtyper using WES data achieved 98.2% (110/112 alleles) accuracy for *RHD* and 94.6% (106/112 alleles) accuracy for *RHCE* alleles (Table 1). Notably, none of the erroneous predictions would have led to an increased risk of Rh alloimmunization. One erroneous prediction in which patient 1, with "RhC" (*RHCE*02/RHCE*01.20.01 or RHCE*Ce/RHCE*ce733G*), was misidentified by WES as "Rhc" (*RHCE*01/ RHCE*01.20.02* or *RHCE*ce/RHCE*48C733G*) could have resulted in the dispensation of C-negative blood to a C-positive patient unnecessarily.

Table 1. Discrepancies between known and WES-predicted genotypes in 56 patients with SCD

Patient	*RH* allele	Known genotypes†	WES-predicted genotypes	Confirmation methods
1	*D*	*RHD*01*	Same	-
	D	Deletion	Same	-
	CE	*RHCE*02 (RHCE*Ce)*	*RHCE*01 (RHCE*ce)*	Serology
	CE	*RHCE*01.20.01 (RHCE*ce733G)*	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*	Serology
2	*D*	*RHD*	*RHD*10.00 (RHD*DAU0)*	Sanger sequencing
	D	*RHD*	*RHD*10.00 or (RHD*DAU0)*	Sanger sequencing
	CE	*RHCE*02 or RHCE*Ce*	Same	-
	CE	*RHCE*01.20.01 (RHCE*ce733G)*	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*	Sanger sequencing
3	*D*	*RHD*01*	Same	-
	D	Deletion	Same	-
	CE	*RHCE*01 (RHCE*ce)*	*An extra RHCE c.105C>T identified*	Sanger sequencing
	CE	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*		Sanger sequencing
4	*D*	*RHD*01*	Same	-
	D	Deletion	Same	-
	CE	*RHCE*01 (RHCE*ce)*	Same	-
	CE	*RHCE*01 (RHCE*ce)*	*RHCE*01.01 (RHCE*ce48C)*	Sanger sequencing

†Determined by standard *RH* genotyping methods of *RH* SNP array and targeted molecular assays, verified by Sanger sequencing or a second independent NGS or serology.

Further validation of the modified RHtyper in the SJLIFE cohort

Next, we validated the modified RHtyper in a second available patient cohort, SJLIFE, consisting of 3030 cancer survivors. Among 2716 patients with racial information, 84.6% (2298) were White, 15.2% (413) Black or African American, 0.11% (3) Asian, 0.04% (1) American Indian or Alaska Native, and 0.04% (1) Native Hawaiian or Other Pacific Islander. The concordance rates between WES and WGS predictions were 96.3% (5837/6060 alleles) for *RHD* and 94.6% (5734/6060 alleles) for *RHCE* (Figure 4; supplemental Tables 2 and 4). Discrepancies included *RHD* zygosity and hybrid alleles (159 alleles, 2.6% of 6060 *RHD* alleles), *RHCE*C* vs *RHCE*c* differentiation (263 alleles, 4.3% of total 6060 *RHCE* alleles; 237 *RHCE*C* misidentified as *RHCE*c*, 3.9% of total *RHCE* alleles; 26 *RHCE*c* as *RHCE*C*, 0.4% of total *RHCE* alleles), *RHCE* c.48G>C zygosity (37 alleles, 0.6%), *RHD* c.1136C>T zygosity (17 alleles, 0.3%), and SNPs and other discrepancies (*RHD*, 47 alleles, 0.8%; and *RHCE*, 26 alleles, 0.4%). For 1036 patients with blood type information, the predicted RhD serological types using WES data were 99.8% (1034/1036 patients), consistent with the clinical serology results; notably, this comparison only assessed whether the modified RHtyper could correctly predict the presence or absence of RhD. The predicted frequency of C antigen was 65.23% (1499/2298 patients) per WGS and 58.96% (1355/2298 patients) per WES for White patients, and 23.24% (96/413 patients) per WGS and 24.21% (100/413 patients) per WES for Black or African American patients, consistent with the known racial distribution (68% of White people and 27% of Black people).[21]

The modified WES-based RHtyper was trained primarily using data from Black or African American patients, whereas most patients in the SJLIFE cohort were White, for whom the frequency of *RH* variation is ~1% to 2%.[21] Therefore, we compared the concordance rates of White vs Black or African American patients in the SJLIFE cohort (Figure 5). Discrepancies were significantly higher among White patients for *RHD* zygosity and hybrid alleles (127 alleles or 2.8% of *RHD* alleles in White patients vs 11 alleles or 1.3% of *RHD* alleles in Black or African American patients; $P = .0157$), and *RHCE*C* vs *RHCE*c* differentiation (227 alleles or 5.0% of *RHCE* alleles in White patients vs 8 alleles or 1.0% of *RHCE* alleles in Black or African American patients; $P < .0001$). In contrast, the discrepancy in *RHD* c.1136C>T zygosity was significantly higher in Black or African American patients (10 alleles or 1.2% of *RHD* alleles in Black or African American patients vs 1 allele or 0.02% of *RHD* alleles in White patients; $P < .0001$), although the overall number of discrepant alleles was very low.

Discussion

The WGS-based RHtyper relies on sequencing coverage profiles to predict the zygosity of alleles and SNPs.[6] This approach alone was less accurate for analyzing WES data because of uneven sequencing coverage and misalignment of sequencing reads. To improve the prediction accuracy using WES data, we optimized RHtyper by leveraging machine learning to target the 4 most affected SNPs and alleles: (1) *RHD* zygosity and hybrid alleles, (2) *RHCE*C* vs *RHCE*c* alleles, (3) *RHD* c.1136C>T zygosity, and (4) *RHCE* c.48G>C zygosity. Machine learning substantially increased the concordance of WES– with WGS–predicted *RH* genotypes when applied to 2 independent large patient cohorts, SCCRIP and SJLIFE, but a few limitations remained.

Manual or automated genotyping of *RHD* and *RHCE* from targeted exome sequencing and WES data has been performed by multiple groups.[9,10,22-25] Prediction of *RHD* zygosity and hybrids and *RHCE*C* vs *RHCE*c* alleles by sequencing coverage have

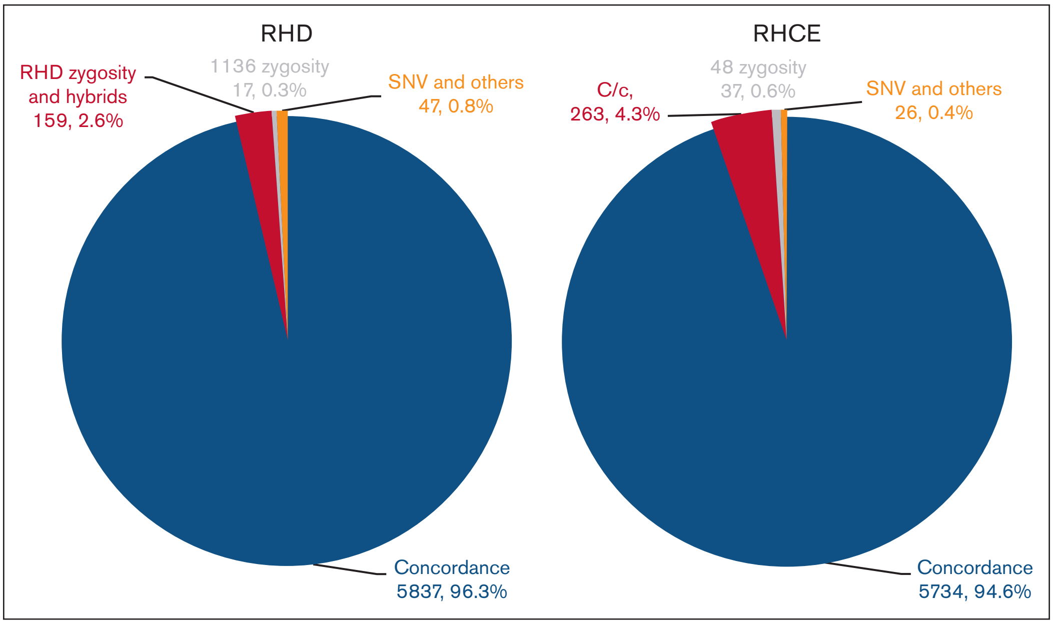
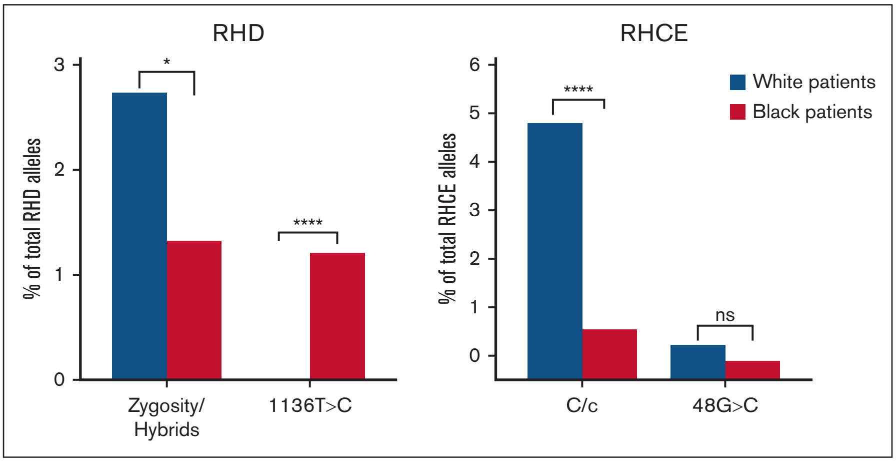


Figure 4. Concordance between WES and WGS predictions for the SJLIFE study. The modified RHtyper achieved high concordance rates between the WES– and WGS–predicted *RH* genotypes of 96.3% for *RHD* and 94.6% for *RHCE* in the SJLIFE cohort consisting of 3030 cancer survivors. The number and percentage of concordant alleles and various types of discordant alleles are shown in parentheses.

consistently been difficult with WES data. Schoeman et al reported that the sensitivity to detect a deletion in *RHD* and *RHCE* was 89.8%, and only 52.8% for duplications using sequencing coverage alone (n = 28).[25] To overcome this limitation, Chou et al and Lane et al determined *RHD* zygosity using *RHCE* as a control, because nearly all individuals have 2 copies of *RHCE,* and *RHCE*C* identification was based on decreased read coverage of *RHCE* exon 2 compared with *RHCE* exons 1 and 3.[9,13] Chou et al reported that the approaches improved the concordance rate to 98% (n = 54).[9] Lane et al developed the first automated algorithm for RBC antigen genotyping using WES data.[13] Using copy number correction factors calculated from 20 individuals with known *RHD* zygosity and C/c antigen status to normalize the sequencing coverage of each exon, the authors were able to correctly genotype the remaining 55 individuals. The improvement strategies used by these studies involved creating predetermined rules based on data from a small cohort of individuals. However, this approach may not be comprehensive enough to capture all the necessary information for accurate prediction in a large number of individuals. WES data were also not reliable in predicting *RHCE* c.48G>C and *RHD* c.1136C>T owing to the misalignment of sequencing reads.[9,25] The algorithm created by Lane et al was able to detect *RHD* c.1136C>T but could not distinguish homozygous from heterozygous ones.[13]

We optimized RHtyper for WES data using machine learning. The learning process allows the incorporation of diverse informative features and has been applied to complicated and high-dimensional data, including genomic sequencing data. It enables accurate predictions based on automated data learning rather than simple rule-based classification. In our study, informative features of per-base coverage and variant allele frequency from hundreds to thousands of exonic positions were used to identify the problematic SNPs and alleles. Training with almost 300 patients with SCD from

Figure 5. Discordance rates of trained SNPs/alleles among White and Black or African American patients in the SJLIFE study. ns, not significant; **P* < .05; *****P* < .001.



the SCCRIP cohort allowed for the recognition of intricate patterns for accurate prediction. Machine learning markedly improved the concordance rates between WES and WGS predictions to 97.2% for *RHD* and 98.2% for *RHCE* in the SCCRIP cohort (n = 396) and 96.3% for *RHD* and 94.6% for *RHCE* in the SJLIFE cohort (n = 3030). Using similar machine learning approaches, RHtyper can be extended to analyze other blood group proteins encoded by highly homologous genes, for example, the MNS blood group.

Discordant predictions between WES and WGS remained despite machine learning. *RHD* zygosity and hybrid alleles, and *RHCE*C* vs *RHCE*c* alleles contributed to most discrepancies. Discordant *RHD* zygosity and hybrid allele calling occurred more often in patients with hemizygous *RHD* deletion or heterozygous *RHD* hybrid alleles, which require sufficient and even coverage for accurate identification and could remain challenging for certain patients even with machine learning. Because the sequencing coverage of *RHD* exon 2 is critical in differentiating *RHCE*C* from *RHCE*c* alleles, we initially suspected that the coverage might be erroneous owing to misalignment mediated by SNPs unique to certain patients. However, a comparison of *RHD* exon 2 and its surrounding intron sequences (50 bp into the surrounding introns) between patients with and without *RHCE*C* vs *RHCE*c* discrepancy revealed no SNPs that would have led to misalignment (data not shown). Furthermore, White patients in the SJLIFE cohort were more likely to have discordant predictions of *RHD* zygosity and hybrid alleles and *RHCE*C* vs *RHCE*c* alleles than Black or African American patients. The skewed discordance could be due to higher frequencies of D-negative and C-positive status in White (15% and 68%, respectively) than in Black or African American patients (8% and 27%, respectively).[21] Racial differences between the training and validation cohorts could also provide an explanation, as all patients in the SCCRIP training cohort were Black or African American and 84.6% of the patients in the SJLIFE validation cohort were White. It is possible that individuals from different races may have slightly different sequencing coverage patterns, or that the informative features used to identify and/or differentiate those alleles vary slightly with race, for which future studies are warranted. Additional training using WES data from White individuals and individuals of other racial and ethical groups is needed to further improve the accuracy of RHtyper.

The clinical implementation of RHtyper may become increasingly relevant as more patients with chronic diseases are being interrogated by WES or WGS. It provides an analysis tool for data that may already exist or be obtained for other clinical care. *RH* genotyping can enhance transfusion safety by facilitating anti-Rh antibody identification and/or, in some cases, improve prophylactic RBC matching strategies. For example, for patients with the hybrid alleles of *RHD*01N.03* or *RHD*DIIIa-CEVS (4-7)-D, RHCE*02.10.01* or *RHCE*CeRN*, which encode partial C antigen, and no conventional *RHCE*Ce* or *RHCE*CE* allele, transfusion with C-negative RBCs is recommended to prevent anti-C formation.[26] Genotyping blood donors, particularly frequent Black donors who support C/E/K-matched RBCs for patients with SCD, may facilitate *RH* genotype–matched blood transfusion and improve transfusion safety in the future. RHtyper achieved high concordance rates in 2 large validation cohorts after incorporating the machine learning models but was not 100% accurate. One limitation is that RHtyper may incorrectly predict *RHCE*C* and *RHCE*c* using WES data. Misidentification of *RHCE*C* as *RHCE*c* may result in C-positive patients receiving C-negative blood, which would not cause any harm to the patient, but from a resource perspective, it would be a poor allocation of C antigen-negative units. Conversely, the misidentification of *RHCE*C* as *RHCE*c* in blood donors may result in exposing C-negative recipients to C-positive blood and potential anti-C formation. Therefore, the use of RHtyper for *RH* genotyping of blood donors would need to be combined with other testing such as standard serologic typing. For clinical application, additional training and validation using samples from multiple racial groups with *RH* genotypes verified by *RH* SNP array, Sanger sequencing, and other molecular methods, as well as serological tests, are essential to further optimize RHtyper prediction.

There are limitations to our study. First, we used WGS-predicted genotypes by RHtyper as a reference. This seemed justified, as we previously demonstrated that the WGS-predicted genotypes were highly accurate compared with genotypes verified by multiple molecular methods and serological types for D and C/c antigens.[6] Second, the SJLIFE cohort was only serologically typed for D antigen; thus, concordance with C antigen typing was not possible. However, the prevalence of C antigen in White and Black or African American patients derived from WGS and WES data were consistent with known frequencies, indicating that the genotyping results were likely accurate.[21]

In conclusion, we optimized RHtyper for WES data by adding machine learning to overcome the variable sequencing coverage and misalignment associated with WES data. The optimization improved *RH* genotyping accuracy and extended the application spectrum of RHtyper to include more widely available WES data.

Acknowledgments

The authors thank the Sickle Cell Clinical Research and Intervention Program and the St. Jude Lifetime Cohort Study teams, as well as the biorepository at St. Jude Children's Research Hospital for sharing the whole-genome and whole-exome sequencing data and patient samples.

This study was supported by the National Institutes of Health/National Cancer Institute (CA021765), the American Lebanese Syrian Associated Charities (Y.Z.), National Blood Foundation Early-Stage Investigator's Award (Y.Z.), and the National Institutes of Health/National Heart, Lung, and Blood Institute HL147879-01 (S.T.C. and C.M.W).

Authorship

Contribution: J.S.H., M.J.W., C.M.W., S.T.C., and Y.Z. designed the research; T-C.C. and G.W. developed and modified RHtyper; J.Y. performed confirmatory Sanger sequencing; J.S.H., M.J.W., and Z.W. provided patient samples and sequencing data; and T-C.C., C.M.W., S.T.C., and Y.Z. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: Z.W., 0000-0001-7556-3869; J.S.H., 0000-0003-4439-7321; S.T.C., 0000-0003-4333-6965; Y.Z., 0000-0001-7254-7564.

Correspondence: Yan Zheng, Department of Pathology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, MS 342, Memphis, TN 38105; email: Yan.Zheng@stjude.org.

References

1. Zheng Y, Chou ST. Transfusion and cellular therapy in pediatric sickle cell disease. *Clin Lab Med*. 2021;41(1):101-119.
2. Chou ST, Jackson T, Vege S, Smith-Whitley K, Friedman DF, Westhoff CM. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood*. 2013;122(6):1062-1071.
3. Waldis SJ, Uter S, Kavitsky D, et al. Rh alloimmunization in chronically transfused patients with thalassemia receiving RhD, C, E, and K matched transfusions. *Blood Adv*. 2021;5(3):737-744.
4. Westhoff CM. The structure and function of the Rh antigen complex. *Semin Hematol*. 2007;44(1):42-50.
5. Chou ST, Evans P, Vege S, et al. RH genotype matching for transfusion support in sickle cell disease. *Blood*. 2018;132(11):1198-1207.
6. Chang TC, Haupfear KM, Yu J, et al. A novel algorithm comprehensively characterizes human RH genes using whole-genome sequencing data. *Blood Adv*. 2020;4(18):4347-4357.
7. Takasaki K, Friedman DF, Uter S, Vege S, Westhoff CM, Chou ST. Variant RHD alleles and Rh immunization in patients with sickle cell disease. *Br J Haematol*. 2023;201(6):1220-1228.
8. Gaspardi AC, Sippert EA, De Macedo MD, Pellegrino J Jr, Costa FF, Castilho L. Clinically relevant RHD-CE genotypes in patients with sickle cell disease and in African Brazilian donors. *Blood Transfus*. 2016;14(5):449-454.
9. Chou ST, Flanagan JM, Vege S, et al. Whole-exome sequencing for RH genotyping and alloimmunization risk in children with sickle cell anemia. *Blood Adv*. 2017;1(18):1414-1422.
10. Lane WJ, Westhoff CM, Gleadall NS, et al. Automated typing of red blood cell and platelet antigens: a whole-genome sequencing study. *Lancet Haematol*. 2018;5(6):e241-e251.
11. Lane WJ, Westhoff CM, Uy JM, et al. Comprehensive red blood cell and platelet antigen prediction from whole genome sequencing: proof of principle. *Transfusion*. 2016;56(3):743-754.
12. Wheeler MM, Lannert KW, Huston H, et al. Genomic characterization of the RH locus detects complex and novel structural variation in multi-ethnic cohorts. *Genet Med*. 2019;21(2):477-486.
13. Lane WJ, Vege S, Mah HH, et al. Automated typing of red blood cell and platelet antigens from whole exome sequences. *Transfusion*. 2019;59(10):3253-3263.
14. Hankins JS, Estepp JH, Hodges JR, et al. Sickle cell clinical research and intervention program (SCCRIP): a lifespan cohort study for sickle cell disease progression from the pediatric stage into adulthood. *Pediatr Blood Cancer*. 2018;65(9):e27228.
15. Howell CR, Bjornard KL, Ness KK, et al. Cohort profile: the St. Jude Lifetime Cohort Study (SJLIFE) for paediatric cancer survivors. *Int J Epidemiol*. 2021;50(1):39-49.
16. Qin N, Wang Z, Liu Q, et al. Pathogenic germline mutations in DNA repair genes in combination with cancer treatment exposures and risk of subsequent neoplasms among long-term survivors of childhood cancer. *J Clin Oncol*. 2020;38(24):2728-2740.
17. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-1760.
18. Palmer LE, Zhou X, McLeod C, et al. Data access and interactive visualization of whole genome sequence of sickle cell patients within the St. Jude Cloud. *Blood*. 2018;132(Suppl 1):723.
19. Wagner FF, Ladewig B, Angert KS, Heymann GA, Eicher NI, Flegel WA. The DAU allele cluster of the RHD gene. *Blood*. 2002;100(1):306-311.
20. Takasaki K, Friedman DF, Uter S, Vege S, Westhoff CM, Chou ST. Variant RHD alleles and Rh immunization in patients with sickle cell disease. *Br J Haematol*. 2023;201(6):1220-1228.
21. Reid M, Lomas-Francis C, M O. *The Blood Group Antigen FactsBook*. Elsevier; 2012.
22. Stef M, Fennell K, Apraiz I, et al. RH genotyping by nonspecific quantitative next-generation sequencing. *Transfusion*. 2020;60(11):2691-2701.
23. Fichou Y, Audrézet MP, Guéguen P, Le Maréchal C, Férec C. Next-generation sequencing is a credible strategy for blood group genotyping. *Br J Haematol*. 2014;167(4):554-562.
24. Stabentheiner S, Danzer M, Niklas N, et al. Overcoming methodical limits of standard RHD genotyping by next-generation sequencing. *Vox Sang*. 2011;100(4):381-388.
25. Schoeman EM, Lopez GH, McGowan EC, et al. Evaluation of targeted exome sequencing for 28 protein-based blood group systems, including the homologous gene systems, for blood group genotyping. *Transfusion*. 2017;57(4):1078-1088.
26. Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv*. 2020;4(2):327-355.