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A Genome-Wide Screen for Large-Effect Alloimmunization Susceptibility Loci among Red Blood Cell Transfusion Recipients with Sickle Cell Disease

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Keywords
Genome-wide association studies · GWAS · African American · Responders · Genomics

Summary
Background: A selective susceptibility of certain individuals to form multiple alloantibodies in response to red cell transfusion is well-recognized in clinical practice, and is a particular problem in persons with sickle cell disease (SCD). The reason for this differential susceptibility is unclear, but inter-individual genetic differences are likely to contribute. **Methods:** We conducted a pilot case-control genome-wide association study using 1,000,000 SNPs in 94 alloimmune responders (cases) and non-responders (controls) with SCD in order to identify loci of large effect size associated with alloimmunization. **Results:** No loci showed evidence of association at a genome-wide significance cut-off ($p < 0.5 \times 10^{-8}$). SNPs in the *ARAP1/STARD10* region showed suggestive association ($p < 1 \times 10^{-6}$), but no association was observed at previously implicated loci *TRIM21* or *HLA*. In analyses of the number of accumulated antibodies, a modest association was found with SNPs in the Toll-like receptor gene *TLR10* ($p < 1 \times 10^{-4}$). **Conclusions:** Alloimmunization in persons with SCD is unlikely to be mediated by loci of very large effect size; however, larger and more comprehensive studies are required to fully evaluate loci with more moderate effects. This study provides a working approach to such future studies in SCD.

Introduction

Transfusion of allogenic blood products is an essential part of modern medicine; however, this process is also an immunologic challenge to the recipient, who is exposed to multiple foreign antigens as a consequence. In the setting of red cell transfusions, this exposure sets the stage for the development of alloantibodies, the presence of which creates the considerable challenge of providing compatible blood products while avoiding the attendant problem of hemolytic transfusion reactions [1]. Given that some 5 million patients receive blood products every year in the USA, including those who receive multiple transfusions as a consequence of inherited or acquired hematological disorders, post-transfusion alloimmunization is a problem of major public health significance [2].

In clinical practice, it is well established that some individuals will form multiple antibodies with almost each transfusion; such 'responders' appear immunologically distinct from 'non-responders' for whom the development of antibodies is a rare and unusual occurrence. This phenotypic dichotomy of 'responders' and 'non-responders' is commonly observed in persons with sickle cell disease (SCD), who often require red cell transfusions both in the acute setting of sickle crises and for their long-term disease management. The rate of red cell alloimmunization in SCD patients is empirically quoted in the range of 30–50% [3, 4] – several orders of magnitude higher than the ~5% figure observed in the general population [2, 5]. Alloimmune responders with SCD are also at increased risk of hyperhemolysis [6], an uncommon but potentially devastating type of delayed transfusion reaction.

The reason some individuals become responders while others do not is not well-understood, but is likely to be multifac-

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torial, involving donor, recipient, and external factors [5–7]. For instance, there is support for genetic ancestry contributing to alloimmunization in SCD patients – the genetic ancestry of individuals with SCD is predominantly African American, whereas blood donors of similar ethnicity are underrepresented in the national donor pool [7–10]. This potentiates the mismatches between donor and recipient red cell antigens that are necessary to stimulate an alloimmune response. Accordingly, expanded red cell antigen phenotyping and closer matching of common antigens between donor and recipient has decreased the incidence of alloimmunization in susceptible groups [11, 12], but a significant rate of alloimmunization remains, strongly implicating other mechanisms in the pathogenesis [13].

Among the potential additional factors is the well-known genetic variation that exists between individuals; for instance, possession of human leukocyte antigen (HLA) types HLA-B35 and HLA-Cw4 alleles have been linked to 'responder' status in persons with SCD [14, 15], and a single nucleotide polymorphism (SNP) in the erythrocyte antigen *Ro52 (TRIM21)* has been associated with the induction and efficiency of alloimmunization in both a transfusion-dependent and age-dependent fashion in patients with SCD [16]. These and other similar reports [17] validate a genetic underpinning to the alloimmune response, but they do so in a disparate, ad hoc fashion, and the primary results have not been replicated in other cohorts.

Advances in microarray technologies designed to interrogate SNPs across the genome have made it possible to perform genome-wide association studies (GWAS). The GWAS approach has emerged as a powerful way of agnostically identifying disease susceptibility loci and has facilitated the identification of biologically important loci in multiple diseases across a range of disciplines [18, 19] including disease severity in SCD [20, 21]. Many of the associations with the greatest magnitudes of effect have occurred in diseases with an immune or autoimmune basis [22–25] or in disorders with a strong gene-environment component [26–28]. These associations have fundamentally changed our understanding of the underlying disease processes [29] and in some cases have facilitated clinically useful genetic testing [30, 31]. Therefore, GWAS are an attractive and robust way of identifying biologically relevant genes and gene pathways contributing to the alloimmune responder phenotype in individuals with SCD; this in turn holds the ultimate promise of new therapeutic targets for those at higher risk. Further, because the GWAS approach includes the identification of both susceptibility *and* protection alleles, relevant loci could directly impact the ability of blood centers to preemptively identify both susceptible recipients for whom extended phenotyping is especially indicated as well as likely non-responders to whom random units can be provided, thereby increasing the number of donors who are available to supply phenotype-matched blood to SCD patients.

As a first foray into alloimmunization genomics, we undertook a pilot case-control GWAS of alloimmune responder status in a cohort of multiply transfused participants with SCD, with the express goal of identifying large-effect susceptibility loci. The genome-wide coverage achieved also allowed us to evaluate previously associated loci and to perform subgroup analyses of allo-antibody accumulation.

Material and Methods

Subjects

The study protocol was approved by the Institutional Review Board of St Luke's Episcopal Hospital and of Baylor College of Medicine. DNA samples used were those taken from patients with a diagnosis of SCD referred to LifeShare for blood group genotyping prior to receiving a red cell transfusion. DNA remaining after completion of the clinical test was retained and de-identified. As these clinical refuse samples were fully de-identified, only limited clinical data was available and the total (or lifetime) number of transfusion units for each individual is unknown; however, all patients had to have received a minimum of two transfusions in the past to be included. Each patient's hemoglobinopathy status was confirmed by genotyping. Samples were obtained from within the LifeShare service area which includes Southeast Texas, the northern half of Louisiana, and Southern Arkansas. Patients were also screened for antibodies using routine serologic techniques. This included incubations at room temperature and 37 °C using Lo-Ion (Immucor, Norcross, GA, USA) and an AHG phase using polyspecific antiglobulin reagent. Patients were characterized as 'responders' (N = 198) if they developed clinically significant alloantibodies after two or more packed red cell transfusions, where clinically significant was defined as 'an antibody that is frequently associated with HDFN (Hemolytic Disease of the Fetus or Newborn), hemolytic transfusion reactions, or a notable decrease in the survival of trans-

Table 1. Cohort (n = 384) and pilot study demographics

Cohort	*384*
Alloimmune category	
Non-responder	186
Responder	198
Alloantibodies, median (min, max, IQR)	2 (1, 11, 1–4)
Gender	
Male	165
Female	211
Unknown	8
Sickle genotype	
HbSS	373
HbSC	11
Age group	
Adult (>18 years)	346
Child (<18 years)	36
Unknown	2
Genotyped individuals	*104*
Alloimmune category	
Non-responder (males)	54 (23)
Responder (males)	50 (20)
Alloantibodies, median (min, max, IQR)	4 (1, 11, 2–5)
Sickle genotype	
HbSS	104

IQR = Interquartile range.

fused RBCs' [32]. 'Non-responders' (N = 186) were those with no alloantibodies after a similar exposure (table 1). We obtained funding to analyze 109 of these DNA samples in the first phase pilot GWAS – 55 'responders' and 54 'non-responders' homozygous for the sickle cell (HbS) mutation. The responder group also included 17 persons with a history of hyperhemolysis syndrome, defined as a post-transfusion drop in hemoglobin below pre-transfusion levels without evidence of bleeding.

DNA Extraction and Genotyping

DNA was extracted from leukocyte buffy coats or whole blood using either a manual method (PureGene®; Qiagen, Venlo, the Netherlands) or the QIACube® (Qiagen). The quantity of double-stranded DNA was assessed in each sample using the PicoGreen (Invitrogen, Carlsbad, CA, USA) assay. All assays were performed according to manufacturer's specifications. Five samples had a DNA concentration below the 50 νg/μl concentration recommended for optimal microarray genotyping and were omitted from further study.

Genotyping was performed according to manufacturer's instructions using the Bead-Station system and HumanOmni1-Quad BeadChips (Illumina, San Diego, CA, USA), which was specifically designed to provide improved SNP coverage in people of West African heritage, as might be expected for participants with SCD. The GenomeStudio software (Illumina) was used to interpret normalized fluorescent intensities as genotypes. HumanOmni1-Quad BeadChips contain approximately 1,140,419 SNPs. SNPs with genotyping efficiency < 90% or out of Hardy-Weinberg equilibrium in controls (at the $p < 10^{-5}$ level) were removed. The final analysis incorporated genotype data from 1,008,655 SNPs.

Sample Quality Control

Known siblings or other related samples were excluded from the initial sample selection. In addition, samples with more than 5% missing data (genotyping efficiency < 0.95) or with evidence of excessive inbreeding (inbreeding coefficient F > 0.1) were removed. Pairs of samples with excess allele sharing suggestive of close familial relationships (parent-offspring, siblings, or 1st degree relatives; PI_HAT > 0.1) were identified by identity-by-descent (IBD); in such cases one member of the pair was removed from analysis (fig. 1).

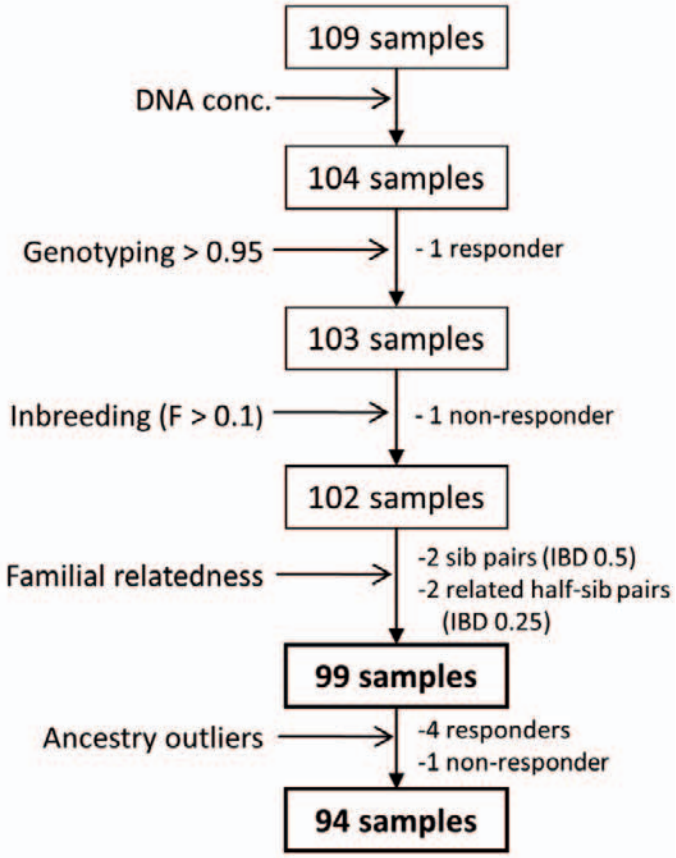


Fig. 1. Sample quality control (QC); Samples were removed from analysis if they i) failed to meet the minimum DNA concentration for the assay according to manufacturer's recommendations (DNA conc); ii) were successfully genotyped at less than 95% of included SNPs (Genotyping); iii) had an inbreeding coefficient (F) suggestive of an excess of homozygotes; iv) had evidence of close-familial pairing (identify by-decent (IBD) or were ancestry population outliers on principal components analysis (see fig. 2).

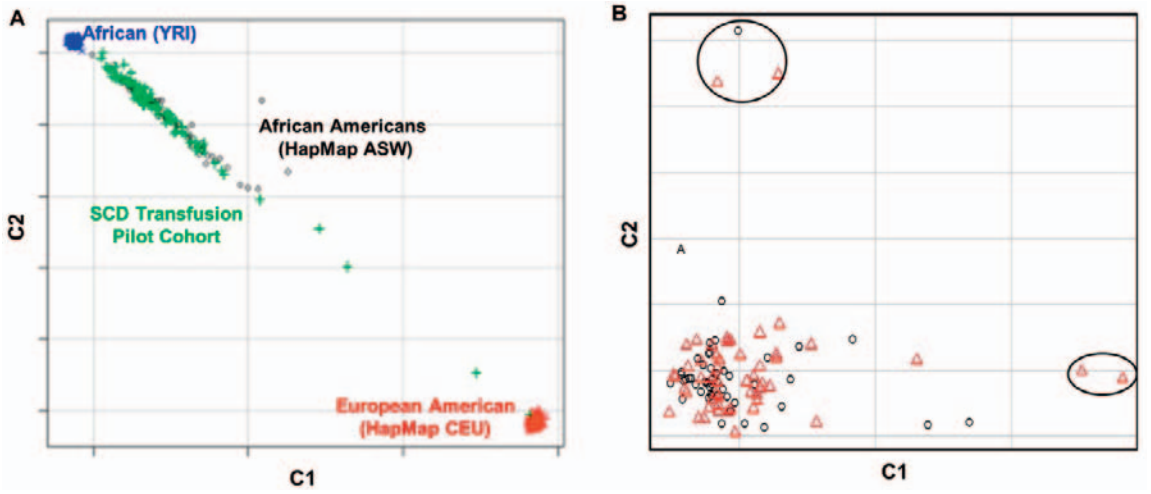
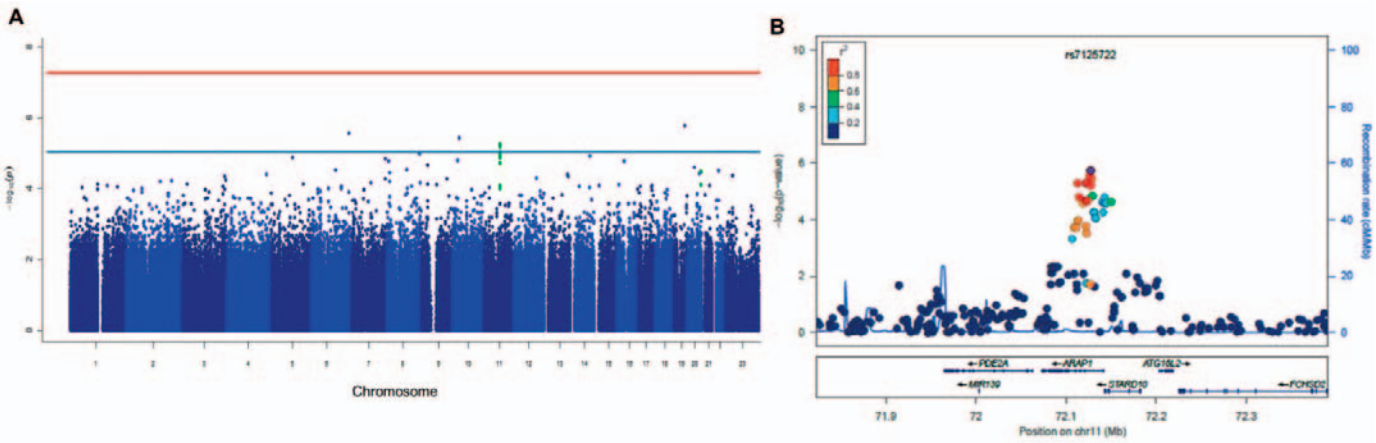


Fig. 2. Multidimensional scaling (MDS) cluster plot of study participants for principal components C1 and C2. **A** Clustering of SCD pilot cohort with HapMap African Americans from Oklahoma and in relation to major continental HapMap groups (YRI = Yoruba from Nigeria; CEU = Ceph from Utah). **B** MDS clustering of cases (black circles) and controls (red triangles) in the pilot cohort. PCA study outliers are circled.



C

Chr	SNP	Position	Allele	Frq Cases	Frq Cntrls	OR	P	Perm P
11	rs12275279	72114944	C	0.42	0.13	4.76	1.2E-05	1.4E-04
11	rs7123876	72122231	A	0.53	0.25	3.40	8.0E-05	3.0E-04
11	rs12289259	72124379	A	0.46	0.16	4.34	1.3E-05	1.4E-04
11	rs7125722	72127364	A	0.42	0.11	5.79	1.9E-06	-
11	rs2365442	72127866	A	0.42	0.11	5.79	1.9E-06	-
11	rs2365441	72127874	T	0.44	0.14	4.95	6.4E-06	-
11	rs67072384	72128372	T	0.44	0.11	6.22	7.2E-07	-
11	rs67075228	72128423	T	0.44	0.11	6.22	7.2E-07	-
11	rs67809272	72128589	A	0.44	0.11	6.22	7.2E-07	-
11	rs72966113	72128618	T	0.46	0.14	5.01	3.5E-06	-
11	rs12294368	72128887	A	0.46	0.14	5.01	3.5E-06	-
11	rs12272109	72131498	G	0.60	0.29	3.67	1.9E-05	3.1E-05
11	rs7122044	72140282	A	0.58	0.26	4.08	6.2E-06	2.8E-05
11	rs1872127	72142733	G	0.55	0.23	4.17	5.5E-06	1.4E-05

Fig. 3. Case control GWAS of responder status. **A** Manhattan plot showing no SNPs below genome-wide significance (red line, $p = 0.5 \times 10^{-8}$), but suggestive SNPs (blue line, $p = 1 \times 10^{-4}$) in the *ARAP1/STARD10* gene region (green dots). **B** Local Manhattan plot of 250 kb region around *ARAP1/STARD10* including genotyped and imputed SNPs. Pairwise LD (r^2) is derived from the HapMap YRI population. **C** GWAS results for genotyped and imputed (shaded) SNPs in the *ARAP1/STARD10* region. OR = Odds ratio; Perm P = permuted p value.

Identification of Population Stratification

Prior to analysis, the remaining individuals were evaluated for shared ancestry using multidimensional scaling (MDS). The principle of MDS is to cluster individuals of similar ancestry by repeatedly adjusting individual genotypes by the amounts attributable to ancestry. The resulting dimensions can be plotted to visualize individuals whose inferred ancestry is outside that of the sample. Clustering was performed with a subset of SNPs pruned using a sliding window to exclude SNPs with strong non-random association (linkage disequilibrium; LD) with surrounding SNPs ($r^2 > 0.2$). Five outliers were identified using this approach (fig. 2). Exclusion of these outliers resulted in a final cohort of 48 'responder' cases and 46 'non-responder' controls.

Statistical Analyses

MDS clustering, IBD analysis, and F statistics (inbreeding) were carried out using PLINK [33], as were basic statistical analyses (χ^2 and permutation). After primary analysis, label swapping adaptive permutation was performed for each SNP. In label swapping, the case/control label is swapped for each individual, and the association statistic is derived multiple times to create a new null distribution with which to compare with the observed data. The 'adaptive' mode essentially curtails the run length by removing SNPs that are highly unlikely to attain statistically significance at each permutation, until a user-defined maximum number of permutations (in this case 1,000,000) is reached for SNPs that continue to be statistically significant. Genome-wide inflation was estimated using the genABEL package [34] in R. Manhattan and MDS plots were generated using a web-based R source code (*http://GettingGeneticsDone.com*) and LocusZoom [35]. Shapeit2 [36] and Impute-2 [37] were used to impute genotype data from the HapMap [38] and the 1000 Genomes Project Consortium [39]. Imputation uses derived patterns of population-specific LD between SNPs to infer genotypes at sites that have not directly been genotyped. This can be a particularly useful approach in populations of African ancestry, where genotyped SNPs may not provide true 'genome-wide' coverage.

In subgroup analyses of the cumulative number of antibodies developed among responders, the distribution of alloantibodies was slightly skewed to the right. A power transformation ($x^{1/4}$) was performed to better fit a normal distribution. Unadjusted linear regression models were fit to the data to derive beta statistics and p values.

Quanto 1.2.4 [40] was used to estimate study power. The study sample size employed had 80% power to determine genetic risk ratios (GRRs) greater than 10 at genome-wide significance ($p < 0.5 \times 10^{-8}$) using SNPs with minor allele frequency (MAF) of 0.15 or greater under an additive disease model.

Results

No Large-Effect Responder Loci, but Suggestive Association in ARAP1/STARD10

For our primary analysis, each SNP with MAF > 0.10 was tested for association (χ^2) under an allelic trend model using 48

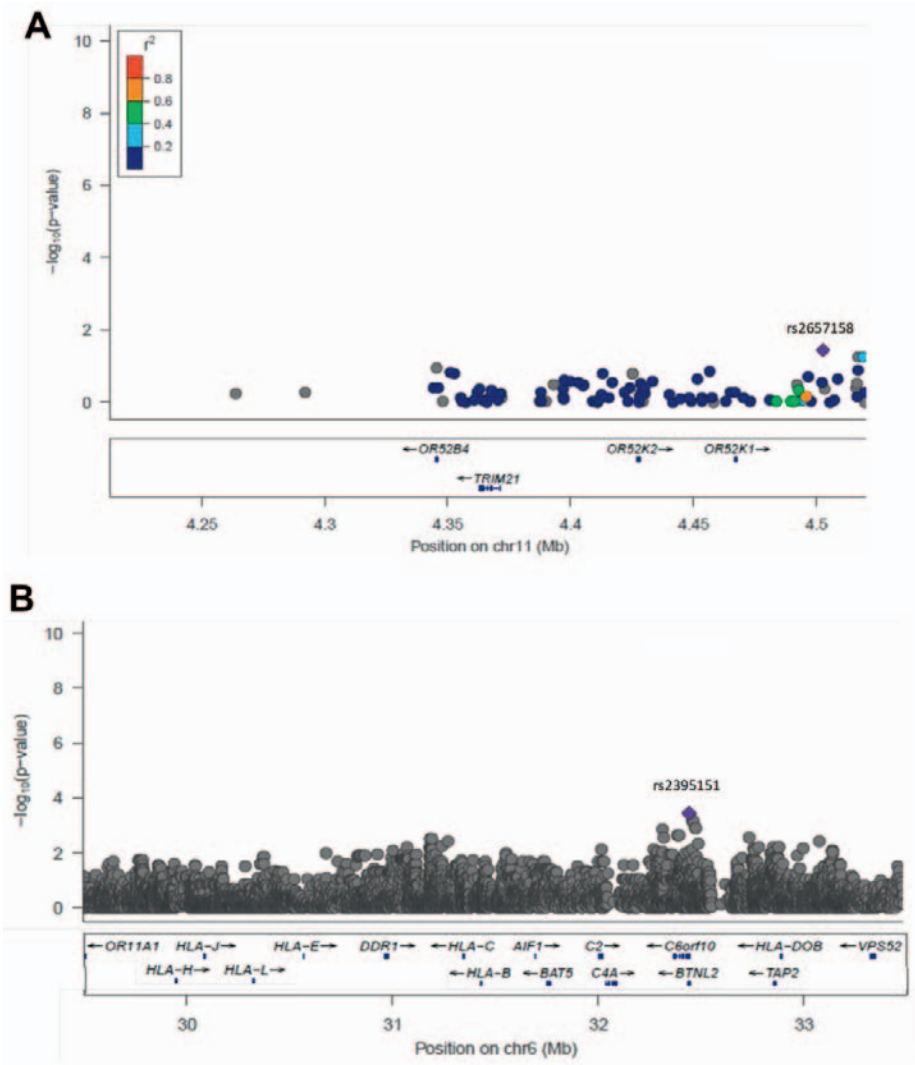


Fig. 4. Local Manhattan plots for previously associated loci *TRIM21* (**A**) and the *MHC* (**B**). In each figure the highlighted SNP (purple diamond) is the strongest association in the region. In **A** pairwise LD (r^2) is derived from the Hapmap YRI population. In **B** the gene list is restricted for space considerations.

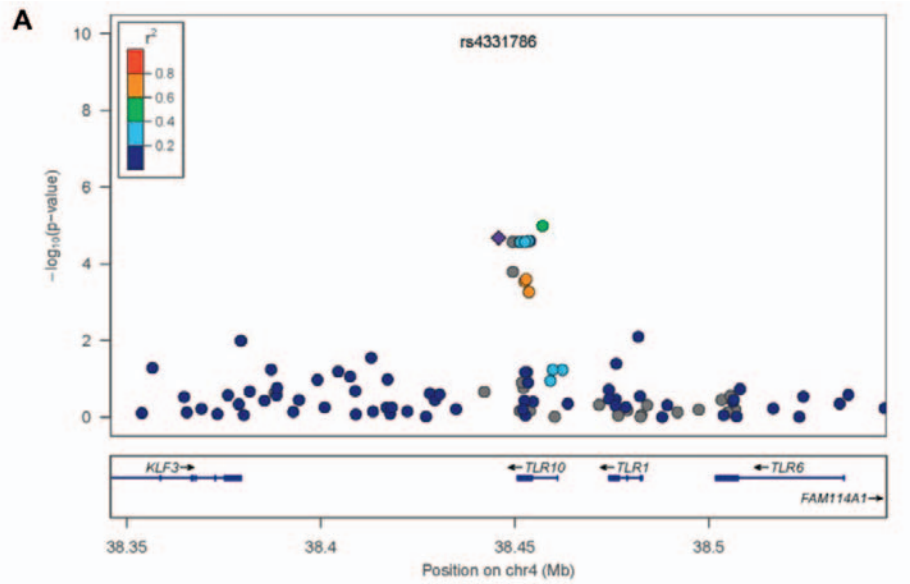
responders as cases and 46 non-responders as controls. The genome-wide inflation factor in this analysis was 0.985, suggesting little residual inflation of the test statistic from genotyping error, cryptic relatedness of individuals, or population stratification. We did not observe any SNPs or clusters of SNPs at or below the accepted threshold for statistical significance in genome-wide association studies ($p < 0.5 \times 10^{-8}$) (fig. 3a).

Next, we sought to evaluate SNPs with p values that were below the 'background' association of most SNPs, but did not meet genome-wide significance. We focused on regions of the genome that had more than one SNP within 100 kb with $p < 1 \times 10^{-4}$; this threshold was deemed as 'suggestive' association. One region met this criteria (fig. 3a) – a cluster of three SNPs (lowest $p = 1.2 \times 10^{-5}$) on the long arm of chromosome 11 at the 3 end of *ARAP1*, extending 5 of *STARD10* (fig. 3c). This modest association persisted after permutation testing (lowest $p = 1.4 \times 10^{-4}$). Manual inspection of the genotyping clusters and the relative local LD pattern in the HapMap YRI from Yoruba, Nigeria [38], suggested that technical (genotyping) error was unlikely to be a significant contributing factor.

Having observed moderately suggestive association with SNPs across this region, we then performed statistical imputation of ~17,983 variants within in a 5 megabase (MB) region centered upon the SNP with the best association in the original genotyping. We then filtered for common variants (MAF > 0.05) with imputation certainty greater than 95%. This resulted in a final test set of 3,795 SNPs. Using a χ^2 test for allelic trend under the same case-control model, five additional SNPs showed similar and, in some cases, stronger association than the genotyped SNPs at this candidate locus (fig. 3b,c), although none reached genome-wide significance.

No Association with Responder Status at Previously Reported Loci

Next, we focused on two regions that have been previously associated with the development of alloantibodies in other studies – *TRIM21* on chromosome 11 and the major histocompatibility complex (MHC) on chromosome 6. At *TRIM21*, none of the genotyped SNPs had a p value less than 10^{-3} (fig. 4a). A similar pattern was observed across the4 MB of



B

Chr	SNP	Position	Ref Allele	MAF	Beta	P
4	rs11735251	38449452	T	0.28	0.514	2.7E-05
4	rs10776482	38451180	G	0.28	0.514	2.7E-05
4	rs10776483	38451435	G	0.28	0.514	2.7E-05
4	rs11096956	38452575	A	0.28	0.514	2.7E-05
4	rs10856837	38453454	A	0.20	0.553	2.5E-05
4	rs9996228	38453912	C	0.20	0.553	2.5E-05
4	rs11466612	38457116	G	0.25	0.565	1.0E-05

Fig. 5. Suggestive association between SNPs across *TLR10* and number of alloantibodies formed. **A** Local Manhattan plot of SNPs within 250 kb of *TLR10*. **B** SNPs showing suggestive association.

the MHC; most SNPs had p values greater than 1×10^{-3} (fig. 4b), with the strongest SNP association ($p < 0.0004$) being found in *c6orf10*, upstream of the MHC class II region some distance from the major HLA types previously associated with the phenotype.

Subgroup Analysis of Alloantibody Accumulation Reveals an Additional Suggestive Locus

As an exploratory subgroup analysis, we incorporated a quantitative approach that focused on SNPs influencing the number of antibodies formed by each responder (N = 48) using unadjusted linear regression models. In this analysis there were no SNPs with evidence of association at or below genome-wide significance; however, using the same criteria for suggestive loci employed in our primary analysis, three regions had more than two SNPs with $p < 1 \times 10^{-4}$ within 100 kb. The most convincing of these included a cluster of eight SNPs in an 11 kb region on chromosome 4p involving the Toll-receptor 10 (*TLR10*) gene (fig. 5).

Discussion

We did not observe any SNP associations with responder status at or below genome-wide significance in our cohort. In the small sample size of this pilot study, genome-wide significance represents a conservative cut-off p value below which false-positive results are highly unlikely. Thus, we can conclude that in this SCD cohort, becoming an alloimmune responder is not influenced by a locus with very large effect size (GRR > 10). Like many other complex traits, the contribution of common genetic variation to responder status is more likely to be the result of loci with more moderate effects. This class of effects is difficult to confidently discern in a pilot GWAS such as this one, and is further compounded by the African ancestry of the cohort. African populations have greater genetic diversity and less LD between SNPs. Because genome-wide genotyping will rarely, if ever, genotype the true causal variant, GWAS studies are dependent upon LD for genotyped SNPs to act as 'proxies' for ungenotyped SNPs. The relative lack of LD in African and African-derived populations thus makes finding more moderate association signals using conventional microarray genotyping platforms more dif-

ficult [41], despite the availability of statistical imputation. Recent interest in the development of Afro-centric SNP genotyping microarray chips should make future GWAS in the African-American SCD population more robust.

The use of a less conservative cut-off allowed us to evaluate evidence for loci with more moderate effects on the phenotype, although such SNPs have a higher possibility of being false-positives. A cluster of SNPs spanning *ARAP1* and the adjacent *STARD10* genes showed suggestive association with responder status in our cohort. *ARAP1* (MIM# 606646) encodes a Rho/Arf GTPase-activating protein (GAP) postulated to play a role in regulating endocytic trafficking of epidermal growth factor receptors (EGFRs) to the cell membrane. *STARD10* (Gene ID:10809) encodes one of a group of proteins characterized by incorporation of a steroidogenic acute regulatory protein(StAR)-related lipid transfer (START) domain that binds specific lipid motifs and implicated in the control of lipid biology and cell trafficking. The related STARD11 protein interacts with the Goodpasture antigen and is known to play a role in the autoimmune response seen in Goodpasture syndrome [42]; there is thus a potential, albeit speculative, biological role for *STARD10* in the processing and presentation of red cell membrane antigens. Larger, well-powered replication studies, utilizing more comprehensive genotyping platforms that are better suited to the African American population, are necessary to validate these findings.

In keeping with recent murine studies [43], our findings suggest that there is little evidence to support a major role for variants in *TRIM21* in responder status. Our sample size was larger than that employed in previous studies; however, our study was not designed to look for more subtle determinants or known covariates of alloimmunization (e.g. age or transfusion dependency) and thus we could not confirm the postulated role of the rs660 SNP in this cohort. It should be noted that rs660 is not represented on the HumanOmni1-quad chip, but also maps to several sites in the current version of the human genome reference sequence (*www.ensembl.org*). The fact that rs660-flanking sequences do not map uniquely in the current genome assemblies raises the possibility that technical factors may underlie the previously reported association. Similarly, we did not find strong evidence for association with SNPs across the MHC in our analyses of this cohort. This would be in line with earlier studies suggesting that restricted HLA allelotypes may not play a significant role in susceptibility to alloimmune transfusion tolerance [44]. However, because our pilot study did not include extended haplotype analyses of the MHC and current genotyping microarrays do not necessarily fully capture African diversity at the MHC, this result does not completely preclude a role for the MHC in the responder phenotype.

In subgroup analysis we employed a quantitative approach to look at potential genetic factors influencing the development of alloantibodies. A quantitative approach for continuous and ordinal traits has increased power to detect association in genetic studies and consequently generally requires a smaller sample size to find similar effect sizes. This approach has been successfully employed in other GWAS [45, 46], including the association of SNPs in *BCL11A* with fetal hemoglobin levels in SCD patients, which was accomplished with a few hundred cases and controls [20]. We did not find evidence for large-effect loci controlling the development of multiple antibodies; however, several SNPs around *TLR10* showed modest association with increasing alloantibodies. Toll-like receptors (TLRs) have a well-established role in the early immune response to invading pathogens, although a specific role for the TLR10 protein has not yet been identified. Recently, murine models of cardiac and skin transplantation have implicated TLRs in the alloimmunity of graft rejection [47], and polymorphisms in *TLR4* have been associated with lung transplant rejection in humans [48]. There is thus growing interest in the role of innate immunity and TLRs in alloimmunization. This again makes it imperative that larger and more comprehensive genomics studies are undertaken to replicate the preliminary results presented here.

This pilot GWAS of alloimmune responder status and alloantibody number did not reveal evidence of a large-effect locus in this SCD cohort, including previously identified loci *TRIM21* and the MHC. However, suggestive association at the putative candidate loci *ARAP1*/*STARD10* and *TLR10* advocate for future genomics studies that will not only evaluate these preliminary results but also engender novel approaches to understanding differential alloantibody susceptibility in SCD. A genomics approach to the problem has recently been endorsed as a viable framework in which to study various aspects of the SCD responder phenotype [49]. The challenge demonstrated by this study is that success in such a venture will require larger cohorts built around a broad expertise and strong collaboration.

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Disclosure Statement

JMM serves is currently an employee of Grifols Inc. and previously served as the principal investigator for grants and research agreements between LifeShare and Novartis Diagnostics or BioArray/Immucor. No other authors have conflicts to declare.

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