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Mini Review

Approaches to Determination of a Full Profile of Blood Group Genotypes: Single Nucleotide Variant Mapping and Massively Parallel Sequencing

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ABSTRACT

The number of blood group systems, currently 35, has increased in the recent years as genetic variations defining red cell antigens continue to be discovered. At present, 44 genes and 1568 alleles have been defined as encoding antigens within the 35 blood group systems. This paper provides a brief overview of two genetic technologies: single nucleotide variant (SNV) mapping by DNA microarray and massively parallel sequencing, with respect to blood group genotyping. The most frequent genetic change associated with blood group antigens are SNVs. To predict blood group antigen phenotypes, SNV mapping which involves highly multiplexed genotyping, can be performed on commercial microarray platforms. Microarrays detect only known SNVs, therefore, to type rare or novel alleles not represented in the array, further Sanger sequencing of the region is often required to resolve genotype. An example discussed in this article is the identification of rare and novel *RHD* alleles in the Australian population. Massively parallel sequencing, also known as next generation sequencing, has a high-throughput capacity and maps all points of variation from a reference sequence, allowing for identification of novel SNVs. Examples of the application of this technology to resolve the genetic basis of orphan blood group antigens are presented here. Overall, the determination of a full profile of blood group SNVs, in addition to serological phenotyping, provides a basis for provision of compatible blood thus offering improved transfusion safety.

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1. Introduction

Blood group systems are comprised of red cell surface antigens (proteins, glycoproteins or glycolipids) which are defined by a human alloantibody [1]. Blood group systems are inherited and controlled by a single gene or cluster of two or three closely-linked homologous genes [1]. The number of blood group systems meeting the classification requirements of the International Society for Blood Transfusion, currently 35, has increased in the recent years as genetic variations defining red cell antigens continue to be discovered. At present, 44 genes that encode the 35 blood group systems have been identified with 1568 alleles listed in the NCBI Blood Group Gene Mutation Database [2]. The most frequent genetic change determining blood type is single nucleotide variation (SNV) resulting in a missense mutation. Inactivating mutations such as deletions and splice-site mutations also result in new epitopes or loss of a red cell surface protein. Additionally, crossover events between closely-linked homologous genes, such as *RHD* and *RHCE* or *GYPA* and *GYPB*, create hybrid alleles often resulting in the expression of partial antigens and/or neo antigens [3,4].

Traditionally the presence, or absence, of a blood group antigen has been determined by phenotyping using antibody-based serological methods. Genotyping of SNVs encoding blood group antigens provides a prediction of phenotype and the application of genotyping methods to blood group typing is a powerful tool to complement, and to overcome some of the limitations of, serology [5–7]. Genotyping can improve the accuracy of typing in cases where serology alone is unable to resolve red cell phenotype, for example in individuals who have weakened antigen expression due to the presence of genetic variants. Furthermore, there are scenarios in which genotyping is the only method available for typing of blood group antigens, such as cases of rare phenotypes where antisera are unavailable [8–10]; in recently- or multi-transfused patients [11,12]; and for the determination of blood type of a fetus via genotyping of cell free fetal DNA in maternal plasma [13,14]. Additionally, genotyping with multiplex or parallel reactions in a single assay is more feasible than serological typing for large-scale population screening studies.

Blood group genotyping by in-house PCR-based assays, either for a single or small number of SNVs, has been undertaken by specialized laboratories since the 1990's. These methods have included PCR-restriction fragment length polymorphism (RFLP), sequence-specific primer (SSP)-PCR, single-nucleotide primer extension, and more recently, real-time (RT)-PCR and high resolution melt (HRM) analyses [15–18]. Although these assays have proven to be accurate, they are limited by their low-throughput, limited multiplex capacity, and the number of manual steps involved. In the 21st century, microarray-based genotyping platforms were developed to overcome these limitations. Discussed in this review are the BloodChip Reference from Progenika (Grifols) and the BeadChip from BioArray Solutions (Immucor), which were among the first to be developed and achieve regulatory accreditation. In more recent times, commercially available SNV typing platforms based on technologies capable of even higher throughput, including Luminex xMAP and single nucleotide primer extension followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), have become available [19,20].

As we progress into the 21st century, massively parallel sequencing (MPS), or next generation sequencing, has emerged as a result of the Human Genome Project and later full genome assemblies for other species [21–23]. This technology can be applied to sequencing the whole genome, the exome, or a panel of selected genes, and with cost decreasing; this technology is being applied in medical diagnostics research [24].

This review is designed to overview two approaches to blood group genotyping, microarray-based genotyping, focusing on two available genotyping platforms, and MPS. This review provides an overview of these technologies as well as the advantages, limitations and examples of the recent applications. It is recognized there are numerous microarrays and MPS platforms available, however, a detailed comparison of these platforms is outside the scope of this review.

2. Preparation of sample material for molecular methods

Irrespective of the molecular approach utilized, genomic DNA extracted without the risk of contamination from the external environment is required as the input material. For minimisation of contamination and for optimisation of workflow, it is preferred that DNA extraction is performed in an automated and high-throughput fashion. Vendors of commercially available DNA extraction platforms vary between jurisdictions; however, the robustness of these platforms is well-accepted. One current limitation of all available genotyping and sequencing platforms is that the DNA extraction step is not integrated into a fully automated process. Thus, DNA extraction remains an independent step performed prior to molecular investigations.

3. SNV mapping by DNA microarrays

3.1. Overview of technology

SNV mapping to predict blood group antigens is most commonly performed by DNA microarrays. DNA microarrays can be defined as miniaturized solid surface assays of high multiplexing power [25]. The solid surface of a DNA microarray commonly consists of a chip, wafer or bead, with probes corresponding to various blood group alleles bound to the surface. During the microarray process, allele-specific hybridization occurs between complementary probes and labeled DNA fragments from a sample, and this is measured to determine genotype.

There are several commercial DNA microarray platforms currently available for blood group genotyping. In this paper we discuss two widely utilized platforms for blood group genotyping, the BloodChip Reference from Progenika (Grifols) and the BeadChip from BioArray Solutions (Immucor). Other available platforms include LIFECODES RBC/RBC-R (Gen-Probe) and ID CORE XT (Progenika) based on Luminex technology [26], GenomeLab SNP stream (Beckman) [27], the HIFI Blood 96 (AXO Science) [28] and the Hemo ID Blood Group Genotyping Panel (Agena Bioscience) [20]. Comprehensive reviews on currently available SNV typing platforms are provided by Veldhuisen et al. and Boccoz et al. [29,30].

The BloodChip Reference was developed between 2003 and 2006 by the Bloodgen project funded by the European Commission [31]. Genotyping by the BloodChip Reference platform involves multiplex PCR amplification, labeling of the amplified products and hybridisation to allele-specific probes on a glass slide [31]. A laser array scanner is used to detect binding at each probe location and software determines the genotype and predicted phenotype by comparing the strength of binding of the labeled PCR products to probes [31]. The BloodChip Reference assay genotypes up to 24 samples in ten hours. The BloodChip Reference microarray contains approximately 6280 spots with 256 internal controls and 40 replicates per allele. The BloodChip Reference v4.1 genotypes 128 SNVs for 33 clinically significant antigens from the ABO, RH, KEL, FY, DO, CO, LU, DI, JK and MNS blood group systems. The desire to predict *RHD* variants was a major focus of the BloodChip Reference design and the number of *RHD* alleles incorporated in the BloodChip assay is extensive [31–33].

The HEA BeadChip was developed and validated by BioArray Solutions and the New York Blood Centre and became commercially available in 2006 [34,35]. Genotyping by BeadChip involves multiplex PCR amplification of DNA followed by denaturation and hybridization to color-coded beads incorporating allele-specific probes with variable 3′ ends [34]. When binding occurs between a complementary PCR product and probe, the probe elongates, producing a fluorescent signal [34]. An image is taken with an automated Array Imaging System and the fluorescent signal intensities determined and correlated with the corresponding probes to provide the basis for genotype and predicted

phenotype [34]. The BeadChip assay genotypes up to 96 samples in five hours. The BeadChip microarray contains approximately 4000 beads comprising seven negative and positive reaction controls and 25 to 40 replicates per allele. Different BeadChip modules are available, with a focus on the range of blood group SNVs to be tested. The human erythrocyte antigen (HEA) BeadChip genotypes 24 alleles encoding 38 antigens from the RHCE, KEL, FY, DO, LW, CO, SC, LU, DI, JK and MNS blood group systems. Additional chips are available (RHD BeadChip and RHCE BeadChip) for targeted red cell genotyping [35].

3.2. Advantages

Genotyping blood group SNVs using microarray technology has many advantages over traditional serological or basic in-house PCR-based tests. Microarrays test for multiple markers simultaneously, providing in-depth information on one blood group system or many blood group systems, depending on the module utilized. Microarrays have a proven track-record for sensitivity and specificity and some platforms, including BloodChip Reference and HEA BeadChip, have obtained Council of Europe marking (accreditation as suitable for use in a diagnostic environment). Furthermore, microarray data analysis software allows rapid interpretation of the signals generated by binding of probes and DNA fragments into an easily understood genotype and predicted phenotype. These automated microarray data analysis processes offer significant advantages over traditional serological and in-house PCR-based tests. This is because for traditional methods, analysis of data is often a more manual and time consuming process and can be subjective or open to interpretation, for example the scoring of red cell agglutination by eye.

3.3. Limitations

The most obvious limitation of all SNV microarray platforms is that they utilize a targeted genotyping approach and thus genotype only alleles incorporated in the microarray. Most platforms are fixed and adaptation, once new alleles are discovered, requires time for specific design and validation, and is at the discretion of the manufacturer rather than the user.

In a small number of cases, genotype does not accurately reflect phenotype. For example, microarray platforms may provide an inaccurate prediction due to the presence of a silencing SNV located outside of the targeted area. In these cases the genotyping platform will detect an apparently normal allele inconsistent with serological results [36,37]. In this area, further design improvements may be required as new variants are reported.

It must also be considered that commercially available platforms may be designed for a specific population. For example the BloodChip Reference was designed for SNVs within the European population whereas the BeadChip was designed with the American population with African-American SNVs in the DO blood group system and *RHCE* gene as a focus. In Australia, we have a varied and multi-ethnic population with a large proportion of individuals of Asian ancestry who may carry alleles in the MNS blood group system and *RHD* gene, not incorporated in these genotyping platforms.

3.4. Applications

Interestingly, few studies have performed direct comparison of commercially available genotyping platforms. Recently in our laboratory we undertook a study involving genotyping of reagent red blood cell donors utilizing three commercially available microarray platforms. Reagent red blood cell donors are extensively serologically phenotyped and it is critical that these phenotypes are accurate. Comparative analysis of commercial SNV typing platforms, including BloodChip Reference and BeadChip, provided evidence that each platform produces reliable data. In this study, sensitivity and specificity of 100% were achieved by each platform on the sample set tested ($n = 196$) [38]. A phenotype genotype discrepancy rate of 10.4% was detected and discrepancies identified were in clinically significant blood group systems [38]. All discrepancies were in samples presumed to be homozygous for antigen expression based on serological typing, however genotyping detected mutations encoding for weakened antigen expression. Overall, this study highlighted the utility of genotyping, specifically for the detection of SNVs which encode for weakened antigen expression of clinically significant blood groups, specifically RhD and Fy^b antigen expression.

The Rh blood group system is highly polymorphic, as demonstrated by Wagner et al. [39,40]. Both the strengths and limitations of SNV-based genotyping were highlighted in a study performed by our group investigating *RHD* variants in a donor population of 2027 apparently RhD negative individuals. While this study detected 171 *RHD* positive donors carrying a range of *RHD* variants, nine individuals carried *RHD* alleles not defined by genotyping on BloodChip Reference. *RHD* exon scanning or Sanger sequencing was required to resolve these rare or novel alleles [41]. An additional two samples remain unresolved following Sanger sequencing, including sequencing of the untranslated and putative cis-regulatory regions of the *RHD* gene [41]. Further investigation is continuing to define the *RHD* variants associated with the altered RhD expression in these individuals.

3.5. Summary

In summary, SNV microarrays are a useful molecular tool for the determination of blood group genotype. However, difficulty in resolution of a case following microarray analysis can occur due to the presence of a novel allele, a rare allele not incorporated in the microarray, or a complex genetic variant, and further investigation must be performed. If the gene causing the unresolved genotype is suspected then Sanger sequencing offers an approach for resolution. The cases presented here exemplify our laboratory's experience that up to 5–10% of complex serological cases cannot be resolved by microarray genotyping and 1–2% of these cases remain unresolved even after analysis by Sanger sequencing.

4. Massively parallel sequencing to define a blood group phenotype

4.1. Overview of technology

MPS is emerging as the molecular method of choice in complex cases which remain unresolved following traditional molecular investigations. Multiple MPS platforms have become commercially available since the release of MPS in 2005 [42]. Although chemistry varies between platforms, in general MPS technologies start with a template library preparation step which involves fragmentation of DNA. The DNA fragments are then ligated to adapters and read in parallel during DNA synthesis to generate data up to the entire genome. Reads are then mapped to a reference sequence, or assembled *de novo*, and variants identified.

4.2. Advantages

Genotyping blood group SNVs using MPS technology has many advantages over serological phenotyping and other molecular methods including in-house PCR-based tests, SNV microarrays and Sanger sequencing. A major advantage of MPS over SNV-based genotyping is the applicability of MPS in detection of unknown polymorphisms. This contrasts with SNV-based genotyping assays which only genotype incorporated SNVs and from Sanger sequencing in which each exon within a gene of interest must be individually sequenced. Additionally, MPS has the potential to offer greater throughput than traditional methods, once optimisation of protocols has been achieved. Another major advantage of MPS is the massive quantity of information obtained in one

experiment, relating either to the genes of interest or the whole genome or exome.

4.3. Limitations

The major limitation of MPS technology is the challenge presented by the substantial quantity of data generated and the associated information technology requirements, including the management of bioinformatics analysis processes and data storage (space and security). In addition, the cost of MPS remains high, though decreasing, and the time-to-result is days to weeks, though also decreasing due to increases in automation and optimisation of workflow and analysis. Optimisation of bioinformatics tools and secure storage spaces to allow effective analysis of MPS data will be essential to the future development of MPS applications. Another important consideration is the detection of SNVs of unknown significance, as unlike targeted microarray approaches, the whole sequence is obtained and variants with an unknown effect on blood group antigen presentation may be detected.

Furthermore, ethical implications relevant to MPS must be considered. For example, if the purpose of MPS is to predict blood group antigens then steps must be taken to ensure that non-relevant information is appropriately managed. There are numerous ways this can be achieved, dependent on the MPS approach utilized, for example, only performing MPS on a selected panel of genes or amplicons, or not performing sequence alignment or analysis of non-relevant genes. Once data has been obtained, safeguards must be implemented to ensure donor and patient confidentiality.

4.4. Applications

Three basic approaches can be utilized for an MPS investigation; whole genome, whole exome or targeted gene sequencing. In the transfusion medicine field the whole exome approach has shown value in resolving the genetic basis of “orphan” antigens. Cvejic et al. recently applied MPS to resolve the genetic basis of Vel, a clinically significant high-frequency blood group antigen [43]. Exome sequencing of five unrelated Vel negative individuals identified the genetic basis of Vel and provided evidence for the establishment of the Vel blood group system [43]. Our group recently utilized MPS to resolve the genetic basis of the low-frequency orphan antigen SARAH (SARA). SARA was discovered in an Australian family in 1990 and has since been described in a Canadian family where SARA incompatibility caused a severe case of Hemolytic Disease of the Fetus and Newborn [44,45]. Our study involved exome sequencing the SARA proband and family [46]. Collecting multiple SARA positive and SARA negative samples from related individuals was vital to the bioinformatics approach as exome sequencing identified a total of approximately 500,000 SNVs [46]. The appropriate SNV filtering steps are dependent on the case. In our study we excluded the previously reported SNVs, non-protein coding SNVs and SNVs not present in all SARA positive individuals and absent in all SARA negative individuals. Following the identification of the SARA SNV, G240T on *GYPA*, we confirmed our findings by performing Sanger sequencing of the candidate gene (GenBank accession number KF973190).

Application of a targeted MPS approach was first undertaken by Stabentheiner et al. who provided proof of principal for MPS genotyping of *RHD* variants [47]. More recently in research studies, MPS has targeted selected panels of two to 18 blood group genes [48–50]. This is an approach likely to increase in utilization as cost continues to decrease. Over the next few years many laboratories will no doubt be developing MPS assays targeting a full panel of blood group genes.

4.5. Summary

In summary, the genetic basis of blood group antigens can be complex. MPS offers a new approach in cases which remain unresolved following traditional investigations including microarray analysis and Sanger sequencing. Although MPS offers advantages over other methods, including high-throughput capacity, there are limitations and challenges to this approach which require further optimisation before wide-spread adoption in the routine setting.

5. Summary and outlook

Molecular methods designed to predict clinically significant blood group antigens are a useful adjunct to overcoming the limitations of serology and to ensure transfusion safety. From a clinical perspective, the molecular method with the most utility is dependent on the complexity of the case. For the majority of cases, genotyping by SNV microarray offers a useful approach for the prediction of blood group antigen phenotype and offers advantages such as automated data analysis. A small number of cases remain unresolved following genotyping by SNV microarray and many of these cases can be resolved by Sanger sequencing. The emergence of MPS is providing an approach to investigate complex cases which remain unresolved. The major benefits of MPS are the capacity to resolve rare and novel variants and the potential for high-throughput testing. At present, on the research front, MPS approaches are being applied to resolve the genetic basis of red blood cell orphan antigens. In the future, routine blood group genotyping by SNV microarrays may be replaced by MPS approaches, however at this time, the requirement for information technology resources remain too extensive for routine use. Regardless of the molecular method used, the determination of a full profile of blood group SNVs, in additional to serological phenotyping, provides a basis for provision of compatible blood thus offering improved transfusion safety.

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