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Frequency of Mi^a (MNS7) and Classification of Mi^a-Positive Hybrid Glycophorins in an Australian Blood Donor Population

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Keywords

Mi^a (MNS7) antigen · MNS hybrid glycophorins · MNS blood group system · Miltenberger · Blood group antigen · Blood group genotyping

Abstract

Background: MNS blood group system genes *GYPA* and *GYPB* share a high degree of sequence homology and gene structure. Homologous exchanges between *GYPA* and *GYPB* form hybrid genes encoding hybrid glycophorins GP(A-B-A) and GP(B-A-B). Over 20 hybrid glycophorins have been characterised. Each has a distinct phenotype defined by the profile of antigens expressed including Mi^a. Seven hybrid glycophorins carry Mi^a and have been reported in Caucasian and Asian population groups. In Australia, the population is diverse; however, the prevalence of hybrid glycophorins in the population has never been determined. The aims of this study were to determine the frequency of Mi^a and to classify Mi^a-positive hybrid glycophorins in an Australian blood donor population. ***Method:*** Blood samples from 5,098 Australian blood donors were randomly selected and screened for Mi^a using anti-Mi^a monoclonal antibody (CBC-172) by standard haemagglutination technique. Mi^a-positive red blood cells (RBCs) were further characterised using a panel of phenotyping reagents. Genotyping by high-resolution melting analysis and DNA sequencing were used to confirm serology. ***Result:*** RBCs from 11/5,098 samples were Mi^a-positive, representing a frequency of 0.22%. Serological and molecular typing identified four types of Mi^a-positive hybrid glycophorins: GP.Hut (n = 2), GP.Vw (n = 3), GP.Mur (n = 5), and 1 GP.Bun (n = 1). GP.Mur was the most common. ***Conclusion:*** This is the first comprehensive study on the frequency of Mi^a and types of hybrid glycophorins present in an Australian blood donor population. The demographics of Australia are diverse and ever-changing. Knowing the blood group profile in a population is essential to manage transfusion needs.

Introduction

The glycophorin genes of the MNS blood group system are clustered in a 350-kb region on chromosome 4q28–q31 (Fig. 1) [1–3]. Glycophorin genes *GYPA*, *GYPB*, and *GYPE* encode GPA, GPB, and GPE, respectively, and share more than 95% sequence homology and gene structure [4, 5]. Homologous exchanges between *GYPA* and *GYPB* result in the formation of *GYP(A-B-A)* and *GYP(B-A-B)* hybrid genes and encode glycophorin molecules expressed on the red blood cell (RBC) surface [6–8]. These hybrid glycophorins display

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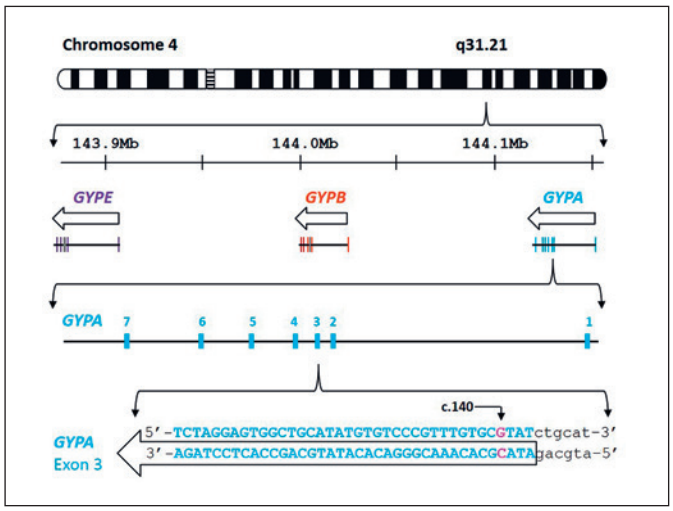

Fig. 1. Schematic diagram of chromosome 4 showing the location and arrangement of *GYPA, GYPB,* and *GYPE* genes. Based on the GRCh38/hg38 assembly, the arrangement and location of the MNS blood group gene cluster is located on Chr4 q31.21 [2, 3]. The GenBank NG_007470 reference sequence was used as the basis for *GYPA* exon 3 sequence [37]. The nucleotide position c.140 is shown on *GYPA* exon 3. The molecular basis for *GYP*Hut* is c.140C>A (p.Thr47Lys) and that for *GYP*Vw* is c.140C>T (p.Thr47Met). Arrows above show the direction of transcription.

a distinct phenotype defined by a profile of antigens including Mi^a [6–8].

Mi^a (MNS7) is an antigen present in 7 hybrid glycophorins, namely: GP.Vw, GP.Hut, GP.HF, GP.Mur, GP.Hop, GP.Bun, and GP.Kip [7–17]. The distribution of these glycophorins varies between population groups. GP.Vw is found more commonly in Caucasians (up to 1.4% in south-east Switzerland) [18] while GP.Mur is more frequent in Asian populations – Malaysians (2.8%) [19], Indians (3.0%) [19], Chinese (6.5%) [20], Vietnamese (6.5%) [21], Filipinos (7.6%) [22], and Ami Taiwanese (88%) [23]. Over a quarter (26%) of Australia's population were born overseas, and several case studies have reported the presence of MNS hybrid glycophorins in Australian individuals [16, 24–26]. Antigens expressed on hybrid glycophorins are immunogenic and may stimulate an immune response when exposed to individuals who do not carry these antigens [6, 7].

In Australia, a limited number of haemolytic transfusion reaction and haemolytic disease of the fetus and newborn cases have been reported due to antibodies against hybrid glycophorins likely stimulated by exposure to GP.Vw and GP.Mur RBCs [7]. However, there are no comprehensive data on the occurrence of Mi^a and the type of MNS hybrid glycophorins present in the current Australian population. The aims of the study were to determine the prevalence of Mi^a and to categorise Mi^a-positive hybrid glycophorin variants in an Australian blood donor population.

Methods

Blood Donor Samples

Blood samples from volunteer Australian blood donors in Queensland were randomly selected for this study between January 2011 and July 2013. A total of 5,098 blood samples were screened for Mi^a using monoclonal antibody anti-Mi^a CBC-172. For blood donors identified carrying the Mi^a antigen, an extra collection of 6-mL EDTA-whole blood sample was requested on their subsequent blood donation for DNA analysis.

RBC Preparation and Genomic DNA Isolation

RBCs from EDTA whole blood samples were washed with PBS and then suspended in PBS to a concentration of 3–5% for use in a standard haemagglutination test (tube method). For molecular biology testing, genomic DNA was extracted from EDTA whole blood samples using a DNA extraction kit (EZ1 DNA Blood 350μL kit; QIAGEN) in a robotic equipment (BioRobot EZ1 Workstation; QIAGEN) according to the manufacturer's instructions. Isolated DNA was quantitated and quality-checked on a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific).

Phenotyping Reagents

All Mi^a-positive RBCs were characterised by serology using a panel of typing antisera (monoclonal and polyclonal antibodies). The list of phenotyping reagents and the haemagglutination techniques applied are shown in Table 1. To further classify Mi^a-positive RBCs, an algorithm was developed to test RBCs sequentially for the presence of low-frequency antigens. The testing algorithm is presented in Figure 2.

Antisera were supplied by the Japanese Red Cross, SCARF, and Immucor as gifts to the Australian Red Cross Blood Service. Epitopes recognised by anti-Mi^a CBC-172, anti-Mur HIRO-138 (64-D6), anti-NEV CBC-181 (64-2A3), and anti-Mi^a GAMA210 have been described in a previous publication report on the Fourth International Workshop on Monoclonal Antibodies [27].

Haemagglutination Techniques

Standard haemagglutination techniques were used in this study. Briefly, one drop of antiserum was added into a test tube followed by one drop of 3–5% RBC suspension. Tubes were mixed and then incubated at room temperature for the saline room temperature (SRT) technique or at 37 °C for the indirect antiglobulin test (IAT).

For the SRT technique using anti-Mi^a GAMA210 monoclonal antibody (Novaclone; Immucor), tubes were incubated for 10 min at room temperature and spun for 15 s at 1,000 *g*. For the SRT technique using anti-Mur antibody, tubes were incubated for 30 min at room temperature and spun for 815 *g*. Agglutination reactions were assessed by visual examination.

For the IAT technique, tubes were incubated at 37 °C for 30 min. After incubation, RBCs were washed four times at 3,500 rpm using a cell washer (Rotolavit; Hettich) and then added with 2 drops of anti-human globulin (Anti-IgG reagent; CSL). Tubes were mixed and then spun at 2,000 rpm (Rotolavit, Hettich) for 15–20 s. Agglutination reactions were examined by visual inspection.

All agglutination reactions for SRT and IAT were scored on a scale of zero (negative) to 4+ (positive).

Murine Anti-Mi^a Monoclonal Reagents: CBC-172 and GAMA210

Monoclonal antibodies recognise different epitopes on Mi^a. Anti-Mi^a CBC-172 recognises amino acid sequence ^{29}HKRDTY-AA^{35} [27] while GAMA210 targets ^{24}QTNDMHKR^{31} or ^{25}TND-KHKRD^{32} [28]. The reactivity profile for these two monoclonal

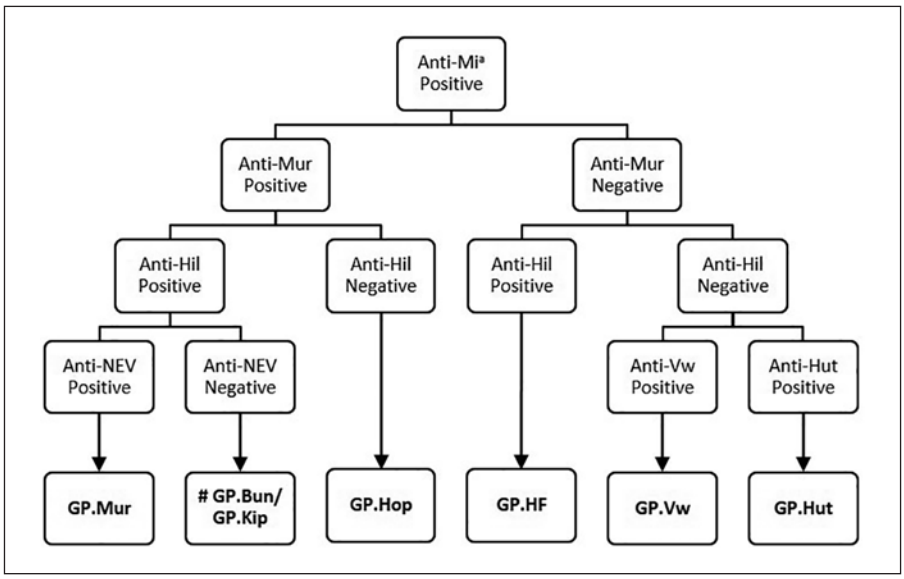

Fig. 2. Phenotyping algorithm for classifying Mi^a-positive MNS hybrid glycophorins using a panel of typing sera. # Phenotyping reagents used above cannot differentiate GP.Bun from GP.Kip RBCs. However, anti-Hop antiserum reacts positive with GP.Bun and negative with GP.Kip RBCs [14, 16, 38]. Anti-Hop antiserum is rare and is only available in specialised or reference testing laboratories. Molecular typing by DNA sequencing or MALDI-TOF MS is a more practical approach for distinguishing GP.Bun from GP.Kip hybrid glycophorins [33].

Table 1. Phenotyping reagents

Antibody	Clone/ID	Type	Technique	Source
Anti-Mi^a	CBC-172	Monoclonal, IgG	IAT	JRC
Anti-Mur	HIRO-138 (64-D6)	Monoclonal, IgM	SRT	JRC
Anti-Hil	Human	Polyclonal, IgG	IAT	JRC
Anti-NEV	CBC-181 (64-2A3)	Monoclonal, IgG	IAT	JRC
Anti-Vw	Human	Polyclonal, IgG	IAT	ARCBS
Anti-Hut	Human	Polyclonal, IgG	IAT	SCARF
Anti-Hop(+Nob)	Human	Polyclonal, IgG	IAT	JRC
Anti-Mi^a	GAMA210	Monoclonal, IgG	SRT	Immucor

Anti-Hop(+Nob), Anek anti-serum, is known to react with Mi^a-positive GP.Hop, GP.Bun, and GP.Kip RBCs. JRC, Japanese Red Cross; ARCBS, Australian Red Cross Blood Service; SCARF, Serum, Cells and Rare Fluids (Exchange program); IAT, indirect antiglobulin test; SRT, saline room temperature.

antibodies were compared using a panel of well-characterised RBCs expressing hybrid glycophorins: GP.Vw, GP.Hut, GP.Mur, GP.Hop, GP.Hil, GP.Bun, and GPB control cells. The IAT technique was used for anti-Mi^a CBC-172 and SRT for anti-Mi^a GAMA210 (Table 1).

Massively Parallel Sequencing for GYPA and GYP(B-A-B) Hybrids GYP*Vw *and* GYP*Hut

DNA was sequenced on a high-throughput, massively parallel sequencing (MPS) platform. MPS was conducted using a DNA sequencing kit (TruSight One Sequencing Panel [TSO]; Illumina). The TSO panel was used for library preparation according to the manufacturer's instructions. TSO amplifies exons and untranslated regions of over 4,800 genes associated with known clinical phenotypes which include the *GYPA* and *GYPB* blood group genes. Targeted sequencing was performed on an MPS sequencing equipment (MiSeq; Illumina) to generate paired-end, 150-bp reads. FASTQ files, MPS data generated by the MiSeq system software, were exported to a bioinformatics software (CLC Genomics Workbench software version 8.5; QIAGEN) for MPS data analysis [29].

High-Resolution Melting Analysis Assay for GYPB and GYP(B-A-B) Hybrids GYP*Mur *and* GYP*Bun

Genomic DNA was isolated from EDTA whole blood using a DNA extraction kit (EZ1 DNA Blood kit; QIAGEN) on a robotic instrument (EZ1 Advanced; QIAGEN). A polymerase chain reaction (PCR) and high-resolution melting (HRM) genotyping assay was used to genotype DNA from Mi^a serology-positive RBCs. One pair of primers, forward primer: P7-F22TT, 5′-ACGCAGTCACCTCATTCTTGTT-3′, and reverse primer: P9-R23GG, 5′-GGCTTTGGAGTAAAAGAGTTGGG-3′, was designed to amplify *GYPB* pseudoexon 3 and the *GYP(B-A-B)* exon 3 hybrid gene [30]. A 270-bp PCR product is expected for *GYPB*, *GYP*Mur*, and *GYP*Bun*. A DNA typing kit (Type-it HRM PCR Kit; QIAGEN) was used to perform the PCR HRM assay. In a PCR reaction mix, 12.5 µL of 2X HRM PCR Master Mix reagent, 5 µL of genomic DNA (10 ng/µL concentration), and 0.9 µL (10 µM) each of forward and reverse primers are combined with water (5.7 µL) to make a final volume of 25 µL. PCR reaction tubes were placed on a real-time PCR cycler (QIAGEN Rotor-Gene Q 5plex HRM). The PCR step was performed under the following conditions: activation step (5 min at 95 °C) fol-

Table 2. Serological profile of Mi^a-positive RBCs

Donor RBCs	Phenotype	Anti-Mia	Anti-Mur	Anti-Hil	Anti-NEV	Anti-Vw	Anti-Hut
Donor 1	GP.Hut	+	0	0	0	0	+
Donor 2	GP.Hut	+	0	0	0	0	+
Donor 3	GP.Vw	+	0	0	0	+	NT
Donor 4	GP.Mur	+	+	+	+	0	NT
Donor 5	GP.Bun	+	+	+	0	NT	NT
Donor 6	GP.Vw	+	0	NT	NT	+	NT
Donor 7	GP.Mur	+	+	+	+	NT	NT
Donor 8	GP.Vw	+	0	NT	NT	+	NT
Donor 9	GP.Mur	+	+	+	+	NT	NT
Donor 10	GP.Mur	+	+	+	+	NT	NT
Donor 11	GP.Mur	+	+	+	+	NT	NT

Additional phenotyping was done on GP.Bun RBCs. In this study, GP.Bun cells reacted positive with Anek antiserum anti-Hop(+Nob) which is consistent with the phenotype reported by Giles et al. [14, 38]. +, positive; 0, negative; NT, not tested.

Table 3. Haemagglutination reaction comparison between two anti-Mi^a monoclonal antibodies against a panel of RBC-expressing MNS hybrid glycophorins

RBC panel	CBC-172	GAMA210
GPB	0	0
GP.Vw	4+	3+
GP.Hut	4+	4+
GP.Mur	4+	4+
GP.Hop	4+	4+
GP.Hil	0	0
GP.Bun	4+	4+

GPB RBCs were used as a negative control. GP.Hil, a GP(A-B) MNS hybrid glycophorin, does not express Mi^a antigen and was used as an Mi^a–negative hybrid glycophorin negative control.

lowed by 40 cycles of denaturation (95 °C for 10 s) and annealing/extension (65 °C for 30 s). At the end of the PCR step, the temperature was gradually increased by 0.1 °C every 2 s from 73 °C to 83 °C [30].

HRM analysis is a post-PCR DNA analysis method used to characterise the dissociation profile of double-stranded DNA. HRM uses intercalating dyes that produce fluorescence only when bound to double-stranded DNA. When the temperature is increased, double-stranded DNA dissociates into single-stranded DNA, losing fluorescence. Total fluorescence and the rate of change in fluorescence was monitored and data acquired on the HRM channel. Rotor-Gene Q Series Software was used to analyse the HRM data. Melt profiles for unknown samples were compared to melt profiles of DNA control samples. A confidence threshold of 90% was set for HRM genotyping call. Genotype assignments for samples with a confidence percentage value below 90% were classified as "Variation" [20, 31, 32].

DNA

Controls Used in the HRM Assay

GYPB homozygote, *GYP*Mur* homozygote, and *GYP*Mur/GYPB* DNA controls used in the HRM assay were genotyped by matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS) and DNA was fully sequenced [33]. These DNA controls have been used in previous publications [20, 30].

Results

Mi^a Antigen Screening

RBCs from 11 out of 5,098 blood samples tested with anti-Mi^a CBC-172 gave a positive agglutination reaction (Table 2). Extended phenotyping of these 11 samples revealed four types of hybrid glycophorins: GP.Hut (n = 2), GP.Vw (n = 3), GP.Mur (n = 5), and GP.Bun (n = 1). The reactivity pattern displayed by each of the 11 blood samples were consistent with previously reported serological profiles for Mi^a-positive hybrid glycophorins.

Comparison of Two Anti-Mi^a Monoclonal Reagents

The reactivity profile for anti-Mi^a CBC-172 against a panel of phenotyped RBCs was compared with a commercially available reagent anti-Mi^a GAMA210. Both showed a negative agglutination reaction with GPB and GP.Hil RBCs and a strongly positive reaction (3+ to 4+) with GP.Vw, GP.Hut, GP.Mur, GP.Hop, and GP.Bun RBCs (Table 3).

Genotyping for Hybrid Glycophorins

DNA samples from 11 Mi^a-positive blood donors were segregated into two groups. Group 1 were the GP.Vw and GP.Hut blood donors (donors 1, 2, 3, 6, and 8) and were genotyped by MPS (Fig. 3). Group 2 were the GP.Mur and GP.Bun blood donors (donor, 4, 5, and 7) and were genotyped by the HRM assay (Fig. 4).

In group 1, sequences were aligned to a reference sequence (Fig. 3a). MPS analysis of the *GYPA/GYPA* DNA

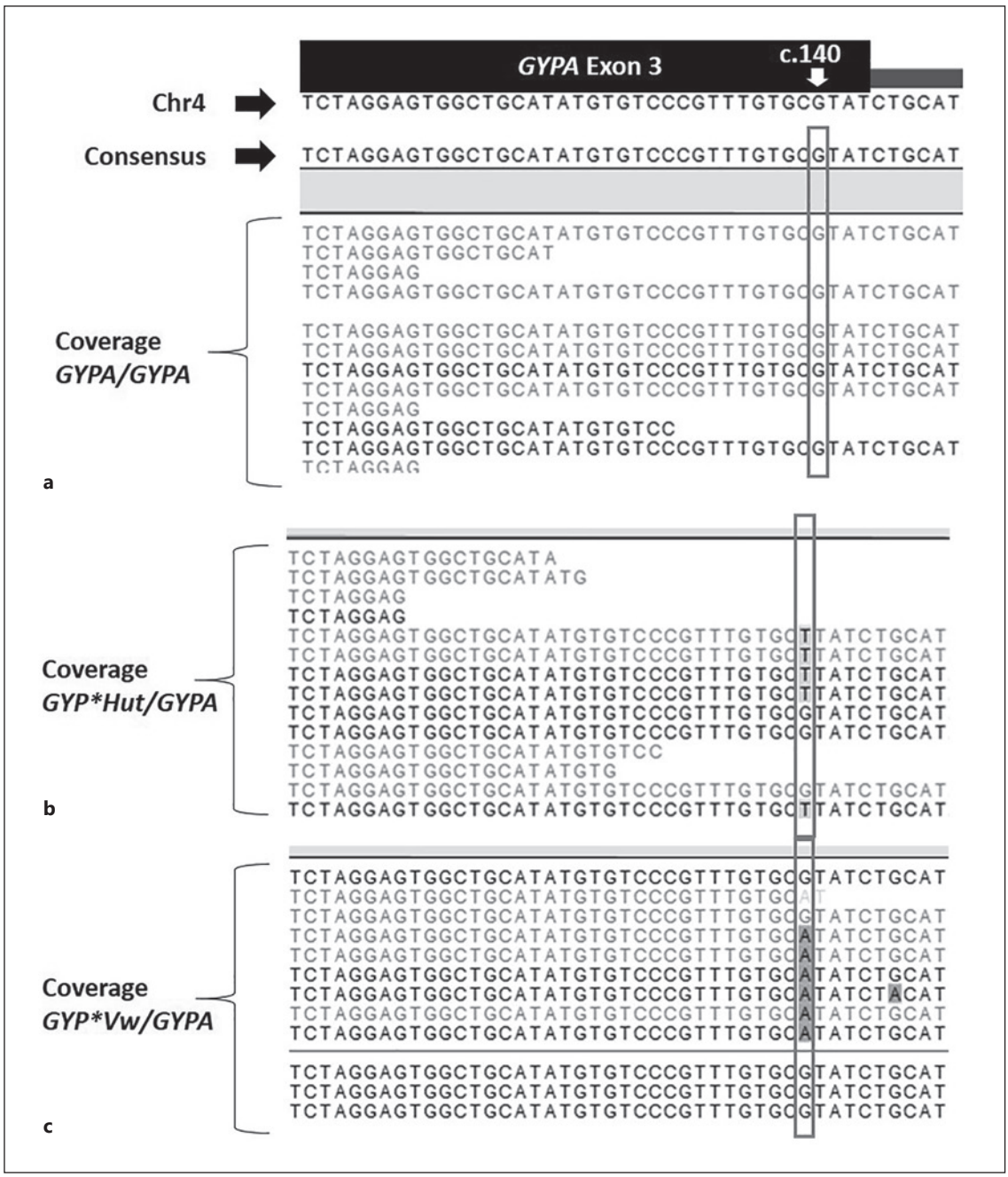

Fig. 3. Sequence alignment for group 1 samples. **a** Representative sequences for *GYPA/GYPA* DNA control aligned to the *GYPA* exon 3 reference sequences. **b** Representative sequences for *GYP*Hut/GYPA* heterozygote – donors 1 and 2. **c** Representative sequences for *GYP*Vw/GYPA* heterozygote – donors 3, 6, and 8. Refer to diagram and legend of Figure 1 for *GYPA* exon 3 sequence. Nucleotide detected at c.140 predicts the base on the complementary strand. Grey box indicates nucleotides in position c.140 for group 1 DNA sequences.

control at position c.140 showed G/G predicting a C/C on the complementary strand (Fig. 3a). Analysis of sequences at c.140 detected T/G (predicting A/C on the complementary strand) for donors 1 and 2 interpreted as heterozygous for *GYP*Hut/GYPA* (Fig. 3b). For donors 3, 6, and 8, A/G were detected at c.140 (predicting T/C on the complementary strand) interpreted as heterozygous for *GYP*Vw/GYPA* (Fig. 3c).

In group 2, HRM analysis showed that the DNA meltcurve profiles for donors 4 and 7 matched the DNA control *GYP*Mur/GYPB* with 99.53 and 99.23% confidence, respectively (Fig. 4). The meltcurve pattern for donor 5 gave a result of 32.5% confidence when compared to *GYP*Mur/GYPB* DNA control. Since 32.5% is below the 90% threshold, the genotype call assigned for donor 5 was "Variation." DNA analysis for donor 5 on the HRM melt-

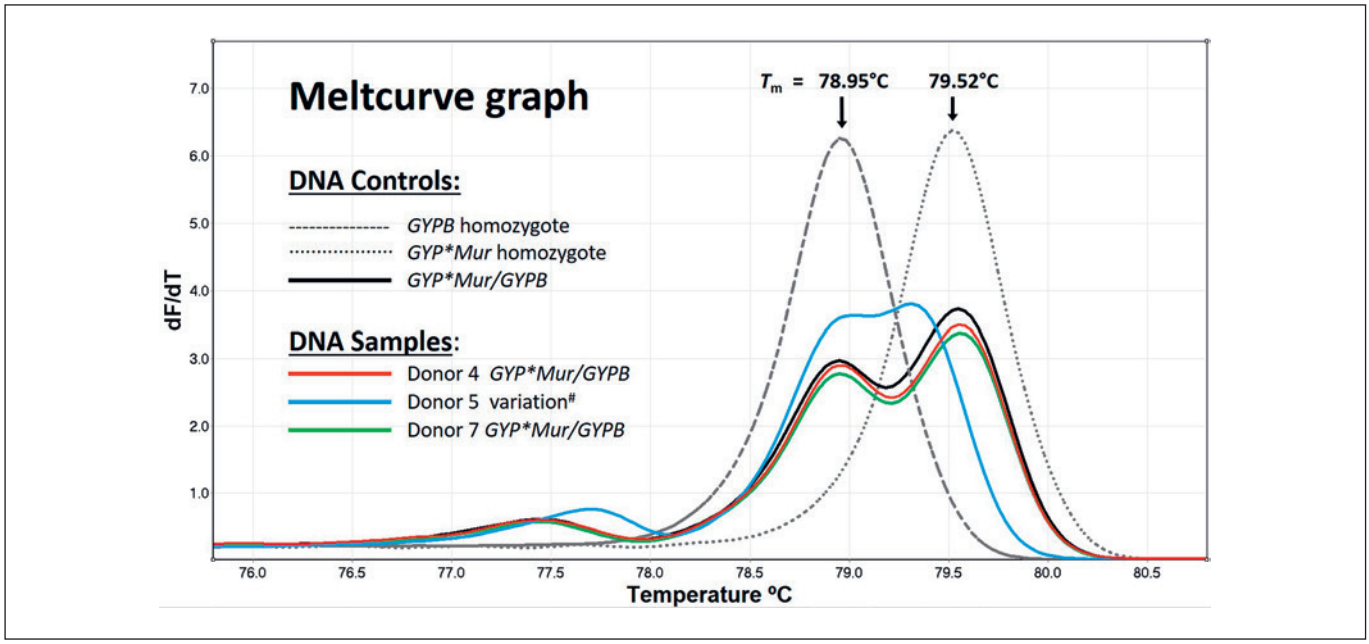

Fig. 4. Meltcurve graph for group 2 samples. Meltcurve graph plots the rate of change in fluorescence (*y* axis) against temperature (*x* axis). The meltcurve profile for DNA controls *GYPB* homozygote (single peak, continuous grey dashes, T_m = 78.95 °C), *GYP*Mur* homozygote (single peak, grey dots, T_m = 79.52 °C), and *GYP*Mur/GYPB* (solid black line, double peaks, T_m = 78.96 and 79.55 °C). High-resolution melting analysis showed that the meltcurve profiles for donor 4 (red line) and donor 7 (green line) are similar to DNA control *GYP*Mur/GYPB* while the profile for donor 5 (blue line) is distinct from all three DNA controls. # DNA from donor 5 had been used in a previous study and had been genotyped as *GYP*Bun/GYPB* [33]. T_m, melting temperature.

Table 4. Phenotyping and genotyping comparison of Mi^a-positive blood donors

Blood donor	Phenotype	Genotype
Donor 1	GP.Hut	*GYP*Hut/GYPA*
Donor 2	GP.Hut	*GYP*Hut/GYPA*
Donor 3	GP.Vw	*GYP*Vw/GYPA*
Donor 4	GP.Mur	*GYP*Mur/GYPB*
Donor 5	GP.Bun	*GYP*Bun/GYPB*
Donor 6	GP.Vw	*GYP*Vw/GYPA*
Donor 7	GP.Mur	*GYP*Mur/GYPB*
Donor 8	GP.Vw	*GYP*Vw/ GYPA*
Donor 9	GP.Mur	Not tested
Donor 10	GP.Mur	Not tested
Donor 11	GP.Mur	Not tested

DNA from donors 9, 10, and 11 was not isolated from their primary blood donation. These donors did not return to donate blood since the preliminary screening. DNA analysis for donor 5 was initially reported in a previous study and had been genotyped as *GYP*Bun/GYPB* [33].

curve graph revealed two peaks showing melting temperature at 78.99 and 79.32 °C. Donor 5 was originally reported as heterozygous for *GYP*Bun/GYPB* in a study assessing the viability of MALDI-TOF MS as a genotyping platform for hybrid glycophorins [33].

Genotyping calls for 8 out of 11 Mi^a-positive blood donors were consistent with the observed phenotypes (Table 4). Three blood donors (Donors 9, 10, and 11) did not present back to the Blood Service for their subsequent blood donation since their RBCs were initially tested for Mi^a between 2011 and 2013 and, therefore, DNA samples from these blood donors were unavailable for genotyping.

Discussion

In this study, 11 out of 5,098 blood samples were Mi^a-positive which represents a frequency 0.22% in the Australian blood donor population. Of the 11 Mi^a-positive MNS hybrid glycophorin blood donors identified, GP.Mur was the most common type followed closely by GP.Vw. Although Mi^a-positive GP.Hop and GP.Kip were not detected in this study, each was reportedly found in an individual in Australia described in two independent case studies [16, 24]. Collectively, data from this study and two case studies indicate that the MNS hybrid glycophorin blood group profile in the Australia population is diverse.

The discovery of these hybrid glycophorin types in the blood donor pool is instructive and practical. RBCs from these blood donors have become a valuable resource of

reagent RBCs for the identification of antibodies against MNS hybrid glycophorins, particularly anti-Mi^a. Anti-Mi^a antibody is clinically significant and reported to cause haemolytic transfusion reactions and haemolytic disease of the fetus and newborn [7]. In an Australian study, peptide ELISA assay detected anti-Mi^a antibody in the serum/plasma of 3.8% blood donors, 4% antenatal patients (5/124), and 1.4% multi-transfused patients (3/215) [34]. Recently, we identified an anti-Mi^a antibody in a patient that reacted positive to a particular packed RBC unit during routine pre-transfusion cross-match. Further serological and molecular studies typed the RBCs as GP.Mur homozygote, S– s+/–, predicting a JENU-negative phenotype [35].

Mi^a is considered a low-frequency antigen (0.23%, 9/3,844) in the Caucasian population [36] but generally appears at a higher frequency in several Asian population groups [11, 20, 23]. Although the Australian demographics are diverse and ever-changing, the frequency of Mi^a at 0.22% suggests that the Australian blood donor population resembles a Caucasian profile. However, for GP.Mur, the frequency reported in this study is 1 in 1,020 in contrast to the historically reported frequency of 1 in 10,020 in a Caucasian population [18].

This is the first comprehensive study to report on the frequency of Mi^a and the type of MNS hybrid glycophorins found in an Australian blood donor population. Knowing the blood group profile in a population is essential to effectively manage transfusion needs.

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Statement of Ethics

Consent was given for the investigation of blood groups at the time of collection of the samples. This study has approval from the Australian Red Cross Blood Service Human Research Ethics Committee (Application No. 2010#07).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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