Characterization of \textit{GYP*Mur} and novel \textit{GYP*Bun}-like hybrids in Thai blood donors reveals a qualitatively altered s antigen

\textbf{Philaphon Jongruamklang,\textsuperscript{1,2} Shane Grimsley,\textsuperscript{3} Nicole Thornton,\textsuperscript{3} Janine Robb,\textsuperscript{4} Martin L. Olsson\textsuperscript{1,5} \& Jill R. Storry\textsuperscript{1,5}}

\textsuperscript{1}Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, Lund, Sweden
\textsuperscript{2}Department of Medical Technology, School of Allied Health Sciences, University of Phayao, Phayao, Thailand
\textsuperscript{3}International Blood Group Reference Laboratory, NHSBT, Bristol, UK
\textsuperscript{4}Quotient, Edinburgh, UK
\textsuperscript{5}Clinical Immunology and Transfusion Medicine, Office for Medical Services, Lund, Sweden

\section*{Background and objectives} The Mi(a+) GP(B-A-B) hybrid phenotypes occur with a prevalence of 2\%–23\% across Southeast Asia. While the s antigen is alleged to be altered, no evidence for specific variants is known. Screening using a monoclonal IgM anti-s mistyped six S–s+ RBC units as S–s–. Further, alloanti-s was identified in an S+s+ patient. Our objective was to investigate the s antigen further.

\section*{Materials and methods} DNA from 63 Thai blood donor samples PCR-positive for a \textit{GYP(B-A-B)} hybrid was sequenced with primers spanning \textit{GYPB} exons 3–4. Flow cytometry was used for semiquantitative analysis of s expression and correlated with the glycophorin genotype.

\section*{Results} DNA sequencing showed that \textit{GYP*Mur} was carried by 56/63 samples (88\%/9\%) of which 5/56 lacked normal \textit{GYPB}: three of these were \textit{GYP*Mur} homozygotes, one was a compound heterozygote carrying \textit{GYP*Mur} and a \textit{GYP*-Bun}-like allele (designated \textit{GYP*Thai}), and the fifth sample carried \textit{GYP*Mur} and another \textit{GYP*Bun}-like allele. Seven samples (7/63) were \textit{GYP*Thai} heterozygotes. IgM monoclonal anti-s (P3BER) did not react with the s antigen carried by GP.Mur or GP.Bun, whereas two IgG anti-s showed enhanced reactivity.

\section*{Conclusions} We confirmed that \textit{GYP*Mur} is the most frequent variant in Thai blood donors and also identified \textit{GYP*Thai} with a frequency of 1\%/1\%. We showed that s antigen on Mi(a+) GP(B-A-B) hybrids is qualitatively altered and should be considered when selecting reagents for phenotyping where such hybrids are prevalent, endemically and in blood centres worldwide.

\section*{Key words:} blood group antigen, glycophorin, GP.Mur.

\section*{Introduction} The MNS blood group system is highly polymorphic. A total of 49 antigens have been described, of which 35 are low-frequency antigens. Many of these are generated by the formation of hybrids that resulted from crossing-over or gene conversion events that occurred between the highly homologous \textit{GYPA}, \textit{GYPB} and occasionally, \textit{GYPE} genes. Typically, gene conversion arises from a mechanism of double-strand break repair in which a short sequence of one gene is copied into the other during gene duplication [1]. In the case of GP(B-A-B) hybrids, this `repair mechanism’ edits a region in \textit{GYPB} corresponding to \textit{GYPA} exon 3 such that an inactive splice site sequence

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in GYPB is replaced by an active GYPA sequence. Consequently, a partial GYPB nucleotide sequence encoded by the so-called GYPB pseudogene 3 is translated and a hybrid protein is produced.

The GP(B-A-B) hybrids are common in Southeast Asia and antibodies to low-frequency antigens carried on these glycoproteins, for example anti-Mi, can cause haemolytic disease of the newborn and/or transfusion reactions [1]. We recently showed that Mi-bearing hybrids were found in 16.7% of Thai blood donors [2], which is comparable with other studies [3–6]. The most common GP(B-A-B) hybrid is GP.Mur but other variants such as GP.Bun, GP.Hop, GP.HF and GP.Kip have also been characterized [1,7]. They differ from each other due to insertion of varying regions of glycophorin A (GPA). The GP.HF hybrid has the longest GPA insert of 24 amino acids, GP.Mur derives nine amino acids from GPA, while GP.Bun, GP.Hop and GP.Kip carry the shortest GPA insert of seven amino acids. The single nucleotide substitution encoded by exon 4 at c.236T/C (equivalent to the S/s defining GYPB c.143T/C) differentiates GP.Hop from the GP.Bun and GP.Kip hybrids (c.236C), and is close to the hybrid crossing-over point [1,8]. GP.Bun and GP.Kip differ by a single amino acid p.Tyr70Ser encoded by c.209A/C (see Fig. S1).

The expression of s antigen is weakened on glycophorin B (GBP) carrying Mit, Mv and sD antigens [9], as Ser encoded by c.209A/C (see Fig. S1). In a study conducted by Lopez et al. [12], the expression of s antigen on the GP.Mur hybrid was suggested by Lopez et al. who described the high prevalence GBP antigen, JENU which is abolished on GP.Mur [11]. In their study, red blood cells (RBCs) from GYP*Mur homozygotes failed to react with a monoclonal anti-s but still reacted with a polyclonal anti-s.

In this study, we have sought to serologically and molecularly characterize glycophorin variants among 63 Thai blood donors that were previously [2] shown to carry a GYP hybrid gene. We hypothesized that s antigen is both quantitatively and qualitatively altered.

Materials and methods

Samples

Collection and preparation of donor blood, genomic DNA and frozen RBCs used in this study have been described in our previous study [2]. We identified 63 samples in which a GYP hybrid gene had been amplified by PCR. These were investigated further by sequencing.

Thawed RBCs from 31 representative samples were selected for serology testing and flow cytometry.

Antibodies

The following antibodies were included in the study: monoclonal anti-s clone P3BER (Immucor, Inc.), clone P3YAN3 (Lorne Laboratories Ltd.), or polyclonal anti-s, ref. Z186 (ALBAsera®, Quotient), polyclonal anti-S, ref. Z182 (ALBAsera®, Quotient), monoclonal anti-Mi, clone GAMAI0 (Immucor Inc.), polyclonal anti-U (in-house reagent) and polyclonal anti-Hop (in-house reagent).

Serology

Hemagglutination was performed using standard serological techniques [13] in gel cards (BioRad, Labex AB) and in tube according to manufacturer’s instructions.

Flow cytometry

A set of 31 samples was selected to determine the s antigen expression on different GP(B-A-B) hybrids predicted by genotyping. RBCs were incubated with either monoclonal anti-s (clone P3BER and clone P3YAN3) or polyclonal anti-s (Z186), followed by either PE-conjugated F(ab')2 fragment Goat Anti-Human IgG (Jackson ImmunoResearch, #109–116–09B) or Goat Anti-Human IgM (Jackson ImmunoResearch, #109–116–129). Data were acquired on a FACScalibur flow cytometer using CellQuest v3.3 (Becton Dickinson) and analysed using FCS Express 6 Flow cytometry software (De Novo Software). Values were taken as median fluorescence intensity (MFI), and all samples were normalized against a well-characterized S+s in-house test RBC sample from our antibody screening panel. The Student’s t-test was used to analyse the results.
GYPB exon-specific amplification and sequencing

The genomic DNA fragments spanning exons 3 and 4 of GYPB were PCR amplified using Expand High FidelityPLUS PCR System (Thermo Fisher Scientific) and forward and reverse primers – GPBx3S, 5'-GGAGAAATTGCTTCATGATAC-3'; and GPBx4AS, 5'-TACCTGGTACAGTGAAAC-GATGG-3'. The PCR products were then eluted and sequenced by Sanger sequencing (GATC Biotech) using GYPB and GPB*Thai allele. Two samples were homozygous for SNV rs7683365 encoding s antigen (GYPB c.143C). The remaining sample was heterozygous, predicting the S**s** phenotype.

Results

Sequence analysis

Sequencing results are summarized in Table 1. GYP*Mur was the predominant hybrid allele and identified in 56/63 samples (88.9%) of which 51 were GYP*Mur/GYPB heterozygotes. Of the remaining five samples, three GYP*-Mur homozygotes were identified. Two samples were identified to be compound heterozygotes: both carried GYP*Mur, and each carried a different GYP*Bun-like allele. Two GYP*Bun-like alleles (hereafter named GYP*Thai and GYP*Thai II) were identified in this study. While the amino acid sequence encoded is predicted to be identical to GP.Bun [GPB(20-45)-GPB{46-69}GYP{70-76}-GPB‘(77-122)], both differ in their breakpoints in intron 3. Sequencing revealed a shorter insertion of GYPB segment in the GYP*Thai allele. The GYP*Bun allele has a GYPB insert of 131 bp, whereas the GYP*Thai allele found in our study has a minimum insertion of 22 bp and a maximum of 53 bp. The GYP*Thai II allele has a minimum insert of 46 bp and a maximum of 117 bp (Fig. 1).

Diagrammatic representation of amino acids for each hybrid protein is shown in Fig. 2a.

Furthermore, heterozygosity for the GYP*Thai allele was identified in the remaining seven (7/63) samples. Thus, the prevalence of this allele in our original study population of 396 samples is 1.1%.

Table 1 Summary of sequencing results for 63 samples previously identified to carry a GYP hybrid gene

| Genotype         | Predicted phenotype | S/s   |
|------------------|---------------------|-------|
| GYP*Mur/GYPB     | s+s                 | 51    |
| GYP*Mur/GYP*Mur  | s+s                 | 3     |
| GYP*Thai/GYPB    | s+s                 | 6     |
| GYP*Thai/GYP*Mur | s+s                 | 1     |
| GYP*Thai II/GYP*Mur | s+s         | 1     |
| Total            |                     | 62    |

Phenotype was predicted based on genotyping results by MALDI-TOF [2].

Sequencing analysis also revealed that 62/63 samples were homozygous for SNV rs7683365 encoding s antigen (GYPB c.143C). The remaining sample was heterozygous, predicting the S**s** phenotype.

Serological and flow cytometric characterization

Samples representative of the genotypes characterized as above were selected for s antigen characterization (see Table 2). All samples carrying a hybrid allele were confirmed Mi(+). As predicted, GYP*Thai samples were found to be Hop+. Whereas, surprisingly, the GYP*Thai II sample was found to be Hop-.

Flow cytometry

We observed that RBCs carrying hybrid alleles demonstrated both qualitative and quantitative changes in s antigen expression with different anti-s. Reactivity of s antigen from GYPB-homozygous samples (GYPB*04/*04) tested with polyclonal anti-s (Z186) showed a mean normalized MFI that is 2.5 times higher than that of heterozygous samples (GYPB*03/*04). Similar results were observed in tests with other anti-s reagents, clone P3BER and clone P3YAN3 (Fig. 3).

GYP*Mur/Mur had significantly lower reactivity when tested with anti-s clone P3BER when compared to GYPB*04/*04 controls: $P = 0.00004$. GYP*Mur

Fig. 1 Comparison of GYPB, GYPB and the GYPB(A-B) hybrids identified in this study. The sequences of two GYP*Bun-like alleles identified in our samples (GYP*Thai and GYP*Thai II) are compared with the known GYP*Mur and GYP*Bun sequences. GYPB and GYPB are shown for reference purposes; the grey highlight indicates GYPB-derived nucleotides. Minimum and maximum sequences are indicated between the breakpoint limits.
heterozygotes were also lower: \( P = 0.00079 \) (Fig. 3a). Reactivity with polyclonal anti-s was significantly increased with the homozygotes \( (P = 0.000016) \) and trending towards a slight increase for heterozygotes \( (P = 0.054) \) (Fig. 3b). Reactivity of GYP*Thai heterozygote RBCs was shown to react similarly to those of GYP*Mur heterozygote RBCs. Notably, four samples that did not carry a GYPB*04 allele were nonreactive with clone P3BER whilst enhanced reactivity was observed with polyclonal anti-s.

A third anti-s (P3YAN3) could not discriminate consistently between s antigen carried on normal GPB or hybrids. Surprisingly, however, one sample identified to be a GYP*Mur/GYP*Thai compound heterozygote had significantly increased reactivity when tested with anti-s clone P3YAN3. This phenomenon was also found in GYP*Mur homozygote RBCs (Fig. 3c).

**Discussion**

Our results demonstrate that the s antigen on glycoporphin hybrids GP.Mur and GP.Bun is both qualitatively and quantitatively altered. The s antigen on these hybrids is not recognized at all by the IgM monoclonal anti-s, P3BER, nor on GP.Hil as shown by one of the index donor samples. This antibody most likely requires a native GPB sequence, and although the differentiating threonine is present on the hybrid molecules, it is insufficient for antibody recognition. This is quite remarkable but in line with early biochemical analysis performed by Dahr [10] who identified the amino acids T44E47T48V52H53 in the native GPB sequence to be involved in s antigen recognition, and possibly the O-glycan carried by p.Thr44. Of these, only p.Thr44 is not present on GP.Mur and GP.Bun (Fig. 2) and these results are in strong contrast to the work of Halverson et al. [14] who reported on the epitope recognition of five different and serologically specific murine monoclonal anti-s as determined by peptide inhibition studies. Their work showed
that for all five antibodies, the reactive epitope appears to be downstream of p.Thr48 and defined by a core peptide sequence, QLVHRF, that could inhibit the antibodies independently of p.Thr48. Furthermore, while a role for p.Thr44 glycosylation in antigen recognition cannot be ruled out, these studies with linear peptides suggest otherwise.

Studies regarding the GP(B-A-B) hybrids and their frequency in Thai populations have also been carried out by others. Palacajornsuk et al. reported that 88% of Mi(a+) samples were GP.Mur and 12% were GP.Bun [5]. Other studies by Chandanayingyong and colleagues [15,16] reported that 93% and 7% were GP.Mur and GP.Bun, respectively. None of these studies report the GYP*Bun-like allele that we identified in our study. However, this allele was first identified by Wei et al. who found one in 51 Mur + Chinese blood donors to carry this allele [17] and refer to another as yet, unpublished sample carrying the same allele. Interestingly, we identified this allele in 8 of 63 Mi(a+) samples (12.6%) which is relatively high in this group and suggests that this is a common variant in Thais. At the phenotypic level, this allele encodes a GP.Bun hybrid that carries the characteristic Hop antigen. More puzzling is the novel GYP*Bun-like allele identified here (GYP*Thai II), which is predicted to express a GP.Bun hybrid protein but which has a much longer insertion of GYPA intron 3. This sample showed an intermediate expression of s antigen that was clearly different from that of GP.Mur or normal s antigen (Fig. 3) and unexpectedly, typed Hop–. The glycoporphins are known to assemble in the membrane as homo- and heterodimers, and since this sample was a compound heterozygote together with GYP*Mur, it is possible that the conformation required for Hop antigen presentation was altered.

Whatever the requirements for antigen presentation, it is clear that the s antigen on GP.Mur and GP.Bun should be considered a variant or partial antigen of clinical significance. In addition to the anecdotal unreferenced report of alloanti-s in a GP.Mur woman reported in Daniels [1], we have also identified alloanti-s in a S+s+ patient with the GYP*Mur/GYPB*03 genotype, supporting our data that the s antigen carried by these hybrids is qualitatively altered. This raises the issue of reagent selection: in populations where these hybrids are prevalent, s phenotyping should be approached differently in patients and donors, analogous to RhD typing in people of European descent. Accordingly, anti-s that does not detect s antigen carried by GP.Mur (e.g. P3BER) should be used for determining the phenotype of patients, whereas anti-s that does not discriminate normal GPB from hybrids should be used for donor testing. This will ensure that patients are not at risk of immunization. A complication of such a paradigm might be the abundance of apparent S–s– patients, when in fact the patient is a GYP*-Mur homozygote, compared to GYP*Mur homozygous donors that will type as S–s+. Determining the Mi\(^a\) phenotype of donors may have significant value, not only in helping to identify compatible blood for patients with rare homozygous (or compound heterozygous) glycoporphin variants but also, to ensure appropriate provision of Mi(a−) blood in a region where this antibody is particularly prevalent [1].

In conclusion, we have further characterized the variant glycoporphin genes identified in our previous study. DNA sequencing not only confirmed that GYP*Mur is the most frequent glycoporphin variant allele among our Thai blood donors, but also identified a GYP*Bun-like (GYP*Thai) glycoporphin variant allele that is also common (Minor Allele Frequency; MAF = 0.011) in this

Fig. 3 Expression of s antigen determined by flow cytometry. Normalization of median fluorescence intensity (MFI) from each sample was plotted according to genotype and predicted phenotype. The bars represent the mean of each group. Three samples that cannot be grouped are presented separately. (a) Reactivity when tested with anti-s clone P3BER. (b) Reactivity with polyclonal anti-s, Z186. (c) Reactivity with monoclonal anti-s clone P3YAN3.

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group. Furthermore, we have shown that the s antigen carried on these hybrids is qualitatively altered and this should be taken into consideration when selecting reagents for phenotyping donors and patients, particularly where such hybrids are prevalent but also in targeted phenotyping of minority blood donors in large metropolitan blood centres worldwide [18].

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Conflicts of interest

The authors declare no conflicts of interest.

Authors’ contributions

PJ, SG and JRS designed the study; PJ and SG performed the experiments; JR provided essential reagents; PJ, NT, SG, MLO and JRS interpreted the data; PJ and JRS wrote the manuscript. All authors contributed to and edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article: Fig. S1 Comparison of GYP A, GYPB and GYP(B-A-B) hybrids exon 3 sequences.

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