MNSs Blood Group Glycophorin Variants in Taiwan: A Genotype-Serotype Correlation Study of ‘Mi^a’ and St^a with Report of Two New Alleles for St^a

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Abstract

Background: Glycophorin variants of the MNS blood group are important in Taiwan. For more than 20 years, screening for the most frequent irregular antibody, anti-‘Mi^a’, has been conducted by using ‘Mi^a’ (+) RBCs, with a significant success. However, the sensitivity and the specificity of this screening strategy have never been validated, and the true incidences of different glycophorin variants in Taiwan have been in controversy. Also, the significance of another less frequent and usually separately reported variant, St^a, has never been evaluated.

Methodology/Principal Findings: We ran a population-based screening (from unselected patients in our hospital) for MNS blood group glycophorin variants by PCR-sequencing method. GP.Mur (Mil.III) was confirmed by sequence from 57 out of 1027 samples (5.6%), and there was no other Miltenberger subtype glycophorin variant found. Glycophorin variant St^a was found from 35 out of 1027 samples (3.4%). In contrast to anti-‘Mi^a’, which is the most frequently identified irregular antibody in Taiwan, the prevalence of anti-St^a was only 0.13% as determined by serologic method. In addition, two new alleles for St^a were found and reported.

Conclusion/Significance: We confirm the long-standing assumption that GP.Mur is the only prevalent Miltenberger subtype in Taiwan. The current anti-‘Mi^a’ screening method used in Taiwan, although neither sensitive nor specific, is still a suitable practice. Although St^a antigen has a high prevalence in Taiwan, routine screening for anti-St^a is not warranted based on current evidence.

Introduction

MNSs blood group antigens are carried on two major membrane sialoglycoprotein, glycophorin A (GPA) and glycophorin B (GBP). They are encoded by glycophorin A gene (GYP(A)) and glycophorin B gene (GYP(B)), respectively. Due to the similarity of GYP(A) and GYP(B), unequal crossing-over or gene conversion can happen, and results in hybrid genes encoding structural variant glycophorins [1]. These glycophorin variants are initially recognized by serologic methods and named as Miltenberger subsystem. Later, the Miltenberger nomenclature was replaced by GP, name terminology with the name denoting the first propositus [2]. Previously known as Miltenberger subtype III (Mi.III), GP.Mur is the glycophorin product of a hybrid GYP(B-A-B) gene on the red blood cell (RBC) membrane. GP.Mur was reported to have a mean frequency of 7.3% among Taiwan population, and is an important blood group antigen in Taiwan [3–6].

In Caucasian, most of the Miltenberger antigens and antibodies are rare. However, antibodies against GP.Mur are the most frequently detected irregular antibodies in Taiwan blood banks. The sera against GP.Mur are mixtures composed of different antibodies, namely anti-Mia, anti-Mur, anti-MUT, anti-Hil, and anti-MINY. Since these polyspecific antibodies are difficult to be separately isolated and specifically identified individually, they have been collectively called anti-‘Mi^a’ in Taiwan. Taiwan Society of Blood Transfusion has issued a guideline of including ‘Mi^a’ (+) cells in the antibody screening panel, and this practice has been conducted for more than 20 years. However, the sensitivity and specificity of this screening strategy have never been validated. Shih et al. used anti-‘Mi^a’ sera to test 200 individuals’ RBCs and found serologically positive in 19 cases [7]. However, only 9 out of those 19 individuals were found to be GP.Mur genotypically positive. This finding indicates that the currently detected anti-‘Mi^a’ sera might be heterogeneous, and might be composed of unknown antibodies against antigen other than those belonging to Gp.Mur.

St^a antigen, first described by Cleghorn in 1962 [8], is another glycophorin variant resulted from hybrid gene GYP(B-A) with a single unequal crossing over at intron 3 of GYP(A) and GYP(B). It
contains the N-terminal part of GYPB and the C-terminal part of GYPA [1]. Currently, four distinct alleles for Sta (type A to D) are identified, differing in the location of crossing-over points [9].

Sta antigen is rarely found in Caucasians, but is significantly highly prevalent (6.36%) in Japan [10]. Reports from other Asia countries such as China and Taiwan also show high frequencies, ranging from 1.63% to 5.2% [7], [11], [12]. Antibodies to Sta antigen had been reported [8], [10]. Sta antigen, like the prevalent GP.Mur antigen in Asian population, is the product of hybrid glycophorin gene, thus may have similarly strong antigenicity as GP.Mur. However, due to the rarity of reports and the lack of large scale studies on either Sta antigen or anti-Sta in the population, the significance of Sta in transfusion medicine, especially in Asian population, has not be determined.

In this study, we want to address the following issues, 1) the genotypic frequencies of each glycophorin variants including Miltenberger subtypes and Sta in Taiwan, 2) to validate efficacy of current anti-'Mia' screening method, and 3) the significance of Sta antigen in Taiwan transfusion practice.

Materials and Methods

Ethics Statement

This study was approved by institution review board (IRB) of Chang Gung Memorial Hospital (approved number: 99-3397A3). All the participants provided their written informed consent to participate in this study.

Table 1. PCR targets and their presumptive product length.

| GYP variants | Primers | Targets | Nucleotides |
|--------------|---------|---------|-------------|
| GYP(A-B)     | Forward 797A | MiV,MiXI | 1225        |
|              | Reverse 2026B |         |             |
| GYP(A-B-A)   | Forward 797A | MiVIII,MiIX | 253        |
|              | Reverse GPABA-E3R | MiII,MiX  |             |
| GYP(B-A)     | Forward 797B | Sta     | 1075        |
|              | Reverse Sta-E4F |         |             |
| GYP(B-A-B)   | Forward F2 and Rccgg | MiII,MiV | 148        |

Table 2. PCR primers used in this study, including their precise locations.

| Primers used in this study | Name of primer | Location       | Sequence                  | Comment     |
|----------------------------|----------------|----------------|---------------------------|-------------|
| 797A*                      | nt 660-679 of IN2 | S'-GCA GTC ACC TCA TTC TTG AC-3' | GYP-A-specific, forward |
| 2026B*                     | nt 116-87 of IN4 | S'-GTT AAC AAC ATA TGC TCT TCT GTT TTA AG-3' | GYP-B-specific, reverse |
| GPABA_E3R**                | nt 110-80 of IN3 | S'-ATG GGT TTT CTG TCA CGA AAG CTT GAG AAC T-3' | GYP-A-specific, reverse |
| 797B*                      | nt 663-682 of IN2 | S'-GCA GTC ACC TCA TTC TTG TT-3' | GYP-B-specific, forward |
| Sta_E4F***                 | nt 38-19 of EX4 | S'-TGG TTC AGA GAA ATG ATG GG-3' | GYP-B-specific, reverse |
| F2***                      | nt 683 of IN2 - nt 4 of EX3 | S'-CCC TTT TTC AAC TTC TTG TAT ATG CAG ATA A-3' | GYP-B-specific, forward |
| Rccgg***                   | nt 28 of IN3 - nt 91 of EX3 | S'-GAG CAA CTA TTT AAA ACT AAG AAC ATA CCG G-3' | GYP-B-specific, reverse |

*From Ref. [7].
**Originally designed.
***From Ref. [13]. IN:intron. EX:exon.
doi:10.1371/journal.pone.0098166.t001

The Frequencies of Glycophorin Variants Including Miltenberger Subtypes and Sta

One thousand and twenty seven (1027) patient’s whole blood samples were obtained from the blood bank of Chang Gung Memorial Hospital. DNA concentrates were extracted from whole blood samples using QIAmp Extraction Kit (Valencia, CA, USA). Genomic DNA extracted from the whole blood samples (0.1 ug/reaction) was thawed on ice and added to a reaction mixture containing 20 pmol of each primer, 200 uM of each dNTP, 0.2 units of Taq polymerase, and 50 uL of reaction buffer (100 ug/uL gelatin, 1.5 mM MgCl2, 10 mM Tris-HCl [pH8.4], and 50 mM KCl). Genomic DNA was amplified by polymerase chain reaction (PCR) using different sets of primers and programs listed in Table 1, Table 2, and Table 3. For GYP(A-B) group, a reference Mi.V concentrate DNA sample was used as positive control. For GYP(A-B-A) group, normal GPA served as the positive control. (Most GYP(A-B-A) hybrids are resulted from gene conversion with a small segment of GYPB between the junction of intron 2 and pseudoexon 3 replacing the homologous segment of GYPB. And, both primers are located in GYPB.) For GYP(B-A-B) and GYP(B-A) groups, we used previously collected and sequence-confirmed Mi.III and Sta blood samples as positive control, respectively. All PCR products were purified by using QIAquick PCR Purification Kit (Valencia, CA, USA). Then, the purified products were sequenced at the Sequencing Center of Chang Gung Memorial Hospital.

doi:10.1371/journal.pone.0098166.t002
Table 3. PCR programs used in this study.

| GYP variants | Denaturation | Annealing | Extension | Cycles |
|--------------|--------------|-----------|-----------|--------|
| GYP(A-B)     | 95 °C 2 mins | 60 °C 2 mins | 72 °C 3 mins | 35     |
| GYP(A-B-A)   | 95 °C 2 mins | 60 °C 2 mins | 72 °C 3 mins | 35     |
| GYP(B-A)     | 95 °C 2 mins | 58 °C 2 mins | 72 °C 3 mins | 35     |
| GYP(B-A-B)   | 95 °C 30 sec | 58 °C 30 sec | 72 °C 2 mins | 30     |

doi:10.1371/journal.pone.0098166.t003

Figure 1. Comparison of the nucleotide sequences spanning genomic regions that contain the crossing-over sites in Intron 3 of the three types of Sta alleles. E and F are newly identified types in this study. Numbering of the nucleotides starts at the first nucleotide of the Intron 3. The locations of each recombination site of Sta variants are boxed. Positions of the nucleotides that are different in ordinary glycophorin genes and the variants are indicated.

doi:10.1371/journal.pone.0098166.g001
The Validation of Efficacy of Current Anti-‘Mi’ Screening Method

Three hundred and eighty-nine (389) randomly collected patient’s blood samples were tested with anti-‘Mi’ sera obtained routinely in our blood bank.

Manual polybrene (MP) method without a supplementary anti-human globulin (AHG) phase was used for serologic testing, as our routine practice. Briefly, RBCs are incubated with the anti-‘Mi’ sera in a low ionic medium at room temperature for one minute. Polybrene, a quaternary ammonium polymer, is then introduced to cause nonspecific red blood cell aggregation. The test tubes are centrifuged, the cell free supernatant fluid decanted, and the Polybrene effect on the cells is neutralized by adding a dilute sodium citrate-glucose solution. The entire procedure is completed in less than three minutes. The hemagglutination results are evaluated macroscopically and microscopically. Reaction intensity of 1+ or more is defined as a positive result. Each blood sample was tested by anti-‘Mi’ of five different individuals.

Genotypes of these tested blood samples were determined by the method mentioned above. The results of serologic test were then compared with genotypic results from PCR-sequencing data. The sensitivity and specificity of the serologic test were then calculated.

The Significance of Stα Antigen in Taiwan Transfusion Practice

Stα(+) genotyped RBCs, as tested in the previous section, were preserved for later use and were included in our blood bank for screening of anti-Stα. Five thousand four hundred and thirty-one (5431) routine samples were tested. MP method with a supplementary AHG phase was used for the serologic test.

Results

The Frequencies of Glycophorin Variants Including Miltenberger Subtypes and Stα

GP.Mur (Mil.III) was confirmed in 57 of 1027 (5.6%) DNA sequenced samples. No other Miltenberger subtypes were found. For Stα group, DNA products were successfully amplified from 35 out of 1027 samples (3.4%). By sequencing, 29 of them belonged to the previous reported Stα type B, and the other 6 samples belonged to currently unreported Stα types. 4 of the 6 have the cross-over point within intron 3, between nucleotides 500 and 533 (we propose it to be called type E). The other 2 have the cross-over point between nucleotides 636 and 676 (we propose it to be called type F). For the comparison of nucleotide sequences, please refer to Figure 1.

The Validation of Efficacy of Current Anti-‘Mi’ Screening Method

By PCR and sequencing, 25 out of the 389 tested blood samples were proved to be Mil.III (GP.Mur) genotype. The prevalence of the Mil.III (GP.Mur) genotype is 6.4% (25/389). Each blood sample was subsequently tested by anti-‘Mi’ of five different individuals. Among the 125 reactions carried out from 25 genetically GP.Mur(+) samples, 84 reactions were positive (sensitivity = 67.2%). 1694 negative results were found from 1820 reactions carried out by genetically GP.Mur(-) samples, and the specificity is 93.1% (1694/1820). Among 1945 serological reactions carried out from all 389 samples, 210 results were positive. Only 84 of these 210 positive results came from genetically GP.Mur(+) samples, and the positive predictive value is 40% (84/210). (Table 4)

The Significance of Stα Antigen in Taiwan Transfusion Practice

Testing 5431 samples for the presence of anti-Stα using Stα(+) genotyped RBCs, 7 antisa were found and the prevalence was about 0.13%. The strength of the reactions except one 3+ case are almost always weak (1+) in MP phase, and all of them included the 3+ case are negative when moving on AHG phase.

Discussion and Conclusions

Screening for anti-‘Mi’ has been incorporated in Taiwan transfusion practice for more than 20 years. The use of ‘Mi’(+) screening RBCs for this purpose is based on the assumption that GP.Mur is the only glycophorin variant reacting with anti-‘Mi’ in Taiwan. However, reports of serologic data and genetic data were conflicting [3], [7], [14]. Furthermore, the efficacy of this screening method had never been validated.

In this study, in accordance with two of the previous reports [3], [14], but against the other [7], we found no other Miltenberger subtypes except GP.Mur in Taiwan. This finding warrants the validity of using ‘Mi’(+) screening RBCs to detect anti-‘Mi’ in current practice. However, our study also showed that this screening method has a low sensitivity of 67.2% (84/125) and positive predictive value of 40% (84/210). The result was fairly consistent with a previous observation. Shih et al. found 19 serologically GP.Mur positive individuals out of 200 samples (9.5%) tested with anti-‘Mia’ sera [7], and we found 210 positive results out of 1945 serological reactions (10.8%). In addition, they found only 9 out of 19 (47.4%) serologically GP.Mur positive individuals were genetically GP.Mur positive, compared with 84 out 210 (40%) by us. Because of the low sensitivity, as much as 32.8% of those individuals with anti-‘Mi’ may not be detected on antibody screening and are on the risk of receiving ‘Mi’(+) transfusion. This problem can usually be avoided by pretransfusion major crossmatch, which is routinely applied in Taiwan transfusion practice. However, for institution utilizing computer crossmatch as pretransfusion incompatibility test, the false negative on anti-‘Mi’ screening may result in clinically significant transfusion reactions.

Genotypic GP.Mur+ individuals can be identified by PCR-sequencing method from extracted genomic DNA as in this study, by direct blood PCR, or by high-resolution melting [14]. Theoretically, RBCs of these individuals will express glycophorin variant GP.Mur on the cell membrane, and these antigens will
react with anti-GP.Mur (e.g. anti-‘Mia’ in Taiwan). Identifying these individuals could prevent transfusion of GP.Mur(+) RBCs to recipients with anti-GP.Mur. However, due to inconsistent expression of Miltenberger antigens, the serologic reaction can be weak or strong, ranging from non-existent, weak reaction requiring microscopic confirmation, to strong grade 3+ agglutination [14]. Although we do not know what will occur clinically when genotypically GP.Mur(+) but serologically nonreactive (to anti-GP.Mur) RBCs are transfused to an individual with anti-GP.Mur, the drastically higher sensitivity of genotyping could offer important safeguard.

The distribution of the Stα antigen, determined by serologic method using anti-Stα, ranges from 0 to 5.2% among different populations of Taiwan [12]. Although three types of Stα were identified as early as in 1991 [15], few studies on Sta and no studies focused on the sensitivity or specificity of anti-Stα were carried out since then. We must emphasize the way we approach to Stα blood group is a reverse of the traditional serology-first (phenotype) strategy, but we consider this method is at least equivalent because of the characteristic of Stα blood group. The expression of Stα is due to the combination of exon 2 of GYPB and exon 4 of GIPA, resulted from gene crossing-over in intron 3. No matter where the alternation site in intron 3 is (resulting in different Stα genetic types), the genetic results on transcription and protein levels are the same. Thus, any Stα genetic types resulting from intron 3 crossing-over of GYPB-A will end up with the same peptide epitope, which is the so called Stα antigen, and should react with anti-Stα. However, our study finds that the reactions are inconsistent and heterogeneous, ranging from no reaction at all to 3+ by MP method, and all of the MP+ cases did not react on AHG phase. We consider the so-called anti-Stα antisemur is nonspecific and cold-agglutinated. Combined the low incidence (0.13%) and the cold-agglutination property of anti-Stα, we consider routine screening of anti-Stα is not indicated, at least in Taiwan, without further evidence.

In conclusion, in this study we confirm the previous assumption that GP.Mur is the only prevalent Miltenberger subtype in Taiwan, and thus validate the current anti-‘Mia’ screening test used in Taiwan blood bank practice. The sensitivity of anti-‘Mia’ screening test is barely fair, but routine pretransfusion major crossmatch may prevent some of the cases and genotyping could act as a safeguard for those slipping through the net. Although the prevalence of Stα antigen in Taiwan is high, the incidence of anti-Stα is low, and the reactions are heterogeneous and probably cold-agglutination. Routine screening of anti-Stα in Taiwan is not necessary based on current evidence.

Acknowledgments

The excellent consulting assistance and sample resources from Mr. Chien-Ting Peng (Department of Laboratory Medicine, Chang Gung Memorial Hospital, Taiwan) is gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: TDC DPC WTW CFS. Performed the experiments: TDC WTW. Analyzed the data: TDC CFS. Contributed reagents/materials/analysis tools: DPC WTW CFS. Wrote the paper: TDC CFS.

References

1. Rearden A, Phan H, Dubnacoff T, Kudo S, Fukuda M (1990) Identification of the crossing-over point of a hybrid gene encoding human glycophorin variant Stat. J Biol Chem 265(16): 9259-9263.
2. Tippett P, Reid ME, Poole J, Green CA, Daniels GL, et al. (1992) The Miltenberger subsystem: is it obsolescent? Transf Med Rev 7(3): 170-182.
3. Broadberry RE, Lin M (1996) The distribution of the Mi.III (GP.Mur) phenotype among the population of Taiwan. Transf Med 6: 145-148.
4. Broadberry RE, Lin M (1994) The incidence and significance of anti-"Mia" in Taiwan. Transfusion 34: 235-241.
5. Lin M, Broadberry RE (1994) An intravascular transfusion reaction due to anti-"Mia" in Taiwan. Vox Sang 67: 320.
6. Lin M, Broadberry RE (1996) In immunohematology in Taiwan. Transf Med Rev 12(1): 56-72. Review.
7. Shih MC, Yang LH, Wang NM, Chang JG (2000) Genomic typing of human red cell Miltenberger glycophorins in a Taiwanese population. Transfusion 40: 54-61.
8. Cleghorn TE (1962) Two human blood group antigens, Ste-a (Stones) and Ri-a (Ridley), closely related to the MNSs system. Nature 219:5: 297-298.
9. Suchanowska A, Smolarek D, Czerskiński M (2010) A new isoform of Sta gene found in a family with NOR polyagglutination. Transfusion 50(2): 514-515.
10. Madden HJ, Cleghorn TE, Allen FH, Rosenfield RE, Mackeprang M (1964) A note on the relatively high frequency of Sta on the red cells of orientals and report of a third example of anti-Sta. Vox Sang 9: 562-564.
11. Daniels G (2002) Chapter 3, MNS blood group system. In: Human Blood Groups, 2nd ed., Blackwell Science. pp. 99-174.
12. Broadberry RE, Chang FC, Jan YS, Lin M (1990) The distribution of the red-cell Sta (Stones) antigen among the population of Taiwan. Transf Med 8: 57-58.
13. Palacajornsuk P, Nathalang O, Tantimavanich S, Rejarchandra S, Reid ME (2007) Detection of MNS hybrid molecules in the Thai population using PCR-SSP technique. Transfus Med 17(5): 169-174.
14. Hsu K, Lin YC, Chang YC, Chon YN, Chia HP, et al. (2013) A direct blood polymerase chain reaction approach for the determination of GP.Mur [Mi.III] and other Hi+ Miltenberger glycophorin variants. Transfusion 53(5): 962-971.
15. Huang CH, Blumenfeld OO (1991) Multiple origins of the human glycophorin Sta gene. Identification of hot spots for independent unequal homologous recombinations. J Biol Chem 266: 23306-23314.