First example of an *FY*01* allele associated with weakened expression of Fy^a on red blood cells

P.A. Arndt, T. Horn, J.A. Keller, R. Young, S.M. Heri, and M.A. Keller

Duffy antigens are important in immunohematology. The reference allele for the Duffy gene *(FY)* is *FY*02,* which encodes Fy^b. An A>G single nucleotide polymorphism (SNP) at coding nucleotide (c.) 125 in exon 2 defines the *FY*01* allele, which encodes the antithetical Fy^a. A C>T SNP at c.265 in the *FY*02* allele is associated with weakening of Fy^b expression on red blood cells (RBCs) (called Fy^X). Until recently, this latter change had not been described on a *FY*01* background allele. Phenotype-matched units were desired for a multi-transfused Vietnamese fetus with α-thalassemia. Genotyping of the fetus using a microarray assay that interrogates three SNPs (c.1-67, c.125, and c.265) in *FY* yielded indeterminate results for the predicted Duffy phenotype. Genomic sequencing of *FY* exon 2 showed that the fetal sample had one wild-type *FY*01* allele and one new *FY*01* allele with the c.265C>T SNP, which until recently had only been found on the *FY*02* allele. Genotyping performed on samples from the proband's parents indicated that the father had the same *FY* genotype as the fetus. Flow cytometry, which has been previously demonstrated as a useful method to study antigen strength on cells, was used to determine if this new *FY*01* allele was associated with reduced Fy^a expression on the father's RBCs. Median fluorescence intensity of the father's RBCs (after incubation with anti-Fy^a and fluorescein-labeled anti-IgG) was similar to known *FY*01* heterozygotes and significantly weaker than known *FY*01* homozygotes. In conclusion, the fetus and father both had one normal *FY*01* allele and one new *FY*01* allele carrying c.265C>T. This new *FY*01* allele, named *FY*01W.01,* is associated with weakened expression of Fy^a on RBCs. ***Immunohematology* 2015;31:103–107.**

Key Words: Duffy, Fy^a, flow cytometry, genotyping, blood group antigen

The Duffy blood group antigens, expressed on the Duffy antigen receptor for chemokines (DARC), are important, not only in the field of immunohematology where Duffy antibodies can cause transfusion reactions and hemolytic disease of the fetus and newborn, but also in the fields of anthropology, genetics, and disease, where the DARC protein is the receptor for the malarial parasite *Plasmodium vivax*.[1] The Duffy gene *(FY)* consists of two exons on chromosome 1.[2–4] The reference allele *FY*02 (FY*B)* encodes for Fy^b, Fy3, Fy5, and Fy6. An A>G single nucleotide polymorphism (SNP) at coding nucleotide (c.) 125 in exon 2 defines the *FY*01* *(FY*A)* allele and results in an asparagine to glycine amino acid substitution in the protein (p.42 Asp>Gly) that results in expression of Fy^a on red blood cells (RBCs) (Table 1). There are several different mutations leading to the FY_{null} phenotype [Fy(a–b–), Fy:–3]. The most common is the *FY*02N.01* allele; a t>c SNP at c.–67 located in a transcription factor binding site called a GATA box hinders binding of the transcription factor GATA-1, which results in loss of *FY*02* expression on RBCs but not in tissues. Another prevalent Duffy variant is the C>T mutation at c.265 on the *FY*02* allele, encoding a p.89 Arg to Cys amino acid substitution that causes weakening of Fy^b, Fy3, and Fy6 expression (Fy^X).[8–10] The microarray assay, HEA BeadChip™ (BioArray Solutions, Immucor, Norcross, GA), tests for these three notable SNPs to predict the Fy phenotype: Fy^a/Fy^b (c.125G/A), GATA (c.–67t/c), and Fy^X (c.265C/T).[11] A GATA box mutation has been described on the *FY*01* allele that results in the Fy(a–b–) phenotype,[12] but the c.265C>T (FyX) change had not been described on the *FY*01* allele until recently.[6,7]

Flow cytometry has been shown to be a useful method for detecting differences in antigen strength (e.g., attributable to zygosity[13]) and has been used to study the expression of

Table 1. Important nucleotide changes for different *FY* alleles

	FY nucleotides				
	Promoter	Exon 2			
Allele	–67	125	265	298	Comments
*FY*02 (FY*B)*	t	A	C	G	Reference allele; Fy^b
*FY*02N.01*	c	A	C	G	Fy(a–b–) erythroid cells only
*FY*02W.01*	t	A	T	A	Fy(b+^{w}), Fy^X
*FY*02W.02*	t	A	T	A	c.145G>T ; Fy(b+^{w}), Fy^X
*FY*02W.03*	t	A	C	G	c.266G>A; Fy(b+^{w})[5]
*FY*02W.04*	t	A	C	G	c.901C>T; Fy(b+^{w})[5]
*FY*01 (FY*A)*	t	G	C	G	Fy^a
FY*01W.01	**t**	**G**	**T**	**G**	**Fy(a+^{w})[6]**
*FY*01W.02*	t	G	T	A	Fy(a+^{w})[7]

The allele in **bold *(FY*01W.01)*** is described in the text.

Duffy antigens on RBCs of different phenotypes or of different ages.[8,10,12,13–20] We describe the first reported example of an *FY*01* allele with the c.265C>T (FyX) change; this allele was shown to be associated with weak expression of Fy^{a} as determined by flow cytometry.[6]

Case Report

The patient was a Vietnamese male fetus with α-thalassemia. A decision was made to provide the fetus with phenotype-matched RBC units but, because of multiple recent intrauterine transfusions, a genotype was needed to determine the fetus' predicted phenotype. Molecular typing results (HEA BeadChip, Immucor) indicated the following phenotype: C+ c– E– e+; K– k+ Kp(a–b+) Js(a–b+) Jk(a+b+); M– N+ S– s+; Lu(a–b+); Di(a–b+); Co(a+b–); Do(a+b+) Jo(a+) Hy+; LW(a+b–); SC:1,–2, but the Fy^{a} and Fy^{b} results were "indeterminate." Further molecular studies were performed on the fetal DNA to determine the Fy phenotype. Blood samples were obtained from both the mother and father for molecular and serologic testing.

Materials and Methods

Serologic Testing

RBCs from the proband's parents were typed with three different sources of anti-Fy^{a} and anti-Fy^{b} (Immucor; Ortho Clinical Diagnostics, Raritan, NJ; American Red Cross, Washington, DC) by the indirect antiglobulin test (IAT). The grading scale included ½+ increments.[21]

Molecular Testing

Genomic DNA was isolated from mononuclear cells using a kit (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA). Microarray testing was performed (HEA BeadChip, Immucor) with software (BASIS 3.3, BioArray Solutions Ltd., Warren, NJ). Amplification and Sanger sequencing of *FY* exon 2 was performed (BigDye Terminator Kit, Life Technologies, Grand Island, NY), and the resulting sequence was aligned to the consensus sequence (Sequencher 5.0, GeneCodes Corp., Ann Arbor, MI). Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) using *BanI* was used to interrogate *FY*01/FY*02* (c.125G>A) as described by Tournamille et al.[22]

Flow Cytometry

A previously described flow cytometric method[23] was used to study the strength of Fy^{a} on the proband's parents' RBCs; known genotyped and/or phenotyped donor or reagent RBCs were tested in parallel as controls. Briefly, 0.1 mL 5% washed RBCs were incubated with 0.2 mL undiluted polyclonal anti-Fy^{a} (Immucor) for 30 minutes at 37°C, washed four times with 0.85% (w/v) phosphate-buffered saline (PBS), then incubated with a dilution of a tagged antihuman globulin (FITC Fab anti-IgG, MP Biomedicals, Aurora, OH) for 30 minutes at room temperature, and washed once with 0.2% bovine serum albumin in PBS. Ten thousand events per sample were acquired by a flow cytometer (FACSort, BD Biosciences, San Jose, CA) using logarithmic amplification of forward scatter, side scatter, and green fluorescence. A gate was set on the forward versus side scatter dot plot to exclude debris, and the median green fluorescence of the gated events was obtained using software (CellQuest Pro, BD Biosciences). In addition to testing by flow cytometry, the RBCs incubated with anti-Fy^{a} were also tested by IAT (Anti-IgG, Ortho Clinical Diagnostics).

Results

Serologic Testing

The mother's RBCs and the father's RBCs both typed as Fy(a+b–); both reacted 4+ with all three sources of anti-Fy^{a}.

Molecular Testing

The molecular testing yielded an indeterminate predicted phenotype for the Duffy antigens on the proband's sample. The microarray assay (HEA BeadChip, Immucor) results showed the fetal sample to be homozygous for c.125G, associated with the *FY*01 (FY*A)* phenotype, and heterozygous for c.265C>T change associated with the *FY*02W.01* and *FY*02W.02* alleles, while being homozygous for c.1-67t. Genomic sequencing of *FY* exon 2 confirmed the genotype results and determined that the c.298G>A SNP associated with the known *FY*02W.01* and *FY*02W.02* alleles was not present. The mother's predicted phenotype by molecular typing was Fy(a+b–), and the sample was negative for the c.265C>T variant. The father's sample, like that of the fetus, yielded indeterminate calls for Duffy antigens on the microarray assay. PCR-RFLP analysis showed the father's sample to be homozygous for c.125G and heterozygous for c.265C/T, concordant with the molecular findings.

Flow Cytometry

Flow cytometry (median fluorescence) results of the controls [5 Fy(a+b–) donors who genotyped as homozygous for *FY*01,* 5 Fy(a+b+) donors who were obligate heterozygotes for *FY*01,* 3 Fy(a+b–) donors who genotyped as *FY*01/ FY*02N.01* and thus were heterozygous for *FY*01,* and 2

Fy(a–b+) donors] and the two Fy(a+b–) parents of the fetus are shown in Figure 1. The median fluorescence results of the donors who were homozygous for *FY*01* were clearly stronger than the median fluorescence results from the donors who were heterozygous, and two distinct groups were apparent. The father's sample, with only one normal *FY*01* allele, gave results that fell clearly in the heterozygous group indicating that his other allele, the new *FY*01* allele, coded for a weakened expression of Fy^a. The median fluorescence results of the mother's sample fell in the gap between the homozygotes and the heterozygotes and could not be clearly distinguished as belonging to either group. The IAT results of all the Fy(a+) controls and the parents' RBCs after incubation with anti-Fy^a were similar (3–3½+).

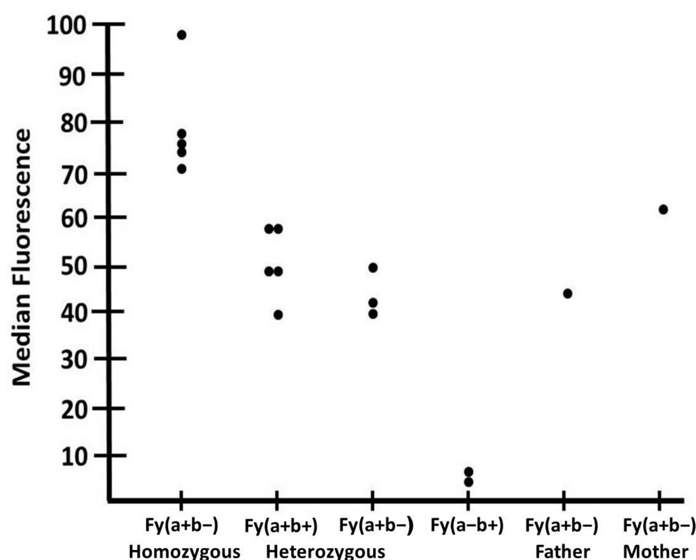

Fig. 1. Median fluorescence (MF) results of RBCs from (1) five *FY*01* homozygotes [phenotype = Fy(a+b–), genotype = *FY*01/FY*01,* MF = 70–98], (2) two groups of *FY*01* heterozygotes [phenotype = Fy(a+b+), genotype not determined (*n* = 5; MF = 40–58), and phenotype = Fy(a+b–), genotype = *FY*01/FY*02N.01* (*n* = 3; MF = 40–50)], (3) two *FY*01* negatives [phenotype = Fy(a–b+), genotype not determined, MF = 5], (4) the proband's father [phenotype = Fy(a+b–), genotype = *FY*01/FY*01W.01,* MF = 45], and (5) the proband's mother [phenotype = Fy(a+b–), genotype = *FY*01/FY*01,* MF = 63].

Discussion

The Duffy blood group system consists of five antigens (the antithetical Fy^a and Fy^b; Fy3, Fy5, and Fy6) located on a multipass membrane glycoprotein known as DARC. The four common phenotypes, Fy(a+b–), Fy(a–b+), Fy(a+b+), and Fy(a–b–), are encoded by the *FY*01, FY*02,* and *FY*02N.01* alleles (Table 1). According to Howes et al.,[1] the *FY*01* allele is most common in Asia, the *FY*02* allele is most common in Europe, and the *FY*02N.01* (null) allele is most common in Africa; heterogeneity of all three alleles is greatest in the Americas. A small percentage (2–3%) of Caucasians have the *FY*02W.01* allele that codes for weakened expression of Fy^b, Fy3, and Fy6. This Fy^X phenotype is caused by the c.265C>T mutation that results in a p.89 Arg to Cys amino acid substitution; this change appears to cause reduced levels of DARC in the RBC membrane.[8] The two alleles currently described with this change are *FY*02W.01* and *FY*02W.02*. Both are associated with a second mutation at c.298G>A, which results in a p.100 Ala to Thr change; the *FY*02W.02* allele has an additional change at c.145G>T, which results in a p.49 Ala to Ser change. Two more recently described alleles in Caucasians, *FY*02W.03* with a c.266G>A (p. 89 Arg to His) change and *FY*02W.04* with a c.901C>T (p.303 Pro to Ala) change, also result in Fy($b+^w$) phenotypes.[5]

Although rare mutations in the *FY*01* alleles have been described to cause null phenotypes,[3,4,24] and there are reports of Southeast Asians with weak Fy^a on RBCs,[25,26] until recently, no *FY*01W* alleles had been reported. In 2015, Lopez et al.[7] reported an Fy(a+b+) Australian Caucasian blood donor who was noted to have weak expression of Fy^a. This phenotype was shown to be associated with a new allele, *FY*01W.02,* which had both the c.265C>T and c.298G>A mutations (as seen with *FY*02W.01* and *FY*02W.02*). In 2013, we reported, at an AABB meeting, a recently transfused Vietnamese patient who was found to have an unusual allele, *FY*01W.01,* with only the critical c.265C>T change.[6] Because of recent transfusions, the proband could not be phenotyped to determine if this allele was associated with weak Fy^a expression, but his parents were willing to be tested. The Fy(a+b–) mother had a normal *FY*01/FY*01* genotype, but the Fy(a+b–) father had the new allele with a normal allele *(FY*01/FY*01W.01).*

As the proband's father typed 4+ with anti-Fy^a due to his *FY*01* allele, it would have been difficult to determine the strength of Fy^a by manual titrations, which have been shown to be poor predictors of antigen strength (e.g., zygosity).[13] Flow cytometry, however, has been used successfully to study Duffy antigens—for example, to demonstrate weak expression of Fy^b, Fy3, and Fy6 on Fy^X phenotype RBCs[8,10,15,17]; to distinguish zygosity (e.g., *FY*01/FY*01* vs. *FY*01/FY*02N.01*)[12,13,15]; and to study Duffy antigen expression on cultured erythroid cells or reticulocytes,[14,16,18,20] after RBC storage and leukocyte reduction,[19] and on ovalocytes.[20] Our flow cytometry studies with known examples of *FY*01* homozygotes and heterozygotes showed clear differentiation between the two groups. The proband's father's RBCs gave results that clearly fell in the heterozygous group, thus indicating that the *FY*01W.01* allele

codes for weak expression of Fy[a]. Interestingly, the proband's mother's RBCs gave results much weaker than any of the five *FY*01/FY*01* homozygous controls (but still stronger than the eight *FY*01* heterozygous controls). She may have an unknown variant *FY*01* allele that codes for slightly weaker Fy[a] expression.

In conclusion, we describe the first example of Fy(a+[w]). The presence of this phenotype was predicted by Tournamille et al.[5] based on the description by Shimizu et al.[25] of some Thai individuals with weak Fy[a]. Unlike the alleles most commonly associated with Fy(b+[w]) and the other allele recently described with Fy(a+[w]), which have at least two mutations (c.265C>T and c.298G>A), the allele we are describing was only associated with one mutation (c.265C>T). This allele was discovered while performing molecular testing on samples from a recently transfused patient. Since the discovery of this allele, two more samples have been identified with the Fy[x] change on an *FY*01* background at the Red Cross National Molecular Laboratory. Both samples were identified by indeterminate calls for the Duffy phenotype on microarray testing, much like the initial case report described here. The first, a female donor, race unknown, demonstrated both c.265C>T and c.298G>A changes on an *FY*01* background (like the *FY*01W.02* allele described by Lopez et al.[7]). The second case is a 49-year-old male patient, race unknown, with only the c.265C>T change located on the *FY*01* background, specifically the *FY*01W.01* allele. As the use of molecular methods increases in the future, it is possible that more examples of *FY*01* alleles associated with Fy(a+[w]) may be discovered.

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