Received: 6 August 2017 | Accepted: 30 August 2017

DOI: 10.1002/jcla.22330

BRIEF REPORT

WILEY

The synonymous nucleotide substitution *RHD* 1056C>G alters mRNA splicing associated with serologically weak D phenotype

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Background: D antigen is one of the most clinically significant blood group antigens. Variation of the *RHD* gene can cause weak D or partial D phenotypes. While most variations are missense substitutions with amino acid changes, those without are called "silent" or "synonymous" substitutions. Synonymous substitutions often have little effect on the protein, not altering the phenotype. However, effect on splicing can affect end-product protein. We report a new synonymous variation, *RHD* 1056C>G, that resulted in weak D phenotype, and predicted its effect with various in silico methods.

Methods: Serologic testing of the D antigen with full sequencing of the *RHD* gene was done. Human Splice Finder was used to predict the effect of this variation, and validation of this method was done with all known *RHD* variations reported in the literature.

Results: *RHD* 1056C>G was predicted to cause the formation of an exonic splicing silencer (ESS) site. The creation of new ESS site potentially inhibits the splicing event, resulting alteration of splicing. This is similar to remodeling of splice acceptor or donor site, as this kind of deep exonic variation could affect the D antigen's quality or quantity. This is in concordance with serologic results, which showed only delayed weak agglutination to anti-D reagents.

Conclusions: The analytic methods we applied showed good correlation with the actual phenotype, along with concordant results when analyzing other known variants reported in the literature. We conclude that *RHD* 1056C>G results in serologic weak D phenotype.

KEYWORDS

genotype, in silico, RHD, silent, splicing, synonymous

1 | INTRODUCTION

D antigen is considered one of the clinically significant blood group antigens, as correct D typing is required for planning transfusion strategies for prevention of alloimmunization, both as a donor and recipient in blood transfusion. Serologic D phenotyping requires care when detecting weak D and partial D phenotypes; these are often the result of amino acid substitutions.[1] Full genotyping of the coding region of the *RHD* gene often reveals these weak D and partial D phenotypes to have single nucleotide changes, and while majority of these variations are missense substitutions with amino acid changes, those without amino acid change are called "silent" or "synonymous" substitutions. Synonymous substitutions often have little effect on the protein, not altering the phenotype. However, these variations can sometimes cause splicing changes, resulting in difference in quality or quantity of the transcripted mRNA, resulting in differences in phenotype.[2] Herein, we report a new *RHD* variant allele (*RHD* 1056C>G, G353G, GenBank accession no. MF737522) that was discovered in a Korean male that

Sejong Chun and Jae Won Yun are equally contributed to this study.

TABLE 1 In silico annotation of single nucleotide variation and prediction of impact for RHD RNA splicing

RHD exon no.	Location of variation	cDNA_change	Phenotype	Oncotator classification	Donor_site (variation %)	Acceptor_site (variation %)	Maximum Entropy (variation %)	ESS_site	ESE_site	References
1	Exofacial	c.147A>G	Weak/partial D	Splice_Site		New site (+50.45), Site broken (−36)	5′ motif (−23.39), 3′ motif (−209.76), 3′ motif (+196.4)	New ESS Site	ESE Site Broken	(15)
2	Membrane	c.165C>T	Weak D/D-positive	Silent				New ESS Site	ESE Site Broken	(4,5)
3	Membrane	c.357T>C	Weak D	Silent					ESE Site Broken	(4,5)
3	Membrane	c.384T>C	NA	Silent					ESE Site Broken	(21)
4	Membrane	c.519C>T	Weak D	Silent			3′ motif (+17.82)	New ESS Site	ESE Site Broken	(4,5)
4	Intracellular	c.576C>T	Weak D	Silent					ESE Site Broken	(4,5)
5	Membrane	c.636C>T	D-positive	Splice_Site	New site (+54.76)		5′ motif (+392.89), 3′ motif (+11.97)			(22)
7	Intracellular	c.960G>A	Weak D	Silent		New site (+65.37)	3′ motif (−24.1)	New ESS Site	ESE Site Broken	(23)
7	Exofacial	c.1056C>G	Partial D	Silent				New ESS Site		This study
7	Exofacial	c.1065C>T	Weak D	Silent	New site (+49.56)		5′ motif (+505.88)	New ESS Site		(16)
8	Exofacial	c.1110C>T	Weak D	Silent				New ESS Site		(20)
8	Membrane	c.1152A>C	Weak D	Splice_Site		Site broken (−31.71)	5′ motif (−15.77), 3′ motif (−66.56), 3′ motif (+314.21)			(4,5)
9	Intracellular	c.1170T>C	Weak D	Silent						(13)
9	Intracellular	c.1227G>A	DEL; extremely weakened phenotype	Splice_Site	New site (+13.88),WT site broken (−10.94)	New site (+59.65), site broken (−35.33)	5′ motif (−34.63)			(11)

ESS, exonic splicing silencer; ESE, exonic splicing enhancer; WT, wild type.
The table was merged from results of Oncotator, wANNOVAR and HSF.

(A)

Reference sequence

RHD Gene > ENST00000328664 Transcript > Exon number: **7** (134 bp) + **100** intronic nucleotides at exon ends

```
  1 tccccctttg gtggccccgg ataccaaggg tgtgtgaaag gggtgggtag ggaatatggg tctcacctgc caatctgctt ataataacac ttgtccacag
101 GGGTGTTGTA ACCGAGTGCT GGGGATTCCC CACAGCTCCA TCATGGGCTA CAACTTCAGC TTGCTGGGTC TGCTTGGAGA GATCATCTAC ATTGTGCTGC
201 TGGTGCTTGA TACCGTCGGA GCCGGCAATG GCATgtgggt cactgggctt acccccccatc cccttaacac tcccctccaa ctcaggaaga aatgtgtgca
301 gagtccttag ctggggcgtg tgcactcggg gcca
```

Total sequence length: 334 nucleotides

Mutant sequence

```
  1 tccccctttg gtggccccgg ataccaaggg tgtgtgaaag gggtgggtag ggaatatggg tctcacctgc caatctgctt ataataacac ttgtccacag
101 GGGTGTTGTA ACCGAGTGCT GGGGATTCCC CACAGCTCCA TCATGGGCTA CAACTTCAGC TTGCTGGGTC TGCTTGGAGA GATCATCTAC ATTGTGCTGC
201 TGGTGCTTGA TACCGTGGGA GCCGGCAATG GCATgtgggt cactgggctt acccccccatc cccttaacac tcccctccaa ctcaggaaga aatgtgtgca
301 gagtccttag ctggggcgtg tgcactcggg gcca
```

Total sequence length: 334 nucleotides

(B)

Human Splicing Finder result

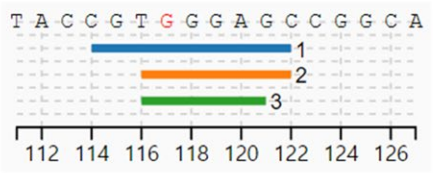
Predicted signal	Prediction algorithm	cDNA Position	Interpretation
New ESS site	1. Intron-identity element[9]		Potential alteration of splicing (creation of a new ESS site)
	2. Motif 2[10]		
	3. Fas-ESS hexamers[8]		

FIGURE 1 Human Splicing Finder (HSF) results for *RHD*:c.1056C>G. (A) Compared to the reference sequence, G sequence was changed to A sequence. Upper case letters are sequences in the exon region; lower case letters are in the intron region. (B) In detailed analysis using HSF predicted three signal for the creation of a new exonic splicing silencer (ESS) site. ESS could inhibit splicing of the pre-mRNA, contributing to alternate splicing. Alternate splicing could affect mRNA quality or stability through whole/partial exon deletion, insertion, frame-shift, and truncation. ESS, exonic splicing silencer

visited our hospital for removal of nasal polyp. The observed synonymous variation was analyzed to result in weakened or partial D expression, and we performed a comprehensive analysis of all synonymous variations in the literature.

2 | METHODS

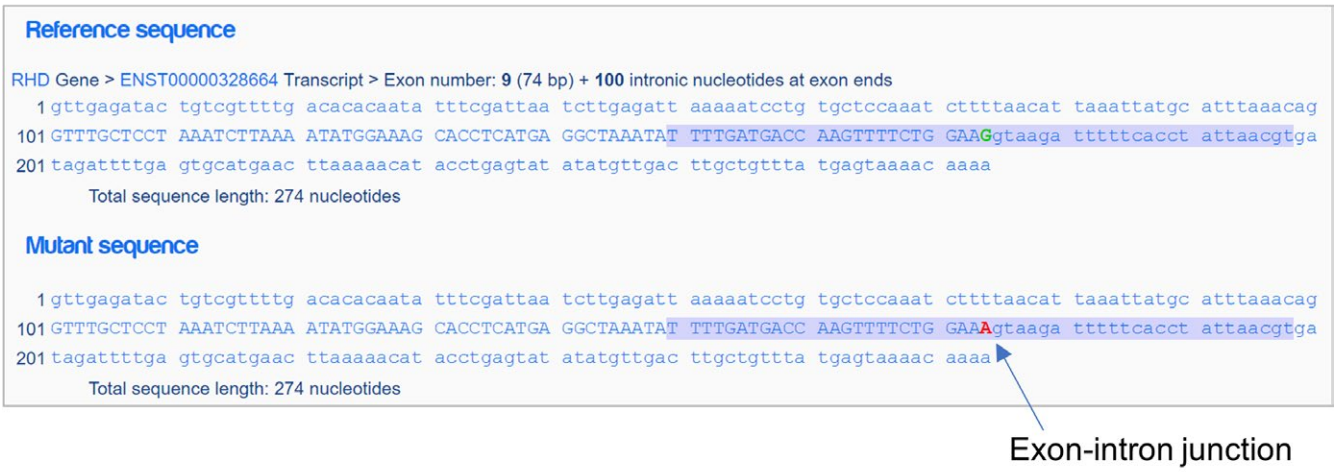
D blood grouping was done with QWALYS-3 (Diagast, Loos, France) and with manual tube methods with anti-D reagents (Ortho Clinical Diagnostics, Raritan, NJ, USA). Genotyping of the RHD gene was done on all exons and adjacent intron regions with previously described protocols.[3] Database of previously reported RHD alleles were retrieved from the blood group antigen gene mutation database (https://www.ncbi.nlm.nih.gov/gv/mhc/xslcgi.cgi?cmd=bgmut/home)[4] and the rhesus base site (http://rhesusbase.info/).[5] To investigate the biological impact of the variations, annotation using wANNOVAR (http://wannovar.wglab.org/)[6] and Oncotator (http://portals.broadinstitute.org/oncotator/)[7] was performed. The evaluation of in silico analysis for splicing variations was done with Human Splicing Finder (HSF) (http://www.umd.be/HSF3/).[8] HSF is frequently used as a tool to predict the effects of variations on splicing signals and to identify splicing motifs. In detail, the tool was designed to perform in silico predictions for formation or disruption of splice donor site, splice acceptor site, exonic splicing silencer (ESS) site, and exonic splicing enhancer (ESE) site. Informed consent for genetic testing was obtained prior to sample collection. The methods applied in this study was in accordance with the Declaration of Helsinki.

3 | RESULTS

3.1 | Serologic and genotype results

Routine preoperation D blood group showed strong 4+ reaction by the automated QWALYS-3 device, with reagents provided by the manufacturer. This was backed up with manual tubes tests. Initially, manual tests did not show agglutination, but later showed weak 1+ reaction was observed with prolonged 15-min incubation. Genotyping

(A)



(B)

Human Splicing Finder Matrices

Splice site type	Impact and Variation %	Motif	New splice site
Acceptor	Site broken -35.33	GTTTTCTGGAAGgt	gttttctggaaaGT
Acceptor	New site 59.65	TTTTCTGGAAGgta	ttttctggaaagTA
Donor	New site 13.88	CTGGAAGgt	CTGgaaagt
Donor	WT site broken -10.94	AAGgtaaga	AAAgtaaga

Maximum Entropy

Splice site type	Impact and MaxEnt Variation %	Motif	New splice site
5' Motif	Broken WT Donor Site -34.63	AAGgtaaga	AAAgtaaga

FIGURE 2 An example of RHD splice site mutation, *RHD*:c.1227G>A. (A) Comparing reference sequence, the G sequence was changed to A sequence. It is located by the exon-intron junction, and Oncotator determined the change as splice site mutation. (B) In detailed analysis using Human Splicing Finder (HSF) predicted several signal for potential splice sites. Using HSF matrices, two acceptor motif changes and two donor motif changes were predicted. Upper case and lower case letters in motif represents exonic sequences and intronic sequences, respectively. Motif change in acceptor or donor site could result whole or partial exon deletion, insertion, frame-shift, and truncation of RHD, which could result in change of RHD protein quality or quantity. In Maximum Entropy (MaxEnt) method, 5′ motif which represents donor site were predicted to be broken with MaxEnt variation, −34.63%. MaxEn, maximum entropy; WT, wild type

of all exons and surrounding intron regions of the *RHD* gene was done. Although zygosity testing was not preformed, hemizygosity was presumed as no observation of heterozygous single nucleotide variants were observed in the extent of the sequenced *RHD* gene was found, and hemizygous background is the most likely cause in D-variants. We observed a single nucleotide change in exon 7, position c.1056C>G compared to the reference cDNA sequence NM_016124.3, which shows no change in amino acid sequence according to the translated isoform (RHD_v001). No other variants were observed to the extent that was sequenced.

3.2 | In silico analysis of all synonymous variations

A review of all reported *RHD* single nucleotide variants without amino acid change was done. Annotation using wANNOVAR and Oncotator was performed for all 14 variations with annotation with the hg19 reference. Interestingly, four cases were found as with splice site mutation around exon-intron junctions (Table 1). All these variations were located within 2 base pairs on the exon side from exon-intron splicing junction. The other nine variations, including this case, were predicted as a silent mutation in wANNOVAR and Oncotator (Table 1). Further

in silico analysis was done with HSF. Using the method, we additionally found two more variations were related with making new donor site or new acceptor site (Table 1).

3.3 | Analysis of *RHD* c.1056C>G

We have also found that seven cases were predicted to be related with ESS site formation or ESE site breakage. The case of c.1056C>G in this study were predicted to be related with new ESS site formation (Table 1), despite being a synonymous variation in amino acid change (Figure 1A). In this case, the creation of new exonic ESS site potentially inhibits the splicing event, resulting alteration of splicing. This is similar to remodeling of splice acceptor or donor site, as this kind of deep exonic variation could affect the D antigen's quality or quantity. In detailed analysis using HSF, the tool predicted three signals for the creation of a new exonic ESS site. ESS could inhibit splicing of the pre-mRNA, contributing to alternate splicing. Alternate splicing could affect mRNA quality or stability through whole/partial exon deletion, insertion, frame-shift, and truncation. Three different prediction algorithms (intron-identity element,[9] Motif 2,[10] Fas-hexamer[8]) were applied with consistent results (Figure 1B). Phenotype of this patient was found to be weakened D expression, and the reaction toward different anti-D reagents differed greatly. This is suggestive of a good match between genotype analysis done in this study and the presented phenotype.

4 | DISCUSSION

This study has shown that all but one known synonymous variations reported to be causative of weak D phenotype was related with exonic splicing donor site or acceptor site alteration. The new case reported by this study, *RHD* c.1056C>G, was analyzed to have splicing changes regarding the exofacial region of the exon 7 to be a main reason. However, further experimental validation such as transcriptome analysis could be required to fully understand the impact of this new variant. Nevertheless, our finding highlights that synonymous variations in *RHD* should be re-evaluated in silico or in vitro when found with weak *RHD* phenotype.

A good example for the validation of our applied methods was done by in silico analysis on the Asia type DEL phenotype. This variation is possibly the best investigated of synonymous variants that cause splicing change in the *RHD* gene; skipping of transcription of exon 9 in *RHD* c.1227G>A individuals was observed,[11-13] and this variant has apparently identical epitopes of the D antigen in extremely low levels.[11,14,15] This variation has a changed sequence placed in the junction region of exon 9 and its following intron (Figure 2A). The prediction of potential splice sites in HSF were inferred from two methods, using HSF matrices (Figure 2B, upper table) and using maximum entropy calculation (Figure 2B, lower table). In Figure 2B, remodeling of splice acceptor and donor site was predicted, suggesting the aberrant splicing of *RHD* exon, identical to previous experiment results. The aberrant splicing is speculated to affect D protein quality or quantity through altering RNA stability, which may have resulted in the extremely low levels of D antigen expression. This variant was also predicted to have multiple splice donor and acceptor changes, compared to other variants (Table 1), implying that the D protein could be furtherly affected in quantity or quality compared to other cases. Indeed, *RHD* c.1227G>A is phenotypically a DEL variant, with D antigens only traceable through elution testing.[14] Such analysis was done on all synonymous variations found in the literature (Table 1); we found that in all 14 cases with synonymous variation and weak D phenotype, 4 cases were found to be related with exon-intron junction variation, suggesting a reasonable prediction of aberrant splicing potentiality. In addition, 10 cases were predicted to be with ESS site breakage or new ESE site formation, suggesting a chance of aberrant splicing change. In one case, the variant with c.1170T>C,[16] no aberrant splicing was predicted in in silico analysis. Although splicing change is a major issue, synonymous variation could have various physiological effects in gene function or expression. Alteration of gene expression or gene function through synonymous variations could be possible by other complex mechanisms such as RNA structure change, RNA translation efficiency change and even protein misfolding.[17] However, these diverse mechanisms were difficult to predict or validate and it was considered beyond the scope of this study.

Location of the variant also seemed to play as a factor in synonymous changes with weakened D antigen expression. Four variations were located in the exofacial region (Table 1). The variations include c.147A>G,[18] c.1065C>T,[19] c.1110C>T [20] and this case, c.1056C>G, all showing weakened D expression according to the original literature that reported these variants. Variations in exofacial region might affect D antibody affinity and specificity to D antigen, potentially changing antigenicity through directly altering epitopes. This implies that some antibodies could show good affinity to D with exofacial variations while others not, which explains the characteristics of different agglutination reaction toward different anti-D reagents. Interestingly, all the cases with evidence of phenotype of partial D expression were found in cases with variations in the exofacial variation (partial D case of c.147A>G and this case, c.1056C>G, Table 1).

In conclusion, we report a new *RHD* allele with apparent synonymous variation which resulted in partial D phenotype, and validated the effect of the variation with in silico methods. We further evaluated other synonymous variations and concluded that good correlation with prediction and actual phenotype.

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How to cite this article: Chun S, Yun JW, Park G, Cho D. The synonymous nucleotide substitution *RHD* 1056C>G alters mRNA splicing associated with serologically weak D phenotype. *J Clin Lab Anal*. 2018;32:e22330. https://doi.org/10.1002/jcla.22330