GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 16, Number 1, 2012

Pp. 50–53
DOI: 10.1089/gtmb.2011.0093

Frequency Determination of α-1,3 Glucosyltransferase p.Y131H and p.F304S Polymorphisms in the Croatian Population Revealed Five Novel Single Nucleotide Polymorphisms in the h*ALG6* Gene

Sandra Supraha Goreta,[1] Sanja Dabelic,[1] Dinko Pavlinic,[2] Gordan Lauc,[1] and Jerka Dumic[1]

The congenital disorder of glycosylation (CDG)-Ic (ALG6-CDG, CDG-Ic) is caused by mutations in the h*ALG6* gene that encodes the N-glycosylation pathway enzyme, α-1,3-glucosyltransferase (NP_037471.2). The aim of our study was to estimate the frequencies of ALG6-CDG related p.Y131H and p.F304S polymorphisms in the Croatian population. Genomic DNA was isolated from blood samples collected from 600 healthy individuals. Functional single-nucleotide polymorphisms rs35383149 and rs17856039 causing p.Y131H and p.F304S, respectively, were genotyped by the TaqMan method and direct sequencing. The frequency of p.F304S polymorphism in the studied cohort was shown to be similar to the frequencies found in other tested populations (27%), whereas the frequency of p.Y131H was found to be three times higher (6.7%). Five novel base substitutions in the h*ALG6* gene were also found: three in exon 5 (c.383T>C, c.390G>A, and c.429G>C) and two in a downstream intervening sequence (IVS5+17C/T and IVS5+34G/A).

Introduction

Congenital disorders of glycosylation (CDGs) are a growing group of autosomal recessive diseases caused by deficient assembly or processing of glycoconjugates (Schachter and Freeze, 2009; Jaeken, 2011). According to a novel classification, CDGs are divided in 4 categories: defects of (i) protein N-glycosylation, (ii) protein O-glycosylation, (iii) lipid glycosylation and glycosylphosphatidylinositol anchor glycosylation, and (iv) defects in multiple glycosylation pathways and in other pathways (Jaeken, 2011). Although CDG was diagnosed in only ~1000 patients so far, based on the frequency analysis for certain common mutations it was assumed that CDGs are frequently misdiagnosed, probably because of the variety of CDG symptoms and the resemblance to some other diseases, especially neurological ones (Jaeken, 2003). The second most frequent type of CDGs, ALG6-CDG, that is, CDG-Ic (OMIM #603147), clinically characterized by psychomotor retardation, dysmorphic features, muscular hypotonia, seizures, and epilepsy (Grunewald *et al.*, 2000; Marquardt and Denecke, 2003; Damen *et al.*, 2004; Sun *et al.*, 2005), is caused by mutations in the h*ALG6* gene (NG_008925) that encodes dolichyl-P-glucose:mannose$_9$-N-acetyl glucosamine$_2$-pyrophosphate-dolichyl (Dol-P-Glc:Man$_9$GlcNAc$_2$-P-P-Dol) α-1,3 glucosyltransferase, an enzyme that participates in the formation of the lipid-linked oligosaccharide precursor of N-linked glycosylation (Burda *et al.*, 1998; Imbach *et al.*, 1999; Newell *et al.*, 2003). The most common mutation in the h*ALG6* gene causing ALG6-CDG is c.998C>T resulting in p.A333V substitution (Grunewald *et al.*, 2000; Newell *et al.*, 2003). Two point mutations c.391T>C in exon 5 and c.911T>C in exon 10 resulting in p.Y131H and p.F304S, respectively, are assumed to be single-nucleotide polymorphisms (SNPs; rs35383149 and rs17856039) (Vuillaumier-Barrot *et al.*, 2001; Westphal *et al.*, 2003). Although p.F304S polymorphism is not a causal mutation of ALG6-CDG, it was shown that it may exacerbate the clinical outcome, especially in patients severely affected by CDG (Westphal *et al.*, 2002). For the p.Y131H substitution, it is still unclear whether the homozygous form c.391C/C is sufficient to cause the disease or some additional genetic alteration has to be present. However, it was shown that this mutation reduces the ability of the gene product to rescue defective glycosylation of an *alg6*-deficient strain of *Saccharomyces cerevisiae* during rapid growth (Westphal *et al.*, 2003).

The allelic frequency for the variant c.911C related to p.F304S was shown to be rather high (0.28) in healthy populations (Vuillaumier-Barrot *et al.*, 2001; Westphal *et al.*, 2002), whereas the frequency of heterozygotes for p.Y131H suggests that homozygotes would occur at the rate of 1:2200, but so far

[1]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia.
[2]DNA Laboratory, Faculty of Medicine, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia.

only one individual, a patient suffering from CDG-like symptoms, was proved to be homozygous for this mutation (Westphal *et al.*, 2003).

This screening study, in which allelic and genotype frequencies of p.Y131H and p.F304S polymorphisms in the h*ALG6* gene in the Croatian population were determined, revealed 5 novel base substitutions in this gene.

Methods

Subjects, sample collection, and DNA isolation

This study included 600 healthy nonconsanguineous individuals (469 men; age median: 38 years; interquartile range: 29–50 and 131 women; age median: 31 years; interquartile range: 24–44). The subjects were healthy blood donors from the entire geographic region of Croatia who all signed informed consent based on the Helsinki declaration. This study was approved by the Ethics Committees of the General Hospitals (altogether ten regional centers were enrolled in the study) where the samples were collected (from the year 2005 to 2007) and by the Ethics Committee of the Faculty of Pharmacy and Biochemistry, University of Zagreb. Genomic DNA was isolated from whole blood samples collected on the Whatman Blood Stain Cards®, according to the modified Chelex® extraction procedure (Goreta *et al.*, 2011). As a positive control, genomic DNA obtained from heterozygotes for c.391T>C and c.911T>C were used.

Allelic discrimination by TaqMan method

All isolated genomic DNA were screened for c.391T>C (p.Y131H) and c.911T>C (p.F304S) by TaqMan® SNP Genotyping Assay (Applied Biosystems). The primers and probes purchased from Applied Biosystems (SNP indicated in *bold lowercase letter*) were as follows: for p.Y131H detection: primers AATTGCTGATCTGCTGATTTACATACCT and GCTGCTTGAAAACCTACCTTTTTCTT, probes VIC-CAAC AACAGTACAAAAC-NFQ-MGB and FAM-ACAACAGT**g** CAAAAC-NFQ-MGB; for p.F304S detection: primers CTTA TTTAAGCTAGATTTATACCTCTACAAAGAAGACAAAT and CCTTTGG AAGAGGGCTGAAGTATTAA, probes VIC-CTCAAAAACGTAAAACAAA-NFQ-MGB and FAM-TCA AAAACGTA**g**AACAAA-NFQ-MGB. Each polymerase chain reaction (PCR) mixture (12.5 μL) contained 5.94 μL of DNA solution, 6.25 μL of Universal PCR master mix, and 0.31 μL of primer/probe mix (final concentration: 900 nM primers and 200 nM probes). The cycles were as follows: stage 1: 50°C, 2 min (1 cycle); stage 2: 95°C, 10 min (1 cycle); and stage 3: 92°C, 15 s and 60°C, 1 min (45 cycles).

DNA sequencing

Sequencing of exon 5 and exon 10 with adjacent parts of intervening sequences (IVS) of the h*ALG6* gene was performed after PCR amplification and purification. Briefly, PCR amplifications were performed by using specific primers and PCR conditions according to the previously published procedure (Imbach *et al.*, 2000), with the modified primer concentration (0.5 μM each) and increased amount of the starting material (5 μL of genomic DNA isolate). After PCR, the purity of PCR products was confirmed on 1.8% agarose electrophoresis, and additionally, the quantity of amplified DNA fragments in PCR mixtures was estimated by using Mass Ruler™ DNA Ladder Low Range (Fermentas). The PCR products were purified with Q1Aquick PCR Purification kit (Qiagen) and sequenced with DNA Sequencing Big Dye Terminator v3.0 kit (Applied Biosystems) according to the manufacturer's instructions on ABI Prism 310 Genetic Analyzer (Applied Biosystems). For effective removal of excess DyeDeoxy™ terminators from completed DNA sequencing reaction before the analysis on the ABIPrism 310 Genetic Analyzer, Centri-Sep™ Spin Columns (Princeton Separation) were used. The data were analyzed by using Sequencing Analysis software®, Version 3.4 (Applied Biosystems).

Statistical analysis

Genotype frequencies were calculated by direct counting. Differences between observed and expected genotype frequencies for calculating Hardy-Weinberg equilibrium were tested by using the χ^2-test. A p-value < 0.05 was considered statistically significant. Data were analyzed by using the statistical software SigmaStat for Windows Version 3.00 (SPSS Inc.).

Results

Screening for the presence of c.911T>C polymorphism (p.F304S) was performed on 600 samples by using the TaqMan SNP Genotyping Assay with commercially available fluorescent probes. The frequency of the heterozygotes was found to be relatively high (0.273). The incidence of c.911C/C homozygotes was 0.073, whereas the allelic frequency of the variant c.911C was 0.209. A statistically significant deviation from the distribution according to Hardy-Weinberg proportions was observed ($p < 0.001$; Table 1). The results were confirmed by direct sequencing of 50 randomly chosen samples.

Among 600 persons genotyped for c.391T>C (p.Y131H) by the TaqMan SNP Genotyping Assay, 560 were shown to be homozygotes wild-types (c.391T/T), 32 were heterozygotes,

Table 1. Genotype and Allelic Frequencies of c.911T>C (p.F304S) Polymorphism in h*ALG6* Gene in Croatian Population

	Observed		*Expected*				
Genotype	n = *600*	*Frequency*	n = *600*	*Frequency*	*Allele*	n = *1200*	*Frequency*
TT	393	0.655	375.4	0.625	T	950	0.791
TC	163	0.273	198.4	0.331	C	250	0.209
CC	44	0.073	26.2	0.044			

$\chi^2 = 11.7$; degree of freedom = 1; $p < 0.001$.

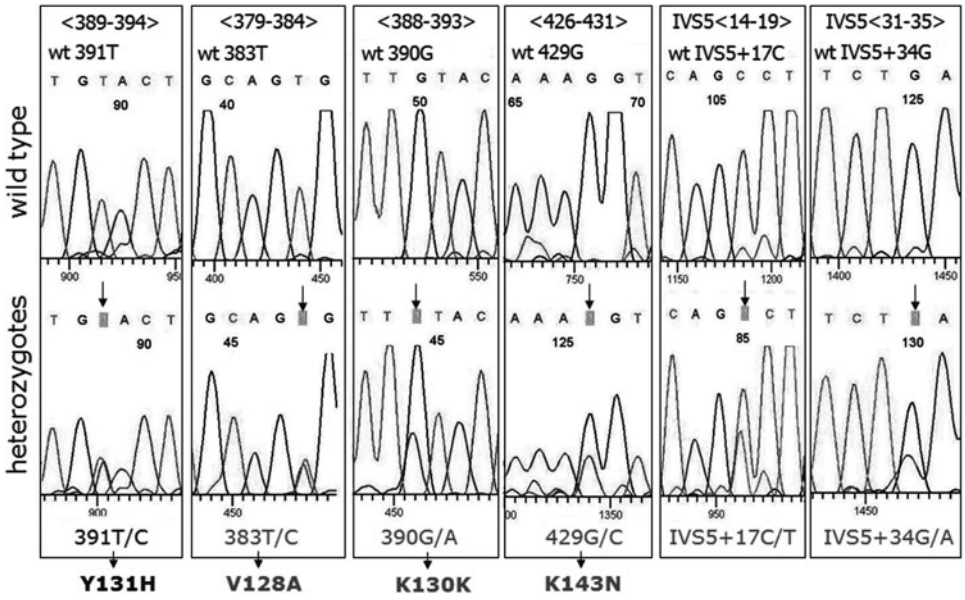

FIG. 1. Five novel single nucleotide polymorphisms detected in the h*ALG6* gene. Sequencing analysis of h*ALG6* exon 5 and parts of intervening sequences (IVS) showing three novel base substitution in exon 5 (c.383T>C, c.390G>A and c.429G>C) and two in IVS of exon 5 (IVS5+17C/T and IVS5+34 G/A), present in the heterozygous form.

whereas 8 of them were apparent c.391C/C homozygotes. Since until now the c.391C/C form was proved to be present in only one individual worldwide, we sequenced all samples with c.391C/C and c.391T/C genotype as well as 50 randomly chosen wild-type samples. Although sequencing analysis confirmed the results obtained for heterozygotes and wild types, it revealed that three of the eight assumed c.391C/C homozygotes were wild types and five were heterozygotes. Interestingly, all of them were carriers of at least one of 5 newly detected base substitutions, all present in heterozygous form; three of them were detected in exon 5 of the h*ALG6* gene and two in the downstream IVS (IVS5+17C>T and IVS5+34G>A; Fig. 1). Variant c.390G>A, settled just 1 base upstream of the already known c.391T>C mutation, results in a degenerated Lys codon at the position 130. Variant c.383T>C, also present very near, causes substitution of valine for alanine at the position 128 (p.V128A), whereas c.429G>C results in a lysine to asparagine substitution at the position 143 (p.K143N). The genotype and allelic frequencies of p.Y131H polymorphism are shown in Table 2. If the calculated frequency of heterozygotes (0.065) is applicable for the entire Croatian population, the estimated incidence of homozygotes would be 1 in 1000.

Discussion

Glycosylation, an enzymatic addition of sugar residues to lipids and proteins, is one of the most abundant and important post-translational modifications, a highly regulated and coordinated process that allows fine tuning of numerous physiological processes. The importance of the correct glycosylation for proper functioning of the organism reflects also in low incidence of CDGs, diseases caused by defects of genes encoding proteins involved in glycan processing. However, in the last 7 years, 33 new CDG types have been recognized; thus, the overall number of these clinical entities increased fourfold (from 12 to 45) (Jaeken, 2011). Additionally, the results of the modest numbers of the population study performed so far, regarding the frequency determination of the several mutations known to cause CDGs when in homozygous form, suggest the existence of a much bigger number of cases than documented. The similarities of CDGs' symptoms to some other diseases might explain the aforementioned discrepancy. To contribute to the knowledge on the frequencies of the N-glycosylation-compromising alleles in general population, we screened 600 healthy Croatian residents for the presence of two ALG6-CDG-related polymorphisms (p.Y131H and p.F304S). The method of our choice was the TaqMan allelic discrimination assay, a genotyping method that proved to be reliable only for the cases where DNA sequences to which reagents bind are precisely and completely known. The surprising finding of the c.391C/C homozygous form by this method, previously only reported once (Westphal *et al.*, 2003), and consequential evaluation of gained results by sequencing, revealed the existence of five novel base substitutions in the h*ALG6* gene that most likely caused incorrect binding of the designed TaqMan primers/probes, thus yielding falsely positive results. Two of them (c.383T>C and c.429G>C) result in substitution of amino acids (p.V128A and p.K143N) located within and nearby the predicted second transmembrane domain of hALG6, respectively (Haeuptle and Hennet, 2009). Whether they have any functional consequences or just contribute to genetic diversity remains to be answered, but it is worth mentioning that majority of so far known ALG6-CDG-related mutations affect amino acids also located in the transmembrane regions of hALG6 (Haeuptle and Hennet, 2009). One, c.911T>C (p.F304S), is reported to exacerbate the clinical outcome in patients severely affected by CDG (Westphal *et al.*, 2002), and speculated to be implicated in other multifactorial disorders that involve N-glycosylation (Freeze, 2001; Freeze and Westphal, 2001). The relatively high frequency ($\sim$0.21) of the c.911C

Table 2. Genotype and Allelic Frequencies of c.391T>C (p.Y131H) Polymorphism in the h*ALG6* Gene in Croatian Population

Genotype	n=*600*	*Frequency* (q)	*Allele*	n=*1200*	*Frequency*
TT	561	0.935	T	1161	0.967
TC	39	0.065	C	39	0.033
CC	0	0.000			

variant allele in the Croatian population observed in this study is similar, but somewhat lower than in other studied populations (~0.28) (Vuillaumier-Barrot *et al.*, 2001; Westphal *et al.*, 2002). Interestingly, a rather unusual deviation from the Hardy-Weinberg equilibrium with a statistically significant increase of homozygotes (both CC and TT) and decrease in heterozygotes was observed, thus indicating that parallel existence of both allelic variants is somehow disadvantageous. On the other hand, the frequency of heterozygotes (0.065) for c.391T>C (p.Y131H), for which it is still unclear whether it is a causal mutation of ALG6-CDG or just a polymorphism, was found to be thrice higher in the Croatian than in the US population (0.021), the only one screened for p.Y131H until now (Westphal *et al.*, 2003). Although the obtained result suggests the incidence of homozygotes of 1 in 1000 in the Croatian population, a much larger number of individuals should be genotyped to confirm this hypothesis. Additionally, it could be speculated that the number of homozygotes would be even higher among the individuals with developmental delay, seizures, and metabolic abnormalities, symptoms similar to those of the patients with ALG6-CDG. Interestingly, certain mutations in genes related to CDGs occur at a high frequency in some populations although some of them are lethal when homozygous, such as R141H mutation (Bjursell *et al.*, 1998; Schollen *et al.*, 2000). It seems that maintenance of such apparently harmful mutations could represent a selective advantage or positive heterosis throughout evolution. Freeze and Westphal (2001) suggested that prevalent functional mutations resulting in impaired glycosylation could provide some resistance to microbial and viral infection that relies on host cell glycosylation, without affecting the host, but, consequently, risking the incidence of CDG. On the other hand, synergistic heterozygosity and multifactorial inheritance, usually combined with environmental factors, might determine susceptibility and/or resistance to the development of many complex genetic diseases, ascribed to multiple defects in different, mostly not yet recognized genes. The significance of the appearance and frequency of p.Y131H, p.F304S, and 5 newly detected base variants in h*ALG6* gene remains to be elucidated.

Acknowledgments

This work was supported by the Ministry of Science, Education, and Sports of the Republic of Croatia, grants #006-0061194-1218 and #309-0061194-2023. The authors would like to thank Prof. H. H. Freeze for providing genomic DNA from p.F304S and p.Y131H heterozygotes.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Jerka Dumic, Ph.D.
Department of Biochemistry and Molecular Biology
Faculty of Pharmacy and Biochemistry
University of Zagreb
A. Kovacica 1
Zagreb 10 000
Croatia

E-mail: jdumic@pharma.hr