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ORIGINAL ARTICLE

Classic galactosaemia in the Greek Cypriot population: An epidemiological and molecular study

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Abstract

Classic galactosaemia is an inherited metabolic disorder of galactose metabolism caused by deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT) resulting from mutations in the *GALT* gene. The objectives of the present study were the determination of the carrier frequency of classic galactosaemia in the Greek Cypriot population and the molecular characterization of the disease alleles. We performed an epidemiological study involving 528 Greek Cypriots originating from all parts of Cyprus. Carriers were identified by measuring GALT activity in red blood cells and were subsequently subjected to mutation analysis. A total of five mutations were identified in patients and carriers of classic galactosaemia: a large deletion of 8.5 kb previously reported by us (55% of alleles), the known mutations p.Lys285Asn (30%), p.Pro185Ser (5%), and c.820+13A>G (5%), and a novel mutation c.378-12G>A (5%). Interestingly, the most common mutation in European populations, p.Gln188Arg, was not identified in this Cypriot cohort. The carrier frequency for classic galactosaemia among Greek Cypriots was estimated to be 1:88, predicting a homozygote incidence of 1:31,000 births. The Duarte 1 and Duarte 2 variants were found to be present at a frequency of 5.5% and 2.5%, respectively.

KEYWORDS

carrier screening, Duarte, galactosaemia, *GALT*, Greek Cypriot, mutation analysis

1 | INTRODUCTION

Classic galactosaemia (OMIM #230400) is the most common inherited disorder of carbohydrate metabolism caused by deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT, EC 2.7.712), resulting from mutations in the *GALT* gene (Holton, Walter, & Tyfield, 2001). The incidence of classic galactosaemia varies in different populations from about one in 16,000 in Ireland (Murphy et al., 1999) to about one in 100,000 in Sweden (Ohlsson, Guthenberg, & von Dobeln, 2012) with an average worldwide incidence of 1:62,000 births (Levy & Hammersen, 1978; Tyfield et al., 1999). Newborns with classic galactosaemia usually develop symptoms within the first week of life, following the initiation of milk feeding. These symptoms include poor feeding, vomiting, diarrhoea, jaundice, liver failure, cataracts, encephalopathy, and septicaemia. Although many of these symptoms resolve if the condition is diagnosed promptly and a galactose-free diet is adopted, long-term complications such as cataracts, speech defects, poor growth, poor intellectual function, neurologic

deficits, abnormalities of motor function, and premature ovarian failure in females may still occur (Fridovich-Keil et al., 2011).

Although the exact pathogenic mechanisms involved in galactosaemia are not fully understood, important advances have been made in this direction (Coelho, Rubio-Gozalbo, Vicente, & Rivera, 2017). De-Souza et al. have shown that the unfolded protein response (UPR) is triggered by endoplasmic reticulum (ER) stress in yeast models of classic galactosaemia and that this UPR activation has a protective role against the cytotoxic effects of galactose (De-Souza et al., 2014). Another mechanism induced by ER stress is the diminished phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signalling, which has been shown to play a role in disease phenotypes such as subfertility and cerebellar ataxia (Balakrishnan et al., 2017). Secondary effects on *N*-glycosylation might also contribute to the pathophysiology of classic galactosaemia (Maratha et al., 2017). The findings of these recent studies open up new possibilities for the treatment of classic galactosaemia.

The *GALT* gene is located on chromosome 9p13, is arranged into 11 exons and spans about 4.3 kb of genomic DNA (Flach, Reichardt, & Elsas, 1990). To date, 336 variants within the *GALT* gene have been identified, according to the entries in the database at the ARUP Institute of Experimental Pathology (http://www.arup.utah.edu/database/GALT/GALT_display.php), and most of these are missense mutations. The p.Gln188Arg (c.563A>G) mutation is the most common mutation in European populations accounting for about 64% of the alleles (Tyfield et al., 1999). The p.Lys285Asn (c.855G>T) mutation is another frequent mutation in European populations with an overall frequency of about 8%, while in some countries of East and Central Europe it is the second most common mutation with a frequency of 25%–40% (Lukac-Bajalo, Kuzelicki, Zitnik, Mencej, & Battelino, 2007; Tyfield et al., 1999). In addition to disease-causing mutations, several variants of the *GALT* gene have been described with various levels of enzyme activity. The most common of these are the two Duarte variants: the Duarte 1, which is associated with increased GALT activity and the Duarte 2, which is associated with reduced GALT activity. Both Duarte alleles share the p.Asn314Asp (p.N314D, c.940A>G) mutation in exon 10 (Reichardt & Woo, 1991). In addition, the Duarte 1 allele carries in *cis* a silent mutation (p.Leu218Leu) while the Duarte 2 allele carries a 4 base deletion in the 5′UTR (c.1-199-116delGTCA) and three intronic variants in introns 4 and 5 (c.378-27G>C, c.507+62G>A, and c.508-24G>A) (Elsas, Lai, Saunders, & Langley, 2001).

In this report, we present epidemiological and molecular data regarding classic galactosaemia as well as the Duarte variants in the Greek Cypriot population.

2 | MATERIALS AND METHODS

2.1 | Subjects

For the epidemiological study, we recruited 528 healthy adult volunteers originating from all parts of Cyprus. The numbers from each district were calculated according to the respective population size. Blood samples were collected after obtaining the approval of the National Bioethics Committee for the project and with signed consent. A further 100 DNA samples from Greek Cypriots from our DNA bank, originating from all parts of Cyprus, were analysed for the novel point mutation and the Duarte variants.

2.2 | GALT enzyme activity measurement

Measurement of GALT activity in washed red blood cells (RBC) was performed using the spectrophotometric method of Kalckar et al. (Kalckar, Anderson, & Isselbacher, 1956), which is based on UDP-glucose consumption.

2.3 | DNA analysis

DNA was isolated from whole blood using the Gentra Puregene Blood Kit (Qiagen), according to the manufacturer's instructions. For bidirectional automated sequencing, *GALT* exons were amplified in eight fragments. Primers used for PCR amplification carried an M13-derived tag used for the subsequent cycle sequencing reaction. Following PCR amplification, products were treated with ExoSAP enzyme and subjected to a cycle-sequencing reaction in 96 well plates. Primers and conditions used are shown in Table 1. Sequencing products were subsequently subjected to ethanol (85%) clean-up using the Beckman Coulter Biomek NX liquid-handling robot. Final elution was in water. All samples were run on an ABI 3730 DNA analyser (Applied Biosystems). Sequence analysis was performed using SoftGenetics “Mutation Surveyor” DNA variant software.

The primers and conditions used for restriction enzyme analysis are shown in Table 1.

3 | RESULTS

The healthy volunteers were divided into three groups according to their GALT activity value: those with an activity higher than 18 μmol/hr/gHb, who were designated as normal, those with an activity less than 15 μmol/hr/gHb who were designated as carriers, and those with an intermediate activity, 15–18 μmol/hr/gHb. Fourteen out of the 528 subjects tested were found to have low GALT activity (<15 μmol/hr/gHb) and were subsequently screened for the two mutations previously identified in the Cypriot galactosaemia patients, the

TABLE 1 PCR primers and conditions used for analysis of the *GALT* gene

GALT mutation	Primer name	Primer sequence (5′-3′)	Tm (°C)	Product size (bp)	Restriction enzyme	Restriction fragments (bp)
p.Lys285Asn	9F	GATGGAGGTTGCTCCCAGTA	55	715	*Tsp509I*[a] 2 hr at 65°C	Mutant: 98,142,475 Normal: 142, 573
	9R	AGCACAAGGGCAACAGAAGT				
New deletion	DEL- 10F	CCACCTAGATGGTGGCTGGAGCTT	68	Deleted: 1.6 kb Normal: 651 bp		
	DEL-9R	ACTTACCCGGCAGTCACTCCAGG				
	DEL-internal	GCGCACGCACATGCAAAGCA				
Sequencing of all 11 exons	M13 forward	CACGACGTTGTAAAACGAC				
	M13 reverse	GGATAACAATTTCACACAGG				
	GALT exon 1: F	CCAGTGTAGTGGCTCTAG	55	487		
	GALT exon 1: R	CTTATGAAACCAGGAAGCAC				
	GALT exon 2: F	GGCCTGCTGGTGGGTGAGAC	55	383		
	GALT exon 2: R	GCCCACCCTAGGGGACCAA				
	GALT exon 3+4: F	CCTGTCCAGTCTTTG	55	359		
	GALT exon 3+4: R	GGGCCGAACCCCAATG				
c.378-12G>A	GALT exon 5+6: F	TGGAGACTCAGCATTGGG	55	480	*Tsp45I*[a] 2 hr at 65°C	Mutant: 176,304 Normal: 75,101,304
	GALT exon 5+6: R	ACAGTGCTGGCTCAGACTC				
	GALT exon 7: F	GTGGACATGGGAACAGGATT	55	489		
	GALT exon 7: R	CGGAGTGTGGTCAGCAAATA				
	GALT exon 8+9: F	TTTGCTGACCACACTCCG	55	461		
	GALT exon 8+9: R	GTTGCAGTTCACTAGGCTG				
	GALT exon 10: F	GGTTGGGTTTGGGAGTAG	55	369		
	GALT exon 10: R	TTTGGCAGTCCCTTCCTG				
	GALT exon 11: F	CATGCCACCATTCTTGGC	55	214		
	GALT exon 11: R	GGCCTTTCTGCTTAATTC				
p.Asn314Asp (N314D)	N314D F	AGATGCTGGGACTGAGGGTGGAGCA ACTTACCCGGCAGTCACTCCAGG GCGCACGCACATGCAAAGCA	60	412	*Ava II*[a] 2 hr at 37°C	Mutant: 74,102,236 Normal: 74,338
	N314D R	GCCTGCACATACTGCATGTGA				
Duarte 1 or LA variant (c.652C>T, p.Leu218Leu)	D1F	TGGGACAGAGGAAATATGCCA	58	270	*Mse I*[a] 2 hr at 37°C	Mutant: 26,74,170 Normal: 26,244

(Continues)

TABLE 1 (Continued)

GALT mutation	Primer name	Primer sequence (5′-3′)	Tm (°C)	Product size (bp)	Restriction enzyme	Restriction fragments (bp)
	D1R	CACCTCTCTCATGGGATAAG AAAGTTAAG				
Duarte 2 4 bp deletion in the 5′ UTR		MLPA kit P156,[b] MRC Holland				

[a]All restriction enzymes from New England BioLabs.

[b]This is an old kit that included primers for the promoter region; the new kit does not include such primers.

TABLE 2 *GALT* mutations/variants identified in obligate carriers and subjects with low GALT activity detected from screening 528 healthy volunteers

Genotype	Random carriers (No.)	Obligate carriers (No.)	GALT activity (all carriers) NR: 15–32 μmol/hr/gHb	Comments
p.Lys285Asn/wt	1	5	Mean: 10.8	Known mutation
GALT gene deletion/wt	1	8	Mean: 11.3	Papachristoforou et al., 2013
p.Pro185Ser/wt	1	_	12	Known mutation
c.820+13A>G/wt	1	_	11.5	Known mutation
c.378-12G>A/wt	1	_	15	New mutation
Duarte 2/wt	10	_	Mean: 14.3	Common variant
GALT gene deletion/Duarte 2	1	_	11	Duarte galactosaemic

Note. DNA mutation numbering is based on GenBank accession No. M96264; NR: normal range; wt: wild type; obligate carriers: parents of eight Greek Cypriot galactosaemia patients (two patients were siblings: one parent was not available).

TABLE 3 Allele frequencies of *GALT* mutations identified in Greek Cypriot carriers and patients

Mutation	Carriers		Patients			
	Number of alleles	Allele frequency	Number of alleles	Allele frequency	Total number of alleles	Allele frequency
GALT gene deletion	2	33.3%	9	64.3%	11	55%
p.Lys285Asn	1	16.7%	5	35.7%	6	30%
p.Pro185Ser	1	16.7%	–	–	1	5%
c.820+13A>G	1	16.7%	–	–	1	5%
c.378-12G>A	1	16.7%	–	–	1	5%
Total	6	100%	14	100%	20	100%

Note. DNA mutation numbering is based on GenBank accession number M96264.

8.5-kb deletion and the p.Lys285Asn mutation (Papachristoforou et al., 2013). One subject was found to carry the 8.5-kb deletion and another subject was identified as heterozygous for the p.Lys285Asn mutation. The 12 subjects that were negative for these two mutations were subjected to bidirectional sequencing of all 11 exons. The known p.Pro185Ser and c.820+13A>G mutations were identified each in one subject, whereas a novel point mutation, c.378-12G>A in intron 4 of the *GALT* gene, was identified in another subject. One hundred random DNA samples originating from all areas of Cyprus were screened for this novel transition by restriction enzyme digestion and all were found to be negative, suggesting that it could represent a novel variant associated with decreased GALT enzymatic activity. The results of the molecular analysis are summarized in Table 2. A total of six galactosaemia carriers were detected (low GALT activity and *GALT* mutation) among 528 random samples, giving a carrier frequency of 1:88, which predicts a homozygote incidence of classic galactosaemia in the Greek Cypriot population of one in 31,000 births. The allele frequencies are shown in Table 3.

Seven out of the 14 subjects with low GALT activity (<15 μmol/hr/gHb) were found to carry the Duarte 2 allele. One additional subject was found to carry both the novel 8.5-kb

deletion and the Duarte 2 allele; thus, this subject is considered to have Duarte galactosaemia (Table 2). One subject with low activity was negative for all mutations and variants tested. Twenty-seven subjects were found to have intermediate enzyme activity (15–18 μmol/hr/gHb). We were able to obtain DNA samples from 16 of these subjects and these were screened for the Duarte variants since these variants are associated with higher enzyme activity than classic *GALT* mutations and, therefore, are more likely to be found in individuals with an intermediate level of GALT activity. However, the possibility of some of these individuals bearing a mild *GALT* mutation has not been excluded. Three of these were found to carry the Duarte 2 allele. To estimate the frequency of the Duarte variants, 100 random DNA samples from healthy volunteers originating from all areas of Cyprus were screened. The frequency of Duarte 1 was found to be 5.5% and the frequency of Duarte 2 was found to be 2.5%, giving a combined frequency of 8%.

4 | DISCUSSION

In the present study, we performed an epidemiological investigation involving 528 Greek Cypriot subjects originating from all areas of Cyprus. A total of six subjects were found to have low GALT activity and to carry a pathogenic mutation in the *GALT* gene, giving a frequency of galactosaemia carriers in the Greek Cypriot population of 1:88, which predicts a homozygote incidence of one in 31,000 births. The incidence in other European countries lies within the range of 1:16,000–100,000 (Tyfield et al., 1999). The highest incidence of classic galactosaemia reported is that for Ireland, about 1:16,000 for the whole population; about 1:34,000 in the non-Traveller community and about one in 430 in the Travellers (Coss et al., 2013; Murphy et al., 1999). Another high incidence is that of Turkey, about 1:24,000 (Ozgul et al., 2013). The lowest incidence is that of Sweden, about 1:100,000 (Ohlsson et al., 2012). The incidence in Greece was originally reported as one in 22,000 (Schulpis et al., 1997), but more recent publications report an incidence of about one in 51,000 (Schulpis et al., 2017).

In a previous study (Papachristoforou et al., 2013), we found two mutations to be responsible for all disease alleles in Greek Cypriot galactosaemia patients (100% detection rate). A novel large deletion of 8.5 kb was found to account for the majority of disease alleles (64%) while the p.Lys285Asn mutation accounted for the remaining alleles (36%). In addition to the two mutations found in patients, three more mutations were detected in the present study, one of them novel. Combining the data from both studies, the allele frequencies are as follows: 8.5-kb deletion 55%, p.Lys285Asn 30%, p.Pro185Ser 5%, c.378-12G>A 5%, and the c.378-12G>A 5%. Interestingly, the p.Gln188Arg, which is the most frequent *GALT* mutation among Caucasian populations (Tyfield et al., 1999), was not found in the Greek Cypriot population. There is a general trend for the relative frequency of this mutation to decrease in Europe in a southeastern direction, starting with the highest frequencies in Denmark (83%) and Ireland (80%) and decreasing to 18% in Italy (Bosch, 2006; Maceratesi et al., 1996; Murphy et al., 1999; Tyfield et al., 1999). Turkey and Greece, however, are exceptions to this trend, with frequencies of about 55% and 44%, respectively (Ozgul et al., 2013; Schulpis et al., 2017).

The p.Lys285Asn *GALT* mutation was found to be the second most common mutation in the Greek Cypriot population, occurring with an allele frequency of 30%, similar to that in populations of East and Central Europe where the frequency is 25%–40% (Lukac-Bajalo et al., 2007; Tyfield et al., 1999). The frequency of the p.Lys285Asn mutation is lower in Western European countries, having a frequency of zero in Ireland and Portugal and 3% in the United Kingdom (Coelho et al., 2013; Murphy et al., 1999; Tyfield et al., 1999), showing a general trend to increase in a southeastern direction, but again Greece and Turkey are exceptions having a relatively low frequency of 3.6% and 9%, respectively (Ozgul et al., 2013; Schulpis et al., 2017). It has been suggested that this mutation has a Slavic origin (Kozak et al., 2000; Lukac-Bajalo et al., 2007). Furthermore, the p.Lys285Asn is more geographically restricted compared to p.Gln188Arg because it has appeared after the main population expansions (Flanagan et al., 2010). The majority of Greek Cypriot carriers bearing the p.Lys285Asn mutation originate from the eastern part of the island, whereas the majority of carriers of the 8.5-kb deletion originate from the western part of the island, pointing to possible founder effects.

We have identified two more known mutations in Greek Cypriot galactosaemia carriers: the p.Pro185Ser mutation and the transition c.820+13A>G in intron 8. Both mutations are registered in the *GALT* database and they were identified in patients from Portugal (Coelho et al., 2013). The p.Pro185Ser substitution affects a conserved amino acid and is associated with 14% of the wild-type activity (Quimby, Wells, Wilkinson, & Fridovich-Keil, 1996). The estimated allele frequency of this mutation in Portugal is 2.6% (Coelho et al., 2013). The c.820+13A>G or IVS8+13 a>g has been previously detected in patients from Portugal and Spain (Gort et al., 2006). The allele frequency of this mutation in Portugal has been reported to be 8% and it is the second most common mutation among Portuguese patients (Coelho et al., 2013). Furthermore, in silico predictions using bioinformatics tools performed by the same authors suggest that the c.820+13A>G is most probably a severe splicing mutation within intron 8, revealing a cryptic donor site and introducing a frameshift, leading to a premature stop codon. Moreover, the same authors confirmed the pathogenicity of the mutation by in vitro and in vivo studies and suggested the use of antisense oligonucleotides as a

novel therapeutic approach in classic galactosaemia (Coelho et al., 2015).

In addition to the large deletion, we have identified another new mutation in the Cypriot population, the c.378-12G>A transition within intron 4 of the *GALT* gene. This mutation has been identified in one DNA sample derived from a volunteer with reduced GALT activity. The transition has not been previously published and is not listed in the *GALT* official database (ARUP Institute of Experimental Pathology). We screened 100 samples from our DNA bank for this transition and they were all negative, suggesting that the c.378-12G>A is not common in our population and therefore could be considered as another novel *GALT* mutation. In silico analysis using the Human Splicing Finder Program (http://www.umd.be/HSF3/HSF.shtml) does not indicate any effects on splicing. However, the possibility exists that the above intronic mutation affects potential enhancer or silencer motifs, which impact on gene expression. Until further functional analysis studies are performed, this transition cannot be considered pathogenic.

Apart from disease causing mutations, a number of milder variants have been described in the *GALT* gene, the most important of which are the Duarte alleles. The Duarte alleles occur with a high frequency in European populations, about 11% (Calderon, Phansalkar, Crockett, Miller, & Mao, 2007b) but with a much lower frequency in Asian populations, about 2% (Suzuki, West, & Beutler, 2001). The Duarte alleles can cause problems in the diagnosis of classic galactosaemia (Barbouth, Slepak, Klapper, Lai, & Elsas, 2006; Calderon et al., 2007a) and for this reason we considered it important to determine their frequency in our population. The frequency of Duarte 1 and Duarte 2 in the Greek Cypriot population was determined at 5.5% and 2.5%, respectively, giving a combined frequency of 8%. The combined frequency is somewhat lower than the average frequency in European populations of 11% (Calderon et al., 2007b) and the same as the "pan-ethnic" frequency of 8% (Carney et al., 2009). The Duarte variants are considered as ancient events, since the N314D has been found in all populations in association with the same linked polymorphisms for Duarte 1 and Duarte 2, and with some variation in the ratio of the two among populations (Flanagan et al., 2010; Tighe et al., 2004). In most populations, the frequency of the Duarte 2 allele is higher than the frequency of the Duarte 1 allele (Milankovics, Schuler, Nemeth, Somogyi, & Fekete, 2009; Tighe et al., 2004). In European populations, the average frequency of Duarte 2 is about 7% and the frequency of Duarte 1 about 4% (Carney et al., 2009), whereas the pan-ethnic frequency is about 5.1% and 2.7%, respectively (Suzuki et al., 2001). In our study, we found that the frequency of the Duarte 1 allele (5.5%) is higher than the frequency of the Duarte 2 allele (2.5%). It is difficult to speculate on the reason for these different frequencies other than attribute them to random genetic drift and the unique genetic composition of the Cypriot population. It is reported that in the Turkish population the frequency of Duarte 1 (2.3%) is also higher than the frequency of Duarte 2 (1.4%) (Ozgul et al., 2013), while Suzuki et al. reported that this is also the case in Native Americans (11.8% and 5.2%), while in African-Americans the frequency of the two alleles is about the same, 1.3% for Duarte 2 and 1.4% for Duarte 1 (Suzuki et al., 2001).

In conclusion, this study provides valuable data on the frequency of classic galactosaemia carriers in the Greek Cypriot population as well as the nature and relative frequency of *GALT* mutations and the Duarte alleles. These data are expected to improve diagnostic services, including carrier detection as well as post- and prenatal diagnosis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

R.P. performed all the biochemical work and most of the mutation analysis and contributed to the writing of the manuscript; P.P.P. helped with the characterization of some of the mutations and contributed to the writing of the manuscript; H.S. and M.W. helped with the set-up of some of the molecular methods and with data analysis; and A.D. designed and supervised the study and contributed to the writing, reviewing, and editing of the manuscript.

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