

Transferrin gene polymorphisms alter the transferrin focusing pattern, making congenital disorder of glycosylation diagnosis difficult

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Background: Several transferrin gene polymorphisms are known to result in a shifted IEF pattern. The aim of this study was to characterize the transferrin gene polymorphisms observed in patients from one referral center. **Materials and methods:** Patients with solely increased pentasialo-Tf were selected. The whole exome sequencing was done from probands (patients) and from DNA available from their parents. **Results:** Two various polymorphisms in the transferrin gene: c.2012G>A, p.Gly671Glu and c.1027C>T, p.Arg343Trp, were found. **Conclusions:** Two transferrin gene polymorphisms: c.2012G>A, p.(Gly671Glu) and c.1027C>T, p.(Arg343Trp) solely correspond to an elevated pentasialo-Tf.

Keywords: transferrin; congenital disorders of glycosylation; polymorphism; isoelectric focusing; whole-exome sequencing

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Abbreviations: CDG, congenital disorders of glycosylation; CNV, copy number variations; IEF, isoelectric focusing; MS, mass spectrometry; SNV, single nucleotide variants; Tf, transferrin; WES, whole exome sequencing

BACKGROUND

Congenital disorders of glycosylation (CDG) comprise a group of rare inherited metabolic disorders caused by a deficient glycosylation of proteins and lipids (Jaeken *et al.*, 2017; Ferreira *et al.*, 2018; Peanne *et al.*, 2018; Francisco *et al.*, 2019). Isoelectric focusing (IEF) of serum transferrin (Tf) is the method of choice for diagnosis of N-glycosylation disorders associated with sialic acid deficiency (Jaeken *et al.*, 2017; Ferreira *et al.*, 2018; Peanne *et al.*, 2018; Francisco *et al.*, 2019). Based on the Tf isoform profile, patients can be classified into CDG type I, CDG type II or a mixed CDG type I/II. Type 1 pattern (CDG-I) is associated with an increased asialo-, disialo-Tf, and decreased tetrasialo-Tf; it indicates an assembly or transfer defect of the dolichol-linked glycan (Lefebvre *et al.*, 2011). Type 2 pattern (CDG-II) indicates a processing defect during Golgi glycosylation; an increased asialo-, monosialo-, disialo- and trisialo-Tf are observed there (Lefebvre *et al.*, 2011).

Several Tf gene polymorphisms are known to result in a shifted IEF pattern, caused by pI differences of the polypeptide chain (Lefebvre *et al.*, 2011; Jaeken *et al.*,

2017; Ferreira *et al.*, 2018; Peanne *et al.*, 2018; Francisco *et al.*, 2019). To improve diagnostics of CDG, mass spectrometry (MS) methods for glycoprotein analysis were developed, however, they are not routinely available (Guillard *et al.*, 2011; Van Scherpenzeel *et al.*, 2016).

The aim of this study was to characterize transferrin gene polymorphisms observed in patients screened for CDG in one referral center, to evaluate their overall prevalence and facilitate interpretation of laboratory findings.

MATERIALS AND METHODS

Serum transferrin (Tf) isoforms isoelectrofocusing

Serum Tf isoforms were analyzed by isoelectrofocusing (IEF) agarose gel electrophoresis according to the modification introduced in our laboratory (Adamowicz *et al.*, 1996; Adamowicz *et al.*, 2007) of the method described by Van Eijk and others (Van Eijk *et al.*, 1982). Since 1995, the CDG selective screening based on serum Tf IEF has been performed at our Institute (CMHI) for patients from the entire country (Bogdańska *et al.*, 2021; Lipiński *et al.*, 2021). During the years of 1995–2020, a total number of 23,183 serum Tf isoform analyses from pediatric patients have been performed. Out of all screened patients, patients were observed with solely increased pentasialo-Tf and patients with solely increased trisialo-Tf, corresponding to Tf gene polymorphisms. The prevalence of those polymorphisms was estimated. The clinical phenotype of patients with Tf gene polymorphisms was not significant.

Molecular analysis

Secondly, in the group of pediatric patients (probands, n=4) and their parents (n=4) in whom the same IEF pattern was observed and DNA was available, molecular analysis was performed. Whole exome sequencing (WES) was done to search for single nucleotide variants (SNV) and copy number variations (CNV). The patients' genomic DNA was extracted from the whole blood sample, and the sequencing library was prepared according to the Agilent Sure-Select Human All Exon V5 protocol. Enriched DNA libraries were sequenced by the Illumina HiSeq4000 instrument. The average depth of coverage for the Tf gene was 100% per 30x.

Table 1. Serum Tf isoforms percentage (%) in patients with Tf gene polymorphism.

Tf isoform	Tf isoform % in patients with Tf gene polymorphism	Reference ranges for Tf isoform %
Asialo-	0	0
Monosialo-	0	0
Disialo-	3.3–4.3	1.5–6.2
Trisialo-	10.1–13.6	7.4–17.1
Tetrasialo-	33.6–36.8	55.7–67.2
Pentasialo-	32.4–37.8	13.2–19.9

The presence of the variant in control populations was verified at 1000Genomes (<http://www.1000genomes.org>), the Exome Variant Server (<http://evs.gs.washington.edu/EVS>), the Exome Aggregation Consortium (<http://exac.broadinstitute.org>), gnomAD (<http://gnomad.broadinstitute.org/>) and MedGen laboratory database that contained records of subjects with the Polish origin.

This study has been approved by an ethics committee of The Children's Memorial Health Institute in Warsaw, Number 23/KBE/2020. Informed consent was obtained from all included patients.

RESULTS

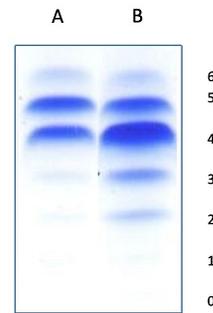
Serum transferrin (Tf) isoforms' isoelectrofocusing

In this study, a total number of 55 pediatric patients with solely increased pentasialo-Tf was identified; the prevalence of this phenomenon was estimated as 0.2%. A solely increased trisialo-Tf was identified in 15 pediatric patients; the prevalence of this phenomenon was estimated as 0.06%.

In the group of 4 children (proband) with solely increased pentasialo-Tf (Table 1) and their parents (n=4) with the same serum Tf IEF profile, from whom DNA was available, molecular analysis was performed. A graphic presentation (IEF profiles) of the serum Tf gene polymorphism resulting in solely increased pentasialo-Tf is presented in Fig. 1.

Whole exome sequencing

In the study group, we identified two polymorphisms in the Tf gene in a heterozygous state: c.2012G>A, p.Gly671Glu (3 probands) and c.1027C>T, p.Arg343Trp (1 proband). A detailed characteristic of those two variants is presented in Table 2.

**Figure 1. IEF of serum transferrin gene polymorphism resulting in increased pentasialo-Tf solely.**

(A) transferrin gene polymorphism (solely increased pentasialo-Tf); (B) normal profile; 0, asialo-Tf; 1, monosialo-Tf; 2, disialo-Tf; 3, trisialo-Tf; 4, tetrasialo-Tf; 5, pentasialo-Tf; 6, hexasialo-Tf.

DISCUSSION

Transferrin (Tf) is a plasma iron transport protein, with two asparagine N-glycosylation sites (Asn432 and Asn630). The glycans are each terminated by two negatively charged molecules of sialic acid that allow discrimination using IEF (Lefeber *et al.*, 2011). The dominant isoform in healthy individuals is tetrasialo-Tf, while pentasialo-, hexasialo-, trisialo- and disialo-Tf occur in much smaller amounts (Lefeber *et al.*, 2011).

In the study presented here we identified two transferrin gene polymorphisms: c.2012G>A, p.(Gly671Glu) and c.1027C>T, p.(Arg343Trp) corresponding to solely elevated pentasialo-Tf IEF pattern.

So far, the c.2012G>A, p.(Gly671Glu) transferrin polymorphism, also known as transferrin B2, represents the best known (De Wolf *et al.*, 2011; Park *et al.*, 2014; Zühlsdorf *et al.*, 2015). The observed IEF pattern could be explained by an amino acid change from an uncharged side-chain of glycine to the acid side-chain of glutamic acid, which adds a negative charge.

The second variant identified in our study, c.1027C>T, p.(Arg343Trp), was previously described by Zühlsdorf and others (Zühlsdorf *et al.*, 2015). The observed IEF pattern could be explained by an amino acid change from the positively charged side chain of arginine to the uncharged side chain of tryptophan.

Park and others (Park *et al.*, 2014) have recently described another variant, c.1775A>C, p.(GluE592Ala), resulting in an increased pentasialo-Tf fraction. There are also other transferrin variants, like c.1765C>T, p.(Pro589Ser), also known as Transferrin C2, or c.829G>A, p.(Gly277Ser) resulting in an increased trisialo-Tf fraction (De Wolf *et al.*, 2011; Kutalik *et al.*, 2011).

In the diagnosis of CDG, it is important to exclude secondary causes of N-hypoglycosylation, as well as transferrin gene polymorphisms (Jaeken *et al.*, 2017;

Table 2. Characteristics of Tf gene polymorphisms.

Variant details	rs121918677	rs150854910
Variant type	single nucleotide variant	single nucleotide variant
HGVS	NM_001063.4:c.2012G>A	NM_001063.4:c.1027C>T
Protein change	p.Gly671Glu	p.Arg343Trp
Molecular consequence	Missense variant	Missense variant
Clinical significance	Transferrin variant B2	not reported in ClinVar
References	Zühlsdorf A <i>et al.</i> , 2015; Park JH <i>et al.</i> , 2014; de Wolf HK <i>et al.</i> , 2011; Kutalik Z <i>et al.</i> , 2011	Zühlsdorf A <i>et al.</i> , 2015

Ferreira *et al.*, 2018; Peanne *et al.*, 2018; Francisco *et al.*, 2019). All described polymorphisms change the charge of transferrin, affecting the IEF pattern. This fact can impose some diagnostic difficulties in patients suspected of CDG. The implementation of MS was reported to be helpful in identification of those polymorphisms (Guillard *et al.*, 2011; Van Scherpenzeel *et al.*, 2016). However, these high-throughput screening technologies are not routinely available. Since the next-generation sequencing became more widely available, molecular analyses comprise an alternative method for transferrin polymorphism identification.

A major limitation of our study is a small number of patients who underwent molecular analyses. The second one is the fact that serum Tf isoforms were analyzed only by IEF. Thus, an MS study will be essential to reveal changes in charge of the transferrin molecules.

CONCLUSIONS

1. Two transferrin gene polymorphisms: c.2012G>A, p.(Gly671Glu) and c.1027C>T, p.(Arg343Trp) correspond to solely elevated pentasialo-Tf IEF pattern.

2. Whole exome sequencing represents a useful tool for identifying transferrin gene polymorphisms.

Authors' contributions

Project administration: Patryk Lipiński, Anna Tylki-Szymańska; Investigation: Patryk Lipiński, Anna Bogdańska, Agnieszka Sobczyńska-Tomaszewska, Anna Tylki-Szymańska Supervision: Anna Tylki-Szymańska; Writing – original draft: Patryk Lipiński; Writing – review and editing: Patryk Lipiński, Anna Tylki-Szymańska.

Competing interests

All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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