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## **Title**

A homozygous variant in *TKFC* abolishing triokinase activities is associated with isolated immunodeficiency

## **Authors**

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## **Keywords:**

*TKFC*, primary immunodeficiency, triokinase, cyclic FMN, DAK, fructose metabolism

## Abstract

**Background:** TKFC (triokinase and FMN cyclase) is a bifunctional enzyme involved in fructose metabolism. Triokinase catalyzes the phosphorylation of fructose-derived glyceraldehyde (GA) and exogenous dihydroxyacetone (DHA), while FMN cyclase generates cyclic FMN. It regulates the antiviral immune response through its interaction with IFIH1 (MDA5). Previously reported pathogenic variants in *TKFC* are associated with either a multisystemic disease including developmental delay, cataracts, cardiomyopathy, liver dysfunction, microcytic anemia, lactic acidosis, and cerebellar hypoplasia or isolated hypotrichosis with loose anagen hairs.

**Methods:** Exome sequencing identified a homozygous novel variant in *TKFC* (c.1624G>A; p.Gly542Arg) in an individual with complex primary immunodeficiency. The variant was characterized using enzymatic assays and yeast studies.

**Results:** The individual presented with recurrent otitis media, refractory rhinosinusitis, chronic active Epstein-Barr virus disease, chronic Herpes simplex virus gingivostomatitis, Varicella zoster virus infection, folliculitis, eosinophilic esophagitis, and food allergies. Clinical investigations revealed hypogammaglobulinemia, increased immunoglobulins A, near absent NK cells and decreased memory B cells. Enzymatic assays showed that this variant displayed inactive DHA and GA kinase activity while maintaining FMN cyclase activity. An allogenic bone marrow transplantation corrected the patient's immunodeficiency.

**Conclusion:** Our report suggests an important role of *TKFC* in immunological function. The pathological features associated with this variant are possibly linked with DHA/GA kinase inactivation through a yet an unknown mechanism.

## Introduction

Triokinase and FMN Cyclase (*TKFC*) (MIM reference number 615844) codes for a bifunctional homodimeric enzyme (1). Its triokinase activity, which is involved in fructose metabolism, catalyzes the phosphorylation of fructose-derived glyceraldehyde (GA) and allows disposal of exogenous dihydroxyacetone (DHA). Its FMN cyclase activity generates riboflavin cyclic 4',5'-phosphate (cyclic FMN), whose role is unknown, from splitting flavin adenine dinucleotide (FAD) (2). The enzyme is structurally composed of intertwined dimers of identical subunits containing two functional domains (N-terminal and C-terminal). Active centers are formed by the N-domain of one subunit and the C-domain of the other. Both domains are required for the triokinase activity, while only the C-terminal domain is required for the FMN cyclase activity (1). *TKFC* was also previously identified as a regulator of antiviral immune response, since its N-domain interacts with MDA5, an RNA helicase acting as a viral RNA sensor during viral replication to induce interferon expression (3). Loss-of-function mutations in *IFIH1*, encoding for MDA-5, cause primary immunodeficiency with susceptibility to severe and recurrent viral respiratory infections in children (4).

Bi-allelic missense variants in *TKFC* have been reported in four individuals with cataracts and a multisystemic disease, termed Triokinase and FMN cyclase deficiency syndrome (TKFCD). Individuals homozygous for c.1628G>T (p.Arg543Ile) or c.1333G>A (p.Gly445Ser), both in the C-terminal domain, displayed developmental delay, cardiomyopathy, liver dysfunction, microcytic anemia, lactic acidosis, and cerebellar hypoplasia (5). An impaired innate antiviral immunity through a *TKFC*-MDA5 pathway was hypothesized in the most severely affected individual but without immunologic studies. Another group reported a fifth individual found to be compound heterozygous for two variants in *TKFC* (c.574G>C (p.Gly192Arg) and c.682C>T (p.Arg228Trp)) in the N-terminal domain and presenting with isolated hypotrichosis with loose anagen hairs (6). Both groups showed abolished triokinase activity.

In this report, we identified an individual with a homozygous variant c.1624G>A (p.Gly542Arg) in *TKFC* C-terminal domain. The individual presented with severe complex primary immunodeficiency, hypogammaglobulinemia, increased immunoglobulins A, near absent NK cells and decreased memory B cells.

## Material and Methods

### Assessment of DNA strand breaks

Breakage calculations were first performed on 25 cells from the individual after 72 hours of culture by Giemsa staining only. Calculations were then performed after 48 hours of culture, on 50 cells in the presence of different concentrations of bleomycin and compared with a control.

### Clonal rearrangements

Using peripheral blood from the individual, PCR analysis with V and J probes, and Southern analysis with JH probe after *EcoRI* and *Hind III* digestion were performed to investigate for IgH clonal rearrangements. PCR analysis was performed using V $\delta$ 2 and D $\delta$ 2.3 probes to look for TCR $\delta$  clonal rearrangements. Southern analysis was performed to elucidate the presence of TCR $\beta$  clonal rearrangement, using C $\beta$  probe after digestion with *EcoRI*, *Hind III* and *BamHI*.

### Exome sequencing

Exome sequencing was performed using HiSeq 4000 and SeqCap EZ MedExome kit (Roche) at the Génome Québec/CHU Sainte-Justine Integrated Center for Pediatric Clinical Genomics (Montreal, Canada). The sequences were aligned using DRAGEN aligner (7) and exome variants were analyzed using Variant Interpreter (both tools were used on the Illumina BaseSpace environment).

### Enzymatic assays

Mutations Gly542Arg, Arg543Ala or the combined Gly542Arg and Arg543Ala were introduced in the wildtype coding sequence of human *TKFC* bearing the common polymorphism Ala185Thr using the plasmid pGEX-6P-3-hF2 as described previously (1). Recombinant proteins were prepared and trikinase activities were assayed spectrophotometrically as described previously (1).

### Saturation kinetics

Saturation kinetics of the FMN cyclase activity of *TKFC* variants was assayed by HPLC as described previously (1). The kinetic parameters  $k_{\text{cat}}$  and  $K_M$  were estimated by non-linear regression of the Michaelis-Menten equation to the datapoints. Variant parameters can be compared to those of wildtype (Thr185) *TKFC*:  $k_{\text{cat}}$ ,  $0.82 \pm 0.21 \text{ s}^{-1}$ ;  $K_M$ ,  $7.0 \pm 2.5 \mu\text{M}$ ;  $k_{\text{cat}}/K_M$ ,  $117,000 \text{ M}^{-1}\text{s}^{-1}$  (1).

### Yeast studies

The yeast mutant  $\Delta\text{dak1}$  harbouring the deletion of *DAK1* gene (equivalent to *TKFC* in humans) was transformed with a high copy number expression vector empty or carrying *TKFC* coding sequences with Ala185Thr (as control), Ala185Thr + Gly542Arg, Ala185Thr + Arg543Ala or the combined Ala185Thr + Gly542Arg + Arg543Ala. The yeast cells, freshly grown on pre-culture plates were inoculated in a medium containing 50 mM DHA as sole carbon source, at an initial optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.25 as described previously (5). The cultures were incubated for 3 days at 28 °C with vigorous agitation.  $\text{OD}_{600}$  values were then recorded.

## **Results**

### *Clinical description*

We investigated a male born prematurely at 35-week gestation to non-consanguineous French-Canadian parents (Supplementary Figure 1). The younger female sibling and the father had no remarkable clinical features, while the mother had a mild seasonal pollen allergy, and the paternal grandfather had recurrent sinusitis.

The individual was seemingly healthy at birth and no infections were noted until 18 months of age. There was no delayed umbilical cord separation. He then developed recurrent labial Herpes simplex virus (HSV) with gingivostomatitis and had recurrent otitis media. At 3 years old, he had a self-limiting varicella episode. He suffered from a chronic rhinosinusitis, that was refractory to antibiotics and Loratadine. Myringotomy and adenotonsillectomy were performed at the age of 5. The individual was diagnosed with a common variable immunodeficiency and received

intravenous immunoglobulins from 5 to 7 years old and subcutaneous immunoglobulin therapy after 7 years old. In addition, the individual reported gastrointestinal symptoms and following endoscopy, he was diagnosed with eosinophilic oesophagitis with obstruction. Intestinal biopsies showed ileal abundant lymphoid tissue and possible rectal granuloma. At 6 years old, abdominal imagings revealed hepatosplenomegaly and multiple voluminous paravertebral lymphadenopathies located at the root of the mesentery, near the aorta. A lymph node biopsy showed mild follicular hyperplasia and was Epstein-Barr virus (EBV) PCR positive and Varicella zoster virus (VZV) PCR weakly positive. There was no monoclonal expansion. The lymphadenopathies and hepatosplenomegaly resolved at 9 years old, but the individual remained with chronically high EBV viral load throughout the years. He had a 94% EBV expression on T-cells and EBV copy number as high as 77 556 581. He received two courses of 4 doses of Rituximab at 9 and 11 years old with no significant impact on EBV replication. Famiclovir was given intermittently during acute HSV episodes. Despite treatment, he suffered frequent HSV outbreaks, otitis media, impetigo, and acute sinusitis. An episode of thoracic zoster infection was reported. He also had mild intermittent asthma and food allergies.

On blood work up, transient lymphopenia was noted. Serological tests were conducted, which revealed a significant reduction in IgG (2,77 g/L), and a significant increase in IgA (4,09 g/L), and IgE (6.54 kU/L) (Table 1). Vaccine responses for tetanus was absent and was low for varicella. A CD8<sup>+</sup> lymphocytosis was detected, but there was a decrease in naive CD4<sup>+</sup> T cells and CD27<sup>+</sup> memory B cells (3%). NK cells were almost completely absent (0.6%). The CD8<sup>+</sup> cells had an abnormal V $\beta$ 2, suggestive of a pathological TCR repertoire. There was a decrease in thymic recent emigrants (38%). Lymphocytes proliferation assays were normal.

Allogenic bone marrow transplant was performed at 17 years old due to the high risk of EBV-associated malignancy, lymphoproliferative disease, and lethal infection. His conditioning treatment consisted of busulfan, fludarabine and Alemtuzumab and he received a 8/8 HLA-identical transplant from an unrelated donor. Engraftment was good with a 100% chimerism after the first month. He was treated for CMV positive PCR, a zoster episode, and a BK virus cystitis. EBV was positive 9 months post-transplant but became negative at 19 months post-transplant without treatment. At 9 months post-transplant, he was diagnosed with late-onset acute cutaneous graft-versus-host disease (GVHD) treated with topical steroids, cyclosporine and UVB phototherapy. A discrete mitral insufficiency was found on cardiac ultrasound. He is now well, working and doing sport.

### *Genetic and immunological investigations*

Assessment of DNA strand breaks induced by bleomycin did not reveal differences compared to a control, excluding Ataxia-Telangiectasia and Fanconi Anemia. Clonal rearrangements for IgH, TCR $\delta$ , and TCR $\beta$  were also negative. Exome sequencing was performed and a candidate *TKFC* homozygous variant of uncertain significance was identified: NM\_015533.4:c.1624G>A (p.Gly542Arg). The individual was also homozygous for the *TKFC* single nucleotide polymorphism (SNP) rs2260655 (NM\_015533.4:c.553G>A; p.Ala185Thr), which was shown to be a fully active variant and is highly prevalent in the human population (8). No other variant in genes associated with immunodeficiency was identified.

### *Characterization of the TKFC variant*

The amino acid Gly542 is highly conserved from bacteria to vertebrates and humans (Figure 1A) and is located in the C-terminal domain of TKFC (Figure 1B). Gly542 is in close proximity to Arg543, which mutation to Arg543Ile was previously reported as pathogenic (5). This residue is also known to be essential for the triokinase activity in humans (6) and for bacterial orthologs (9,10). The charged guanidinium group near the ATP  $\gamma$ -phosphoryl (Figure 1C) stabilizes the bipyramidal transition state during transfer on DHA. The adjacent Arg542 in the TKFC mutant is likely to create a strong sterical hindrance to ATP binding (Figure 1D), thus suggesting a loss of triokinase activities in the Gly542Arg mutant. The variation p.Gly542Arg is also predicted to be pathogenic by Mutation taster, MetaRNN (score 0.9639), and CADD (score of 31), among other tools.

Enzymatic assays were conducted to elucidate the impact of the Gly542Arg variant on triokinase activity and on FMN cyclase activity. The mutant TKFC and the wild-type enzyme both contained the variant Ala185Thr, in order to recapitulate the individual's homozygous common polymorphism rs2260655 which does not affect triokinase activity (8). The Gly542Arg variant resulted in an inactive DHA and GA kinase with less than 1% of wild-type activity (Figures 1E and 1F). In addition, we constructed the double mutant Gly542Arg + Arg543Ala to test whether removing the sterical hindrance from Arg543 could partially restore the enzymatic activity. Arg542 could not replace Arg543; the double mutant also showed less than 1% of wild-type DHA and GA kinase activity (Figure 1E and 1F).

By contrast, FMN cyclase activity remained unaltered with the Gly542Arg mutant when compared with the wildtype (Figure 1H). The double mutant Gly542Arg+Arg543Ala also showed a FMN cyclase activity similar to wildtype. These results suggest that the substrate FAD binds the active site in a different way to ATP, and agrees with this reaction not involving a phosphoryl transfer but occurring through an internal attack (1,11). In fact, it is likely that FMN cyclase does not require a closed active site (2).

Yeast studies were also conducted to monitor the effect of *TKFC* variants on growth in a culture medium with DHA as sole carbon source. It was previously shown that the overexpression of the human TKFC in  $\Delta$ ak1 cells allows the yeast cells to grow on DHA as sole carbon source as previously reported (5) and that the replacement of residue Ala185 by the common polymorphism Thr185 had no effect on the growth competence (8). Overexpression of Thr185 indeed allowed growth of yeast cells, while the Gly542Arg mutant and the Gly542Arg+Arg543Ala double mutant did not restore growth as previously observed for the Arg543Ala mutant (Figure 1G) (8). Thus, the data obtained with the yeast model support the results of the enzymatic assays.

## Discussion

There have been two previous reports of homozygous *TKFC* variants in humans in the literature (5,6). One individual was reported with compound heterozygous missense variants in *TKFC* N-terminal domain (c.574G>C (p.Gly192Arg) and c.682C> T (p.Arg228Trp)), and an isolated hair phenotype (6). In contrast, the *TKFC* homozygous variants c.1628G>T (p.Arg543Ile) and c.1333G>A (p.Gly445Ser), both located in the C-terminal domain, have been linked to cataracts, developmental delay, liver dysfunction and microcytic anemia (5). One individual carrying the Arg543Ile variant also had cerebellar hypoplasia, and fatal cardiomyopathy with lactic acidosis following a febrile illness. It was speculated that this more severe phenotype could be caused by innate immune deficiency.

In this report, we describe an individual with a homozygous variant (c.1624G>A (p.Gly542Arg)) in the gene *TKFC* who presented with an isolated severe complex primary immunodeficiency. Interestingly, *TKFC* N-terminal domain was previously shown to interact with MDA5 (3). MDA5 is an RNA helicase acting as a viral RNA sensor during viral replication and as an inducer of interferon expression. Loss-of-function mutations in *IFIH1*, the gene encoding for MDA5, cause primary immunodeficiencies, leading to susceptibility to severe viral respiratory infections in children (4). A disturbance in this interaction may cause interferon dysregulation and impaired response to interferon signaling. The immunodeficiency observed in the individual described herein could be caused by a perturbation of MDA5 effect on interferon induction.

It is interesting to note that the individual is also homozygous for the rs2260655 SNP (c.553G>A; p.Ala185Thr) in *TKFC*. This variant was reported to have a null triokinase activity as it was unable to switch the fructose metabolism from toxic oxidation to lipogenesis when overexpressed in *TKFC*<sup>-/-</sup> mice (12). However, the direct measurement of triokinase activity was not performed in this study. In contrast, we and another group have observed in previous studies that this variant has fully active triokinase activity *in vitro* and yeast  $\Delta$ ak1 cells transformed with human *TKFC* with this variant grow normally on DHA as sole carbon source (1,8,13). In addition, this variant is highly prevalent in non-Africans populations (8). Therefore, we conclude that the Ala185Thr variant is not responsible of the phenotype of this individual and is benign.

Finally, our report is the first to establish a link between a *TKFC* variant and immunological dysfunction in human. We suggest the variant to be pathogenic because of DHA/GA kinase inactivation. More studies are needed to understand the implication of DHA/GA kinase in immune function.



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## Conflict of interest

No conflict of interest to declare.

## Author contributions

CM, CTL, BM, AO, JCC, JMR and RAB wrote the manuscript. AC, JC, MJC, BM and JCC performed experiments. JMR and PMC made bioinformatic analysis. JLB, FT, EH, PT and MD provided clinical information and material. JLM supervised the exome sequencing project. AO, BM, JCC, JMR and PMC designed the study and interpreted the data.

## Figure Legends

**Figure 1:** Effect of the Gly542Arg mutation on TKFC structure and enzymatic activity

- A.** Conservation of Gly542 in *TKFC* homologs from bacteria to vertebrates and humans.
- B.** Molecular model of wildtype homodimeric TKFC. The model, described in (6), is the result of a combination of homology modeling (1,6) and molecular dynamics simulation (2). The TKFC subunits are differentially shown in light or dark grey. The active sites, with ATP and DHA bound (yellow and green), are formed by the C-terminal and N-terminal domains of two subunits. The left-hand site (C1-N2) is modeled in a closed, active conformation, while the right-hand one (N1-C2) is in an open conformation. Amino acid Gly542, changed to arginine in the individual, is shown in red color. The position of previously reported variants are highlighted (cyan (5) or pink (6)). These amino acids are located in either the N- or the C-terminal domains.
- C.** Detail of the active site showing ATP bound to the C-domain near Arg543, and located at reaction distance of the triose acceptor (DHA) which is covalently bound to His221 in the N-terminal domain.
- D.** Same as (C) with the Arg542 variant, showing that Arg542 is not compatible with ATP, as both would occupy the same space. This could explain the reduction of the triokinase activity in the variant, as Arg542 will preclude the protein to reach the active conformation with ATP bound.
- E-F.** Effect of TKFC variants on the DHA kinase (E) and GA kinase (F) activity. Mutants Gly542Arg, Arg543Ala or the combined Gly542Arg and Arg543Ala (combined) were introduced in the wildtype coding sequence of human *TKFC* bearing the common polymorphism Ala185Thr. Recombinant proteins were prepared and triokinase activities were assayed spectrophotometrically. Error bars represent standard deviation of triplicate assays.
- G.** Effect of TKFC variants on yeast growth.  $\Delta$ dak1 yeast cells were transformed with a high copy number expression vector empty or carrying *TKFC* coding sequences as in (E) and (F). The yeast cells were inoculated in a medium containing 50 mM DHA as sole carbon source and incubated for 3 days at 28 °C with vigorous agitation. OD<sub>600</sub> values were then recorded. Growth assays were repeated twice, and the data averaged. Error bars represent

standard deviation.

- H.** Saturation kinetics of the FMN cyclase activity of TKFC variants. The activity was assayed by HPLC. The kinetic parameters  $k_{\text{cat}}$  and  $K_{\text{M}}$  were estimated by non-linear regression of the Michaelis-Menten equation to the datapoints. Error bars represent standard deviation of triplicate assays. Variant parameters can be compared to those of wildtype (Thr185) TKFC:  $k_{\text{cat}}$ ,  $0.82 \pm 0.21 \text{ s}^{-1}$ ;  $K_{\text{M}}$ ,  $7.0 \pm 2.5 \mu\text{M}$ ;  $k_{\text{cat}}/K_{\text{M}}$ ,  $117,000 \text{ M}^{-1}\text{s}^{-1}$  (1).

**Table 1:** Hematologic and immunologic features

**Supplementary Figure 1:**

Pedigrees and Genotypes of the Family

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**Table 1**

<b>Biological parameter</b>	<b>Patient</b>	<b>Normal values</b>
Blood count, g/L		
Total white blood cells	5.4	5.4-15.5
Polynuclear neutrophils	1.8	1.5-7.1
Lymphocytes	2.6	1.5-5.5
Monocytes	0.6	0-0.9
Eosinophils	0.4	0-0.4
Hemoglobin	131	115-135
Platelets	278	140-440
Lymphocyte count		
Total count, $\mu\text{L}$	2,600	1500-5100
CD3 <sup>+</sup> count, $\mu\text{L}$	1950	1320-3619
CD3 <sup>+</sup> CD4 <sup>+</sup> , $\mu\text{L}$	1196	736-2303
CD45RA <sup>+</sup> /CD4 <sup>+</sup> (%)	71	57-90
CD45RA <sup>+</sup> CD31 <sup>+</sup> /CD4 <sup>+</sup> (%)	52	39-77
CD3 <sup>+</sup> CD8 <sup>+</sup> , $\mu\text{L}$	832	350-1107
CD3 <sup>+</sup> TCR $\gamma\delta$ , $\mu\text{L}$	78	< 300
CD19 <sup>+</sup> , $\mu\text{L}$	968	285-1084
CD27 <sup>+</sup> /CD19 <sup>+</sup> (%)	4	> 10
CD3 <sup>-</sup> CD56 <sup>+</sup> , $\mu\text{L}$	3	56-630
IgG, g/L	2.77	4.86-14.06
IgA, g/L	4.09	0.25-2.22
IgM, g/L	0.90	0.23-2.38
Anti-tetanus IgG, UI/mL	< 0.1	>0.2
Anti-polio total antibodies		
Polio 1	< 1/8	> 1/8
Polio 2	1/180	> 1/8
Polio 3	< 1/8	> 1/8
PHA proliferation (cpm)		
unstimulated	1300	
stimulated	31025	
OKT3 proliferation (cpm)		
unstimulated	2768	
10 ng	26675	