The insulin-sensitivity sulphonylurea receptor variant is associated with thyrotoxic paralysis

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Abstract

Thyrotoxicosis is the most common cause of the acquired flaccid muscle paralysis in adults called thyrotoxic periodic paralysis (TPP) and is characterised by transient hypokalaemia and hypophosphataemia under high thyroid hormone levels that is frequently precipitated by carbohydrate load. The sulphonylurea receptor 1 (SUR1 (ABCC8)) is an essential regulatory subunit of the β-cell ATP-sensitive K⁺ channel that controls insulin secretion after feeding. Additionally, the SUR1 Ala1369Ser variant appears to be associated with insulin sensitivity.

We examined the ABCC8 gene at the single nucleotide level using PCR-restriction fragment length polymorphism (RFLP) analysis to determine its allelic variant frequency and calculated the frequency of the Ala1369Ser C-allele variant in a cohort of 36 Brazilian TPP patients in comparison with 32 controls presenting with thyrotoxicosis without paralysis (TWP).

We verified that the frequency of the alanine 1369 C-allele was significantly higher in TPP patients than in TWP patients (61.1 vs 34.4%, odds ratio (OR) = 3.42, P = 0.039) and was significantly more common than the minor allele frequency observed in the general population from the 1000 Genomes database (61.1 vs 29.0%, OR = 4.87, P < 0.005). Additionally, the C-allele frequency was similar between TWP patients and the general population (34.4 vs 29%, OR = 1.42, P = 0.325). We have demonstrated that SUR1 alanine 1369 variant is associated with allelic susceptibility to TPP. We suggest that the hyperinsulinaemia that is observed in TPP may be linked to the ATP-sensitive K⁺/SUR1 alanine variant and, therefore, contribute to the major feedforward precipitating factors in the pathophysiology of TPP.

Key Words
- ABCC8
- genetic susceptibility
- thyrotoxicosis
- periodic paralysis

Introduction

Thyrotoxic periodic paralysis (TPP) is an acute manifestation of thyrotoxicosis and is characterised by transient episodes of flaccid muscle paralysis, hypokalaemia and hypophosphataemia that occur only in the presence of high thyroid hormone levels (Dias da Silva et al. 2002, Lin et al. 2003, Lin 2005, Maciel et al. 2011). TPP is the most frequent cause of acquired flaccid paralysis in adults (20–40 years of age) and demonstrates a strong male predominance, with a male to female ratio of approximately 30:1 in Brazilian patients (Silva et al. 2004, Maciel et al. 2011). Although its incidence is higher in Asian populations, TPP occurs in populations of every ethnicity (Okinaka et al. 1957, McFadzean & Yeung 1967, Ober 1992).

The pathogenesis of TPP remains unclear. Some authors have suggested that TPP results from a...
combination of environmental factors, genetics and thyrotoxicosis (Silva et al. 2004, Lin 2005, Maciel et al. 2011). Indeed, attacks of muscle weakness may be triggered by the ingestion of sweet snacks, alcohol consumption, or after vigorous physical exercise (Chang et al. 2013). Recently, a collaborative study identified mutations in a previously unreported gene that was named KCN18 in 33% of unrelated TPP patients (Ryan et al. 2010). This gene encodes an inwardly rectifying potassium (Kir) channel subunit Kir2.6, and is primarily expressed in skeletal muscle and transcriptionally regulated by tri-iodothyronine (T₃) (Ryan et al. 2010). However, the absence of Kir2.6 mutations in the majority of TPP patients indicates that other susceptibility genes remain yet to be identified.

Both T₃ and insulin can activate the 3Na⁺/2K⁺ ATPase pump. Results from several studies have indicated that the activity of the 3Na⁺/2K⁺ ATPase pump is increased in thyrotoxicosis and is further exacerbated in patients with TPP (Chan et al. 1991, 1994). Results from clinical studies also indicated that the serum insulin level is elevated before and during the paralysis episodes (Shishiba et al. 1972, Hamada et al. 1985). Chan et al. (1994) demonstrated the role of insulin in the pathogenesis of TPP. These authors showed an increased insulin response in a glucose tolerance test and a higher platelet 3Na⁺/2K⁺ ATPase activity in TPP patients compared with healthy control subjects and thyrotoxicosis patients without paralysis (Chan et al. 1994). Recently, we have proposed a feedforward model for TPP pathophysiology (Maciel et al. 2011). According to this model, in a genetically susceptible thyrotoxic patient, the combination of an over-activated 3Na⁺/2K⁺ ATPase pump with hyperinsulinaemia and abnormally increased glucose-stimulated insulin secretion result in reduced outward K⁺ efflux in skeletal muscle, which causes hypokalaemia and culminates in the depolarisation-induced acute loss of muscle excitability (Kung 2006, Rolim et al. 2010, Maciel et al. 2011). Among seven hyperinsulinaemia genome loci, the ATP-sensitive potassium (KATP) channel genes ATP-binding cassette transporter sub-family C member 8 (ABCC8) and KCNJ11 are strong candidates for new TPP susceptibility studies.

Therefore, based on the thyrotoxic distress in muscle and the serum insulin response to a carbohydrate load in TPP subjects, we screened genes related to the metabolic equilibrium of glucose in muscle cells, including KATP channel genes, especially those regulated by T₃, which arbitrate the slow-to-fast-twitch muscle fibre change. Therefore, we investigated whether variants of KATP channels (i.e. Kir6.2 and sulphonylurea receptor 1 (SUR1)) that participate in insulin secretion and glucose uptake in muscle could be associated with TPP. In this study, we explore the relationship between the ABCC8 gene variant Ala1369Ser and susceptibility to TPP.

Materials and methods

Subjects

We recruited 68 male thyrotoxic patients, of whom 36 presented with TPP and 32 were control subjects presenting with Graves’ disease without a history of paralysis (i.e. thyrotoxic without paralysis; TWP). TPP patients have been treated at the Endocrinology Outpatient Service of Sao Paulo Hospital since 1997. Additionally, 1092 genotypes of variant frequencies (SNPs) were obtained from the 1000 Genomes database as a population genetics control (Flicek et al. 2013).

TPP was diagnosed based on clinically significant acute muscle weakness attacks that were accompanied by hypokalaemia and thyrotoxic symptoms. All TPP patients exhibited suppressed levels of TSH, increased levels of thyroid hormone (total and free thyroxine (T₄)) and hypokalaemia with serum potassium levels of <3.5 mmol/l (normal range: 3.5–5.0 mmol/l). Patients with another established aetiology of paralysis or hypokalaemia (e.g. paralysis that was secondary to drugs or renal or intestinal K⁺ wasting) were excluded. Peripheral blood samples were collected after a written informed consent had been obtained from each subject. The protocol was approved by the local research ethics committee (CEP-UNIFESP 0940/11).

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes of TTP and control subjects using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Spectrophotometric DNA quantification was performed using a NanoDrop 2000 spectrophotometer. We searched for the ABCC8 gene variant Ala1369Ser (rs757110) using PCR-restriction fragment length polymorphism (PCR-RFLP) screening and subsequent direct sequencing analyses.

The ABCC8 gene region encompassing the Ala1369Ser variant (rs757110) was amplified using the following primers: forward, 5'-GGG AAG AGT CCA AGG AGG AG-3'; and reverse, 5'-CAG GAG ACT GCG ATG TCT GA-3'. We amplified the ABCC8 gene using PCR and 200 ng of genomic DNA, 12.5 µl of Promega PCR Master Mix and
10 pmol of each primer in a total reaction volume of 25 µl under the following conditions: initial DNA denaturation at 94 ºC for 5 min followed by 35 cycles of 30 s of denaturation at 94 ºC, 30 s of annealing at 55 ºC and 45 s of extension at 72 ºC, with 10-min final extension at 72 ºC to complete the reaction. Each 5-µl PCR product was analysed on a 1% agarose gel and then purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK).

PCR fragments were sequenced using BigDye Terminator v3.1 mix and the identical primers that were used for amplification and were subsequently analysed on an ABI Prism 3130x Capillary Electrophoresis Sequencer (Applied Biosystems). We performed PCR-RFLP analysis using the MwoI restriction enzyme (New England Biolabs, Beverly, MA, USA) and NEBcutter 2.0 (http://tools.neb.com/NEBcutter2/) (Vincze et al. 2003). Restriction digests were performed in a 20-µl reaction mixture containing 10 µl of PCR product, 2 µl of 10× NEBuffer 3, 0.5 µl of MwoI and 7.5 µl of sterile deionised water and were incubated at 60 ºC for 1 h. The A-allele variant contains two MwoI restriction sites and is digested to 195-, 106- and 92-bp fragments, whereas the C allele contains a third MwoI restriction site and is digested to 287-, 106- and 92-bp fragments. The resultant DNA fragments were then separated according to size using a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME, USA) and were visualised using GelRed (Biotium, Hayward, CA, USA) staining under u.v. light.

**Statistical analyses**

The allele and genotype frequencies were compared between TPP patients and TWP controls using the χ²-test whenever appropriate. The association between the ABCC8 Ala1369Ser variant and the risk of TPP was estimated by the odds ratio (OR) and considering the 95% CIs, using the non-risk genotype (AC+AA) as a reference. Statistical significance was defined as an α risk < 0.05, and Hardy–Weinberg equilibrium was evaluated. Statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 3.0 (GraphPad Software, La Jolla, CA, USA).

**Results**

We studied 36 TPP patients and 32 sex-matched controls. The anthropometric measurements and clinical and biochemical features of the TPP and TWP subjects are summarised in Table 1. The TWP control group is similar to the TPP group regarding the percentage of mixed Black/Caucasians (41 and 43%, respectively) and Caucasians (34 and 37%, respectively), different in the percentage of black patients (25 and 6%, respectively), whilst Asian (11%), and Indian (3%) patients were only present in the TPP group. The mean age of TPP patients was 28.56 ± 7.73 years (range: 19–51 years) and the mean age of TWP patients was 40.77 ± 11.57 years (range: 20–66 years). In TPP patients, the initial serum potassium level measured before treatment was 2.25 ± 0.55 mmol/l (range: 1.2–3.4 mmol/l), whereas the initial serum potassium level was within the normal range in the TWP control patients (4.52 ± 0.39 mmol/l; range: 4.0–5.2 mmol/l). There was no difference in the serum TSH and free T4 levels between the TPP and the TWP control patients.

In contrast to the BMI that was determined at the time of thyrotoxicosis diagnosis, the BMI that was determined at the time of the last follow-up visit while in a euthyroid state was higher in TPP patients than in the TWP controls (30.05 ± 5.34 vs 26.93 ± 2.73 kg/m², P = 0.041).

The distribution of the Ala1369Ser (rs757110) genotype frequency was examined in TPP patients and TWP subjects; representative electropherogram sequences and restriction analyses are shown in Fig. 1. Genomic DNA obtained from TPP patients (n = 27) and population controls (n = 32) was genotyped. As shown in Table 2, the frequency of the C allele was significantly higher in

**Table 1** Summary of the clinical features of TPP and TWP patients. Age at diagnosis, potassium levels and anthropometric data are expressed as the median, total number and range when the clinical data were available

<table>
<thead>
<tr>
<th>Feature</th>
<th>TPP</th>
<th>TWP</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.56 ± 7.73 (n: 36)</td>
<td>40.77 ± 11.57 (n: 31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range: 19–51 years)</td>
<td>(range: 20–66 years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium (3.5–5.0 mmol/l)</td>
<td>2.25 ± 0.55 (n: 29)</td>
<td>4.52 ± 0.39 (n: 12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TSH (0.35–5.0 µu/l)</td>
<td>0.03 ± 0.03 (n: 36)</td>
<td>0.03 ± 0.02 (n: 32)</td>
<td>0.433</td>
</tr>
<tr>
<td>fT4 (0.7–1.5 ng/dl)</td>
<td>5.29 ± 2.84 (n: 36)</td>
<td>4.43 ± 1.72 (n: 32)</td>
<td>0.192</td>
</tr>
<tr>
<td>BMI at diagnosis (kg/m²)</td>
<td>23.98 ± 3.241 (n: 17)</td>
<td>24.06 ± 3.10 (n: 29)</td>
<td>0.936</td>
</tr>
</tbody>
</table>
TPP patients than in the TWP control subjects (61.1 vs 34.4%, OR=1.42, P=0.325, 95% CI=0.70 to 2.89). Thus, the C-allele Ala1369 variant of the \textit{ABCC8} gene is associated with TPP susceptibility.

**Discussion**

In this case–control study, we demonstrate for the first time, to our knowledge, that the frequency of the Ala1369 variant is significantly higher in TPP patients than in TWP patients, revealing an association between the insulin-sensitivity-related p.Ala1369Ser genetic variant of \textit{SUR1} and TPP. \textit{SUR1} is the \(\beta\) subunit of a KATP channel encoded by the \textit{ABCC8} gene that is crucial for the maintenance of glucose homeostasis (McTaggart et al. 2010). The closure of the KATP channel is a metabolic prerequisite for insulin secretion. KATP channels are large macromolecular complexes that form hetero-octamers, consisting of four inwardly rectifying potassium channel (Kir6.x) subunits that generate the pore surrounded by four regulatory SUR subunits (Inagaki et al. 1995). We focused on \textit{SUR1} because of the clinical observation of hyperinsulinaemia in TPP and, among all genes involved in the pathogenesis of hyperinsulinaemia, on the basis of published literature, 45% of mutations are found in \textit{ABCC8} and \textit{KCNJ11} (KATP channels).

KATP channels demonstrate widespread tissue distribution and are abundant in skeletal muscles (Spruce et al. 1985, Ashcroft 1988). Kir6.2 represents the predominant Kir6.x isoform in skeletal muscle and is expressed with SUR2A to form the KATP metabolic channel complex. Moreover, Tricarico et al. (2006) demonstrated that \textit{SUR1}, which is encoded by the \textit{ABCC8} gene, is also expressed in skeletal muscle in a manner that is dependent on the muscle fibre type: fast-twitch muscles demonstrate significantly higher expression than slow-twitch muscles. Moreover, \textit{ABCC8} is highly expressed in fast-twitch muscle fibre in which T3 has a seminal role (Tricarico et al. 2006, Marsili et al. 2010).

The \textit{ABCC8} gene is composed of 39 exons and is located on chromosome 11p15.1. Several \textit{ABCC8} variants have been described previously. In this study, we screened exon 33–37, where most mutations causing hyperinsulinaemia exist and focused on the most important \textit{SUR1} variant that confers insulin sensitivity, which is largely known as the non-synonymous variant p.Ala1369Ser (Hamming et al. 2009, Villareal et al. 2009).

Interestingly, the \textit{SUR1} p.Ala1369Ser variant appears to be in linkage disequilibrium (LD) with the p.Glu23Lys
variant in the neighbour \textit{KCNJ11} gene, which encodes the potassium channel Kir6.2 and is related to type 2 diabetes (T2D) susceptibility (Gloyin et al. 2003, Chistiakov et al. 2009). Thus, carriers of the alanine allele (Ala1369) of \textit{ABCC8} may also carry the lysine (Lys23) variant of \textit{KCNJ11}. We have also demonstrated previously that the variant Lys23 was found in the majority among 22 TPP patients (Silva et al. 2004). A functional study provided evidence for an altered channel when these two variants are co-expressed (Lys23/Ala1369). This altered channel demonstrated an increased KATP channel activity in pancreatic β-cells (Hamming et al. 2009). These authors also demonstrated that the SUR1 p.Ala1369Ser variant may be responsible for T2D risk by decreasing the ATP sensitivity (Chan et al. 2009). The mechanistic basis of muscle uptake (Miki et al. 2013). an enhanced insulin response of the pancreatic β-cells after glucose intake in pregnant women, although this variant did not correlate with basal blood glucose levels.

KATP channels also modulate glucose uptake in skeletal muscle (McTaggart et al. 2010), in which they occur as hybrid assemblies of Kir6.2/SUR2A and Kir6.2/SUR1 subunits that are organised as homomeric complexes; SUR2B may also contribute to the functional channels (Tricarico et al. 2006). The mechanistic basis of muscle glucose metabolism in skeletal muscle remains poorly understood apart from its known insulin-enhancing glucose uptake. To illustrate this complexity, one study has reported enhanced peripheral insulin sensitivity in Lys23 (KK genotype) carriers using hyperinsulinaemic euglycaemic clamp, which revealed that hepatic insulin sensitivity is ~40% greater in subjects with the Lys23 variant, and these subjects demonstrate increased insulin sensitivity after oral glucose (Villareal et al. 2009). However, results from studies of null mice indicated that increased KATP channel activity would be expected to decrease insulin-dependent glucose uptake (Miki et al. 2002).

In our study, the frequency of the Ala1369 variant was significantly higher in TPP patients than in TWP patients and normal control populations, which reveals a new link between genetic susceptibility to TPP and the insulin response. The combination of hyperinsulinaemia and abnormally increased glucose-stimulated insulin secretion in TPP patients has been demonstrated in other studies (Chan et al. 1994, Soonthornpun et al. 2009). In fact, Soonthornpun and colleagues demonstrated that TPP patients exhibited lower insulin sensitivity. Using a euglycaemic hyperinsulinaemic clamp and a 75-g oral glucose tolerance test, these authors demonstrated that patients with TPP may be more resistant to insulin and, therefore, may be more likely to have hyperinsulinaemia (Soonthornpun et al. 2009). These authors also found that TPP patients exhibited a higher BMI and greater waist circumference than age/sex-matched controls who presented with TWP. Our results also indicated that the current BMI of patients with TPP was higher than that of patients with pure hyperthyroidism, confirming the results of Soonthornpun et al. (2009). Additionally, a Chinese study revealed that non-obese type 2 diabetic patients with the Ala1369 variant have a better hypoglycaemic response to anti-diabetic sulphonylurea drug gliclazide than Ser1369 patients, reinforcing the existence of insulin-response genetic predisposition for those bearing this variant (Feng et al. 2008).

Table 2 Genetic association analysis of the SUR1 p.Ala1369Ser variant observed in the TPP and control groups. The allele (C/A) and genotype (AA, CC, AC and CC or AC) for the p.Ala1369Ser (rs757110) in thyrotoxic periodic paralysis (TPP) patients, thyrotoxic without paralysis (TWP) control patients and the population genetics control (1000 Genomes Project/1KGP) are listed with frequencies, odds ratio (OR), CI of 95% and \( P \) value for comparisons when available. ORs and CIs were calculated using the non-risk genotype (AC + AA) as a reference.

<table>
<thead>
<tr>
<th>Allele</th>
<th>TPP (n = 27)</th>
<th>TWP (n = 32)</th>
<th>OR</th>
<th>95% CI</th>
<th>( P ) value(^a)</th>
<th>Population genetics control (n = 1092)</th>
<th>OR</th>
<th>95% CI</th>
<th>( P ) value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21</td>
<td>42</td>
<td>2.20</td>
<td>0.61 to 8.00</td>
<td>0.344</td>
<td>1561</td>
<td>623</td>
<td>3.94</td>
<td>2.26 to 6.86</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>22</td>
<td>0.80</td>
<td>0.29 to 2.64</td>
<td>0.692</td>
<td>623</td>
<td>574</td>
<td>3.94</td>
<td>2.26 to 6.86</td>
</tr>
</tbody>
</table>

\(^a\)Comparing TPP with TWP patients.
\(^b\)Comparing TPP with the population genetics control.
In this study, the C-allele of the Ala1369 risk variant was present at a higher frequency in TPP patients than in the population genetics control that consisted of 1092 multi-ethnic subjects from the 1000 Genomes project. TPP is known to be more prevalent in Asian populations (Lin 2005). Interestingly, the TPP-risk C-allele is also more common in the Asian population than in the general population (41 vs 29%) (Fliceck et al. 2013). This finding may explain the prevalence of TPP susceptibility in Asians, followed in frequency by Brazilian patients; additional rs757110 frequency (risk C-allele SNP) investigations using other ethnic cohorts may provide additional evidence to support this idea.

Although we have found a statistical correlation between C-allele backgrounds and insulin sensitivity among TPP patients, published data regarding this SNP still remains very controversial. Data from two other T2D populations with different backgrounds did not reach the same genetic predisposition findings when assessing severe hypoglycaemia response to sulphonylurea treatment in German-Caucasians (Holstein et al. 2012) and Japanese (Sato et al. 2010) patients. Certainly, our cohort and these groups of T2D patients are metabolically different and were pharmacologically challenged. Besides, TPP patients are far from being hypoglycaemic despite their mild hyperinsulinemic state. In fact, after being cured of thyrotoxic catabolic conditions, the latest measured BMI (kg/m²) in our TPP patients was 30.05 ± 5.34 (n: 16) compared with 26.93 ± 2.73 (n: 29) observed in the TWP patients (P < 0.041).

During the genotyping and matching of our cohort of TPP and controls (TWP) for ABCC8 variant, two Asian GWAS studies were published, indicating that a new TPP locus (17q24.3) has emerged (Cheung et al. 2012). None of the genes related to insulin response is found within this locus, indicating that ABCC8 hyperinsulinaemia genetic predisposition is either an additional phenotype-modulating trait or an ethnic-dependent linked genetic association. The latter possibility might also be the explanation for the recent study by Chu et al. (2012), which demonstrated that TPP and non-thyrotoxic sporadic periodic paralysis shared the same susceptible genetic variant rs623011 on 17q24.3 independent of thyroid hormone.

Given the significant OR for its genetic susceptibility risk, it is conceivable that the SUR1 Ala1369 variant plays a role in insulin sensitivity, thus mediating the response to the carbohydrate-load triggering factor for thyrotoxic paralysis.

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