

# Novel lincRNA Susceptibility Gene and Its Role in Etiopathogenesis of Thyrotoxic Periodic Paralysis

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Thyrotoxic periodic paralysis (TPP) is a life-threatening neuromuscular complication of thyrotoxicosis characterized by muscle weakness and hypokalemia and with an unclear etiopathogenesis. However, the 17q24.3 locus had been genetically linked to TPP, in which the genetic variant rs312691 (TC genotype) in long intergenic noncoding RNA (lincRNA) *CTD-2378E21.1* is located downstream of inward-rectifier potassium (Kir) channel genes [*KCNJ2* and its antisense *KCNJ2 (AS-KCNJ2)*]. A TPP patient with a suppressed thyroid-stimulating hormone level, a high free thyroxine level of (5.8 ng/dL), and low serum potassium level of (2 mEq/L) was evaluated for Kir channel expression during and after recovery from thyrotoxicosis. We observed that circulating lincRNA and Kir expression varied in accordance with thyroid status and TC genotype. To endorse this association of a lincRNA-rs312691 variant with a genetic risk of TPP, an additional series of 37 patients with TPP and 32 patients with thyrotoxic without paralysis (TWP) were assessed. We verified that the risk of minor allele C was greater in TPP than in TWP (odds ratio, 5.289;  $P = 0.0062$ ), and protective major allele T was more frequent than observed in the 1000 genome controls (odds ratio, 11.90;  $P < 0.0001$ ). *AS-KCNJ2* was downregulated during thyrotoxicosis in the TWP controls carrying allele T and were upregulated in those with TPP with risk allele C. Moreover, *KCNJ2* (Kir2.1) expression was reduced during thyrotoxicosis and restored in euthyroid status. We further excluded any other coding variant by performing targeted exome sequencing mutational screening in 17q24.3. Our data suggest that high lincRNA *AS-KCNJ2* and *CTD-2378E21.1* expression, possibly driven by the triiodothyronine regulatory mechanism, reduces the Kir2.1 expression observed during thyrotoxicosis. This finding could contribute to the understanding of the reduced inward-rectifying current observed during muscle weakness in genetically susceptible TPP patients.

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**Freeform/Key Words:** thyrotoxic paralysis, lincRNA, *KCNJ2*, Kir channel expression, Kir antisense

Thyrotoxic periodic paralysis (TPP) is a neuromuscular complication of thyrotoxicosis that can be caused by either excessive endogenous or excessive exogenous thyroid hormone (triiodothyronine and thyroxine) levels, resulting in transient attacks of muscle paralysis, hypokalemia, and hypophosphatemia [1]. TPP occurs more frequently in Asian and South-American populations, although it can affect individuals of all ethnicities [2, 3].

Abbreviations: *AS-KCNJ2*, antisense *KCNJ2*; cDNA, complementary DNA; Kir, inward-rectifier potassium; lincRNA, long intergenic noncoding RNA; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; TPP, thyrotoxic periodic paralysis; TWP, thyrotoxic without paralysis; WES, whole exome sequencing.

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The etiopathogenesis of TPP remains unclear. It is believed that TPP results from a combination of a genetic susceptibility and thyrotoxicosis. It has been reported that mutations in the *KCNJ18* gene were identified in 33% of TPP patients [4]. However, two-thirds of TPP patients do not present with mutations in *KCNJ18*, highlighting the need to search for other genetic susceptibility mechanisms.

Two different genome-wide association studies, in Thai [5] and Chinese [6, 7] cohorts, have reported noncoding polymorphic variants located at 17q24.3, indicating the presence of another genetic locus associated with TPP. These genetic variants are located far downstream of the three inward-rectifier potassium (Kir) channel genes *KCNJ16* (Kir5.1), *KCNJ2* (Kir2.1), and its reverse-strand antisense *KCNJ2* (*AS-KCNJ2*).

To confirm the previous Asian findings describing a novel susceptibility locus to TPP, we assessed the etiopathogenic questions regarding the genetic susceptibility in Western patients with sporadic TPP and also evaluated its effect on 17q24.3 surrounding channel genes under thyrotoxicosis by assessing *in vivo* Kir channel expression.

## 1. Subjects and Methods

### A. TPP Patients and Controls

We recruited 69 thyrotoxic patients: 37 with TPP and 32 controls who had presented with Graves disease without paralysis (*i.e.*, thyrotoxic without paralysis [TWP]), from the thyroid outpatient clinic at Universidade Federal de São Paulo (São Paulo, SP, Brazil). TPP was diagnosed based on acute muscle weakness attacks accompanied by hypokalemia, thyrotoxicosis, and suppressed thyroid-stimulating hormone levels. Research approval is registered under the protocol CEP-UNIFESP 0940/11.

### B. Polymerase Chain Reaction Sequencing Genotype

Genomic DNA was extracted from the peripheral blood of TPP patients and controls. The frequency of rs312691 (T>C risk genotype) variant was evaluated using polymerase chain reaction (PCR) sequencing, as previously reported [8].

### C. Mutational Screening Using Target Exome Sequencing of Genes in 17q24.3 Locus

Libraries were prepared from genomic DNA from 3 TPP patients and 14 controls. Exons were captured using the SureSelect Human All Exon Kit (Agilent Technologies, Santa Clara, CA). Edge BioSystems (Gaithersburg, MD) was used to perform exome sequencing, variant calling, and annotation. The sequences were aligned to the GRCh37/hg19 human genome assembly using the CLC Bio Genomic Workbench (Qiagen Bioinformatics, Redwood City, CA) and studied using the whole exome sequencing (WES) pipeline for paralogous *KCNJ* genes, as previously reported [9].

### D. Quantitative Real-Time PCR

For real-time quantitative PCR (RT-qPCR) assay, total RNA was extracted from peripheral blood samples from hospitalized patients, 1 euthyroid patient, 2 TWP patients, and 1 TPP patient in the thyrotoxic and euthyroid state. We used 2  $\mu$ g of total RNA for synthesizing complementary DNA (cDNA), followed by qPCR of *KCNJ2* (NG\_008798.1), *KCNJ2-AS1* (NR\_036534.1), *CTD-2378E21.1*, and ribosomal protein S8 (*RPS8*, NM\_001012) as the reference gene. All reactions were performed in both technical and biological triplicates. The primers are described in Supplemental Table 1. The values between oligo-dT primed and nonprimed cDNA for RT-qPCR (net) were measured for preventing spurious cDNA synthesis [10], and calculated using the  $2^{-\Delta\Delta C_t}$  method.

### E. Statistical Analysis

The allele and genotype frequencies were compared using a  $\chi^2$  test or an independent Student's *t* test. The association was estimated using the odds ratio (OR) and the 95%

confidence interval (CI) by adopting the nonrisk TT genotype as the reference. RT-qPCR was performed using analysis of variance with a *post hoc* Tukey test. Statistical significance was defined as an  $\alpha$  risk of <0.05 using SPSS version 22.0 (IBM Corp, Armonk, NY), and GraphPad Prism (GraphPad, San Diego, CA).

## 2. Results

### A. Clinical Features and Genotype Frequency (rs312691) in TPP and TWP

A typical patient with TPP presenting with suppressed TSH, free thyroxine (5.8 ng/dL), and low potassium 2 mEq/L was evaluated during and after thyrotoxicosis. The clinical and biochemical diagnostic findings of a series of TPP and TWP groups are summarized in Supplemental Table 2.

PCR sequencing genotyping (Fig. 1) revealed that the frequency of the C allele was significantly greater in TPP patients than in TWP controls (56% vs 34.4%; OR, 11.90; 95% CI, 3.66 to 38.61;  $P < 0.0001$ ). In addition, a statistically significant difference was observed between TPP and the population genetic control (1000 genomes) group (56% vs 32%; OR, 5.28; 95% CI, 1.641 to 17.05;  $P < 0.0062$ ). No difference was found in the C allele frequencies high FT4 [between the TWP and 1000 genome group (34.4% vs 32%;  $P = 0.1384$ ; Table 1)].

### B. Downregulation of Kir2.1 Channel Expression During Thyrotoxicosis Follows an Enhanced Expression of lincRNA (CTD-2378E21.1)

We performed a case-control study to verify the role of the TC risk genotype regulatory variant under thyrotoxic conditions on the expression of *KCNJ* channels downstream of reverse-oriented lincRNA *CTD-2378E21.1*. The data showed that *AS-KCNJ2* was downregulated during thyrotoxicosis in the TWP control patients, who carry the major population allele T (case 3), and is upregulated in TPP patients carrying the minor allele C (cases 2 and 4). We observed that sense *KCNJ2* expression is different in rs3126691 C allele carriers compared with T allele carriers in the thyrotoxic state. In case-control 4, an inverted expression pattern between lincRNA *CTD-2378E21.1* and *KCNJ2* was present in thyrotoxic and euthyroid state (Fig. 2).

### C. Target WES for Mutational Screening of 17q24.3 Genes

To exclude any gene mutations that could interfere with ion-channel coding gene expressions or TPP events, mutational screening was performed using target exome sequencing of genes in 17q24.3. We observed two missense variants in the *SLC39A11*: A287V (rs61736066) and

**Table 1. Allele Frequency of rs312691 Regulatory Variant in lincRNA *CTD-2378E21.1* (17q24.3)**

Genotype	TPP (Cases)			TWP (Controls)			1KGP Population Genetics (Random Controls)		
	CC (n = 10)	TC (n = 24)	TT (n = 5)	CC (n = 4)	TC (n = 14)	TT (n = 14)	CC (n = 115)	TC (n = 469)	TT (n = 508)
Allele C		0.56 (n = 44)			0.344 (n = 22)			0.32 (n = 699)	
Allele T		0.44 (n = 34)			0.656 (n = 42)			0.68 (n = 1485)	
Odds ratio	11.90 <sup>a</sup>		5.289 <sup>b</sup>		2.25 <sup>c</sup>			—	
95% CI	3.66–38.61 <sup>a</sup>		1.641–17.05 <sup>b</sup>		0.8313–6.090 <sup>c</sup>			—	
P value	< 0.0001 <sup>a</sup>		0.0062 <sup>b</sup>		0.1384 <sup>c</sup>				

The allele frequencies for the T>C variant were calculated in Brazilian TPP, TWP, and 1000 Genome database project (1KGP) control groups.

<sup>a</sup>Allele frequency compared between TPP and TWP patients.

<sup>b</sup>Allele frequency compared between TPP and 1KGP controls.

<sup>c</sup>Allele frequency compared between TWP and 1KGP controls.

A287T (rs141628946). Although these variants were found in 2 of 3 TPP patients and none in 14 WES controls, they have an unclear clinical significance and are unlikely to be causal (Supplemental Table 3).

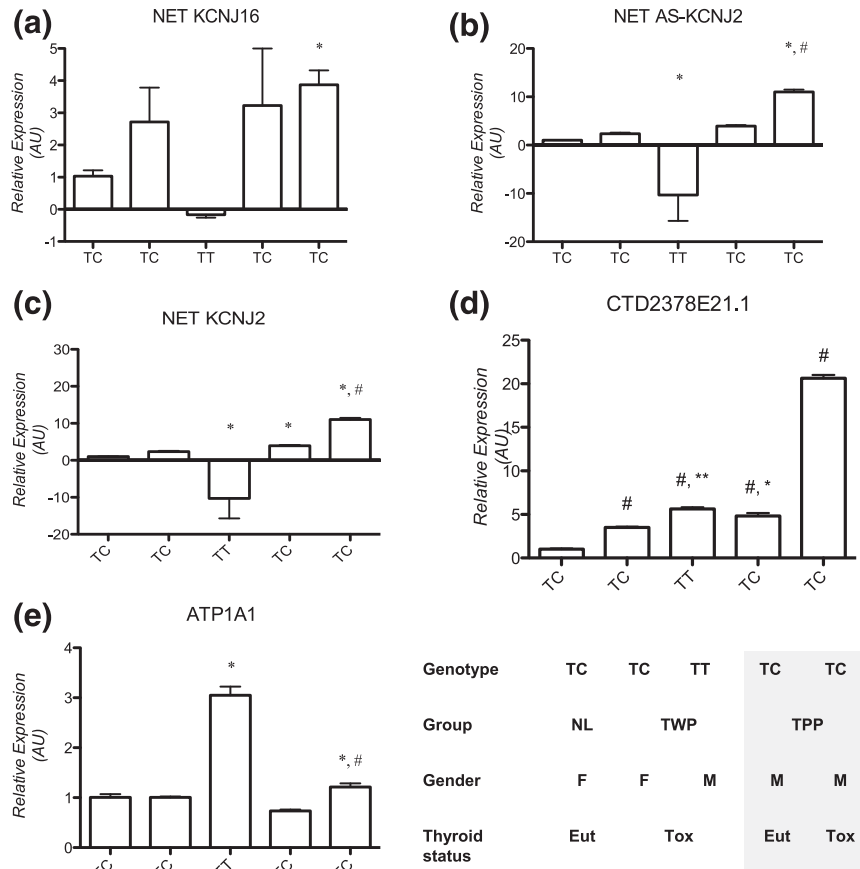
### 3. Discussion

In the present study, we assessed three issues regarding TPP etiopathogenesis: (1) whether the rs312691 genetic variant (CT-risk genotype) is present in Western patients; (2) if there are mutations present in 17q24.3 surrounding genes linked to the CT genotype; and (3) to test, *in vivo*, the hypothesis of sense *KCNJ2* (Kir2.1) expression would reflect differential expression of lincRNA *CTD-2378E21.1* and *AS-KCNJ2* in patients bearing the CT genotype during and after recovery from thyrotoxicosis.

To the best of our knowledge, our study has demonstrated for the first time in non-Asian TPP patients that the frequency of the TPP risk allele, which was previously identified in three independent Asian populational studies [5–7], is significantly greater in Brazilian TPP patients than in TWP patients or 1000 population genetic controls. Variants located far downstream of *KCNJ2* in the desert gene 17q24.3 locus are found in linkage disequilibrium, albeit with slightly different likelihood ratios [5–7]. Besides, no deleterious mutation was observed in ion-channel genes within 17q24.3 region using WES, reinforcing a more convincing association between the risk C allele and susceptibility to TPP.



**Figure 1.** Position and chromatogram representation of the risk allele C of rs312691 within lincRNA CTD-2378E21.1 found in association with TPP. The C risk is located ~150 kb downstream from the *KCNJ2* gene (Kir2.1 channel), which might affect the transcription of the gene and result in periodic paralysis during thyrotoxicosis. The top two chromatograms demonstrate the heterozygous TC genotype and the bottom ones, the homozygous TT and CC genotype.



**Figure 2.** Expression analysis of Kir channel and lincRNAs in the novel TPP susceptibility locus 17q24.3. Gene expression of human *KCNJ2* (Kir2.1) and lincRNAs (*CTD-2378E21.1* and *AS-KCNJ2*) during and after resolved thyrotoxicosis related to rs312691 TT/TC genotype and thyrotoxic (Tox) status were analyzed by measuring peripheral blood circulating messenger RNA relative expression in arbitrary units (AU). In addition to the high triiodothyronine level in blood serum, RT-qPCR of *ATP1A1* gene expression was used as a positive control gene for the thyrotoxic state. Allele C of rs312691 variant is the TPP risk one. Statistical significance comparing normal euthyroid condition (NL) with TPP in thyrotoxic (Tox; #) and euthyroid (Eut; \*) status. Right box in light gray designates a TPP case-control patient and thyrotoxic patient compared with the recovered euthyroid status for *KCNJ2*, lincRNA *CTD-2378E21.1*, and antisense *AS-KCNJ2* channel gene expression. F, female; M, male; NL, normal euthyroid control.

Based on the location of rs312691 in lincRNA *CTD-2378E21.1*, upstream of the Kir2.1 channel, we hypothesized that this lincRNA would contribute to the pathogenesis of TPP by adding the TPP risk variant as a *cis*-regulatory element modifier. Triiodothyronine-dependent transcriptional factors such as NKX2.2/2.5, PAX6, MEF2, and CREBP1/2 are *in silico* predicted to bind this region. By the time of submitting our findings, an elegant Taiwanese study reported on lincRNA *CTD-2378E21.1* as a novel disease-associated gene for sporadic periodic paralysis, which acted negatively on mouse *Kcnj2* (*mKcnj2*) expression using an *in vitro* heterologous system [11].

Moreover, our study measured the Kir2.1 expression of a TPP patient during and after recovery from thyrotoxicosis and compared the expression with that of a TWP patient, carrying either the TT or TC genotype. Our data reinforce the findings of Song *et al.* [11], in which they verified reduced *mKcnj2* expression driven by transfecting human lincRNA *CTD-2378E21.1* in a mouse myoblast C2C12 cell line. The findings from both studies reinforce that Kir2.1 expression should be critical for muscular excitation–contraction coupling by reducing depolarization excitability [12]. We hypothesized that this mechanism can be mediated by its

antisense (Kir2.1) transcript, which was observed in our study. We have observed that *AS-KCNJ2* is downregulated during thyrotoxicosis in TWP controls who carry the allele T and is upregulated in TPP patients with risk allele C. Likewise, other loss-of-function mutations in the *KCNJ18* (Kir2.6) gene placed in the first TPP genomic locus (17p11.1) also cause reduce skeletal muscle cell excitability [4, 13].

We found two variants in the *SLC39A11* gene using WES. This gene encodes the solute carrier family 39 member 11 that is a channel with metal ion transmembrane transporter activity. Although both variants, p.Ala280Val (rs61736066) and p.Ala287Thr (rs61736066), found in 2 of 3 and 1 of 3 TPP patients, were predicted to be damaging (Provean/SIFT/PolyPhen-2), their MAF is high in the genetic control population worldwide, varying from 6% in African subjects to 22% in Japanese subjects using the Exome Aggregation Consortium (A = 0.1046 of 12,507), 1000 genomes (A = 0.1050 of 526), and Grand Opportunity Exome Sequencing Project (A = 0.0757 of 985), with an average heterozygous  $\pm$  standard error of  $0.187 \pm 0.242$ . To date, the variants found in *SLC39A11* have only been linked to glioma and bladder tumor risk [14, 15]. Additional studies expanding the number of TPP and TWP subjects with different genetic background are needed to link single nucleotide polymorphisms in *SCL39A11* with the risk association of clinical significance.

Therefore, given the significant OR for the genetic susceptibility of rs312691 in lincRNA *CTD-2378E21.1* observed in Western patients and in *in vivo* findings seen in a TPP patient during and after resolved thyrotoxicosis, it is conceivable that hormonal-induced dysfunctional expression of Kir2.1 in skeletal muscle might result in a reduced depolarizing inward current detected in TPP.

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