K_{ATP} CHANNEL EXPRESSION AND GENETIC POLYMORPHISMS ASSOCIATED WITH PROGRESSION AND SURVIVAL IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

The ATP-sensitive potassium (K_{ATP}) channel directly regulates the microglia-mediated inflammatory response following CNS injury. To determine the putative role of the K_{ATP} channel in amyotrophic lateral sclerosis (ALS) pathology, we investigated whether ALS induces changes in K_{ATP} channel expression in the spinal cord and motor cortex. We also characterized new functional variants of human ABCC8, ABCC9, KCNJ8 and KCNJ11 genes encoding for the K_{ATP} channel, and analyzed their association with ALS risk, rate of progression and survival in a Spanish ALS cohort. The expression of ABCC8 and KCNJ8 genes was enhanced in the spinal cord of ALS samples, and KCNJ11 increased in motor cortex of ALS samples, as determined by real-time polymerase chain reaction. We then sequenced the exons and regulatory regions of K_{ATP} channel genes from a subset of 28 ALS patients and identified 50 new genetic variants. For the case-control association analysis, we genotyped five selected polymorphisms with predicted functional relevance in 185 Spanish ALS (134 spinal ALS and 51 bulbar ALS) patients and 493 controls. We found that bulbar ALS patients presenting the G/G genotype of the rs4148646 variant of ABCC8 and the T/T genotype of the rs5219 variant of KCNJ11 survived longer that other ALS patients presenting other genotypes. Also, the C/C genotype of the rs4148642 variant of ABCC8 and the T/C genotype of the rs148416760 variant of ABCC9 modified the progression rate in spinal ALS patients. Our results suggest that the K_{ATP} channel plays a role in the pathophysiological mechanisms of ALS.

KEY WORDS: Amyotrophic lateral sclerosis, ALS, microglia, K_{ATP} channel, neuroinflammation, motor neuron disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease whose etiology and progression are not clearly understood. ALS is a multisystem neurodegenerative disease with heterogeneous clinical presentations [1, 2]. As a general ALS pathological hallmark, the loss of specific neurons in the spinal cord (motor neurons from the corticospinal, spinal or bulbar regions), motor cortex, and brainstem results in muscle atrophy and paralysis [3]. However the sensory, cerebellar, extrapyramidal, and autonomic systems are also affected [4]. As a reflection of the large phenotypic variability of ALS, about 45% of ALS patients present moderate cognitive deficits and another 20% of them also develop frontotemporal dementia [5, 6].

The factors accounting for these different disease courses remain unknown. Among all the pathological mechanisms proposed as possible explanations for ALS heterogeneity, neuroinflammation is one of the few common mechanisms present in most ALS patients [7, 8]. The microglial reaction is associated with degenerating motor neurons in the ventral horn of the spinal cord, corticospinal tract and motor cortex [9]. Activated microglia release pro-inflammatory, neurotoxic and neurotrophic factors that stimulate astrocytes and recruit immune cells from the periphery [10], triggering a chronic inflammatory process. At the molecular level, the nature of neuroinflammation in ALS is heterogeneous. The phenotype of reactive microglia depends at each moment on a plethora of signals from the microenvironment, which in turn vary with the time and intensity of motor neuron damage [11]. This spectrum of microglial phenotypes determines the neuroinflammatory process and interferes with ALS progression [8]. Endogenous factors that modulate the microglial response to those signals may therefore account, at least in part, for the different ALS progression rates and patient survival.

The ATP-sensitive potassium (K_{ATP}) channel directly regulates microglial activity and is a major component of the inflammatory response following CNS injury [12–16]. First detected in cardiomyocytes, the K_{ATP} channel is also expressed by other cell types

such as pancreatic β-cells and neurons from various brain regions, where it controls important cellular processes by coupling the electrical activity of the cell to its energy availability [17, 18]. Surveillant microglia show low K_{ATP} channel expression in the human and rodent CNS, whereas expression is increased in activated microglia in Alzheimer's disease patients, rats suffering hypoxia-ischemia and mice with experimental autoimmune encephalomyelitis [19–21]. In reactive microglia, the K_{ATP} channel regulates the reactive state of the cell, controls the release of inflammatory compounds and modifies the phagocytic activity [22, 23]. Pharmacological activation of the K_{ATP} channel is neuroprotective and has anti-inflammatory effects in experimental autoimmune encephalomyelitis [15, 21]. It also curtails neuroinflammation associated with excitotoxicity, ischemia, trauma and neurotoxicant exposure [24–26].

The K_{ATP} channel is a large hetero-octameric protein complex comprising four regulatory and four pore-forming subunits. Sulfonylurea receptor (SUR) 1 and 2 constitute the regulatory subunits of the channel and are encoded by the ABCC8 and ABCC9 genes respectively. The inwardly rectifying potassium channel (Kir) subunits 6.1 and 6.2 form the pore and are encoded by the KCNJ8 and KCNJ11 genes respectively [27]. Given the central role of the K_{ATP} channel in the control of cellular processes, it is not surprising that single nucleotide polymorphisms (SNPs) in its encoding genes have been associated with pathological situations. Polymorphisms in the ABCC8 and KCNJ11 genes have been linked to type II diabetes and with hyperinsulinaemic hypoglycaemia [28, 29], while functional variants in the ABCC9 gene have been associated with different cardiomyopathies and hypertension [30].

In the CNS, ABCC9 gene polymorphisms have been linked to sleep disorders, hippocampal sclerosis of aging and depression [31–33]. To our knowledge, no relationship has been established between functional variants of the ABCC8, KCNJ8 and KCNJ11 genes and any human pathology involving neuroinflammation. In this study we hypothesized that K_{ATP} channel expression is increased in the spinal cord and

motor cortex of ALS patients, and that putative functional variants of genes encoding for this channel may modify the rate of progression and outcome of ALS.

MATERIALS AND METHODS

Human post-mortem nervous tissue

Human post-mortem tissue samples of motor cortex and cervical and thoracic spinal cord were used to assess K_{ATP} channel expression via real-time reverse transcription polymerase chain reaction (qRT-PCR). These samples were obtained from the Neurological Tissue Bank of the IDIBAPS-Biobanc, (Barcelona, Spain) according to European ethical guidelines and following approval by the appropriate Research Ethics Committee. Samples were collected at autopsy from individuals who had suffered a clinical history of sporadic ALS (n=6), according to the El Escorial diagnostic criteria [34], and from non-ALS controls (n=6). Age, sex, clinical onset of symptoms, and time of death were characterized in all cases (Table 1). For histology procedure, 6-μm serial sections were obtained from paraffin-embedded tissue samples. In these sections, hematoxylin-eosin staining was performed to evaluate ALS neuropathology and neuronal loss in motor cortex and spinal cord. Some other samples were fresh-frozen and stored at -80ºC for qRT-PCR analysis.

Population and clinical data for the genetics study

A Spanish cohort of 185 ALS patients (wALS) meeting the revised El Escorial criteria for ALS diagnosis was included in the genetic study. A total of 750 non-affected and non-related control subjects were recruited from the Spanish National DNA Bank (Salamanca, Spain). The control group was created from a subset of 493 randomly selected controls subjects, matched for age and sex, to assess the risk of disease. Control and ALS patients were Caucasian Spanish citizens. In all cases informed consent to participate in studies of genetic epidemiology was collected. Sample collection was conducted in accordance with European ethical guidelines and

supervised by the appropriate Research Ethics Committee. A control group of 147 Caucasian individuals (46 of then of Iberian origin) with no neurodegenerative disease and no family history were collected from the HapMap (www.hapmap.org) and 1000 Genomes (www.1000genomes.org) projects.

All ALS patients were clinically characterized, monitored and followed-up on an quarterly basis using the homogeneous criteria of Dr. Gámez at the ALS Unit of the Hospital Universitari Vall d'Hebron. Sex, age at onset, initial topography and survival time were ascertained for each patient (Table 2). Survival time was defined as the period between onset of clinical symptoms and the date of the need for non-invasive ventilation for more than 23 h/day, the date of tracheostomy ventilation, or the date of death.

ALS patients were classified according to their symptom onset as spinal (Spinal ALS) or bulbar (Bulbar ALS). The rate of progression of patients was determined as previously described [35, 36]. In brief, they were ranked according to the mean slopes of ALSFRS-R decline and FVC decline. Patients were then divided into three groups: fast progression (first tertile for ALSFRS-R and FVC values), mean progression (second tertile), and slow progression (third tertile).

Real-time RT-PCR

Total RNA from spinal cord and motor cortex samples was isolated using the TRIzol reagent (Invitrogen, Paisley, UK) following the manufacturer's instructions. Real-time RT-PCR procedures were as previously described [24, 37]. Briefly, RT reactions were carried out using random primers and 2 µg of mRNA was synthesized using the First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). The RT reaction was performed at 42°C for 60 minutes followed by an additional 5 minutes at 70°C. SensiFAST™ SYBR Hi-ROX One-Step mix (Bioline, UK) was used to conduct real-time PCR. The two-step amplification program was: 2 minutes at 95°C for denaturation and polymerase activation, 40 cycles of 5 seconds at 95°C for denaturation, and a final

extension for 1 minute at 60°C. For each target gene, the expression level was determined using a standard curve (efficiency between 93 to 100%) and normalized to the GAPD mRNA level. Reactions were performed in triplicate to reduce variability. Supplementary Table 1 presents the primer sequences for target genes and endogenous controls used in the study. The ΔΔCt method was used to analyze the data as described by Bookout et al. [38]. Data are presented as mean ± standard error of the mean (SEM). Changes in expression were analyzed using the Student's t test. Homogeneity of variance was checked using Levene's test. Differences were considered to be significant at $p < 0.05$.

DNA purification and identification of variants

A subset of 28 subjects from the wALS cohort (Table 2) was randomly selected to complete sequencing of exons and regulatory regions of the four genes coding for K_{ATP} channel subunits: ABCC8 (SUR1 subunit), ABCC9 (SUR2 subunit), KCNJ8 (Kir6.1 subunit) and KCNJ11 (Kir6.2 subunit). Genomic DNA was extracted from whole blood samples of patients using the QIAamp DNA Mini Kit (QIAGEN, USA), following the manufacturer's instructions. Gene sequences were analyzed for mutations in the coding exons, adjacent intronic regions and regulatory promoter sequences by next generation sequencing (NGS). To capture the sequenced regions, specific probes were synthesized using the SureSelect Target Enrichment kit (Agilent). Massive sequencing was performed using the Genome Analyzer IIx platform (Illumina, San Diego, CA, USA). Genetic variants were detected using the genomic DNASTAR suite (Madison, WI, USA), visually confirmed using the IGV software (www.broadinstitute.org) and manually curated.

Selection of functionally relevant variants and genotyping

The frequencies of the SNPs identified in the ABCC8, ABCC9, KCNJ8, and KCNJ11 genes by sequencing in 28 ALS cases were compared with their respective frequencies in Caucasian and IBS populations registered on 1000genomes and dbSNP

databases, using DeFinetti software (http://ihg.gsf.de/ihg/snps.html, [39]). The SNPs showing statistically different frequencies were selected for case-control validation. The DeFinetti software was also used to calculate the Hardy-Weinberg (HW) equilibrium and to analyze the putative association of the identified variants with the disease. False positives due to the multiple test analysis were corrected by the Bonferroni test and significant differences were assumed at $p<0.0002$. The SNPs with statistically significant differences between cases and control populations were analyzed *in silico* using the SNP Function Prediction [40] and the SNP-Nexus [41] software to predict changes in protein coding regions, splicing sites, and transcription factor binding sites in the promoter.

Variants rs4148646, rs4148642, rs148416760, rs5219 and rs1800467 were genotyped in DNA samples of the wALS cohort and controls (Table 2). Genotyping was performed using the KASPar SNP Genotyping System (LGC Genomics, UK) according to the provider's standard procedures and using the primer set described in Supplementary Table 2. Fluorescence was measured at 25ºC in a 7300 real time PCR System (Applied Bioscience, USA). Genotype calling was carried out using 7300 system SDS software v1.4 (Applied Bioscience, USA) and Klustercaller software (LGCgenomics, UK).

Case-control statistical analysis and power

The study design was based on a pragmatic, case-control, and retrospective clinical analysis with a cohort of 185 ALS patients (134 spinal ALS and 51 bulbar ALS) and a cohort of 493 controls (>2.5 controls per case) as previously described [35]. Selected variants of ABCC8, ABCC9, and KCNJ11 genotypes were used as predictors of the risk of suffering ALS, the age at onset, the rate of progression of symptoms, and the survival time.

The HW equilibrium was calculated using Fisher's exact test implemented in the SNPassoc package in R Software [42]. The odds ratio (OR) and the 95% confidence

interval (95% CI) were estimated using generalized linear models (for either quantitative or binary traits), using the SNPassoc package. Analyses were performed using five different inheritance models (dominant, recessive, additive, co-dominant and over-dominant). The SPSS software (IBM, Spain) was used to perform ordinal logistic regression. Analyses were adjusted by sex. P-values were corrected by a factor of 7.3 to correct for multiple test comparisons using five different inheritance models [35, 36, 42]. Using this criterion, the uncorrected level for statistical significance was established at $p<0.0069$. Both, uncorrected and corrected (adj-p) p-values are shown.

Survival analysis was performed using Kaplan-Meier curves and COX multivariable regression (SPSS software), including all dead patients and those receiving either non-invasive ventilation for more than 23 hours/day or ventilation by tracheostomy. Sample size and statistical power were calculated using Quanto software [43]. This study had a prior statistical power of 80% (alpha = 5%) for detecting a difference of 21 months in survival between groups of patients (main population survival= 53.47 ± 49.2 months, minor allele frequency = 0.379), and a beta of 98% (alpha = 5%) for detecting a difference of > 29 months. For the risk of suffering ALS, this study had a power of 80% (alpha = 5%) for detecting an OR > 1.45 between cases and controls.

RESULTS

K_{ATP} channel expression in the spinal cord and motor cortex of ALS cases

Hematoxylin-eosin staining revealed typical ALS-associated pathophysiological features in the spinal cord and motor cortex of ALS patients. We found loss of large motor neurons in the anterior horns of cervical and thoracic spinal cord (Fig. 1a-d). In these areas, most of the surviving motor neurons were swollen and presented hyaline conglomerate inclusions (Fig. 1b, d); some of them also contained Bunina bodies (Fig. 1d). We also observed cell vacuolation, spongiosis and increased glial density in the corticospinal tracts of ALS spinal cord samples. Motor cortex sections of ALS patients

contained fewer Betz cells than controls. In this area, some of the surviving Betz cells presented hyaline conglomerate inclusions (Fig. 1e, f).

To analyze the effects of ALS on K_{ATP} channel expression, we used RT-PCR to quantify the mRNA level of every channel subunit in the spinal cord and motor cortex. We observed low expression of SUR1 (mean C_T = 27.95 ± 1.38), SUR2 (mean C_T = 27.77 ± 1.48), and Kir6.2 (mean C_T = 28.14 ± 1.70) subunits in control samples, while Kir6.1 subunit expression was slightly higher (mean C_T = 25.24 ± 0.96). In the spinal cord of ALS cases, we found a significant 2.27-fold increase in SUR2 ($t = 7.48$, $p = 0.002$) and a 1.86-fold increase in Kir6.1 ($t = 3.05$, $p = 0.038$) relative to controls. Kir6.2 expression was 3.26-fold higher ($t = 2.95$, $p = 0.042$) in the motor cortex of ALS cases in comparison with controls (Fig. 1g, h).

Identification of K_{ATP} channel genetic variants in ALS patients

We selected DNA samples of 28 subjects from the wALS cohort for NGS analysis of genes coding for the K_{ATP} channel. The complete sequencing of ABCC8, ABCC9, KCNJ8 and KCNJ11 gene exons and regulatory regions accounted for 30,368 bp of sequence (Supplementary Table 3). We identified a total of 238 genetic variants in all four genes. Fifty of these identified variants were new; 23 of them were located in the ABCC8 gene, 18 in the ABCC9 gene, 5 in the KCNJ8 gene, and 4 in the KCNJ11 gene (Supplementary Table 4). With respect to their location, we found six new variants in the coding region of the ABCC8 gene (variants F6S, A4P, A977T, M1394V, L1422P, T1509P) involving the prediction of an alternative amino acid in the translated SUR1 protein (Supplementary Table 4).

Analysis of the functional consequences of the identified genetic variants revealed that 44 known SNPs have putative effects on the sequence or expression of the coded proteins (Supplementary Table 5). Seventeen SNPs were located in predicted transcription factor binding sites in the promoters of the ABCC8, ABCC9 and KCNJ11 genes. Also, three SNPs were located in splicing sites of the primary transcript and

another 14 SNPs may potentially alter the splicing process of these genes. Four known SNPs in the coding region produce non-synonymous changes that were predicted as "not damaging" to protein function. Eleven variants were located in predicted miRNA target sites of the primary transcript. None of the new SNPs found in the KCNJ8 gene were predicted to be functionally relevant.

We selected 123 polymorphisms with a predicted functional relevance and obtained their allele frequencies from the dbSNPs and 1000 genomes databases. Then, we assessed the genetic association of these SNPs in the Spanish ALS cases and the European or world populations. We found significant differences in the frequency of 55 SNPs ($p<0.05$) between ALS cases and either European controls of Caucasian origin or different ethnic groups. After multiple test correction ($p<0.0002$), the number of SNPs that differed in population frequency between ALS cases and controls was reduced to 18. On the basis of the functional prediction and the allele frequency differences between ALS cases and controls, we selected the following five variants for a large case-control genetic study: variants rs4148646$^{C/G}$ and rs4148642$^{C/T}$ of the ABCC8 gene, variant rs148416760$^{T/C}$ of the ABCC9 gene, and variants rs5219$^{C/T}$ and rs1800467$^{C/G}$ of the KCNJ11 gene.

K_{ATP} channel gene variants and disease risk or age at onset

We obtained the genotype of the selected polymorphisms from 185 ALS patients (134 spinal ALS and 51 bulbar ALS) and 493 Spanish controls. All five genetic variants were in the HW equilibrium in all groups ($p=0.20$ for variant rs4148646 and $p=0.72$ for variant rs4148642 of ABCC8; $p=1$ for variant rs148416760 of ABCC9; and $p=0.26$ for variant rs5219 and $p=0.05$ for variant rs1800467 of KCNJ11). None of the selected variants was associated with disease risk, age at onset, or familial/sporadic origin in any of the analyzed groups (wALS, spinal ALS and bulbar ALS).

K_{ATP} channel gene variants and survival

We performed single marker analyses associating variants in ABCC8, ABCC9, and KCNK11 genes with the survival time (months) of wALS, spinal ALS, and bulbar ALS patients. We found no association of the analyzed ABCC9 gene variants with ALS survival time.

Single marker analyses of the ABCC8 s4148646 variant revealed differences in the survival time in bulbar ALS. Patients with the G/G genotype survived for longer (116.6 ± 38.4) than patients with the C/C (42.0 ± 11.4) and C/G (30.9 ± 3.9) genotypes under a recessive inheritance model (Table 3). Kaplan-Meier survival curve analysis also revealed differences between patients with the G/G genotype (median = 153.0 months, 95% CI [0, 401]) and those with the C/G or C/C genotypes (median = 24.0 months, 95% CI [14.6, 35.3]) in the bulbar ALS group under a recessive model (log Rank = 5.551; HR = 0.25, 95% CI [0.07, 0.86], p = 0.028.) (Fig. 2a). This difference was not significant in wALS and spinal ALS patients.

Single marker analyses of the rs4148642 variant under a dominant model revealed a tendency for differences between genotypes in bulbar ALS patients. In this case, patients with the C/C genotype had a shorter survival time (62.8 ± 13.7 months) than patients with the C/T (29.5 ± 4.5 months) or T/T (20.0 ± 2.5 months) genotypes, but the difference did not reach significance after multiple test correction (p = 0.024). Similar values were observed in the spinal ALS group under a recessive model (not shown).

Single marker analyses associating variants in the KCNJ11 gene with survival time also revealed some differences in bulbar ALS, but not in wALS or spinal ALS patients. In bulbar ALS, patients with T/T genotype of the rs5219 variant had a longer survival time (100.8 ± 35.1 months) than patients with the C/C (42.0 ± 11.4 months) or C/T (31.3 ± 54.1 months) genotypes, under a recessive inheritance model (Table 3). Kaplan-Meier survival curve analysis also showed differences between patients with the T/T genotype (median = 153.0 months, 95% CI [0, 398]) and those with the C/T or C/C genotypes (median = 24.00 months, 95% CI [9.9, 38.0]) in the bulbar ALS group

under a recessive model (log Rank = 5.083; HR = 0.31, 95% CI [0.10, 0.91], p = 0.034) (Fig. 2b).

K_{ATP} channel gene variants and progression of ALS symptoms

We performed single marker analyses associating variants in the ABCC8, ABCC9, and KCNK11 genes with the rate of progression of symptoms in wALS, spinal ALS, and bulbar ALS patients. We found no association between the analyzed KCNJ11 gene variants and the progression of ALS symptoms.

Single marker analyses of the ABCC8 rs4148642 variant revealed some differences between wALS and spinal ALS patients, under a dominant inheritance model (Table 4). In the wALS group, the frequency of the C/T and T/T genotypes (38%) was higher in patients showing a slow progression of ALS symptoms, compared with the wild type C/C genotype frequency (26.6%). In spinal ALS patients, the wild type C/C genotype was even more common in patients with a fast rate of progression (48,2%) than the C/T and T/T genotypes (16,1%). This difference that showed a significant protective effect in spinal ALS (OR=0.31; 95%IC [0.15 – 0.62]; p=0.001) was not significant in bulbar ALS patients.

Single marker analyses of the rs148416760 variant in the ABCC9 gene also revealed some differences in wALS and spinal ALS patients, under a dominant inheritance model (Table 4). In the wALS group, 83.3% of patients with the T/C genotype showed a fast progression of ALS symptoms, whereas only 37.3% of patients with the T/T genotype showed that fast progression. In spinal ALS patients, 100% of patients with the T/C genotype showed fast progression of symptoms. This significant effect in spinal ALS patients (OR=27.06; 95%IC [1.45 – 504]; p<0.001) was not observed in bulbar ALS patients.

DISCUSSION

This study provides evidence that ALS increases the expression of the ABCC8 and KCNJ8 genes in the spinal cord and KCNJ11 in the motor cortex of patients. Moreover, we report for the first time on the association of the rs4148646$^{G/G}$ and rs5219$^{T/T}$ variants of the ABCC8 and KCNJ11 genes respectively with longer survival times in bulbar ALS; and on the association of the rs4148642$^{C/C}$ and rs148416760$^{T/C}$ variants of the ABCC8 and ABCC9 genes respectively with changes in the progression rates in spinal ALS patients. These results suggest a role for the K_{ATP} channel in the course of ALS, both by modifying the survival time and the rate of progression of symptoms.

Due to the low number of samples and the complex regulation of translational mechanisms and protein synthesis, the expression changes here described by RT-PCR are not conclusive and further experiments are needed to validate and better understand these different expression patterns. Nevertheless, our results may reflect diverse mechanisms of regulation of K_{ATP} channel expression associated with the control of microglial activity during ALS. The expression of ABCC8, ABCC9, KCNJ8 and KCNJ11 increases in the CNS in several pathological situations, and has been associated with the molecular control of neuroinflammation [12–14, 16, 21]. For example, increase of astroglial ABCC9 expression in human brain samples has been associated with risk genotypes for hippocampal sclerosis of aging [44]. In addition, pharmacological activation of the K_{ATP} channel curtails the microglial reaction and exerts neuroprotective and anti-inflammatory effects [25, 45]. The increased expression of ABCC9 and KCNJ8 found in the spinal cord of ALS cases suggests the involvement of the K_{ATP} channel in the control of neuroinflammation processes associated with this disease. However the main subunits assembling functional K_{ATP} channels in brain microglia are encoded by ABCC8 and KCNJ11 [12, 23, 24] and in the motor cortex we only found increased expression of KCNJ11. Differential ALS-induced changes in gene expression in the motor cortex and spinal cord have already been described for genes such as human sirtuins [46], a protein family involved in the control

of neuroinflammation [47], and rat glutamate transporter 1 variants [48]. These tissue-dependent variations in gene expression have been proposed to explain in some extent susceptibility of tissues to cell loss and to modify ALS progression [48].

Genes encoding K_{ATP} channel components have been found to be highly polymorphic, especially the ABCC8 gene [49]. Variations in ABCC8 and KCNJ11 genes compromise the molecular mechanism of insulin release and have been associated with type II diabetes mellitus and with hyperinsulinemic hypoglycemia (see [50] for a review). Also, functional variants in the ABCC9 gene have been associated with different cardiomyopathies, hypertension [51, 52], sleep disorders, hippocampal sclerosis of aging and depression [31–33]. We identified 286 variants after mutational analysis of exons and regulatory regions of these genes in Spanish ALS patients. Forty-five of these variants are new; most of them located in ABCC8 and ABCC9, and could potentially modify the expression of these genes. Most noticeable is that five of the new variants found in the ABCC8 gene involve changes in the SUR1 protein sequence that may have functional consequences. SUR subunits of the K_{ATP} channel are the pharmacological target for the treatment of several pathologies. For example, sulfonylureas block the K_{ATP} channel by binding to SUR and are used for the treatment of type II diabetes [53], while potassium channel openers (KCOs) also bind to SUR, activate the channel and are indicated for the treatment of hypoglycemia and malignant hypertension [54]. The new polymorphisms herein described in the ABCC8 gene may thus modify the affinity of those compounds for the channel and account for differential drug efficacy among patients. Additional molecular and cellular experiments are needed to characterize the effects of these polymorphisms on K_{ATP} channel activity and to clarify their putative pharmacological relevance.

Our preliminary association analysis of 123 polymorphisms in the ABCC8, ABCC9, KCNJ8 and KCNJ11 genes identified 18 variants with different frequency distributions between a Spanish ALS cohort and controls of either Caucasians or different ethnic groups worldwide. On the basis of their putative functional relevance, we selected five

of these variants for a large case-control study, but the remaining 13 polymorphisms may still be associated with ALS. However as these differences in genotype distribution may also reflect unequal allele frequencies between the Spanish cohort and the European or world populations, new experiments with a higher number of ALS cases and appropriate controls are needed to validate these associations.

In our case-control study, the rs4148646$^{G/G}$ and rs5219$^{T/T}$ variants of ABCC8 and KCNJ11 respectively were associated with longer survival times in ALS patients with bulbar onset under a recessive genetic model. These associations were not significant in patients with spinal onset, which implies a role for the K_{ATP} channel in tissue susceptibility to cell loss and ALS pathogenesis. Three of the bulbar-onset ALS patients with rs4148646$^{G/G}$ and rs5219$^{T/T}$ variants of ABCC8 and KCNJ11 genes had mild or moderate impaired bulbar function, and upper motor neuron predominant involvement. Interestingly, this subgroup of bulbar ALS onset has longer median survivals if they are offered non-invasive mechanical ventilation [55, 56].

Polymorphism rs4148646 is an intron variant of the ABCC8 gene (intron 38-39) with unknown function and no previously described pathological association. Intronic polymorphisms do not vary protein sequence or function but they are proposed to modify gene expression or mRNA splicing processes [57]. In this context, some intronic variants of the ABCC8 gene have been associated with type II diabetes mellitus [29]. The rs4148646$^{G/G}$ variant of ABCC8 may hence modify the availability of SUR1, which is required to ensemble functional channels. It is also possible that the rs4148646$^{G/G}$ polymorphism could be a genomic marker in linkage disequilibrium with other adjacent functional variants. Meanwhile the rs5219 variant of KCNJ11 involves a protein sequence change (E23K) and has been associated with type II diabetes mellitus in Asian Indian and Chinese populations but not in Caucasians [29, 58–60]. The association of these two variants with a longer survival time in bulbar ALS patients may be related to the role of the K_{ATP} channel in the control of microglia-mediated inflammatory processes. ABCC8 and KCNJ11 encode for SUR1 and Kir6.1, which are

the main subunits of the functional K_{ATP} channel in microglia [12, 23, 24]. Thus, ALS patients harboring rs4148646$^{G/G}$ or rs5219$^{T/T}$ variants may present reactive microglia with a reduced cytotoxic profile, resulting in curtailed neuroinflammation and a less severe neurodegenerative process. In this context, survival time has recently been associated with a genetic variant of the CX3CR1 gene in sporadic ALS patients [35]. In the CNS, CX3CR1 encodes for a microglial receptor that mediates microglia-neuron interactions [61], which also implies a role for reactive microglia in ALS pathogenesis [8].

Our study also found an association between the rs4148642$^{C/C}$ and rs148416760$^{T/C}$ variants of the ABCC8 and ABCC9 genes respectively and changes in the rates of progression in spinal ALS patients but not in patients with bulbar onset. This difference may have clinical relevance that could help identify the patient's risk of requiring gastrostomy tube feeding or mechanical ventilation. It also suggests a role for SUR subunits of the K_{ATP} channel in tissue susceptibility to cell loss and ALS progression. Polymorphism rs4148642 in the ABCC8 gene is also an intronic variant (intron 30-31), while the rs148416760 SNP of ABCC9 is located in the 3'-untranslated region (UTR). Both polymorphisms have unknown function and no previously described pathological association. Two intronic variants of ABCC9 have been previously associated with hippocampal sclerosis of aging, a pathology involving TDP-43 [32]. Due to their location, these variants were not genotyped in our study but they are not in linkage disequilibrium with the rs148416760 polymorphism in the IBS population, suggesting that they are not associated with the progression rate of ALS. However a large case-control study would be necessary to validate this hypothesis. ABCC9 genetic regulation is complex and includes different short and long variants in the 3'-UTR region of mRNA [62] that may determine the translational efficiency and mRNA stability [63] and are proposed to be associated with pathological situations [62]. Thus, the rs4148642 and rs148416760 polymorphisms herein associated with the progression of ALS symptoms may modify the availability of SUR1 and SUR2 and thus the assembly of functional

K_{ATP} channels, with major consequences for the control of microglial activity and neuroinflammation.

This study presents some limitations due to the low incidence of bulbar onset in the ALS population, and to the low allelic frequency of the rs148416760 variant of ABCC9. It is interesting to note that, in spite of these sample size limitations, our results reach statistical significance and suggest an association of the analyzed ABCC8, ABCC9 and KCNJ11 variants with the progression and outcome of ALS. Finally, this study opens new questions to be addressed in a future functional study that must include samples of ALS patients with different genotypes and clinical phenotypes. Thus, the relationship between neuroinflammation parameters and the distribution, expression and protein concentration of the K_{ATP} channel need to be analyzed in a larger number of samples from ALS patients presenting different disease onsets and genetic polymorphisms.

In conclusion, our study reports for the first time an association of ABCC8, ABCC9 and KCNJ11 genetic variants with the rate of progression of symptoms and survival time of ALS patients, which indicates that the K_{ATP} channel plays a role in the course of the disease. Variants rs4148646$^{G/G}$ and rs4148642$^{C/C}$ of ABCC8, rs148416760$^{T/C}$ of ABCC9 and rs5219$^{T/T}$ of KCNJ11 may therefore be used as genetic markers of prognosis in the survival and progression of ALS. Furthermore, our study points to the K_{ATP} channel as a putative pharmacological target for the treatment of ALS. To validate these hypotheses, further experiments are needed to characterize at the molecular, cellular and pharmacological levels the role of the K_{ATP} channel in the control of microglial activity in the CNS areas progressively affected by ALS.

DISCLOSURES

MP, NM and MJR applied for a PCT application “Diazoxide for use in the treatment of amyotrophic lateral sclerosis (ALS)” (Application number PCT/EP2011/064061). The other authors declare that they have no conflict of interest.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

The local IRBs at the Vall d’Hebron Research Institute, the DIBAPS Biobank, and the Spanish National DNA Bank approved the protocols of this study. It was conducted according to the principles set out in the Declaration of Helsinki. All patients and/or close relatives gave their written informed consent to participation in the study in paper format, and a blood sample for genetic analysis was obtained from all of them. CNS tissue donors gave their informed written consent for the use of CNS tissue for research purposes. The categorization of ALS patients was performed according to their clinical features (familial or sporadic ALS forms, bulbar or spinal or respiratory onset, rate of progression and survival time). No experiments with animals were performed in this study.

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FIGURE LEGENDS

Fig. 1. Pathophysiological features and K_{ATP} channel expression changes in spinal cord and motor cortex of ALS patients. **(a-f)** Hematoxylin-eosin staining of transverse sections of thoracic spinal cord **(a-d)** and motor cortex **(e-f)**. Arrows mark large neurons with hyaline conglomerate inclusions in the anterior horn **(d)** and Beth cells in motor cortex **(f)**. Some cells also contained Bunina bodies (arrowhead in **d**). Bars: 100 μm in a and c; 25 μm in b, d, e and f. **(g, h)** Graphs show the ALS-induced fold change of K_{ATP} channel expression quantified by qRT-PCR in the spinal cord **(g)** and motor cortex **(h)**. ABCC8 and ABCC9 genes encode for the SUR1 and SUR2 subunits, while KCNJ8 and KCNJ11 genes correspond to the Kir6.1 and Kir6.2 subunits respectively. Student's t test; *, $p < 0.05$; **, $p < 0.01$

Fig. 2. Kaplan-Meier survival curves for bulbar ALS patients with different ABCC8 and KCNJ11 genotypes. **(a)** Curves for ABCC8 rs4148646 genotypes according to a recessive genetic model; log Rank = 5.551; HR = 0.25, 95% CI [0.07, 0.86], p = 0.028. **(b)** Curves for KCNJ11 rs5219 genotypes according to a recessive genetic model; log Rank = 5.083; HR = 0.31, 95% CI [0.10, 0.91], p = 0.034.

Table 1. Detail of the cases included in the gene expression study.

	Controls	sALS
Nº cases	6	6
Women	2	2
Men	4	4
Age at onset, years (range)	-	46 (34-58)
Age at death, years (range)	61 (40-82)	58 (38-79)
Topography	-	Spinal
Post-mortem delay, hours (range)	7.3 (4-12)	9.2 (6-12)

Table 2. Demographic data of the subjects included in the genetic study

	Controls	wALS	Spinal ALS	Bulbar ALS
Subjects*	493 (750)	185	134	51
Women (%)	227 (46.0%)	89 (48.1 %)	61 (45.5%)	28 (54.9%)
Men (%)	266 (54.0%)	96 (51.9%)	73 (54.5%)	23 (45.1%)
Age (range)	57.8 ± 4.0 (28-99)	60.0 ± 13.2 (28-91)	57.7 ± 12.7 (28-91)	65.8 ± 12.7 (38-87)
Women**	60.9 ± 16.5	60.2 ± 12.8	58.2 ± 11.4	64.5 ± 14.8
Men**	55.1 ± 10.9	59.8 ± 13.6	57.4 ± 13.8	67.4 ± 9.6
Age at onset**	-	55.5 ± 13.7	53.0 ± 13.3	62.2 ± 12.6
Age at death**	-	62.0 ± 12.4	59.1 ± 12.1	67.2 ± 11.2
Mortality	-	106 (57.3%)	72 (53.7%)	34 (66.7%)
ALS type				
Familial	-	45	32	13
Sporadic	-	140	102	38

*, n (control cohort); **, mean ± SD.

Table 3. Single marker analysis for ALS patient survival time (months) according to the topography onset under a recessive model.

Gene / variant	Onset	Genotype	n	Median ± SD	Diff (95% CI)	p value
ABCC8 / rs4148646						
	wALS	C/C-C/G	123	55.5 ± 4.6	13.8 (-11.4, 39.1)	0.284
		G/G	20	68.5 ± 14.4		
	Spinal ALS	C/C-C/G	88	63.6 ± 5.9	-8.46 (-39.5, 22.6)	0.595
		G/G	14	54.5 ± 13.6		
	Bulbar ALS	C/C-C/G	34	35.4 ± 5.2	81.4 (42.7, 120.0)	0.0002
		G/G	5	116.6 ± 38.4		
KCNJ11 / rs5219						
	wALS	C/C-C/T	122	55.3 ± 4.6	16.7 (-8.5, 42.1)	0.196
		T/T	20	71.2 ± 14.1		
	Spinal ALS	C/C-C/T	88	63.0 ± 5.9	-1.5 (-33.8, 30.6)	0.924
		T/T	13	61.2 ± 13.9		
	Bulbar ALS	C/C-C/T	33	35.8 ± 5.3	66.0 (27.7, 104.2)	0.0017
		T/T	6	100.8 ± 35.1		

Table 4. Single marker analysis for rate of progression of ALS symptoms according to the topography onset under a dominant model.

Gene / variant	Onset	Genotype	Progression subgroup n (%)			Effect compared to Fast progression		
			Fast	Normal	Slow	OR (95%IC)	p value	model p value
ABCC8 / rs4148642								
	wALS	C/C	39 (49.4)	19 (24.1)	21 (26)	0.49 (0.27, 0.88)	0.017	0.016
		C/T + T/T	23 (29.1)	26 (32.9)	30 (38)			
	Spinal ALS	C/C	27 (48.2)	14 (25)	15 (26)	0.31 (0.15, 0.62)	0.001	0.001
		C/T + T/T	9 (16.1)	21 (37.5)	26 (46)			
	Bulbar ALS	C/C	10 (47.6)	5 (23.8)	6 (28,6)	1.76 (0.56, 5.54)	0.331	0.329
		C/T + T/T	14 (60.9)	5 (21.7)	4 (17,4)			
ABCC9 / rs148416760								
	wALS	T/T	57 (37.3)	46 (30.1)	50 (32)	7.23 (0.92, 57.0)	0.061	0.038
		T/C	5 (83.3)	0 (0)	1 (16.7)			
	Spinal ALS	T/T	31 (28.7)	36 (33.3)	41 (38)	27.06 * (1.45, 504)	<0.001	0.001
		T/C	5 (100)	0 (0)	0 (0)			
	Bulbar ALS	T/T	24 (55.8)	10 (23.3)	9 (20.9)	0.27 * (0.01, 6.88)	<0.001	0.081
		T/C	0 (0)	0 (0)	1 (100)			

* Estimation using OR over a 2x2 table

Figure 1

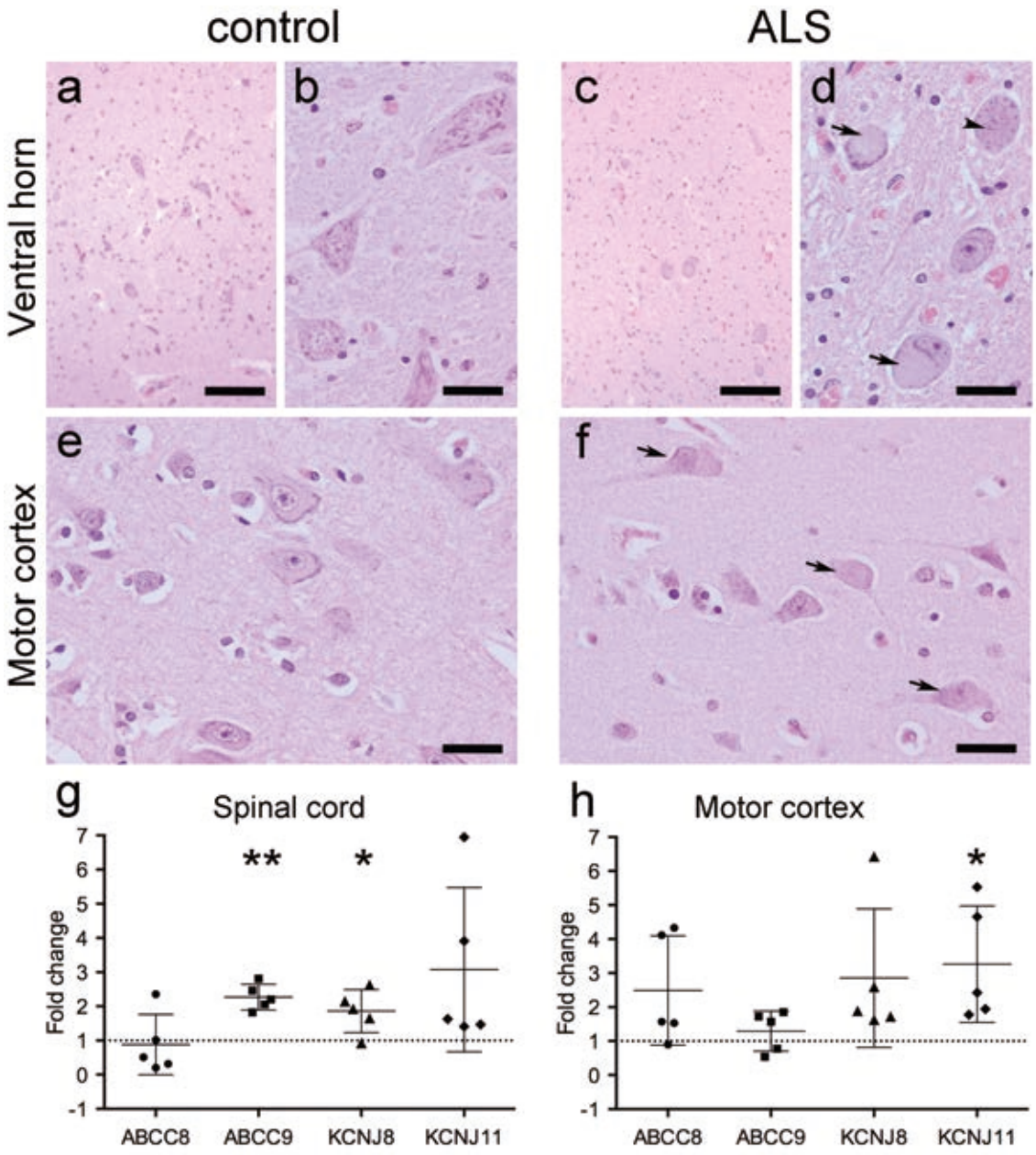
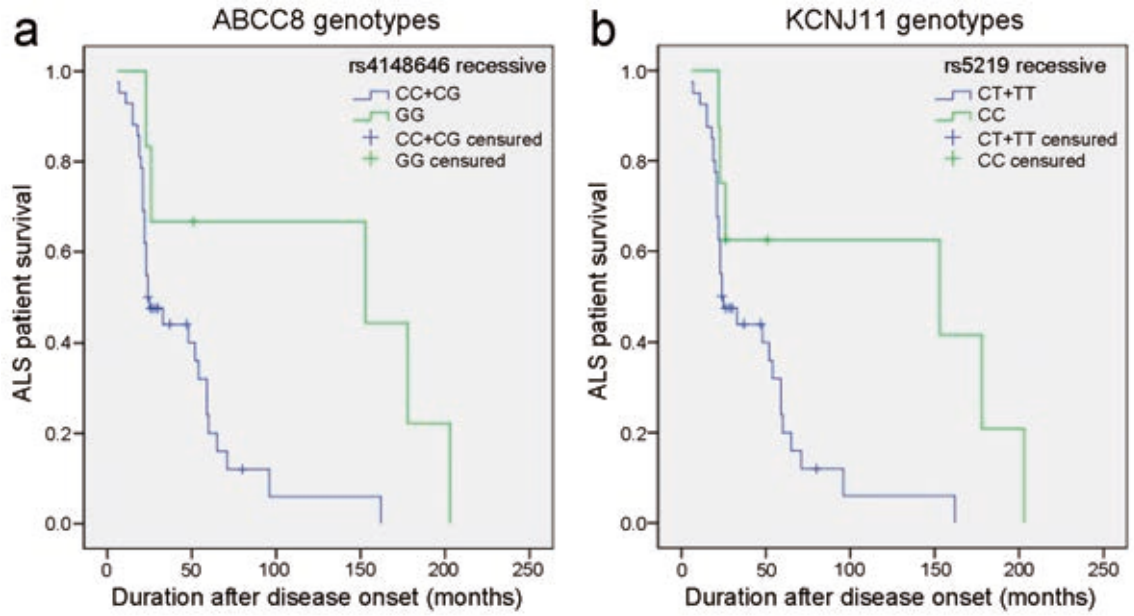

control
ALS
a
b
c
d
Ventral horn
e
f
Motor cortex
g
Spinal cord
Fold change
**
*
ABCC8
ABCC9
KCNJ8
KCNJ11
h
Motor cortex
Fold change
*
ABCC8
ABCC9
KCNJ8
KCNJ11

Figure 2


a
ABCC8 genotypes
rs4148646 recessive
CC+CG
GG
CC+CG censured
GG censured
ALS patient survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Duration after disease onset (months)
b
KCNJ11 genotypes
rs5219 recessive
CT+TT
CC
CT+TT censured
CC censured
ALS patient survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Duration after disease onset (months)

Supplementary Table 1 Primer sequences included in the qRT-PCR study

Target	Gene	Sequence (5'-3')	Size	Accesion nº
GADP	GAPD	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTCG	238 bp	NM_002046.2
SUR1	ABCC8	F: GTCCAGATCATGGGAGGCTA R: CAGAAGACAGCCCCTGAGAC	182 bp	NM_000352
SUR2	ABCC9	F: CATCCATTAGTGGGTCTTCG R: GATGTAAGCCTTGACGTGCT	229 bp	NM_020298
Kir6.1	KCNJ8	F: CATGGAGAAAAGTGGAATGG R: TGGAGAATCAAAACCGTGAT	174 bp	NM_004982
Kir6.2	KCNJ11	F: TTTTCTCCATTGAGGTCCAA R: TTGCTGAAGATGAGGGTCTC	190 bp	NM_000525

Supplementary Table 2 Primer sequences used for genotyping of K_{ATP} channel variants

Gene ID	Allele X primer	Allele Y primer	Common primer	Allele X	Allele Y
ABCC8_rs4148646	CAGGGACAGGACTGGCCTG	CAGGGACAGGACTGGCCTC	CTGTGCACTGATGACGGCCACAA	G	C
ABCC8_rs4148642	CCTGGGGCTCCAGCCTTC	CTCCTGGGGCTCCAGCCTTT	CCACTCCAGCCCTGCAACTCAT	C	T
ABCC9_rs148416760	GGCAGGCCAGCTAAACTTTCTG	GGCAGGCCAGCTAAACTTTCTA	GCTCAAGATCATAACCACAGCTAATCATT	C	T
KCNJ11_rs5219	GGCACGGTACCTGGGCTC	GGGCACGGTACCTGGGCTT	ATACGTGCTGACACGCCTGGCA	C	T
KCNJ11_rs1800467	CAGGTCGCTGGGTGCCAC	CAGGTCGCTGGGTGCCAG	TCATTGATGCCAACAGCCCACTCTA	G	C

Supplementary Table 3 Characteristics of the gene sequences selected for NGS

Target	Gene	Number of exons	Promoter (bp)	Size of the obtained sequences (bp)
SUR1	ABCC8	39	1000	9062
SUR2	ABCC9	44	1000	11993
Kir6.1	KCNJ8	2	1000	4746
Kir6.2	KCNJ11	3	1000	4567

Supplementary Table 4 Variants identified in the genomic sequence of 28 ALS patients.

Gene	Identified Variants	New Variants	3'UTR	5'UTR	Non-synonymous	Synonymous	Promoter	Essential splice site
ABCC8	92	23	rs73419228	rs77498130	rs8192690	rs1799859	rs8192694	rs1799854
ABCC9	105	18	rs10770871 rs2900493 rs829063 rs1283784 rs76908314 rs11835091 rs16924306 rs114906131 rs4762865 rs76760708 rs77492551 rs79461374 rs76728072 rs117295320		rs61688134	L1220L rs61001398 rs58386780 rs2287626 rs35404804 rs10770865	22076371 G/A rs10734715 rs7316271 rs35677639 rs117917449 rs2277404 rs4762865 rs2900493 rs76728072 rs79461374	21958999 I/D rs4762720 rs697250 rs3759236 rs113122586

Gene	Identified Variants	New Variants	3'UTR	5'UTR	Non-synonymous	Synonymous	Promoter	Essential splice site
KCNJ8	20	5	21918438 G/A	21927562 T/C 21927552 T/C 21927548 T/C 21927544 T/G rs74069152	rs34811413	rs34524779 rs34093632 rs16924297	rs76901046	
KCNJ11	21	4	rs5212			rs8175351	rs35271178	

New variants are shown in red

Supplementary Table 5 Predicted functional consequences of the identified genetic variants according to FuncPred

Gene	SNP_ID	Position	Allele	Predicted function	Protein	TFBS	Splicing (ESE or ESS)	nsSNP	Polyphen	Disease association
ABCC8	rs4148646	17393643	G>C	Intronic	--	Y	--	--	--	Diabetes type 2
ABCC8	rs4148642	17397953	C>T	Intronic	--	--	--	--	--	Diabetes type 2
ABCC8	rs60824529	17453137	G>A	CDS	N386N	--	Y	N	--	NA
ABCC9	rs148416760	21800268	T>C	3' UTR	--	--	--	--	--	NA
KCNJ11	rs1800467	17387284	C>G	CDS Promoter	L270V L183V	ER-α PPAR-γ1	--	Y	Benign	Diabetes type 2*
KCNJ11	rs5219	17388025	C>T	CDS 5' UTR	K23E	PPAR-γ1	--	Y*	Possibly damaging	Diabetes type 2*
KCNJ11	rs57778283	17390038	C>T	5' upstream	--	Y	--	--	--	NA
KCNJ11	rs57988393	17390057	A>C	5' upstream	--	Y	--	--	--	NA

NA, Non associated; *, According to PharmGx; related to GRCh38.p7