Overexpression of KLC2 due to a homozygous deletion in the non-coding region causes SPOAN syndrome

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Abstract

SPOAN syndrome is a neurodegenerative disorder mainly characterized by spastic paraplegia, optic atrophy and neuropathy (SPOAN). Affected patients are wheelchair bound after 15 years old, with progressive joint contractures and spine deformities. SPOAN patients also have sub normal vision secondary to apparently non-progressive congenital optic atrophy. A potential causative gene was mapped at 11q13 ten years ago. Here we performed next-generation sequencing in SPOAN-derived samples. While whole-exome sequencing failed to identify the causative mutation, whole-genome sequencing allowed to detect a homozygous 216-bp deletion (chr11.hg19:g.66,024,557_66,024,773del) located at the non-coding upstream region of the KLC2 gene. Expression assays performed with patient’s fibroblasts and motor neurons derived from SPOAN patients showed KLC2 overexpression. Luciferase assay in constructs with 216-bp deletion confirmed the overexpression of gene reporter, varying from 48 to 74%, as compared with wild-type. Knockdown and overexpression of klc2 in Danio rerio revealed mild to severe

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curly-tail phenotype, which is suggestive of a neuromuscular disorder. Overexpression of a gene caused by a small deletion in the non-coding region is a novel mechanism, which to the best of our knowledge, was never reported before in a recessive condition. Although the molecular mechanism of KLC2 up-regulation still remains to be uncovered, such example adds to the importance of non-coding regions in human pathology.

Introduction

Hereditary spastic paraplegias (HSPs) are common neurodegenerative genetic disorders in which patients present progressive spasticity and lower limbs weakness. Up to date, more than 70 loci have been associated with HSPs and at least 50 genes have been identified (1). In 2005, our group identified in a geographic isolate in the backlands of Northeastern Brazil, 26 Caucasian individuals belonging to consanguineous families with an autosomal recessive (AR) complicated form of HSP, which associates spastic paraplegia, optic atrophy and neuropathy (SPOAN syndrome, OMIM #609541) (2). This condition is characterized by onset of progressive spastic paraplegia in infancy, and progressive motor and sensory axonal neuropathy in late childhood/early adolescence leading to severe motor disability. All patients are wheelchair bound after 15 years old, with progressive joint contractures and spine deformities. Patients also have subnormal vision secondary to apparently non-progressive congenital optic atrophy, dystarthisia starting in the third decade of life and exacerbated acoustic startle response. Patients show no intellectual impairment. Ten years after the gene mapping, more than 70 individuals from this cluster, three unrelated affected individuals from Southern and Southeast Brazil, and a pair of Egyptian siblings were diagnosed with SPOAN. Although, all patients share the same haplotype spanning 2.3 Mb into chromosome region 11q13, Sanger sequencing of candidate genes failed to reveal the causative gene (3). Here we describe the SPOAN causative mutation, a small deletion in the non-coding region that causes gene overexpression. Gain of function in a recessive condition is a novel mechanism that, to the best of our knowledge, was never reported before.

Results

Next-generation sequencing and SPOAN mutation

Whole-exome sequencing (WES) was performed in genomic DNA from one Brazilian and one Egyptian patient diagnosed with SPOAN syndrome. We identified six homozygous variants at the critical region, but population frequency and segregation analysis excluded four variants, while the remaining two were SNPs located in non-coding region, suggesting that these two were unlikely to be associated to the clinical phenotype (Supplementary Material, Table S1). Although WES failed to reveal the SPOAN mutation, the sequencing allowed us to refine the critical interval on chromosome 11q13 to 1.77 Mb, between markers rs508548 (A>G at 65,626,289 position in CFL1) and an undescribed variant located at 67,395,410 (G>C in NUDT8). Next, using whole-genome sequencing (WGS), we identified a homozygous 216-bp deletion (chr11:hg19:g:66,024,557_66,024,773del), located at the non-coding upstream region of kinesin light chain-2 (KLC2) (Supplementary Material, Fig. S1). This variant was detected in homozygosity in all affected Brazilian individuals (n = 73), and in the Egyptian affected siblings, while it was not present in homozygosity in 111 healthy Brazilian relatives. This 216-bp deletion was also absent in 474 Brazilian healthy controls and is not described in the 1000 genomes database.

Gene expression analysis

To verify if the deletion affects the expression level of genes located in SPOAN critical region, we performed expression array using cDNA from fibroblasts. Several genes (n = 23; Supplementary Material, Table S2) showed differential expression in patients compared with controls (P < 0.01). Unexpectedly, this assay revealed KLC2 overexpression. Quantitative reverse transcription PCR (RT-qPCR) performed using fibroblast cDNA samples confirmed the expression array results (Fig. 1A). We next generated induced pluripotent stem-cells (iPSC) which were differentiated into motor neurons (MN). RT-qPCR using MN samples revealed KLC2 up-regulation in SPOAN patients compared with healthy controls, confirming the over expression observed in the previous experiments (Fig. 1C). Also we investigated KLC2 expression in blood, using a larger number of cDNA samples from healthy controls, heterozygotes and affected individuals. This assay did not reveal any difference in expression levels between heterozygotes compared with SPOAN’s and to healthy controls (Fig. 1E).

To investigate if the 216-bp deletion is the cause of KLC2 up-regulation, we performed luciferase gene reporter assay using three cell lines (HEK293T, U87MG and MN), which were transfected with two constructs: a KLC2 wild-type promoter and KLC2 216-bp deleted regulatory region driving the Luciferase gene. In the three cell lines, the construct with the 216-bp deletion produced a luciferase activity increment compared with wild-type promoter, varying from 48 to 74% (Fig. 1F).

Klc2 knockdown and overexpression in Danio rerio

We then used Danio rerio as an animal model to study the ‘in vivo’ effect of klc2 knockdown and overexpression. Knockdown regulation was achieved by microinjecting zebrafish embryos with two different klc2 morpholinos (translation blocking morpholino [MO[klc2-TB]] and splice morpholino [MO[klc2-S]]) and one each at doses of 4 and 6 ng. Mild phenotype was defined for embryos showing curly-tail and circular swimming whereas severe phenotype for embryos with dramatically shortened and twisted tail and that were unable to swim. Both phenotypes became evident at 48-h post fertilization (hpf) (Fig. 2A). In all cases, statistically significant differences were observed between mismatch-MO and specific-MO injected embryos. For both morpholino strategies when comparing to the respective mismatch-MO controls, an increase in lethality and/or frequency of phenotypes was mainly observed in detriment of normal phenotype. Furthermore, this difference was more evident when higher amount of either MO[klc2-TB] or MO[klc2-S] was injected (Fig. 2B). Phenotype rescue assays were performed by coinjection of 100 pg of mRNA[klc2-eGFP] and splice morpholino at 6 ng (Fig. 2C), and an improvement of ~33% (P < 0.01), from severe to mild phenotype, was consistently observed (Fig. 2D).

As SPOAN syndrome seems to result from KLC2 up-regulation, we mimicked this condition in zebrafish by microinjecting mRNA[klc2-eGFP] in specific concentrations in embryos. Fluorescent embryos displayed similar phenotype to klc2 morphants (Fig. 3A). A high lethality (over than 70%) was observed in embryos microinjected with mRNA[klc2-eGFP] at 200 pg at 24-hpf stage and we excluded this concentration data in phenotype analysis (Fig. 3B). We observed higher frequency of curly-tail phenotype in...
embryos microinjected with mRNA^{klc2-eGFP} compared with control (mRNA^{eGFP}), being statistical significant in embryos microinjected at 150 pg mRNA concentration (P < 0.05) (Fig. 3C).

**Discussion**

We previously mapped the SPOAN gene, responsible for a syndromic form of AR spastic paraplegia, at 11q13 (2,3). Based on next-generation sequencing, we were able to uncover a new causative mechanism for this condition. We observed that a small deletion in KLC2 non-coding region is responsible for the gene up-regulation and SPOAN phenotype. Additionally, BSCL2 and FLRT1, two genes previously associated with HSP and located nearby but outside the 11q13 critical region, were excluded as candidates (4,5). The Egyptian patients reported in this study as SPOAN carried the c.T2023C (stop loss) homozygous mutation in FLRT1, and were previously assigned by Novarino et al. (Family 709) as SPG68. However, here we suggest that 216-bp deletion, shared by all SPOAN patients, is probably the causative mutation in both Egyptian siblings, rather than the reported FLRT1 mutation.

KLC2 codes for KLC2, a protein involved in anterograde axoplasmatic transport of organelles and macromolecules cargoes (6–10). KLC2 is a part of kinesin protein-1 complex (11), which binds to kinesins heavy chain in a stoichiometric ratio of 1:1 (12), being highly expressed in neurons. Several neurodegenerative diseases show impairment in axonal transport (13,14) and some kinesins heavy chains (KIF5A, KIF1A and KIF1C) have been associated with HSP (15–18). Animal models have also shown that disturbance of axonal transport proteins cause neurodegenerative disease and axon degeneration (19–21). Although the disease mechanism described here involves a homozygous deletion in a non-coding region, all these observations strongly suggest that KCL2 is the causative gene for SPOAN.

According to the RepeatMask database, KLC2 upstream region was generated by a non-LTR retrotransposon (L3/CR-1) insertion. DNA footprint and alignment of L3/CR-1 did not show conservation among distant species, but the high conservation observed among primates suggests it was inserted during the divergence of primates from other mammals. In several human populations, KLC2 surrounding region (10-kb up- and downstream) and three described SNPs surrounding the mutation location have low fixation index (FST) (Supplementary Material, Fig. S2) (rs116801155, rs190099601 and rs76627914 with FST of 0.0044, 0.0002 and 0.0427, respectively), indicating a high conservation in humans. Surprisingly, the small deletion in its non-coding upstream region causes KLC2 overexpression, suggesting a novel molecular mechanism never report before, a gain of function in recessive condition. Intriguingly, the 216-bp deletion overlaps 9-bp of 5′-untranslated region (5′-UTR) of the largest KLC2 transcript (NM_001134775.1), which means that this mutation is located at KLC2 promoter region (upstream of the transcription start site [TSS]) and it should cause gene downregulation instead gain of function. Although this region has characteristics of a promoter (enrichment of H3Kme3, DNase I hypersensitive sites [DHS], RNA pol II binding sites, etc.), transcription factors complexes that bind at this region may act as transcriptional repressor, which could explain the gene up-regulation. Additionally, this
deletion overlaps an unspliced antisense long non-coding RNA (lncRNA, AU311830.1) and regulatory elements: DHS, several transcription factors binding sites (TFBS), histone marks and DNA methylation (Supplementary Material, Fig. S1). Thus, a disruption of this non-coding and regulatory region might alter the expression level of downstream genes, which can explain SPOAN gain of function.

Expression analysis showed an unexpected KLC2 overexpression from fibroblast and MN SPOAN samples. Because SPOAN is a recessive condition, we tried to check the KLC2 expression pattern in heterozygous samples. Whole-blood samples collected from a large number of heterozygotes did not reveal increased KLC2 expression, when compared with homozygotes and healthy controls. These results suggest a tissue-specific effect since 216-bp deletion causes KLC2 up-regulation in fibroblast and MN cell-lines, but not in blood. Also, luciferase assay showed that reporter constructs with 216-bp deletion have increased luciferase activity when compared with the wild-type. These results support the hypothesis that the 216-bp deletion located at non-coding region is likely the responsible for the KLC2 overexpression.

Zebrafish has been an interesting animal model used in genetic studies due to its fast embryonic development and the fact it carries several human orthologues genes. The percentages of lethality and animals with curly-tail phenotype observed in morphants in this study were similar to those reported in several reports that employed zebrafish for other HSP (22–28). Microinjection of mRNAKLC2-eGFP in zebrafish embryos showed a similar phenotype of klc2 morphants, which reinforces our hypothesis that klc2 is an essential gene for MN function and development. Thus, we hypothesize that imbalance of KLC2 gene expression results in neurodegenerative phenotype in humans.

Gene overexpression had been associated with several neurological disorders but none of them have AR inheritance. For example, duplication or triplication of PLP1 cause Pelizaeus-Merzbacher disease (OMIM #312080) (29–33) and PMP22 duplication causes Charcot-Marie-Tooth disease type 1A (OMIM #118220), a hereditary demyelinating neuropathy (34,35). Variants detected upstream APP region were associated with up-regulation of APP protein in Alzheimer disease and Down syndrome patients (36). Additionally, downregulation or complete disruption of protein synthesis is usually the common mechanism in HSP in which functional studies have been conducted. For instance, this is the case in X-linked [e.g. L1CAM (37)], autosomal dominant [e.g. ATL1 (38) and SPAST (39)] and AR conditions [as SPG20 (40) and FA2H (41)].

In short, several unexpected and surprising results were observed during SPOAN syndrome molecular investigation. Although the molecular mechanism of this up-regulation still

Figure 2. Effect of klc2 knockdown in zebraﬁsh. (A) (a and d) Embryos microinjected with control splicing blocking morpholino: (a) misMOKLC2-SP4ng (d) misMOKLC2-SP6ng. (b, c, e and f) Embryos microinjected with splicing blocking morpholino: (b and c) MOKLC2-SP4ng (e and f) MOKLC2-SP6ng. Normal (a and d), mild curly-tail (b and e) and severe curly-tail (c–f) phenotypes were recorded at 48-hpf. (B) Frequencies of observed phenotypes among morphants. Number of microinjected embryos: MOKLC2-SP4ng (292); misMOKLC2-SP4ng (305); MOKLC2-SP6ng (103); misMOKLC2-SP6ng (249); MOKLC2-SP8ng (283); misMOKLC2-SP8ng (234); MOKLC2-SP9ng (423); misMOKLC2-SP9ng (370). P < 0.01, χ² test. (C) Fluorescent embryo co-injected with 6 ng MOKLC2-SP and 100 pg mRNAKLC2-eGFP (selected by fluorescence at 24-hpf) showing mul-curly tails was recorded at 48-hpf. Scale bar 200 µm. (D) Embryos co-injected with 6 ng MOKLC2-SP and 100 pg mRNAKLC2-eGFP (n = 70 embryos) showed a partial rescue of morphant phenotype compared with MOKLC2-SP6ng (n = 30 embryos). P < 0.01, χ² test.
remains to be uncovered, it adds another example of the importance of non-coding regions in human pathology.

**Materials and Methods**

**Patients**

Clinical information regarding SPOAN patients in the geographic cluster detected in northeastern Brazil was detailed elsewhere (2,3). Additionally, we evaluated another three Brazilian patients, with different ancestors from northeastern Brazil and two Egyptians siblings with the identified 216-bp deletion and same clinical symptoms. Blood samples were used for DNA extraction from all patients, from several obligate carriers and from unaffected siblings. Fibroblasts were obtained from dermal biopsies from five patients, one heterozygote and four Brazilian healthy controls, following informed consent under protocols approved by the Biosciences Institute, University of São Paulo (Protocol CEP 010/2003).

**Molecular analysis**

Previous studies conducted by our group using Sanger sequencing did not identify deleterious variants in exons of candidates genes located in the critical region for SPOAN (LRFN4, KLC2 and CCS) (3). To have a more comprehensive and detailed view over this region, WES was performed using DNA samples from two SPOAN subjects using Agilent SureSelect Human All Exon 50 Mb Kit and sequenced in Illumina HiSeq2000 (Illumina, San Diego, CA, USA). Alignment against reference GRCh37 was performed with BWA (42); genotyping with GATK (43); SNP and InDel annotation with AnnoVAR (44) and CNV detection with the R package ExomeDepth (45). The WES coverage achieved at the candidate region was 40× and 77× in the Egyptian and Brazilian samples, respectively. The 216-bp deletion was not detectable by WES. Variants detected in the mapped linkage region were filtered by their frequency, compared with 1000 Genomes database, NHLBI GO Exome Sequencing Project (ESP), Exome Aggregation Consortium (ExAC) and with sequences obtained from 1484 Brazilian controls.

Whole-genome sequencing was performed in DNA from a third affected patient (a distant cousin from Brazilian series) using Illumina TruSeq DNA kit. Alignment against reference GRCh37 was performed with BWA (42); genotyping with GATK (43); SNP and InDel annotation with SnpEff (46) and CNV detection using R package ExomeDepth (45) restricted to exon regions (using bedfile template of the Agilent V4Plus kit), Findel and additional manual screening in the target linkage region. The achieved coverage at the candidate region was 26×. Variants were filtered by comparison with 1000 Genomes database, SPOAN mutation (chr11:hg19:g.66,024,557_66,024,773del) was checked for cosegregation in affected and family health controls (also checked in 474 unrelated health controls) by PCR followed by agarose gel electrophoresis using primer ID 1 (Supplementary Material, Table S3).

**Induced pluripotent stem-cells (iPSC)**

Retrovirus vectors containing the Oct4, c-Myc, Klf4 and Sox2 human cDNAs were obtained from Muotri’s group and the
Human RNA extraction and cDNA synthesis

RNA extraction from fibroblasts (n = 5 affected; n = 1 heterozygote; n = 4 healthy controls) and MN (n = 3 affected; n = 1 heterozygote; n = 3 healthy controls) was performed with TRIZOL® reagent (Invitrogen) and Norgen Biotek RNA/DNA/Protein Purification Kit (Norgen Biotek Corp., Ontario, Canada); RNA from whole-blood (n = 3 affected; n = 1 heterozygote; n = 6 family healthy controls) was performed with accutase (Chemicon, EMD Millipore, Darmstadt, Germany) and plated onto poly-ornitine/laminin-coated dishes (Sigma) with NMN media plus FGF and EGF. Homogeneous populations of neural progenitor cells (NPCs) were achieved after 1-2 passages with accutase in the same condition. To improve cell differentiation, brain-derived neurotrophic factor (20 ng/ml), glial cell-derived neurotrophic factor (20 ng/ml), insulin-like growth factor-1 (20 ng/ml), RI (5uM) and SHH (100 ng/ml; neuronal maturation medium) were added to neuronal cultures for 5 weeks. NPCs were differentiated in MN following a protocol modified from study described elsewhere (48).

Expression array

Fibroblast cDNA samples were submitted to array expression assay using GeneChip® Scanner 3000 7G System (Affymetrix, Santa Clara, CA, USA). The results of expression array were normalized by Robust Multi-array Average (49) and statistical method (test-T) was performed using CLCbio Genomics Workbench, adjusted by Bonferroni and false discovery rate (FDR). Data were submitted to GEO (accession number: GSE67527).

Quantitative reverse transcription PCR (RT-qPCR)

KLC2 primers for RT-qPCR were detailed in Supplementary Material, Table S3 (primer ID 2). RT-qPCR was normalized to GAPDH and was performed using LightCycler® 480 (Roche Diagnostics). KLC2 expression data were calculated using 2^-ΔΔCT method (50). Mann–Whitney test (Nonparametric) was performed using GraphPad Prism version 5.00 (San Diego, CA, USA). Each experiment was performed in triplicate and each sample was replicated twice.

Zebrafish animal model

Adult zebrafish were maintained at 28°C on a 14 h light/10 h dark cycle and the embryos were obtained by natural mating. Zebrafish presents only one klc2 gene in its genome (ZFIN ID: ZDB-GENE-030131-2670), which turns appropriate the use Danio rerio as animal model in this study. The use of Danio rerio in this study was approved by the Committee on the Ethics of Animal Experiments of Pharmacology and Biochemistry Sciences department of National University of Rosario, Argentina (No. 429/2014).

Zebrafish RNA extraction and cDNA synthesis

Total RNA was extracted from whole embryos at different embryonic stages (6, 24, 48 and 72-hpf). RNA extraction was performed using TRIZOL® reagent (Invitrogen), following the manufacturer’s protocol. First-strand cDNA was synthesized using SuperScript Reverse Transcriptase (Invitrogen) with a specific primer (primer ID 3) for Danio rerio klc2 gene transcript (Ensembl EN-SRAG00000075485). The complete klc2 CDS was amplified by PCR using primers ID 4, forward including EcoRI and reverse including SacI restriction sites.

Plasmids and DNA constructs

The complete CDS sequence from klc2 (mRNA^5') was cloned using EcoRI and SacI sites into an engineered version of pc2S+MT as described elsewhere (52). This plasmid was used to...
transcribe mRNA^{klc2-eGFP} coding for KLC2 fused to eGFP. Plasmid without klc2 insert was used to transcribe mRNA^{eGFP} as a control. For mRNA^{klc2-eGFP} and mRNA^{eGFP} transcription, plasmids were linearized by NotI and the SP6 promoter was used for in vitro transcription using mMAGMA® mRNA Machine® Kit (Ambion, Applied Biosystems). The mRNA^{klc2-eGFP} was used to perform the overexpression assay and for rescue of morphant’s phenotype.

Knockdown and overexpression assays
Microinjection of morpholino oligonucleotides (MO) in the yolk of embryos at one- to two-cell stage were performed in specific concentrations (4 and 6 ng). Translation blocking morpholino (MO{klc2-T}R) sequence was 5’-GGTGCGACATACCACCTGGACACACA-3’ (misMO{klc2-T}R was 5’-GGGTACATGCCCGCCGTCCAG-3’) and splicing blocking morpholino (MO{klc2-S}P) sequence was 5’-CGGTGTGTGTGTGCCTCC-3’ (misMO{klc2-S}P was 5’-CGTGTCCTGCCTCC-3’). MO{klc2-S}P target exon 2 of klc2 gene. The rescue of phenotype was performed by co-injecting 6 ng MO{klc2-S}P and 100 pg mRNA^{klc2-eGFP} in the yolk of embryos staged at one- to two-chi. Chi-square and Fisher’s exact tests were performed using GraphPad Prism version 5.00 (San Diego, CA, USA).

Overexpression of klc2 gene in zebrafish was performed by microinjecting mRNA^{klc2-eGFP} at specific concentrations (100, 150 and 200 pg), as described in previous study (53). Same concentrations of mRNA^{eGFP} were microinjected in zebrafish embryos to be used as controls. Both microinjected embryos (mRNA^{klc2-eGFP} and mRNA^{eGFP}) were selected by fluorescence at 24-hpf stage and evaluated at 48-hpf under MVX10 Olympus Microscope, and recorded with MVXTV1XC Olympus digital camera. Chi-square test was performed using GraphPad Prism version 5.00 (San Diego, CA, USA).

Supplementary Material
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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