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SYSTEMS BIOMEDICINE FOR UNRAVELING THE MOLECULAR BASIS AND MODELING CORTICOSPINAL MOTOR NEURON DISEASE

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1. Abstract

The current knowledge on hereditary spastic paraplegia (HSP) and related motor neuron disorders suggests that axonal degeneration is a final consequence of disturbances in diverse processes such as myelin composition, neuron growth, cell adhesion and signaling, mitochondrial energy production, nucleotide metabolism, microtubule assembly and transport, endoplasmic reticulum biogenesis or lipid metabolism.

The goals of this project have been: 1) Construction of a research database of thoroughly characterized Spanish families with motor neuron disorders whose clinical or genetic data suggest a dysfunction in myelin and/or lipid metabolism; 2) Identification, metabolic and functional characterization of novel sequence variants and/or novel genes involved in motor neuron diseases; and 3) Development of in vitro and in vivo models of motor neuron degeneration.

To achieve these objectives, we have carried out next generation sequencing studies (NGS) in 88 families with undiagnosed motor neuron disease and we have reached the diagnosis in 46 families, we have discovered 7 new candidate genes, and in 35 families we have not found any candidate gene. The yield of molecular diagnosis has been 60%. To find new metabolites and metabolic pathways involved in motor neuron diseases, we have conducted metabolomic and lipidomic integration studies and identified, in patients with adrenomyeloneuropathy, a lipidomic signature that is defined by high levels of phospholipids, glycerolipids, sphingolipids or sterols. In addition, we have generated two models of motor neuron degeneration in vivo (zebrafish), knock-outs of *plp1* and *degs1*, which will help us to better understand the pathophysiology of these diseases in humans. The first patients of one of them, DGS1 deficit, have been diagnosed and published by our group.

2. Results obtained

1. Next generation sequencing (NGS) and bioinformatic analysis:

We have **sequenced the whole exome** of 88 families with undiagnosed motor neuron disease. Having reached a diagnosis in 46, we found 7 new candidate genes and in 35 families we found no candidate gene. The molecular diagnostic yield was 60%.

Among the diagnosed cases, we found 6 frameshift, 27 missense, 3 non-frameshift deletions, 6 splicing and 4 stop-gain variants. Of these, 25 had never been previously described.

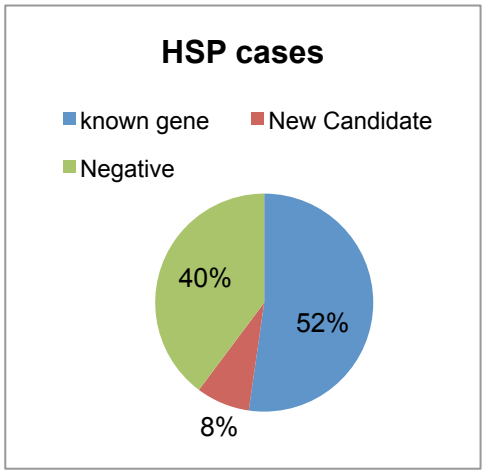
We carried out **segregation studies** for all the families in which we found candidate genes in the exome analysis. In total we performed the validation by Sanger of 228 variants from 128 different genes, in all the members of the family of which we had DNA. For the evaluation and classification of the variants we followed the guidelines of the ACMG (American College of Medical Genetics) and the AMP (Association for Molecular Pathology) (Richards S et al., Genet Med., 2015, Amendola LM et al., Am. J Hum Genet., 2016). According to these guidelines, we identified 24 pathogenic variants, 16 probably pathogenic variants and 6 variants of unknown significance (VUS).

We have conducted **in vitro validation studie**s in 8 genes:

CAPN1: western blot and other functional tests. These studies have confirmed this gene as the cause of the disease in the patient under study.

GFAP: transfection and microscopy studies. These studies have confirmed this gene as the cause of the disease in the patient under study.

SCN4A: to evaluate the existence of splicing anomalies, we have performed minigene transfection assays (Fichou Y et al., Transfusion, 2015) that have ruled out this gene as the cause of the disease in the patient under study.



SPG11: to confirm if the mutations are in a heterozygous compound, as we do not have DNA from the parents, we have carried out cDNA studies since it is a splicing mutation. These studies have confirmed this gene as the cause of the disease in the patient under study.

ERBB4: to evaluate the existence of splicing anomalies, we have performed minigene transfection assays, since this gene is not expressed in blood or fibroblasts. The studies have ruled out this gene as the cause of the disease in the patient under study.

PNKP: to evaluate the existence of splicing anomalies, we have carried out cDNA studies and minigene transfection assays. The studies have ruled out this gene as the cause of the disease in the patient under study.

In addition, we are currently conducting validation studies in 2 cases of variants in genes not linked to a disease so far. In one of the cases we are carrying out cDNA and complementation studies in yeast, in collaboration with a group from Strasbourg. In the other case, we are performing cloning and transfection studies, as well as other studies of protein expression and effect on cell transport in fibroblasts.

In 29 cases in which we have not reached any diagnosis with whole exome sequencing, we have proceeded to sequencing the whole genome. We are currently carrying out the bioinformatic study of these cases, which is highly complex due to the huge amount of data generated by these studies. As in the case of the exome, the candidate genes will be validated by Sanger even though the project has been completed.

2. Metabolic characterization of genes involved in motor neuron disorders.

In order to elucidate a metabolic signature indicative of motor neuron degeneration, we have focused our studies on patients with one of the most representative diseases with motor neuron degeneration, X-linked adrenoleukodystrophy (X-ALD). X-ALD is a hereditary neurometabolic disorder caused by mutations in the *ABCD1* gene and characterized by a progressive spastic paraplegia that affects the corticospinal tracts and adrenal insufficiency. Different phenotypes are described within the X-ALD: a) Adrenomyeloneuropathy (AMN), which begins in adulthood, mainly affects the spinal cord in the form of spastic paraparesis and has no important inflammatory character; b) Cerebral AMN (AMNc), AMN patients who develop dementia, behavioral disorders and presence of inflammatory response in white matter; c) Infantile cerebral adrenoleukodystrophy (ALDc), with onset between 3 and 10 years of age and characterized by an inflammatory response that causes demyelination.

In this part of the study, we have used cerebrospinal fluid (CSF) samples of X-ALD patients with adult forms and we have applied metabolomic and lipidomic methods based on mass spectrometry.

To analyze the characteristic metabolic profiles of each of the phenotypes studied, we have performed a multivariate analysis of the metabolic composition and, specifically, of lipid species present in these samples. Initially we applied a principal component analysis, followed by a hierarchical grouping analysis. These tests have shown that there is a common lipidomic signature in AMN and cAMN patients, which seems to be defined by higher levels of phospholipids, glycerolipids, sphingolipids or sterols. With the ANOVA test we have identified 108 differentially expressed molecules between the groups. Subsequently, we have integrated the results obtained in the studies of metabolomics and lipidomics, to perform an enrichment analysis in order to find new affected metabolic pathways. We have identified 5 ceramides increased in X-ALD. Ceramides belong to the sphingolipid family and are powerful signaling molecules involved in both physiological and physiopathological cellular processes. There are some evidences that the aberrant accumulation of ceramides is neurotoxic and participates in neuronal death in neurodegenerative diseases. In addition, all the glycerolipids identified in this study are increased in X-ALD. These results reinforce the idea that lipid metabolism plays a crucial role in motor neurodegeneration and offers good candidates for future investigations of other motor neuron diseases.

3. Development of *in vivo* models of motor neuron degeneration.

To validate the pathogenicity of the candidate genes, we have used the zebrafish as a model, due to its advantages for functional analysis.

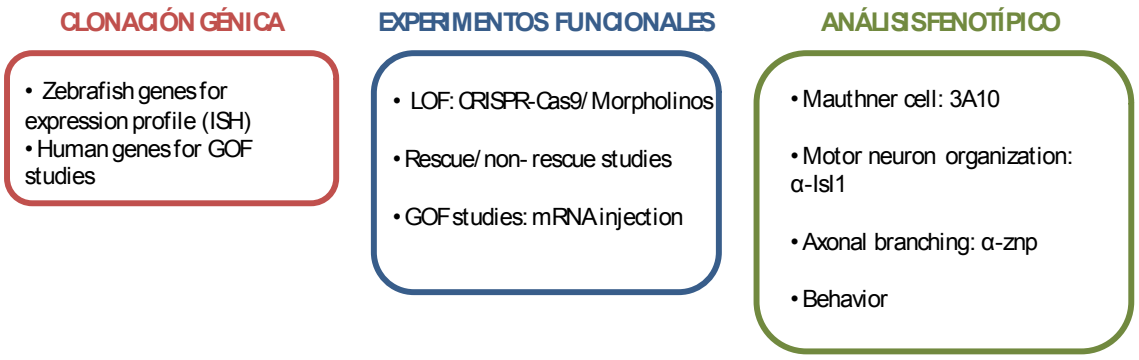
First, we identified candidate genes and performed phylogenetic studies with the best hits in order to know the number of ortholog genes in zebrafish. Studies of sub-functionalization of the orthologs were carried out, and once the best ortholog was identified expression profile studies of the given genes were analyzed.
Second, three consecutive functional approaches were considered and undertaken according the candidate gene requirements:

i) To generate transient gene loss-of-function fish by injection with splicing-defective morpholinos,

ii) To generate stable knock-out zebrafish lines using the CRISPR/Cas9 genome editing system. Although this is a very interesting technique, several reports have demonstrated that genetic compensation happens upon gene-edition in the case of genes playing a crucial role for the organism.

iii) In the cases than the patient mutation results in a gain-of-function (GOF) of the gene, embryos were injected with mRNA of the given gene.

Upon analyses of mutations found in patients, the chosen targeted genes are the following: *plp1b*, *pgm2*, *dync1, degs1* and *fus*. After preliminary analysis involving studies in the structure of gene/protein we observed that some mutations resulted in no observed phenotype so we decided to focus our analyses on *plp1b* and *degs1* genes. According to the goals of the project, once the genes mutated in patients were identified and it was decided to proceed into the in vivo analyses, we generated the following pipeline:



3.1. Phylogenetic studies

One of the patients displayed a mutation in the ***hPLP1*** gene. Although PLP1 is a gene, whose mutations can result either in HSP or ALS (attending to the location of the mutation) and is well studied, little is known about the mitochondrial activity and locomotion behavior defects associated to the different mutations resulting in HSP. After the phylogenetic studies, two ortholog *plp1* genes were found in zebrafish, *plp1a* and *plp1b.* Spatiotemporal expression studies performed by RT-PCR and in situ hybridization we observed that z*plp1a* and z*plp1b* were expressed during embryogenesis, *plp1b* is the only *plp1* gene expressed in the territory of interest (oligodendrocytes). These results will be published this year (Joya et al., in preparation).

The other mutated gene we concentrated on is ***hDEGS1***. *DEGS1* encodes for a desaturase enzyme which catalyzes the conversion of dihydroceramide (DhCer) to ceramide (Cer), in the *de novo* ceramide biosynthesis pathway. Phylogenetic studies showed high degree of identity between *zdegs1* and *hDEGS1*. In this case, *degs1* expression was enriched in the larval brain and spinal cord, especially in oligodendrocytes.

3.2. Designing the functional strategy to be used for generation and characterization of the mutant fishes

In the case of *PLP1* we designed a CRISPR-Cas9 strategy with the aim of knocking down all the different variants of *plp1b* present in zebrafish.
Due to the complexity of the protein, for *DEGS1* we decided to downregulate the gene by the use of splice-blocking morpholino (MO-DEGS1 e2i2).
Fishes were grown in heterozygosis and carriers were screened. Now we have the F1 running and well established and embryos are obtained by mating adult males and females.

3.3. Phenotypic screening

We divided the phenotypic analyses in:
- Effects in cells: Mauthner cell (3A10 staining), motor neurons (anti-Isl1), motor neuron axonal outgrowth and branching (anti-znp).
- Behavior analysis: spontaneous locomotion behavior.
- Mitochondrial activity based in the use of Tg[UAS:mitoDsRed].

Our studies have shown that the zebrafish model MO-DEGS1 presents a 10-fold increase in the DhCer/Cer ratio, a reduced number of oligodendrocytes (altered myelination) and locomotor disability, consistent with the patients' phenotype. In addition, we have also shown that the drug FTY720 improves the phenotype of the fish. In collaboration with international groups, we have managed to collect 19 patients with mutations in *DEGS1*, with a phenotype compatible with Spanish cases. The description of the patients and the results obtained in the fish have been recently published (Pant et al, 2019, J Clin Invest). We are currently starting a compassionate use with FTY720 in some of the patients diagnosed.

3. Relevance and possible future implications

Despite some encouraging examples, successful diagnosis will not always, or even (at present) often, lead to improved treatments. The reality is that the majority of known Mendelian diseases cannot be effectively treated, at least as for now. Nevertheless, the importance to affected families of receiving a specific, correct diagnosis after years of uncertainty should not be understated. Individuals with spastic paraplegia often require full-time care from a young age which is a heavy burden for the parents and family. It is fascinating to see the dedication and love that show families in caring for people with complex neurological deficits over many years. For them, a simple knowledge of the real explanation for the disorder can provide comfort. The correct diagnosis can also facilitate the provision of appropriate state health and social services. Of course, the hope is that knowing the correct diagnosis will also allow a more targeted approach to future therapies as they are discovered. Early application of next-generation sequencing (NGS) can bring to a close an often previously tedious, expensive, and emotionally wrenching ‘‘diagnostic odyssey’’; for all of the reasons listed above, the use of NGS is simply good medical practice. There are likely few therapeutic areas set to benefit more from this new paradigm in Clinical Genetics than neurological disorders, particularly those affecting children. There are several interconnected reasons for this: much neurological illness has already been shown to have a genetic basis; it is often difficult to predict the genetic defect on clinical grounds; new causative variants are being described weekly; and it is expensive and burdensome to test on a gene-by-gene basis. In addition, the global burden of unexplained neurological disorders is immense. NGS can facilitate individualized molecular diagnosis in patients and families with hitherto undiagnosed and unexplained disorders.
The traditional diagnostic model in the evaluation of an individual with a putative genetic disorder includes formulation of a diagnostic hypothesis that may include a diverse range of possibilities. These possible diagnoses are then tested by a variety of biochemical (blood, urine, cerebrospinal fluid (CSF)), structural (MRI), functional (EEG), and specific gene analyses. A recent study examined the economic implications of the Whole Exome Sequencing (WES)-based diagnosis in the context of 500 patients evaluated using traditional genetic tests (Shashi et al., 2014, Genet Med. 16:176-82.). This work showed that if the diagnosis is not clinically apparent at the first visit, then the cost on average per successful genetic diagnosis using traditional tests is approximately $25,000. The cost of WES, on the other hand, is now well under €500

per sample. Thus, when used in an appropriate setting, WES has the potential to provide significant cost benefit to the healthcare budget and to society. This project has allowed the diagnosis of 46 families that would have remained undiagnosed with traditional methods. The implementation of this methodology in the early stages of diagnosis could have spared very high costs to the public health service. For this purpose, a collaborative study is being carried out with the participation of the main hospitals in Catalonia, funded by the Catalan government, which will end at the end of 2019 (The implementation of genomic-based personalized Medicine in non-diagnosed neurological rare diseases, SLT002 / 16/00174-PERIS).

4. Generated references (La Marató de TV3 appears in the acknowledgments)

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