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Genetics of amyotrophic lateral sclerosis

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Résumé

La sclérose latérale amyotrophique (SLA) est la maladie des neurones moteurs la plus fréquente, affectant 4-6 individus par 100,000 habitants à l'échelle mondiale. La maladie se caractérise par une faiblesse et une atrophie musculaire suite à la dégénérescence des neurones du cortex moteur, tronc cérébral et moelle épinière. Les personnes atteintes développent les premiers symptômes à l'âge adulte et la maladie progresse sur une période de trois à cinq ans. Il a été répertorié qu'environ 10% des patients ont une histoire familiale de SLA; 90% des gens affectés le sont donc de façon sporadique. La découverte il y a 19 ans de mutations dans le gène zinc/copper superoxide dismutase (*SOD1*), présentes dans 15-20% des cas familiaux de SLA et environ 2% du total des individus affectés, a été l'événement déclencheur pour la découverte de variations génétiques responsables de la maladie. La recherche sur la génétique de la SLA a connu une progression rapide ces quatre dernières années avec l'identification de mutations dans de nouveaux gènes. Toutefois, même si certains de ces gènes ont été démontrés comme réellement liés à la maladie, la contribution d'autres gènes demeure incertaine puisque les résultats publiés de ceux-ci n'ont pas, à ce jour, été répliqués. Une portion substantielle de cas reste cependant à être génétiquement expliquée, et aucun traitement à ce jour n'a été démontré comme étant efficace pour remédier, atténuer ou prévenir la maladie.

Le but du projet de recherche de doctorat était d'identifier de nouveaux gènes mutés dans la SLA, tout en évaluant la contribution de gènes nouvellement identifiés chez une importante cohorte multiethnique de cas familiaux et sporadiques. Les résultats présentés sont organisés en trois sections différentes. Dans un premier temps, la contribution de mutations présentes dans le gène *FUS* est évaluée chez les patients familiaux, sporadiques et juvéniles de SLA. Précisément, de nouvelles mutations sont rapportées et la proportion de mutations retrouvées chez les cas familiaux et sporadiques de SLA est évaluée. De plus,

une nouvelle mutation est rapportée dans un cas juvénile de SLA; cette étude de cas est discutée. Dans un deuxième temps, de nouvelles avenues génétiques sont explorées concernant le gène *SOD1*. En effet, une nouvelle mutation complexe est rapportée chez une famille française de SLA. De plus, la possibilité qu'une mutation présente dans un autre gène impliqué dans la SLA ait un impact sur l'épissage du gène *SOD1* est évaluée. Finalement, la dernière section explique la contribution de nouveaux gènes candidats chez les patients atteints de SLA. Spécifiquement, le rôle des gènes *OPTN, SIGMAR1* et *SORT1* dans le phénotype de SLA est évalué.

Il est souhaité que nos résultats combinés avec les récents développements en génétique et biologie moléculaire permettent une meilleure compréhension du mécanisme pathologique responsable de cette terrible maladie tout en guidant le déploiement de thérapies suite à l'identification des cibles appropriées.

Mots-clés : Sclérose latérale amyotrophique, maladie des neurones moteurs, dégénération neuronale, génétique humaine, mutations rares, séquençage de gènes candidats, *SOD1*, *TARDBP*, *FUS, OPTN, SIGMAR1*, *SORT1*.

Abstract

Amyotrophic lateral sclerosis (ALS) is the most common of motor neuron diseases, affecting 4-6 individuals per 100,000 individuals worldwide. ALS is characterized by muscle weakness and atrophy caused by the degeneration of neurons located in the motor cortex, brain stem and spinal cord. This fatal disease generally has an adult onset and progresses over a three to five year period. While 10% of patients affected have a family history of the disease, 90% of cases do not and are considered sporadic. The finding of mutations in the zinc/copper superoxide dismutase gene (*SOD1*) gene 19 years ago in about 15-20% of familial ALS (FALS) patients and approximately 2% of overall cases developed the interest of identifying rare genetics variants causing the disease. The ALS research field experienced a rapid progression during the last four years as mutations in new genes have been identified. While mutations in some of those new genes have been clearly linked to ALS, the role of others is still questionable and so far has not been positively replicated in other populations. Importantly, a significant portion of cases still need to be genetically explained and, unfortunately, there is still no effective treatment to cure, attenuate or prevent the disease.

The aim of this Ph.D research project was to identify new ALS mutated genes while analysing the causative role of other newly identified genes in a large familial and sporadic ALS cohort of different origins. The results presented here are categorized into three different sections. First, the contribution of *FUS* mutations to familial, sporadic and juvenile ALS is analysed. Specifically, new *FUS* mutations are reported in ALS cases and the proportions of variants present in the tested familial and sporadic ALS cohorts are assessed. In addition, a new mutation is reported in a juvenile ALS patient, and this interesting case is discussed. Second, new genetic avenues are explored for the *SOD1* gene. Precisely, a new and complex *SOD1* mutation is reported in a French ALS family.

Moreover, the possibility that other ALS mutated genes influence *SOD1* splicing events is evaluated. Third, the contribution of new candidate genes is evaluated. Precisely, the contribution of *OPTN, SIGMAR1* and *SORT1* genes to the ALS phenotype is assessed.

Hopefully, our different findings combined with recent developments in genetics and molecular biology will permit a better understanding of the pathological mechanisms involved in the disease and will lead to the identification of the right targets in order to develop appropriate therapeutics for ALS patients.

Keywords : Amyotrophic lateral sclerosis, motor neuron disease, neurodegeneration, human genetics, rare mutations, candidate genes sequencing, *SOD1*, *TARDBP*, *FUS, OPTN, SIGMAR1* et *SORT1*.

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Nucleotides:

A	Adenine
G	Guanine
T	Thymine
C	Cytosine

Amino acids:

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamine acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine
X	Stop

Abbreviations :

aa	amino acids
AAS	Anabolic/androgenic steroids
AD	Alzheimer's Disease
AFM	Association Française contre les Myopathies
ALS	Amyotrophic Lateral Sclerosis
ANG	Angiogenin
AOA2	Ataxia-Ocular Apraxia-2
ASSP	Alternative Splice Site Predictor
ATXN2	Ataxin 2
Aut/schizo	Autistic/schizophrenic
BDGP	Berkeley Drosophila Genome Project
BMAA	Beta-N-Methylamino-L-alanine
bp	base pair
c.	coding
C9ORF72	Chromosome 9 Open Reading Frame 72
cDNA	complementary deoxyribonucleic acid
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CHMP2B	Chromatin-Modifying Protein 2B
CHOP	DNA damage-inducible transcript 3
chr.	chromosome
CIHR	Canadian Institute of Health Research
cM	centiMorgan
CSF	Cerebrospinal Fluid
CMAP	Compound Muscle Action Potential
CRH	Corticotropin Releasing Hormone
c-terminal	carboxyl-terminal
dbSNP	Single nucleotide polymorphism database
DCTN	Dynactin

del	deletion
DNA	Deoxyribonucleic acid
DPP6	Dipeptidyl-Peptidase 6
EEG	Electroencephalography
ELF-MF	Extremely Low Frequency Magnetic Fields
ELP3	Elongation Protein 3
EMF	Electromagnetic Fields
EMG	Electromyography
ER	Endoplasmic Reticulum
F	Female
FALS	Familial Amyotrophic Lateral Sclerosis
FGGY	FGGY carbohydrate kinase domain
FIG4	SAC1 lipid phosphatase domain
FRDA	Friedreich Ataxia
FRSQ	Fonds de la Recherche en Santé du Québec
FTD	Frontotemporal Dementia
FTLD	Frontotemporal Lobar Degeneration
FUS	Fused in Sarcoma
FUS/TLS	Fused in Sarcoma/Translocated in Liposarcoma
GRN	Granulin
GWAS	Whole Genome Association Studies
HD	Huntington's Disease
HGMD	Human Gene Mutation Database
hnRNPs	Heterogeneous ribonucleoproteins
HPA	Hypothalamic-Pituitary-Adrenal
HRE	Hypoxia-Response Element
HSP	Hereditary Spastic Paraplegia
IAHSP	Infantile onset Ascending Hereditary Spastic Paraplegia
IBMPFD	Inclusion Body Myopathy with Paget's Disease of Bone

ins	insertion
ITPR2	Inositol 1,4,5-triphosphate receptor type 2
JALS	Juvenile Amyotrophic Lateral Sclerosis
JPLS	Juvenile Primary Lateral Sclerosis
Kg	Kilogram
LMN	Lower Motor Neurons
LOD	Logarithm of odds
LOH	Loss-of-heterozygosity
M	Male
Mb	Megabase
MND	Motor Neuron Disease
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
n.	number
NCBI	National Center of Biotechnology Information
Nd1-L	Actin-stabilizing protein
n-terminal	amino-terminal
NTG	Normal Tension Glaucoma
OP	Organophosphate
OPTN	Optineurin
p.	protein
PBP	Progressive Bulbar Palsy
PCR	Polymerase Chain Reaction
PD	Parkinson’s Disease
PDB	Paget’s Disease of Bone
PDC	Parkinsonism-Dementia Complex
PGRN	Progranulin
PLS	Primary Lateral Sclerosis
PMA	Progressive Muscular Atrophy

POAG	Primary Open-Angle Glaucoma
RefSeq	Reference Sequence
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RRM	RNA-Recognition Motif
SALS	Sporadic Amyotrophic Lateral Sclerosis
SBMA	Spinal Bulbar Muscular Atrophy
SE	Selenium
SETX	Senataxin
SIFT	Sorting Intolerant From Tolerant
SIGMAR1	Sigma Nonopioid Intracellular Receptor 1
SLA	Sclérose Latérale Amyotrophique
SMA	Spinal Muscular Atrophy
SNAP	Sensory Nerve Action Potential
SNP	Single Nucleotide Polymorphism
SOD1	Superoxide Dismutase 1
SORT1	Sortilin Transcription Factor Binding Site 1
SPG11	Spastic Paraplegia 11
TARDBP	TAR DNA Binding Protein
TDP-43	TAR DNA Binding Protein 43
UBQLN2	Ubiquilin 2
UCSC	Universiy of California at Santa Cruz
UK	United Kingdom
UMN	Upper Motor Neurons
UNC13	Unc-13 homolog A
US	United States
UTR	Untranslated Region
VAPB	VAMP-associated protein type B
VCP	Valosin-Containing Protein

VEGF	Vascular Endothelial Growth Factor A
wt	wild type

I dedicate this work to my two little angels, Victoria and Angelic, and to my precious husband Luc. Thank you for your understanding, patience, love and support along this demanding journey. I love you with all my heart.

"On the mountains of truth you can never climb in vain: either you will reach a point higher up today, or you will be training your powers so that you will be able to climb higher tomorrow."
Friedrich Nietzsche

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Chapter 1 : Introduction

1.1 Physiology, clinical manifestations, and epidemiology of amyotrophic lateral sclerosis

1.1.1 Physiology

Amyotrophic lateral sclerosis, commonly named Lou Gehrig's disease or Charcot's disease is also called motor neuron disease (MND) in the United States, which actually refers to a larger spectrum of heterogeneous diseases affecting the motor neurons. Specifically, motor neuron diseases include classic amyotrophic lateral sclerosis (ALS), progressive bulbar palsy (PBP), spinal bulbar muscular atrophy (SBMA), progressive muscular atrophy (PMA), spinal muscular atrophy (SMA), hereditary spastic paraplegia (HSP) and primary lateral sclerosis (PLS). These seven disorders are all characterized by a progressive degeneration of motor neurons but differ in terms of where the neuronal death takes place. PBP, SMA, SBMA, and PMA result from the death of lower motor neurons (LMN) while HSP and PLS are explained predominantly by the degeneration of upper motor neurons (UMN). ALS affects both the UMN and LMN of the corticospinal tract located in the motor cortex, brainstem and anterior horn of the spinal cord. Specifically, neuronal degeneration in the cortex deprived the LMN located in the brain stem and spinal cord to receive executive command from the brain, while neuronal death in the anterior horn of the spinal cord causes denervation of skeletal muscles. The term amyotrophy in ALS actually refers to the atrophy of the denervated muscles. As the motor neurons located in the anterior and lateral corticospinal tract degenerate in patients, they are replaced by gliosis. The terms lateral sclerosis refer to the hardening of this region because of gliosis.[1]

The first symptoms of motor neuron degeneration in ALS patients are usually experienced during mid-adulthood and progresses very rapidly, usually on a 3 to 5 years period. Affected individuals finally deceased following the denervation of respiratory muscles.

The cause of neuronal death in ALS is currently unknown, and no effective treatment to prevent, slow down or stop neurodegeneration exists to date. Riluzole is the only treatment that brings a small beneficial effect, slightly extending the life of patients of two to three months.[2] Significant efforts have been deployed for decades worldwide to better understand the pathological mechanisms involved in ALS and eventually develop therapeutics. Noteworthy, findings about the pathology of other neurodegenerative diseases contributed to the identification of common molecular events between different but somehow related neuronal diseases. Specifically, various neurodegenerative disorders have been associated to the pathological accumulation of misfolded proteins in neurons and glial cells, specific aggregation protein components being a characteristic of different neurological conditions. Importantly, aggregations of TDP-43, FUS or SOD1 proteins are detected immunohistochemically in neurons of ALS patients. Aggregations containing one of these proteins are found exclusively without the presence of the other two, dividing the molecular pathology of ALS into three distinctive groups.[3-10] A common view is that misfolded endogenous proteins form inclusions then aggregates, this way initiating the disease and contributing to its progression by the acquisition of a toxic gain of function. Specifically, these new acquired properties include increasing hydrophobicity and/or sequestration of important cellular components into the aggregates, inhibiting proteasomes, generating oxidative species, and/or influencing other pathways.[11] Another possibility is that the functional non-misfolded portion of the protein is not sufficient to efficiently perform its role in the neuronal cell, the misfolded protein not being able to perform its function. This loss of function also prevents other proteins to perform their roles after being recruited into the aggregates. One last possibility is that toxicity of aggregates do not

initiate the disease per say but is actually a defensive response from the cell to protect itself following another unknown toxic event.[12]

1.1.2 Clinical manifestations

Patients experiencing UMN degeneration develop muscle weakness and spasticity, hyperreflexia and pseudobulbar palsy, while LMN death is characterized by muscle weakness and atrophy, cramps, hyporeflexia and fasciculations.[13] A progressive spreading of symptoms must be observed within the same and/or other regions of the body, and the ALS diagnosis is made only after the certitude that the UMN are involved along with LMN. However, early diagnosis could be difficult since patients often have symptoms overlapping with other MND at the first stages of the disease.[14] Also, in some patients, either UMN or LMN are predominantly involved throughout the disease progression, making the diagnosis even more difficult. Patients are usually classified into four different categories using the El Escorial criteria: suspected, possible, probable, or definite ALS.[15]

ALS diagnosis is usually made by excluding all other neurological disorders after obtaining evidence from electrophysiological, imaging, cerebrospinal fluid, or serological studies[16]. Specifically, patients undergo complete neurological, motor, cranial nerves, sensory and cerebellar examinations, cervical and spinal magnetic resonance imaging (MRI), electromyography (EMG), lumbar puncture, and toxicology/biochemical blood screenings. Indeed, cortical morphology analyses revealed specific cortical thinning in the precentral gyrus of ALS patients in addition to relative thinning in the temporal regions of individuals experiencing a rapid progression of the disease.[17] Consistent white matter reduction is also recorded in the corpus callosum while grey matter reduction is specifically observed in primary and supplementary motor areas, as well as in the anterior cingulate and temporal lobe regions of ALS patients.[18] Electrophysiologic studies evaluate muscles

denervation and motor nerve conduction using compound muscle action potential (CMAP) and sensory conduction velocities using sensory nerve action potential (SNAP). ALS patients show muscles denervation while their sensory functions are mainly intact. A complete physical examination is needed to evaluate the atrophy, spasticity and weakness of muscles in limbs asymmetrically/unilaterally or symmetrically/bilaterally. Precisely, muscle weakness is first experienced in the lower or upper limbs in about 70-75% of affected individuals, making spinal onset the most common form of ALS onset. About 20-25% of cases develop the first symptoms in the bulbar region, while approximately 3% of patients experience a respiratory onset. Presence of hyperreflexes is also investigated, as well as the existence of fasciculations, clonus and Babinski signs, and absence of Hoffman signs. Considering that up to 50% of ALS cases have some cognitive deficits,[19] the cognitive functions of patients are also assessed by an extended neuropsychological examination. The progression of symptoms is regularly evaluated by clinicians, monitoring drastic weight loss because of muscular atrophy, and difficulty eating after the development of dysphagia and dysarthria.

Detailed information is collected concerning the health status of family members constituting previous, current and following generations. It is estimated that 90% of ALS patients do not report any previous familial history of motor neuron symptoms, the disease mainly affecting people in a sporadic way. The remaining 10% of patients do have additional family members with similar symptoms or with other related neurodegenerative diseases. While ALS is an adult-onset disease, some ALS families have been reported with other affected members developing the disease before 25 years of age, which is considered a juvenile form of amyotrophic lateral sclerosis (JALS).[20] Also, other families were reported with members affected with dementia such as Alzheimer's disease (AD) or frontotemporal dementia (FTD),[21] or members developing Parkinsonism or Parkinson's diseases (PD)[22]. While some studies reported that neurodegenerative disease aggregates within ALS kindred,[22, 23] it was recently demonstrated after a large prospective population-

based study in the Netherlands that familial aggregation of ALS, dementia and PD is significantly lower than previously assumed.[24]

Familial and sporadic ALS patients are almost undistinguishable based on clinical manifestations.[25] The only two variations that exist concern the age of onset and the sex distribution. Familial amyotrophic lateral sclerosis (FALS) tends to develop ten years earlier than sporadic amyotrophic lateral sclerosis (SALS), with a mean age of onset around 45 years. Also, sporadically affected males tend to be slightly more predominant, with a ratio of 1.3-1.6:1 female,[25] while males and females are equally affected in ALS families.

1.1.3 Epidemiology

ALS is the most common of MND worldwide. It newly affects about 1-2 individuals per 100,000 inhabitants each year, approximating its prevalence to 4-6 cases per 100,000 individuals.[26, 27] While it is perceived that the incidence of ALS tend to increase and major fluctuations have been reported both in men and women, no consistent trend was reported through a decade.[28] In addition, it was demonstrated that the apparent incidence increase is explained by growing ascertainment cases because of better ALS diagnosis.[29, 30] Change in the reported incidence could also be explained by the increasing age of the general population because of longer lifespan expectation, this way increasing the mortality frequency from ALS, especially in women.[31, 32] While the mean age of onset is 45 years of age for the familial cases and 55 years of age for the sporadic cases, a study using a large sample of affected individuals confirmed an increased incidence in the 60 to 69 year age group.[30] Precisely, ALS is uncommon in individuals under 30 years old, but significantly more common among people in their 50s, with a sharp increase until the seventh decade. Considering that the mean age of onset peaks in mid-adulthood but very young or very old

onsets have been reported, it cannot be concluded that ALS is an age or aging related disorder.

The struggle to diagnose ALS was explained in the previous section, raising the difficulty to accurately estimate incidence, specifically in certain age group. It is particularly true for the elderly developing motor neuron symptoms, considering the difficulty to differentiate ALS from other various comorbidities affecting muscle strength, motion and cognitive functions often seen in this age group. Indeed, musculoskeletal pain and fatigue are the most frequently reported symptoms by older adults,[33] and ALS tends to be more frequently misdiagnosed among patients over 60 years old, especially those living in large cities.[34] Importantly, elderly are also less likely to encounter neurological services, and delivery of routine medical services is not optimal for this age group.[35]

Another changing issue is the male to female ratio of affected individuals. Past reports estimated the ratio to be 2.6:1 in the 60s and 70s but recently decreased to 1.1:1 in the 90s.[30, 36, 37] This can be explained by the higher incidence of women seeking medical advice and receiving diagnosis[38] or by the higher lifespan expectancy of women, which increases their probability to eventually develop the disease. Another possibility is the changing lifestyle of women because of the socioeconomic modifications of the last century, which has become more comparable to men's lifestyle. Women are increasingly exposed to occupational and environmental risk factors, which may explain the decrease in the male to female ratio. Another interesting report described an increased risk for ALS among smokers,[39] especially females,[40] underlying another important contributing factor.

While it is commonly stated that the mean disease duration range between three to five years, precisely 70-80% of cases decease within five years.[41] The remaining 20-30% experiences an extremely fast or an unusually slow progression of symptoms. A marked variation in terms of disease progression during the first three years after onset has been reported, suggesting a wider progression spectrum of the disease. Again, problems with the differential diagnosis between ALS and other motor neuron disorders might in part explain this variation. An important contributing factor is the site of onset of the first symptoms, with bulbar and respiratory onsets usually ending in shorter disease duration since respiratory muscles are affected more quickly. Another explanation is the contribution of modifier genes, influencing the resulting phenotype in each patient by accelerating or reducing its progression rate. Finally, certain essential motifs in a given protein known to cause ALS might be prone to accelerate neuronal degeneration when mutated if the genetic change is translated into a non-functional protein. It is also possible that other mutations located in the same gene might only partially deprive the cell of the protein function, resulting in a slower disease progression. The only variables that have been clearly shown to independently predict ALS outcome are age at the onset,[29, 42-46] site of onset,[42, 45-49] and speed of symptoms progression.[47, 50] In summary, increased survival was predicted by a younger age of onset, a spinal onset, less severe symptoms at the time of the first visit, and a body mass index loss lower than 5%.[46] Another study predicted a longer survival for patients with the predominant involvement of upper motor neuron.[51]

1.2 Etiology of amyotrophic lateral sclerosis

1.2.1 Genetic component to ALS: familial versus sporadic ALS

Familial cases of ALS represent 10% of overall cases. [25] This estimation is based on the analysis of a few large pedigrees with a Mendelian autosomal dominant inheritance and a complete penetrance. The remaining portion of patients is considered to be affected

sporadically, with no previous reports of motor neuron symptoms in other family members. This classification is indeed made by default, and some proposed to name sporadic ALS isolated ALS. In fact, it could be difficult to obtain accurate or complete information about other kindred who are sometimes affected with another neurodegenerative disorder which is often seen in ALS families. Apparent sporadic onset can be found in very small ALS families, the number of chances to develop the disease for other members is this way reduced by the family size. Also sometimes a patient has lost contact with some family members and is not aware of the health status of those members. Also, some siblings of apparent sporadic cases are reluctant to report symptoms, are sometimes misdiagnosed, or deceased before developing the first ALS signs, the sporadic patient actually being part of a familial syndrome. Adoption or illegitimacy could also shuffle the cards. Recessive form of the disease, ALS onset in children before the onset of symptoms in one of their parent who is a mutation carrier, or incomplete penetrance in families can also lead to a sporadic categorization of cases.[52]

This being said, the active investigation of genealogies actually evaluated the prevalence of FALS to 17-23%.[53-56] Considering that FALS cases actually represent about 20% of overall cases, that the clinical manifestation of SALS and FALS is almost undistinguishable, and that mutations identified in FALS have also been found in SALS cases this way confirming the contribution of genetics to sporadic ALS, it is clear that the identification of FALS genes would extend our understanding of the ALS pathology affecting both sporadic and familial cases. Moreover, recently, one group assessed the relative risk for ALS in families counting more than 6,000 Swedish individuals who have been first classified as sporadic ALS patients. They also evaluated the concurrence of ALS in more than 86,000 Swedish twin pairs. They reported a significant higher risk for siblings or children of ALS patients to develop the disease, and concluded that a major genetic role contributes to familial ALS.[57] Based on these assumptions, it is reasonable to claim that most, if not all ALS cases can be explained by genetic predispositions. Consequently,

significant progress in the genetics of ALS has been made during the last two decades. Precisely, mutations in 13 different ALS genes have been identified among 15 different ALS loci, and two different ALS-FTD genes have been identified among three different ALS-FTD loci (see table I). These are described thoroughly in the next section.

1.2.2 Genetic component to ALS: loci and genes identified

1.2.2.1 ALS1: *SOD1*

Section of the editorial entitled: *SOD1 mutations : more to learn*. To be published in the Canadian Journal of Neurological Sciences, March 2012,39 :2.

In 1989, genetic analysis using 150 families with classical ALS helped identify two regions of possible linkage on chromosomes 11 and 21.[58] Further evidence of linkage on chromosome 21q22.1-q22.2 was published in 1991, this way identifying the first ALS locus currently known as ALS1.[59] In 1993, an international consortium reported 11 different *SOD1* missense mutations in 13 out of 18 dominantly inherited ALS families.[60] Since then, about 168 disease-causing mutations (Human Gene Mutation Database: http://www.hgmd.org),[61] 87% of which are nucleotide substitutions, have been identified among the 153 amino acids of this five-exon gene. The remaining 13% of mutations are deletions, nonsense or splicing mutations which affect the length of the protein. [61-64] Even if most *SOD1* mutations are transmitted in an autosomal dominant manner, a few families have been reported with a reduced penetrance or a recessive transmission among members.[65] Moreover, compound heterozygotes have also been reported, with two different heterozygote *SOD1* mutations in the same patient.[66] An intriguing report recently described a patient affected with both familial ALS and cerebellar ataxia and an *SOD1* mutation.[67] Rare *SOD1* mutated cases with frontotemporal dementia (FTD), cognitive impairment, and

autonomic dysfunction have also been reported.[68-70] Interestingly, some substitutions affecting particular *SOD1* amino acids are associated with a slow disease progression, while other amino acid substitutions sometimes located in the same region of the gene, are found in patients with a very fast progression.[64, 71, 72] The effect on rate of progression is thought to be related to the fact that some mutants lead to a stable protein while others are highly unstable.[55, 64, 73-75] Approximately 42 mutations have been reported in SALS cases, representing 25% of the total *SOD1* variation and about 1% of SALS cases. Precisely, 15-20% of familial cases result from *SOD1* mutations, hence variations in this gene explain approximately 1-2% of overall cases.[60]

The superoxide dismutase 1 (SOD1) protein is ubiquitously expressed and is mainly located in the cytosol of cells, catalyzing the reduction of the superoxide anion to O_2 and H_2O. Most mutations reduce dismutation,[76] but some have normal or only slightly reduced dismutase activity.[73, 74] Based on the dominant inheritance and the fact that *SOD1* knockout mice have no motor neuron phenotype while overexpression of mutant *SOD1* does,[77, 78] it is agreed that mutant *SOD1* acquires a novel cytotoxic function which promotes neurodegeneration. This toxic gain-of-function has been proposed to involve different mechanisms including protein aggregation and misfolding, oxidative stress, mitochondrial dysfunction, microglia activation, glutamate excitotoxicity, and defects in axonal transport.[79] Specifically, the presence of protein misfolding and aggregation is a recurrent observation in cells of ALS patients, which may inactivate or impair normal processes such as proteasomal degradation or chaperone function.[80] Some observations also suggested that *SOD1* might be involved in RNA processing after the finding that mutant *SOD1* impaired the post-transcriptional processing of *VEGF* mRNA, which encodes an important neuroprotective factor, this way provoking a significant decline in *VEGF* expression.[81] In addition, SOD1 toxicity has been found to modify wild type (wt) SOD1 by inducing it to misfold.[82] Moreover, it was demonstrated that proximity to mutant SOD1 in non-neuronal cells such as microglia and astrocytes is necessary for the toxicity of neighboring motor

neurons.[83] Though extensive research has been conducted to understand the specific pathways involved, it is still unclear how mutant *SOD1* leads to the ALS phenotype.

1.2.2.2 ALS2: *ALSIN*

In 1994, a locus on chromosome 2q33-q35 was reported after a linkage analysis using a large ALS family from Tunisia.[84] In 2001, two different homozygous deletions in the *ALS2* gene causing a loss-of-function of its encoded protein were identified in one amyotrophic lateral sclerosis and two primary lateral sclerosis autosomal recessive families with members experiencing a juvenile onset of the diseases with a slow progression.[85, 86] While no *ALS2* mutations were reported in adult-onset typical ALS, variants were observed in infantile–onset ascending spastic paralysis (IAHSP).[87] The 34-exon *ALS2* gene encodes the GTPase regulator alsin, which plays a role in intracellular endosomal trafficking. [88]

1.2.2.3 ALS3: chromosome 18q21

A locus on chromosome 18q21 was identified in 2002 after performing a genome scan using a large European family with 20 members affected with classical ALS.[89] The disease was transmitted in an autosomal dominant fashion, and all affected members developed typical ALS. While a maximum lod score of 4.5 was obtained, no causative mutations in the ALS3 region have been identified to date.

1.2.2.4 ALS4: *SETX*

A locus on chromosome 9q34 was identified in 1998 and was named ALS4.[90] After testing 19 genes in the region, *SETX* autosomal dominant mutations have been identified in juvenile FALS and SALS patients experiencing a slow progression of the disease without

the involvement of bulbar and respiratory muscles.[91] Interestingly, autosomal recessive mutations have also been identified in patients with spinocerebellar ataxia, specifically with ataxia-ocular apraxia 2 (AOA2), ataxia with elevated levels of alpha-fetoprotein, distal amyotrophy, and peripheral neuropathy.[92, 93] In fact, it is believed that this mutated type of recessive ALS is an intermediate form of motor neuron disease, standing between ALS and spastic paraplegia, while involving lower limbs and excluding the bulbar region. No mutations were reported in adult-onset ALS. The *SETX* gene encodes for the senataxin protein that contains a DNA/RNA helicase domain in its c-terminal, suggesting a role in DNA repair and RNA processing.[91] It was also suggested that the gene play a role in the coordination of transcriptional events.[94]

1.2.2.5 ALS5: *SPG11*

Mapping of the ALS5 locus to chromosome 15q15.1-q21.1 was obtained in 1998 using five families from Europe and North Africa with affected members developing typical ALS at an earlier age of onset. This form of juvenile ALS was believed to be the most prevalent form of recessive ALS.[95] *SPG11* (*spatacsin*) mutations were first identified in 2010 in juvenile ALS patients characterized by a long-term survival.[96] Compound heterozygous deletions were recently identified by our group after the whole exome sequencing of two affected family members with a recessively inherited juvenile motor neuron disease.[73] Spatacsin is a transmembrane protein ubiquitously expressed in the nervous system which is phosphorylated upon DNA damage. While mutations in the *SPG11* gene were previously involved in spastic paraplegia,[97] it is interesting to note that, in this study, one affected family member displayed atypical juvenile ALS and the other developed classical hereditary spastic paraplegia (HSP), this intra-familial phenotypic heterogeneity reinforcing the idea that motor neuron diseases are part of a continuum.

1.2.2.6 ALS6: *FUS*

In 2003, three ALS families linked to chromosome 16q12 were published by three different groups. [98-100] The discovery of mutations in a DNA/RNA binding protein in 2008[101, 102] prompted geneticists to look for mutations in genes encoding proteins having similar functions. Consequently in 2009, two groups reported mutations in the *FUS* gene located on chromosome 16p11.2 which encodes another DNA/RNA binding protein, this way elucidating the ALS6 locus.[6, 103] This discovery was made after the identification of a family of Cape Verdean origin with a possible recessive inheritance pattern. A cluster was identified on chromosome 16, and sequencing revealed a homozygous missense mutation (H517Q) in exon 15 of the *FUS* gene in all affected members of this family. Additional screening identified 13 other dominant mutations in 24 different families. No *FUS* mutations were found in sporadic ALS cases, but later reports established the frequency of *FUS* mutations to be present in about 4% of familial cases, 1% of sporadic patients, and less than 5% of overall cases.[62, 104] More than 52 different mutations have been identified so far in this 15 exons gene (Human Gene Mutation Database: http://www.hgmd.org).[61] Interestingly, almost all mutations are clustered in the c-terminal of the protein, mostly lying in the final 17 amino acids of FUS. While the associated phenotype is typical ALS, mutations in juvenile ALS patients as well as in FTD and essential tremor cases have been reported.[64, 71, 104, 105] ALS patients with *FUS* mutations seem to develop the first symptoms earlier, have a higher rate of bulbar onset, and experience a more rapid progression when compared to patients with *SOD1* mutations.[71]

The fused in sarcoma (FUS) protein is ubiquitously expressed and is predominantly located in the nucleus of cells. However, FUS immunoreactive inclusions have been detected in the neuronal and glial nuclei and cytoplasms of patients affected not only with ALS, but also with FTLD as well as Huntington's, Alzheimer's, and Parkinson's disease,[106]

this observation defining a new proteinopathy in neurodegeneration. The spectrum of FUS RNA targets still has to be defined in order to establish the normal function of the protein.

1.2.2.7 ALS7: chromosome 20p13

Linkage to chromosome 20 was established in 2003 using one typical ALS family with a dominant mode of inheritance from the Boston area.[100] The best LOD score was obtained using markers on the distal short arm of the chromosome. However, the linkage was not reproduced with any other ALS families, and was obtained after genotyping only two affected individuals of the same generation. The ALS7 locus was claimed to be probable but less secure at the time of publication, and the gene have not yet been identified.

1.2.2.8 ALS8: *VAPB*

A locus on chromosome 20q13, now known as ALS8, was identified using eight different families from Brazil.[75] Founder studies demonstrated a common Portuguese ancestor to all families. The dominant P56S missense mutation in the *VAPB* gene was identified in all affected members,[76] which has been demonstrated to induce the formation of insoluble cytoplasmic aggregates of the mutant protein. Interestingly, the same mutation gave rise not only to ALS, but also alternate phenotypes including late-onset spinal muscular atrophy, progressive bulbar palsy, and progressive muscular atrophy. [76] The same mutation has also been identified in other ALS patients of different origins including German, Japanese and American, but surprisingly has not been found in Portuguese.[62, 79, 107, 108] Only one other mutation in the gene has been reported to date to cause ALS. [78] The VAMP-associated protein type B participates in intracellular transport and is mainly located in the endoplasmic reticulum.

1.2.2.9 ALS9: *ANG*

Linkage analysis using Scottish and Irish families permitted the identification of a region on chromosome 14q11.2, making this locus the ninth to be discovered in ALS.[83, 109] The finding that mutant *SOD1* binds to *VEGF* and alters its expression prompted the screening of candidate genes located in the ALS9 locus sharing the same metabolic pathway. Indeed, to date, about 21 variants have been identified in the *ANG* gene (Human Gene Mutation Database: http://www.hgmd.org).[61] However, only one dominant variant was actually shown to cosegregate with the disease in a unique Dutch family with one member affected not exclusively with ALS, but also with Parkinsonism and FTD.[81] The same variant was identified in French, North American, Irish, Scottish and Swedish patients affected with classical ALS,[62, 94, 110, 111] making it the most common *ANG* mutation reported to date. Functional expression studies demonstrated a loss of angiogenic function of the mutant protein.[110]

1.2.2.10 ALS10: *TARDBP*

Mutations in the *TARDBP* gene[101, 102] were first reported after the discovery that its encoded protein TDP-43 is the principal constituent of neuronal cytoplasmic inclusions in ALS and FTD patients,[112] this way identifying the ALS10 locus on chromosome 1p36.22. More than 49 dominant mutations have been identified so far in adult onset ALS (Human Gene Mutation Database: http://www.hgmd.org),[61] mostly lying in the c-terminal portion of the protein. Mutations have been reported in about 5% of familial ALS cases, 0.5-2% of sporadic patients, and approximately 5% of overall cases.[101, 102, 113, 114] While most *TARDBP* mutated patients are affected with typical ALS, some develop alternate phenotype including FTD, progressive supranuclear palsy, Parkinson's disease and chorea.[67, 93]

The *tar DNA-binding protein* (*TARDBP)* gene encodes a nucleic DNA/RNA binding protein that is redistributed to the cytoplasm of neurons and glial cells when mutated. TDP-43 is involved in DNA/RNA processing, a common function that later guided the screening of the *FUS* gene located in the ALS6 locus. [68-70] TDP-43 immunoreactive inclusions have been observed both in the nucleus and cytoplasm of neurons and glial cells, defining a unique proteinopathy, distinct from the one observed in patients with FUS inclusions.[115] Postranslational alterations of TDP-43 such as hyperphosphorylation, ubiquitination and cleavage have been reported to modify its interaction with other proteins involved in RNA metabolism,[96, 106] hence influencing pre-mRNA splicing, RNA stability and axonal transport.[116] Some of these interactions were found to dependent on TDP-43 RNA-binding, whereas others are RNA-independent. [117] Specifically, some TDP-43 interacting proteins cluster into two different interaction networks: a nuclear or splicing cluster and a cytoplasmic or translation cluster.[117] This suggests that TDP-43 assumes different roles in RNA metabolism, and acts in the nucleus as well as the cytoplasm. Additional TDP-43 RNA targets need to be identified in order to better understand the pathway in which they are involved, as well as to determine their precise role in neurodegeneration.

1.2.2.11 ALS11: *FIG4*

Mutations in the *FIG4* gene located on chromosome 6q21 have been first identified in 2007 in severe cases of Charcot-Marie-Tooth disease characterized by an early onset and involving both sensory and motor neurons.[118] The finding of mutations in patients affected with a disease involving the motor neurons prompted the screening of *FIG4* in ALS and PLS patients. Nonsynonymous variants were found in nine patients out of 473, mutations being present in about 2% of the tested cohort. Among them, seven patients were diagnosed with classical ALS and two had PLS. Six of the dominant variants identified were shown to be deleterious.[119] No other *FIG4* mutations reports have been published since then and

further screening in other populations is needed to properly establish the genetic contribution of *FIG4* to the ALS pathogenesis. FIG4 is a phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) that might play a role in autophagy in the nervous system, while mutations have been proposed to contribute to inclusion body diseases.[120]

1.2.2.12 ALS12: *OPTN*

Homozygosity mapping using six ALS patients from Japanese consanguineous families helped define in 2010 the ALS12 locus on chromosome 10p13. After sequencing 17 candidate genes in the region, variants were identified in *OPTN*,[121] a gene in which mutations were previously associated with primary open-angle glaucoma (POAG).[122] Additional mutations were identified by the same group in familial and sporadic classical ALS cases causing both recessive and dominant traits. Subsequent screening of *OPTN* in patients of European origin did not confirm the genetic implication of *optineurin* in ALS, at least for this specific population,[123, 124] and more reports are needed. Mutated OPTN cytoplasmic distribution has been however demonstrated to differ from wt OPTN, and OPTN-immunoreactive cytoplasmic inclusions have been observed. OPTN have also been demonstrated to be recruited in TDP-43 or SOD1 inclusions.[121]

1.2.2.13 ALS13: *ATXN2*

An extended (CAG)n repeat in *ATXN2* was first reported in 1996 in patients affected with spinocerebellar ataxia-2.[125] While normal chromosomes contain 14 to 31 repeats interrupted by one to three CAA repeats, chromosomes of patients contain a pure stretch of 34 to 57 CAG repeats. The CAG is highly unstable during transmission, and its size is negatively correlated with the age of symptoms onset in 50% of cases.[125] The ALS13 locus on chromosome 12q24.12 was attributed to the *ATXN2* gene in 2010 when it was

discovered that an intermediate repeat length ranging from 27 to 33 glutamines was significantly associated with ALS.[126] Additional reports confirmed the same association, and it was also shown that intermediate repeats were CAA interrupted.[127-130] It was also demonstrated that polyQ expansions in *ATXN2* enhance its interaction with TDP-43, and that both *ATXN2* and TDP-43 relocalize to stress granules after oxidative stress. [126] Ataxin-2 is primarily located in the Golgi apparatus. While expression of full-length extended ataxin-2 disrupted the normal morphology of the Golgi complex,[131] it is still unclear how intermediate repeat length influences the risk to develop ALS.

1.2.2.14 ALS14: *VCP*

In 2004, missense mutations were found in the *VCP* gene located on chromosome 9p13.3 in patients affected with FTD or inclusion body myopathy with Paget disease of bone (IBMPFD).[132] The valosin-containing protein (VCP) is associated with a spectrum of essential cell protein pathways including cell cycle, homotypic membrane fusion, nuclear envelope reconstruction, postmitotic Golgi reassembly, DNA damage response, suppression of apoptosis, and ubiquitin-dependent protein degradation.[132] In 2010, exome sequencing revealed a heterozygous mutation as a cause of adult-onset ALS with or without FTD in an Italian family,[133] this way identifying the 14th locus for the disease. While a few additional mutations were reported in FALS and SALS cases,[133-136] none were found in the Australian ALS population.[137] It can be concluded that mutations in *VCP* is not a common cause of ALS.

1.2.2.15 ALS15: *UBQLN2*

Linkage analysis using a large ALS family composed of 19 affected members with either a juvenile or an adult disease onset permitted the identification of the ALS15 locus

on chromosome Xp11.21.[20] The disease was transmitted in a dominant fashion among the family members, with a reduced penetrance in females and no evidence of male-to-male transmission. Therefore, 41 candidate genes out of 191 coding genes in the region were sequenced, and a single cosegregating mutation was identified in the *UBQLN2* gene. Additional FALS or FALS-FTD cases were screened for mutation in this intronless gene, and four novel mutations were identified. The five missenses found by this group all affected proline residues located in a single PXX repeat region. Ubiquilin-2 was shown to be present not only in inclusions located in the hippocampus and pyramidal neurons, but also to be involved in inclusion formation in X-linked ALS.[20] These observations are critical, considering that protein inclusions are a persistent landmark in neurodegenerative diseases, and especially in ALS and FTD. Further mutation reports are however needed.

1.2.2.16 ALS-FTD: *CHMP2B*

Linkage to chromosome 3p11.2 was established using a large Danish family with autosomal dominant FTD.[138, 139] In 2005, the mutation responsible for the disease in this family was reported to be located in the *CHMP2B* gene. The chromatin modifying protein 2B (CHMP2B) is part of an endosomal secretory complex which is believe to participate in endosomal trafficking.[140] Another variant in the same gene was additionally found in an unrelated individual with unspecific dementia.[141] While two different groups did not find any *CHMP2B* causative mutations in their tested FTD cohort,[142, 143] two other groups found one truncated mutation in one autosomal dominant FTD patient and one missense mutation in an ALS patient.[144, 145] Mutations in the *CHMP2B* gene are found rarely in ALS or FTD patients, and its causative role in neurodegeneration is questionable.

1.2.2.17 ALS-FTD: chromosome 9q21-q22

Genomic screening was first conducted using 16 families from the Boston data set with members affected with either dominant ALS or ALS-FTD, which permitted the identification of a locus on chromosome 9q21-22. Subsequent analysis was performed to independently confirm the region involved by genotyping four other families from the Chicago data set with members affected with ALS and/or FTD.[146] The mutated gene causing the two phenotypes in these families still needs to be identified.

1.2.2.18 ALS-FTD: *C9ORF72*

Three different groups contributed to the identification of a major locus on chromosome 9 by reporting three overlapping loci after conducting linkage analysis using different autosomal dominant families with members affected with ALS and/or FTD along with TDP-43 proteinopathy.[21, 147, 148] Additional reports helped refine the minimum linkage region to 3.7 Mb, containing only five known genes.[149-153] The common coding and non-coding region was extensively sequenced by many for several years, but no causative mutations were identified. Several association studies conducted in the ALS and FTD sporadic population confirmed the implication of the chromosome 9p locus in both diseases.[154-157] Rigorous efforts finally permitted the identification of important heterozygote hexanucleotide repeat expansions in the chromosome 9 open reading frame 72 (*C9ORF72)* gene in three of the reported families linked to the chromosome 9p.21 locus. [158, 159] The gene has three identified transcripts and the expansions are either located in the first intron or the promoter region of the gene. Specifically, the maximum size of the hexanucleotide repeat in control participants was 23 units, while it ranged approximately between 700 and 1600 in patients. The same noncoding but highly conserved GGGGCC expansions were further found in a significant proportion of familial and sporadic ALS and/or FTD cases. Precisely, 11.7% of familial FTD and 23.5% of familial ALS patients were found with the repeat, making this variant the most common genetic abnormality

identified to date for those two familial forms of diseases.[158] In addition, repeat expansions were present in almost one-half of Finnish familial ALS cases and in about one-third of familial ALS cases of European descent.[159] While the C9ORF72 protein is uncharacterized and its domains are unknown, it is expressed in a variety of tissues and is mainly a cytoplasmic and synaptic protein in neurons.[159] Using lymphoblastoid cell lines, it was demonstrated that the hexanucleotide repeat expansions in *C9ORF72* are associated with a decreased expression of C9ORF72 mRNA.[158] The same group observed RNA foci in neuronal tissues of ALS and FTD patients. It is suggested that these expansions interfere with the normal expression of the protein, causing the loss of one alternatively spliced *C9ORF72* transcript and the formation of nuclear RNA foci. It is also suggested that variations in the repeat length influence the resulting expressed phenotype, [158] explaining the development of ALS and FTD in some familial cases, while other family members develop either ALS or FTD. Finally, anticipation might be present in C9ORF72 extended FALS patients, considering that younger members of Finnish ALS-FTD families experienced a very early age of onset. More reports are needed to establish more precisely the proportion of C9ORF72 extended patients.

Table I Summary of ALS associated loci

Name	Locus	Gene	ALS Onset	Inheritance	Alternative phenotypes	References ALS locus reports	References 1st ALS mutation reports
ALS1	21q22.11	*SOD1*	Adult	Dominant Recessive	Cerebellar ataxia Cognitive impairment Frontotemporal dementia Autonomic dysfunction	Siddique,1991	Rosen,1993
ALS2	2q33.1	*ALSIN*	Juvenile	Recessive	Infantile-onset ascending spastic paraplegia Juvenile primary lateral sclerosis	Hentati,1994	Yang,2001, Hadano,2001
ALS3	18q21	Unknown	Adult	Dominant	Unknown	Hand,2002	Unknown
ALS4	9q34.13	*SETX*	Juvenile	Dominant	Ataxia-ocular apraxia 2 Cerebellar ataxia Motor neuropathy	Chance, 1998	Chen, 2004
ALS5	15q21.1	*SPG11*	Juvenile	Recessive	Hereditary spastic paraplegia	Hentati,1998	Orlacchio, 2010
ALS6	16p11.2	*FUS*	Adult Juvenile	Dominant Recessive	Frontotemporal dementia Essential Tremor Parkinsonism	Sapp, 2003 Ruddy, 2003 Abalkhail,2003	Kwiatkowski, 2009 Vance, 2009
ALS7	20p13	Unknown	Adult	Dominant	Unknown	Sapp, 2003	Unknown
ALS8	20q13.32	*VAPB*	Adult	Dominant	Late-onset spinal muscular atrophy Progressive bulbar palsy Progressive muscular atrophy	Nishimura, 2004	Nishimura, 2004
ALS9	14q11.2	*ANG*	Adult	Dominant	Frontotemporal dementia Parkinsonism	Hayward, 1999 Greenway, 2004	Greenway, 2008
ALS10	1p36.22	*TARDBP*	Adult	Dominant	Frontotemporal dementia Progressive supranuclear palsy Parkinson's disease Chorea	None	Sreedharan, 2008 Kabashi, 2008
ALS11	6q21	*FIG4*	Adult	Dominant	Primary lateral sclerosis Cognitive impairment Charcot-Marie-Tooth disease	None	Chow, 2009
ALS12	10p13	*OPTN*	Adult	Dominant Recessive	Primary open-angle glaucoma	Maruyama, 2010	Maruyama, 2010
ALS13	12q24.12	*ATXN2*	Adult	Dominant	Spinocerebellar ataxia 2	None	Elden, 2010
ALS14	9p13.3	*VCP*	Adult	Dominant	Frontotemporal dementia Inclusion body myopathy Early-onset Paget disease	Johnson, 2010	Johnson, 2010
ALS15	Xp11.21	*UBQLN2*	Adult Juvenile	Dominant	Frontotemporal dementia	Deng, 2011	Deng, 2011
ALS-FTD	3p11.2	*CHMP2B*	Adult	Dominant	Frontotemporal dementia	None	Parkinson, 2006
ALS-FTD	9q21-22	Unknown	Adult	Dominant	Frontotemporal dementia	Hosler, 2000	Unknown
ALS-FTD	9p21.2	*C9ORF72*	Adult	Dominant	Frontotemporal dementia	Morita, 2006 Vance, 2006 Valdmanis, 2007	DeJesus-Hernandez,2011 Renton , 2011

1.2.3 Association studies in ALS

Whole genome association studies (GWAS) have been proven to be a powerful tool in genetics after the results obtained from research on different complex diseases. In GWAS, thousands of single nucleotide polymorphisms (SNPs) are genotyped across the genome in a large number of samples without any preconceived assumption about diseases. Many association studies have been conducted in different populations worldwide using sporadic cases of ALS, but not all have been conclusive. The first report failed to significantly identify hits but contributed to future studies by making the SNP data freely available on the internet. [160] Those data were used as a replication set in a follow-up study identifying *FGGY* as a candidate gene for ALS.[160] Another GWAS was conducted by a group from the Netherlands, reporting an association with the *ITPR2* gene.[161] This study also combined the results obtained with the data available on the internet after the first GWAS, and failed to re-associate the *ITPR2* gene with sporadic ALS. A novel association with the *DPP6* gene was however identified,[162] this way replicating an already published Irish study.[163] However, the Nertherlands and Irish groups included data from the same cohorts, which explains the replicated association of the *DPP6* gene. One subsequent study replicated this association in an Italian cohort,[164] while data obtained using samples from Poland, France and Canada did not.[165, 166] Another study attempted to replicate the associations with hits obtained from previous GWAS, but was unable to confirm any conclusive association.[167] Association of the gene *ELP3* to ALS was also reported after performing a GWAS using three different populations.[168] Two susceptibility loci were also identified without replicating previous associated genes or SNPs, including one within the boundaries of the *UNC13A* gene and another on chromosome 9p21.2.[157] The identification of the susceptibility region on chromosome 9p21.2 was the first conclusive data obtained from a GWAS in ALS research, considering that a major ALS-FTD locus was known to exist in the same region after several linkage studies. Subsequently, two additional groups reported an association between sporadic ALS and a region on chromosome 9p21 in the Finnish and British populations.[154, 155]

Unfortunately, GWAS findings in one population are rarely replicated in others, and no genes have been concretely associated to ALS. Intriguingly, genes identified using candidate-based approaches have not been the top hits in GWAS. Indeed, the published associations to chromosome 9p were the only replicated and conclusive GWAS in ALS research. The availability of several thousand cases with definite diagnosis is a key element for GWAS to identify genetic factors responsible for a disease. The high degree of allelic and non-allelic heterogeneity in SALS cases is a limiting factor for the identification of causative genes, considering that different disease-causing alleles may exist within the same gene. Moreover, the identification of rare highly penetrant mutations in genes such as *TARDBP* or *FUS* in SALS patients is problematic given that GWAS can only detect common low penetrant variants. The whole genome or exome sequencing approaches seem to be more suitable for the identification of such novel rare causative variants in ALS, and the results that will be obtained from such approaches in the next few years will hopefully highlight unknown genetic contributions to familial and sporadic ALS.

1.2.4 Environmental component to ALS

The first association of an environmental factor contributing to the emergence of ALS was with the Chamorro indigenous people of Guam, who presented an extremely high incidence of the disease, who consumed a lot of flying fox and consequently accumulated cycad neurotoxins in their organism including beta-N-methylamino-L-alanine (BMAA) shown to be produced across the cyanobacterial order.[169, 170] Later, a report of an ALS cluster near Lake Mascoma associated the increased disease incidence to the presence of cyanobacteria in the surrounding water area.[171] Many in vivo studies using mice, rats, monkeys and chicks demonstrated that exposure to BMAA induce neurodegenerative symptoms.[170] Results obtained from zebrafish also demonstrated a disruption in neuronal

development resulting from BMAA exposure. In vitro studies using rodents also concluded that BMAA predominantly acts on motor neurons by increasing the generation of reactive oxygen species (ROS) and Ca(2+) influx along with disrupting mitochondrial activity. Consequently, neuronal death is believed to result from excitotoxic mechanisms.[170] No other robust association have been reported since then, and the search for other causative evidence connecting environmental risk factors or neurotoxic chemicals to sporadic ALS has not been fruitful. Nevertheless, some epidemiologic trends suggested that several environmental influences might be linked to the ALS etiology. Several environmental toxic exposures are believed to induce the liberation of free radicals, which might lead to oxidative stress. Precisely, free radicals are ROS produced normally in biological systems. Human cells have developed a complex system of defence mechanisms to eliminate excessive ROS accumulation which otherwise has the ability to damage lipids, proteins, and DNA. When cellular antioxidant defences are unable to keep the ROS levels below a toxic threshold because they are overwhelmed with free radicals or they are encoded in insufficient amount, cells experience oxidative stress.[172] A few environmental factors have been proven to increase ROS levels and hence the risk to develop ALS. These include lead, mercury, and selenium exposure, contact with pesticides and insecticides, intense physical activities, head injuries, electromagnetic fields (EMF) exposure and tobacco smoking.[173] These studies are briefly explored in the next section.

1.2.4.1 Toxicity and ALS

Since most ALS cases are sporadic, many studies have been devoted in the last decades to the possible contribution of neurotoxic chemicals to ALS phenotype. Accumulated evidence confirmed the potency of metals to induce toxicity to cells and consequently cause a number of pathologies. Precisely, many metals are able to catalyse the formation of ROS and indeed damage key proteins, leading to protein denaturation and

aggregation as well as to the inability of the ubiquitin/proteasome system to eliminate dysfunctional proteins.[174]

The role of lead among other metals in the ALS pathology is the most studied in the field.[175] Lead is not normally present in the human body and is a known neurotoxicant, not a neuroprotectant.[176] Interestingly, low exposure has been shown to liberate free radicals, to induce oxidative damage to essential biomolecules and to affect antioxidant defence systems of cells.[177] Paradoxically, higher exposure to lead has been associated with an increased survival in humans.[178-180] Further studies are however needed to establish the possible neuroprotective role of lead in ALS.

Neurotoxicity after mercury exposure is also well documented,[181] yet its contribution to ALS has not been clearly demonstrated. Case studies with patients affected with ALS symptoms along with inorganic mercury intoxication have been reported,[182, 183] while retrospective case–control studies failed to report an association between mercury or other heavy metals in the pathogenesis of the disease.[184, 185] Interestingly, G93A-SOD1 mice experienced an early onset of hind limb weakness after a chronic exposure to methylmercury, suggesting that an exposure to a toxic metal could hasten the onset of symptoms in individuals carrying a genetic polymorphism for ALS.[186] Deposition of mercury in motor neurons of the spinal cord, brainstem and cerebral cortex were observed in rodents after being exposed to mercury.[187, 188] Of interest, a cluster of affected cases was reported in a region of Lake Michigan, where residents consumed lots of fish species in which a high level of mercury has been found.[189] These reports propose that mercury exposure might contribute to the etiology of ALS.

Another metalloid associated with ALS is selenium (Se), a trace element having both toxic and nutritional properties in humans. Selenium safe range of exposure based on its biological reactivity is controversial, depending on its organic/inorganic state and oxidation level. However, it seems that its safe range is significantly lower than previously believed.[190] While organic Se is found in food and is believed to be harmless,[191] inorganic Se found in groundwaters is fifty times more toxic.[192] Two independent studies suggested a causative relationship between Se exposure and ALS. Precisely, one group from the US reported a cluster of four individuals (out of 4,000 inhabitant) who developed ALS along with Se intoxicated farm animals in a region affected by naturally occurring selenosis.[193] Another group from Italy also reported four individuals affected with ALS (out of 5,182) in a region where the municipal tap water was taken from two wells high in selenium. A causative relationship was suggested since no other life-style or occupational factors correlated with the disease.[194, 195] One study also documented a correlation between Se contaminated areas in China and the appearance of neurological symptoms in the population, among other consequences.[196] Laboratory models as well as experimental studies demonstrated that the nervous system and more specifically motor functions, are influenced by Se levels.[190] Precisely, it was shown that the toxicity of inorganic Se affects selectively the motor neurons in pigs and cows.[192, 197, 198] An increased level of iron and selenium was also observed in the brain of patients affected with Parkinson's disease.[173]

While associations between elevated ALS risk and lead, mercury and Se toxicity have been suggested in the literature, no clear causative evidence has been published so far. This can be explained by the fact that exposure to certain metals is not sufficient to develop the disease, and individual's genetic makeup is required to induce epigenetic changes that will eventually cause ALS.[199] It is well known that the main unifying mechanisms of action of metals are the interference with cellular reduction/oxidation (redox) regulation and the subsequent induction of oxidative stress.[200] These mechanisms are known to be perturbed

in ALS. However, the clear causative contribution of metalloids to the disease still needs to be demonstrated.

The organophosphate (OP) class of pesticides is extensively used in agricultural and household settings and its role have been investigated as a potential risk factor for ALS,[201] considering that pesticides have been previously associated to other neurodegenerative diseases.[202] It was suggested that SALS patients have an impaired ability to detoxify these pesticides, which could be explained by changes in genes part of the metallothionein or paraoxonase families.[201, 203] While inconsistencies related to OP exposure and mutations in one of the paraoxonase gene have been reported,[204] many studies using human and rodent subjects support this theory.[205-209] An Australian case-control study associated sporadic ALS to solvent and chemical exposure in addition to industrial and overall herbicide or pesticide exposure, showing a dose-response effect.[210] A systematic review of the literature on environmental exposure to chemicals and metals permitted the identification of two well-designed studies reporting a significant association between pesticides exposure and increased ALS risk.[211] However, another large study conducted in the USA did not observe any conclusive association. Nonetheless, this group found a substantially increased risk of the disease in individuals exposed to formaldehyde, and they demonstrated a dose-response relationship with increasing years of exposure.[212]

Another risk factor that has been associated to ALS is cigarette smoking. One study concluded that individuals who have already smoked have a twofold risk to develop the disease, while current smokers have a threefold increased risk. The risk level was positively correlated with smoking duration. While it is possible that the formation of free radicals during the metabolism of one or several of the 3,800 compounds present in cigarettes can induce oxidative stress on neurons, it is also probable that pesticides in tobacco is responsible for this toxicity.[213]

1.2.4.2 Environmental interactions, genes and epigenetics

ALS has been re-named in the USA after the American Major League baseball player Lou Gehrig died from this MND. A substantial increase in disease frequency among soccer and football players has been curiously observed since then.[214, 215] The possible association between physical activity and ALS risk was evaluated, but no significant difference was found among cases and controls. However, patients reported having participated more frequently in organized sports in high school.[216] It was recently demonstrated that anabolic/androgenic steroids (AAS), drugs known to enhance muscle mass that are frequently illegally abused by athletes to increase their performances actually increase the risk for ALS. Precisely, a group used mice overexpressing human mutant *SOD1* at different stages of the disease and after monitoring gastrocnemius muscles, they found that the expression of certain genes associated to muscle atrophy was up-regulated before the symptoms onset. It was concluded that AAS may intensify some of the alterations induced by *SOD1*.[217] In the same line, it was demonstrated that the combination of intense exercise with other factors such as drugs or ischemia caused by head injuries actually increase the production of ROS.[28] It was also reported that victims of head injuries have an increased risk of developing a neurodegenerative disease such as AD and PD.[218, 219] Considering that head injuries are frequent in soccer and football players, it can explain their increased risk to develop ALS. While some studies suggested such association,[220, 221] another demonstrated that head injury is not a significant associated factor.[222]

War veterans can also be victims of head injuries, which could explain the twofold increased risk of developing ALS for Persian Gulf War USA Army and Air Force personnel. [223, 224] Veterans are also known to smoke cigarettes more than the control population, a practice also known to increase the chances to develop the disease.[213] Of interest, a study demonstrated that head injuries alone were associated to an increased risk

to develop ALS in veterans and not smoking.[225] Noteworthy, the veterans affected with ALS developed the first symptoms at a significantly earlier age than expected. [226, 227] Some studies suggested an association between the increased number of affected individuals and exposure to organophosphates, describing ALS veterans with significantly lower serum concentrations of PON1, [228, 229] a member of the paraoxonase family in which changes are known to impair the ability to detoxify this pesticide.[204] Another group proposed that Gulf War veterans have been extensively exposed to cyanobacteria by inhaling cyanotoxins that are carried by dust at specific time of the year during the war.[230] These reports suggest that neurological symptoms in those veterans possibly result from environmental chemical exposures during the war.

The emergent relationship between environmental-induced oxidative stress and epigenetic modifications influencing critical genes is currently at the center of many studies. In fact, it has been well established that epigenetic changes occur due to environmental factors, this way altering genes without any previous DNA sequence variation.[231-233] Specifically, gene expression depends on epigenetic regulations, which are influenced by mechanisms such as chromatin condensation, several histone modifications, and covalent alterations of DNA by methylation.[174] Precisely, the epigenome works as an interface between the inherited genome and the changing environment. While metal exposure can damage neuronal cells through both oxidative and non-oxidative mechanisms such as the formation of DNA adducts, metals can also result in substantial changes in DNA methylation and histone modifications. These changes can lead to epigenetic silencing, modification or reactivation of gene expression.[234] A Swedish study using a large cohort of workers from the engineering industry with a large proportion of resistance welders who were regularly exposed to extremely low frequency magnetic fields (ELF-MF) reported an increased risk for Alzheimer's disease and ALS.[235] However, it has been underlined that further studies are needed, which should consider investigating the separate effect of EMF exposure and electrical shocks to make more accurate interpretations.[236] In

fact, it is possible that exposure to electromagnetic fields modifies DNA methylation and histone structure. More interest has been given to epigenetics in the last decade, and its role in neurodegeneration still has to be clearly defined.

Chapter 2 : Contribution of *FUS* mutations to ALS

2.1. Mutations in *FUS* cause FALS and SALS in French and French Canadian populations. Published in Neurology. October 2009;73(15):1176-9.

2.2. Identification of novel *FUS* mutations in sporadic cases of amyotrophic lateral sclerosis. Published in Amyotrophic Lateral Sclerosis. March 2011;12(2):113-7.

2.3. Identification of a *FUS* splicing mutation in a large family with amyotrophic lateral sclerosis. Published in Journal of Human Genetics. March 2011;56(3);247-9.

2.4. Novel *FUS* deletion in a patient with juvenile amyotrophic lateral sclerosis. Published in Archives of Neurology on January 16, 2012.

2.1 Mutations in *FUS* cause FALS and SALS in French and French Canadian populations

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2.1.1 Rationale

In 2003, three different groups conducted linkage analysis using ALS families from the United Kingdom (UK) and the United States (US), and identified the ALS6 locus on chromosome 16q12.[98-100] The US group later localized a family of Cape Verdean origin with four members affected with ALS. They were informed that the maternal grandparents of the proband were first cousins, which raised the possibility of a recessive inheritance of the disease. They conducted a loss-of-heterozygosity (LOH) mapping and identified a major cluster on the same ALS6 locus, spanning 4Mb and containing 56 genes. They finally found a mutation in exon 15 of *FUS*, the last exon of the gene. Affected individuals were homozygous for the mutation, while unaffected members were heterozygous. Additional screenings of FALS samples helped identify twelve additional dominant *FUS* mutations in 16 different families. Interestingly, the mutations were almost all located in the c-terminal of the protein. However, no mutations were found in 293 sporadic patients.[6] One group from the UK tested their own FALS British samples and identified three different mutations in eight ALS families. The mutations were again localized in the c-terminal of the protein.[103] Overall, the two groups estimated that mutations in *FUS* cause about 5% of familial ALS, while the proportion of sporadic ALS cases mutated in *FUS* still has to be determined.

The main interest in the *FUS* gene was about its molecular functions. In fact, the prominent resemblance between *FUS* and *TARDBP* was demonstrated in a 2009 review.[237] Both have been implicated in various steps of gene expression regulation such as transcription, RNA splicing, RNA transport, and translation.[238, 239] Also, both contain RNA-binding motifs, structurally resemble a family of heterogeneous ribonucleoproteins (hnRNPs), directly bind to RNA in addition to single- and double-stranded DNA, and are implicated in RNA maturation and splicing.[237]

Because of these three publications, we decided to screen for *FUS* mutations a sub-group of our own familial and sporadic ALS cohort. We wanted first to evaluate the proportion of *FUS* mutations in our FALS patients of European origin in order to see if the published results could be replicated in a more heterogeneous population, and second to see if mutations can be found in sporadic patients. Moreover, we noticed that our lab had already screened autistic and schizophrenic patients for *FUS* mutations in the past. We re-analysed these sequences and used them as an unrelated disease-control group for comparison with the ALS group.

2.1.2 Contribution of authors

Belzil & Valdmanis : Study design, data generation and analysis, statistical analysis, manuscript writing.

Daoud & Kabashi : Manuscript revision.

Noreau, Gauthier & Hince : Technical support, manuscript revision.

Desjarlais : Clinical information organization, manuscript revision.

Bouchard, Lacomblez, Salachas, Pradat, Camu, Meininger & Dupré : Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

2.1.3 Abstract

Background: The identification of mutations in *TARDBP* and more recently the identification of mutations in the *FUS* gene as the cause of amyotrophic lateral sclerosis

(ALS) is providing the field with new insight about the mechanisms involved in this severe neurodegenerative disease. Methods: To extend these recent genetic reports, we screened the entire gene in a cohort of 200 ALS patients. An additional 285 patients with sporadic ALS were screened for variants in exon 15 for which mutations were previously reported. Results: In total, three different mutations were identified in four different patients, including one three bp deletion in exon 3 of a SALS patient, and two missense mutations in exon 15 of one FALS and two SALS patients. Conclusions: Our study identified sporadic patients with mutations in the *FUS* gene. The accumulation and description of different genes and mutations definitely helps develop a more comprehensive picture of the genetic events underlying ALS.

2.1.4 Introduction

The profile of genes mutated in amyotrophic lateral sclerosis (ALS) has expanded considerably since 2006. The primary causative gene remains the zinc copper superoxide dismutase gene (*SOD1*) as mutations in *SOD1* explain ~15-20 percent of familial ALS (FALS) cases, which altogether represents ~two percent of the combined sporadic (SALS) and FALS cases.[60] However, several mutations were recently reported in the TAR-DNA binding protein (*TARDBP*) gene encoding the TDP-43 protein at the ALS10 locus.[240] The *TARDBP* mutation search was initiated following the discovery that TDP-43 is a major constituent of aggregates found in patients with ALS or frontotemporal dementia (FTD).[112] Mutations are almost exclusively situated at the glycine-rich C-terminal portion of *TARDBP* in its sixth and last exon. The identification of mutations in *TARDBP* helped convince researchers studying the ALS6 locus on chromosome 16 that the *FUS* gene was a good candidate for harboring mutations in patients with ALS.

The presence of a homozygous mutation in a recessive family with ALS from Cape Verde combined with heterozygous changes in dominant ALS pedigrees that helped map the ALS6 locus led to the conclusion that *FUS* was the causative gene in that region.[6, 103] The product encoded by the *FUS* gene (FUS) has a function similar to that of the TDP-43 protein. It is an RNA-binding protein with hnRNP properties and it has a prior history of involvement in the nervous system: mouse glutamate receptors help regulate the localization of *FUS* to dendritic spines.[241] Moreover, *FUS* helps in actin organization of dendritic spines via mRNA transport of the actin-stabilizing protein Nd1-L.[242] Initially, *FUS*/*TLS* (fusion/translated in liposarcomas) was identified as a t(12;16) translocation product which combines its N-terminal portion with the C-terminal portion of the *CHOP* gene leading to round cell liposarcomas.[243] *FUS* knockout mice have been generated and no neurological defects were reported.[244, 245]

We sought to validate the results recently obtained on chromosome 16 by sequencing the *FUS* gene in a panel of FALS and SALS cases. This led to the identification of two mutations that were previously reported[4] and one novel mutation.

2.1.5 Materials and Methods

2.1.5.1 Standard Protocol Approvals, Registrations, and Patient Consents

Protocols were approved by the ethics committee on human experimentation of the Centre Hospitalier de l'Université de Montréal and the Comite d'Ethique de la Salpetriere. All patients gave written informed consent after which patient information and blood were collected.

2.1.5.2 Subjects

Patients were collected from the province of Quebec, Canada (n = 100) and from France (n = 100) between 2004 and 2009. DNA was extracted from peripheral blood using standard protocols. A total of 80 FALS and 120 SALS patients were screened for the 15 coding exons of the *FUS* gene as well as 190 ethnically matched controls and 285 patients with schizophrenia or autism as part of an unrelated project. An additional cohort of 285 SALS patients were screened for variants in exon 15, considering that most of the mutations already identified are located in the c-terminus of the protein.[4]

2.1.5.3 Gene Screening

Primers were designed using the ExonPrimer software from the UCSC human genome browser website (www.genome.ucsc.edu). Twelve sets of primers were sufficient to cover the 15 exons in *FUS* (NM_004960.2). Primer sequences and amplification conditions are listed in supplementary material table III. PCR products were sequenced at the Genome Quebec Innovation Center. Variants were tested in patients and controls using the same procedure of direct sequencing.

2.1.5.4 Protein sequence alignment

Cluster analysis was performed using the Clustal W method. The closest homologue in several species was retrieved by use of NCBI's BLAST program (supplementary material figure 2).

2.1.5.5 Phosphorylation sites prediction

The phosphorylation site prediction scores corresponding to the deletion p.S57 were obtained using the NetPhos neural network-based method (supplementary material table IV).

2.1.6 Results

The complete sequencing of the *FUS* gene in 200 patients with ALS and the sequencing of exon 15 for an additional 285 SALS cases led to the identification of two missense mutations and one three bp deletion in three SALS and one FALS patients (table II). These mutations were not found neither in 285 patients with schizophrenia or autism which were used as a non-ALS disease cohort, nor in 190 controls matched for age and ethnicity. A three bp heterozygous deletion (c.169_171delTCT, p.S57del) was identified in a patient with sporadic ALS. This TCT deletion results in the loss of the serine-57 residue and an overall decreased phosphorylation score (supplementary material table IV). Two mutations were present at amino acid 521: an arginine to cysteine (c.1561 C>T, p.R521C) in one SALS patient, and an arginine to a histidine (c. 1562 G>A, p.R521H) in one SALS and one FALS case (figure 1). Notably, these two missense mutations are the same as reported by Kwiatkowski et al. (2009).[6] We were unable to test for segregation in the FALS patient as no additional family members were available. All the patients with mutations had a typical ALS profile. No documented history of FTD or cognitive impairment was present in these patients.

A rare missense variant (c.188A>G, p.N63S) was detected in a SALS case; however, it was also present in eight of 190 controls suggesting that it is a benign polymorphism. This particular base pair change is not well conserved across species and

the serine residue is present in the rhesus monkey. Additionally, a nine bp deletion (c.676_684delGGCGGCGGC) was detected in exon 6 which results in the loss of three glycine residues (p.G226_G228del). This variant was detected in a patient with ALS as well as one in 190 controls (supplementary material table V).

The entire *FUS* gene was sequenced in 285 patients with schizophrenia and autism, and only one coding variant was detected, a GGC insertion in exon 6, resulting in the introduction of a glycine residue (c.684_685insGGC, p.G228_G229insG). This is at the same location where a nine base pair deletion was detected in a patient with ALS and a control individual suggesting that this particular glycine stretch is prone to expansion/contractions. Thus, the frequency of *FUS* variants is not particularly high in a population of non-ALS patients (supplementary material table V).

2.1.7 Discussion

Our study identified three SALS cases with mutations in the *FUS* gene. These patients were labelled sporadic considering that the cases were isolated and that the family history was negative.

Two mutations which were previously described by Kwiatkowski et al. (2009)[6] were also identified in this study, the p.R521H and p.R521C mutations. One new deletion was also identified in a SALS individual. The overall percentage of mutations identified in this study was one of 80 FALS or 1.25%, and three of 405 SALS or 0.74%. This is less than the original reports. Also, our study only detected heterozygous changes while the other reports described homozygous and heterozygous changes.[6, 103]

Future identification of more sporadic cases with missense and deletion mutations in *FUS* will definitely provide a more comprehensive picture of the proportion of mutations involved in ALS pathology. The accumulation and description of different mutations in ALS cases and future detection of mutations in more SALS patients will help understand the genetic mechanisms involved in this neurodegenerative disease. The identification of new genes represent a highly informative event for the selection of candidate genes to be investigated in the future, considering that the genetic factors underlying a substantial proportion of ALS cases remains unknown. Further investigation of the function of those genes will progressively stimulate the development of drug treatment and therapy for the disease.

2.1.8 Acknowledgments

We would like to thank the patients involved in this study, Mélanie Benard, Isabelle Thibault and Pierre Provencher for sample collection and organization, and to acknowledge support from the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARS), the Association Française contre les Myopathies (AFM), the French Group on MND, and Genethon for DNA extraction and cell lines.

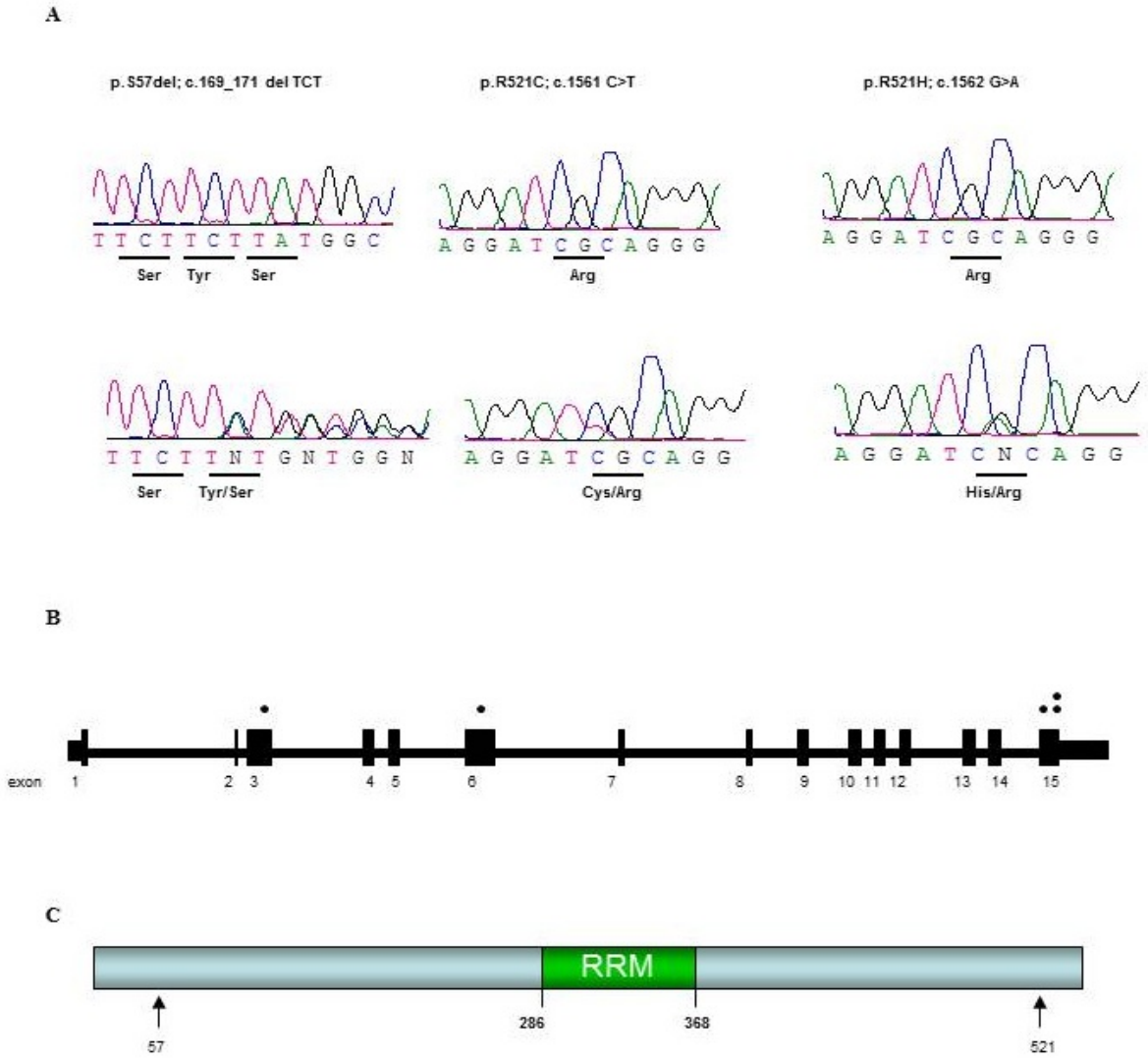
2.1.9 Tables and Figures

Table II Clinical and genetic profile of ALS patients with mutations in the *FUS* gene

Variant	Amino Acid change	Nucleotide change	Exon	Chr16 Position*	Origin	Family history	Sex	Age of onset (years)	Duration (months)	Site of onset
1	p.S57del	c.169_171 del TCT	3	31,101,465	France	SALS	M	80	10	Spinal
2	p.R521C	c.1561 C>T	15	31,110,240	France	SALS	M	26	27	Spinal
3	p.R521H	c. 1562 G>A	15	31,110,241	Quebec	FALS (affected father)	M	54	30	Spinal
4	p.R521H	c. 1562 G>A	15	31,110,241	France	SALS	F	32	72	Spinal

* human genome build 36.1

Figure 1 Sequence traces and position of mutations in *FUS*



(A) Sequence trace for the wildtype allele is presented over top of the sequence of the mutated allele. The amino acid that is changed is listed below. (B) Schematic (not to scale) of the *FUS* gene. Dots represent the exons in which mutations were identified. In the lower panel, the amino acid position of the mutations is indicated by the arrows. The position of the RNA-recognition motif (RRM) is also highlighted in green.

2.1.10 Supplementary material

Table III Primers and conditions for *FUS*

Exon(s)	Forward Primer	Reverse Primer
1	CTGCTCAGTCCTCCAGGC	TCCCACTGAAAACGAAAAGC
2+3	CAGTGCTTGAGTTAAGGAATTTAGC	AGGACCAGACTCCGTCTCC
4	CTGAGAGGCTGGCTTTATGAG	ACTGCTCCTACTGCTGGTCC
5	GGACTCCACTAAAAGTGAAAGG	AGCCTCAGCAACAGAGACAG
6	TCATTGCCTGGCACTTGTC	GGCTTCAGGGAGATTCATGC
7	CTACCCATGTTTGGGGAATG	AAGACATCTGCAAATGAACAATC
8	CCTGTTGACTAACGGCTCATC	AGTTTCAAAGAACATCCAGGC
9	TTGCTTGATGGATACTAGGTGC	TGCTGGCAACCATTAAAGAC
10+11	TTTGGGAATTATAAACCTCATGTTC	CCATGCAAGCCTTTACCATC
12	GCTTGCATGGAATGGGTTAG	TACTTCTTTGGAAAACACGCAC
13+14	TCCTCACTGTATCTCTAAAGTCACC	TCTCAACAAAACCCTGTTATCC
15	AGGTAGGAGGGGCAGATAGG	GGAAGGTTACAAAATAACGAGGG

PCR was performed using 50ng DNA, 20 pmol of each primer, 10X buffer, 0.25 nM dNTPs and 0.15 ul of Taq (Qiagen). For each exon, a denaturation step of 5 minutes was first performed at 94°C. Then a touchdown protocol was used which consisted of an initial cycle of 30 seconds denaturation at 94°C, 30 seconds annealing at 59°C and 45 seconds elongation at 72°C. This was followed by nine cycles in which the annealing temperature was decreased each time by 0.5°C. Then 25 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 59°C and 45 seconds elongation at 72°C were run. A final extension at 72°C was performed for 7 minutes. For exons 6, 9, and 12, 1x Q solution (Qiagen) was incorporated in the PCR mix.

Table IV Phosphorylation site prediction scores of deletion in *FUS*

Amino acid change	Putative effects	Putative site of phosphorylation	Context	Wildtype score	Variant score
p.S57del	Probable decreased phosphorylation	S54	YGQS**S**YSSY	0.451	0.203
		S56	QSSY**S**SYGQ	0.974	0.924
		S57	SSYS**S**YGQS	0.788	0.00
		Y58	GQSS**Y**SSYG	0.554	0.105
		Y61	SYSS**Y**GQSQ	0.521	0.575

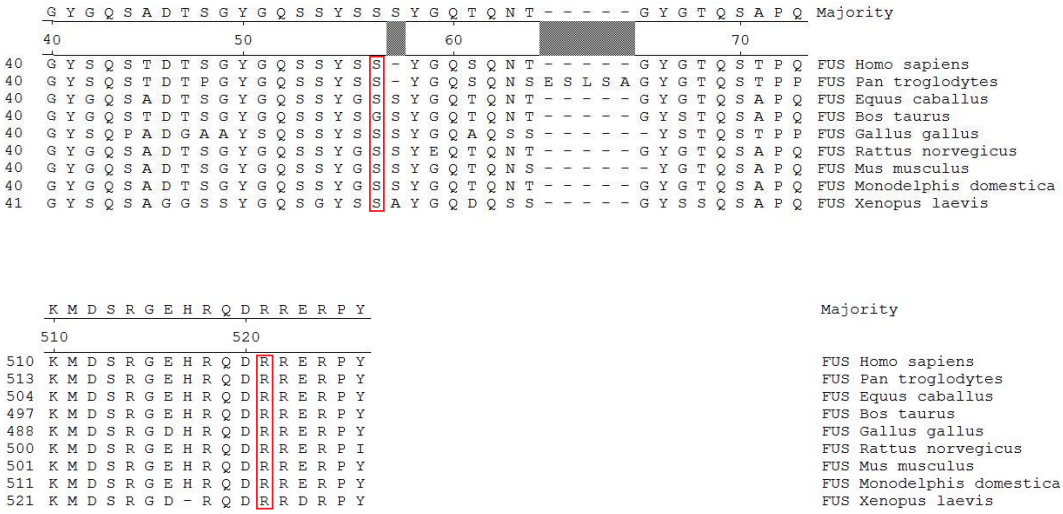
*Reference: Blom, N, Gammeltoft, S, Brunak, S. Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 1999; 294: 1351-1362.

Phosphorylation scores range from 0.00 (minimal probability) to 1.00 (high probability). [246]

Table V *FUS* variants found in controls and aut/schizo patients

Gene	*Exon*	*Nucleotide change*	*Amino acid change*	*dbSNP*	*ALS x/200*	*Control x/190*	*Aut/Schizo x/285*
FUS	3	c.62C>A	p.P21H	n/a	0	1	0
FUS	3	c.147C>A	p.G49G	rs741810	0	81	146
FUS	3	c.153C>T	p.G51G	rs61733962	0	2	0
FUS	3	c.188A>G	p.N63S	n/a	1	8	0
FUS	6	c.676_684 delGGCGGCGC	p.G226_G228del	n/a	1	1	0
FUS	6	c.684_685 insGGC	p.G228_G229insG	n/a	0	0	1
FUS	15	c.-4G>C	5'UTR	n/a	0	1	0
FUS	15	c.-2A>C	5'UTR	n/a	0	1	0

Figure 2 Protein sequence alignment of *FUS* in different species



The proteins used for comparison and their accession numbers include: Homo sapiens (NP_004951.1), Bos taurus (Q28009), Equus caballus (XP_001500625), Gallus gallus (XP_001235919), Monodelphis domestica (XP_001364792), Mus musculus (AAH58247), Pan troglodytes (XP_001158561), Rattus norvegicus (XP_001060226) and Xenopus laevis (NP_001080383). The ClustalW method was used for sequence alignment.

2.2 Identification of novel *FUS* mutations in sporadic cases of amyotrophic lateral sclerosis

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2.2.1 Rationale

When conducting the first *FUS* mutation screening, we used only a sub-group of our ALS cohort. Nevertheless, we were the first lab to report *FUS* mutations in sporadic ALS cases. More importantly, two sporadic patients were found with two different substitutions at amino acid 521 located in the last exon of the *FUS* gene, and those same mutations were previously reported in familial ALS cases. Interestingly, the two sporadic mutated patients started to develop the first ALS symptoms at an unusually young age (26 and 32 years of age), and this definitely retained our attention. Considering that our sporadic ALS cohort is mostly of French and French-Canadian origin, that the three different sporadic mutations identified in the previous paper were in patients from France, and that the first two reports mostly tested Anglo-Saxon samples, we believed that we might find additional mutations by screening more sporadic cases. This way, our study would contribute to better evaluate the contribution of *FUS* mutations in apparently sporadic ALS patients. Furthermore, finding new mutations would help characterize the role of the FUS protein in the ALS pathology and better understand the neurodegeneration mechanism involved in this motor neuron disease.

It is however difficult to evaluate if a specific mutation found in a sporadic patient actually cause the disease, since no segregation analysis can be conducted. The variant can only be confirmed to be absent in the patient's parents if their DNA is available but still, no conclusion can be made in regards to the variant role in the disease unless it has been previously reported in familial cases. Bioinformatics softwares are available to predict the effect of genomic variants,[247, 248] whether damaging or benign, but these are only predictions and no firm conclusions can be made. Because of this, we decided to screen for *FUS* mutations two groups of 475 participants each: one SALS group and one control group. Our aim was to compare the number and type of mutations identified in the two groups, and evaluate if variations were more common in the disease affected group than the

control group. This way, we would be more confident that variants found in sporadic cases actually cause the disease.

2.2.2 Contribution of authors

Belzil : Study design, data generation and analysis, statistical analysis, manuscript writing.

Daoud : Manuscript revision.

St-Onge : Data generation and analysis, manuscript revision.

Anne Desjarlais : Clinical information organization, manuscript revision.

Bouchard, Dupré, Lacomblez, Salachas, Pradat, Meininger, Camu : Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

2.2.3 Abstract

Objective: Mutations in the *FUS* gene have been recently associated with amyotrophic lateral sclerosis. While most of the variants have been identified in patients with a family history of the disease, a few mutations were also found in sporadic patients. Considering this, we wanted to evaluate the frequency of mutations in the coding region of the *FUS* gene in a sporadic ALS cohort compared to a control population. Methods: We tested 475 SALS cases of European origin and 475 matched controls for coding variations in the 15 exons of the *FUS* gene. Results: Rare novel variants were identified in a total of five SALS patients: one missense, one deletion, one frameshift, and one nonsense substitution. Two of the four variants are located in the carboxy terminal of the protein where the previously

reported variants were mostly clustered. Conclusion: *FUS* gene mutations are rare in SALS, with four new *FUS* variants identified in five different SALS cases. These findings will help evaluate the proportion of *FUS* variations in the SALS population, and to better understand its contributing role to ALS pathology.

2.2.4 Introduction

Amyotrophic lateral sclerosis (ALS) is the most common of the motor neuron diseases. It is an adult onset disease characterized by neuronal death in the motor cortex, brain stem and spinal cord, and patients usually die from respiratory failure three to five years after the appearance of the first symptoms. Ninety percent of affected individuals do not have a family history for the disease, and are considered sporadic cases (SALS)[249]. In 1993 mutations in the *SOD1* gene were found to be responsible for 15 to 20% of the ten percent of patients with a family history of ALS (FALS), while coding variations in *SOD1* is believed to account for 2-7% of SALS cases[65]. Mutations in the *TARDBP* gene was identified in 2008 as being responsible of 1 to 3% of all ALS cases[101], after its protein TDP-43 was found to be an important constituent of aggregates in the neurons of ALS and frontotemporal dementia (FTD) patients[112]. In 2009, mutations in the *FUS* gene were reported to cause approximately 4% of FALS cases[6, 103] and less than 1% of sporadic cases[250]. Considering that the proportion of SALS cases with mutations in the *FUS* gene has not been precisely established, we wanted to assess whether variations in the *FUS* gene may be responsible for a fraction of sporadic cases of ALS. We found four new variants that were exclusively present in five different sporadic ALS samples, while only one new missense was present in one control, suggesting that variations in the *FUS* gene are apparently a rare cause of SALS.

2.2.5 Materials and Methods

2.2.5.1 Standard Protocol Approvals, Registrations, and Patient Consents

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. Protocols were approved by the ethics committees and the institutional review boards of the relevant institutions. All patients gave written informed consent after which patient information and blood were collected.

2.2.5.2 Subjects

Patients were collected from France and Quebec (n = 475) between 2004 and 2009 and did not have a known history of ALS in their family. DNA was extracted from peripheral blood using standard protocols. All 475 SALS patients were screened for the 15 exons of the *FUS* gene. All 15 exons were also sequenced for 475 ethnically matched controls.

2.2.5.3 Gene Screening

Primers were designed using the ExonPrimer software from the UCSC human genome browser website ((http://genome.ucsc.edu/cgi-bin/hgGateway) and have been previously published[250]. Twelve sets of primers were sufficient to amplify and sequence the 15 coding exons of *TLS/FUS* (NM_004960.3). The amplified intronic region flanking each exon included at least 50 bp. The amplification was conducted by polymerase chain reactions (PCRs) using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per manufacturer's instructions. PCR products were amplified with the same procedure of direct sequencing in patients and controls, and were sequenced

at the Genome Quebec Innovation Center (Montréal, Québec, Canada) using a 3730XL DNA analyzer. Mutation surveyor software (version 3.10) was used for mutation detection analyses (SoftGenetics, Pennsylvania, USA).

2.2.5.4 Protein sequence alignment

Cluster analysis was performed using the Clustal W method. The closest homologue in several species was retrieved by use of NCBI's BLAST program (figure 3B-C-D, figure 4B-D). The proteins used for comparison and their accession numbers include: Homo sapiens (NP_004951.1), Pan troglodytes (XP_001158561), Equus caballus (XP_001500625), Bos taurus (Q28009), Gallus gallus (XP_001235919), Rattus norvegicus (XP_001060226), Mus musculus (AAH58247), Monodelphis domestica (XP_001364792) and Xenopus laevis (NP_001080383).

2.2.6 Results

The entire coding region of the *FUS* gene was amplified and sequenced from the DNA of 475 SALS patients and 475 control participants. 21 SALS as well as 25 control samples were removed from the total considering that more than 50% of their sequence amplification failed. Thus, a total of 454 SALS and 450 control samples were used for the analysis. Overall, more than 96% of the twelve amplicons tested for each sample were successfully sequenced and analyzed (supplementary table VI). In total, twelve coding variants and thirteen synonymous substitutions were identified (table VI). A novel heterozygous missense mutation (p.P18S; c.52C>T) was of particular interest since it was found in two unrelated French cases: one SALS patient with a classical ALS phenotype and one patient with an ALS phenotype characterized predominantly by lower motor neurons degeneration. The substituted proline was highly conserved across nine species (figure 3B)

and was absent from 450 control participants. In addition, a heterozygous deletion of eighteen nucleotides (c.430_447delGGACAGCAGCAAAGCTAT) leading to an in frame deletion of six amino acids (p.G144_Y149del) was identified in one French SALS patient (figure 4A). Those six amino acids (GQQQSY) are all well conserved across nine different species, except for G144 that is not conserved in the chicken and the Q147 that is not conserved in the chicken and the horse. Considering that the wild type protein has a total of 526 amino acids, this deletion would results in a protein with 520 amino acids. A heterozygous adenine duplication (c.1506dupA) was also observed in one Canadian SALS individuals, resulting in a shifting of the reading frame (figure 4C) which is predicted to cause a stop codon at position 516. The end product has a total of 515 amino acids since the last well conserved 25 amino acids of the wild type protein are replaced by fourteen new amino acids (EQRWLWPWQDGFQG; figure 4D). A known polymorphism (rs10684) at nucleotide c.1509 (G>A) was present in this individual. This explains why only an adenine is seen in the chromatogram at this position (figure 4C). Moreover, a heterozygous nonsense substitution (c.1555C>T) was identified in one SALS sample of French-Canadian origin at amino acid 519, when a glutamine is replaced by a stop codon (Q519X). The last eight amino acids of the protein, which are very well conserved across the nine species, are deleted from the end product, resulting in a truncated protein of 518 amino acids (figure 3D). The SALS patient having the mutation displayed a very early age of onset and experienced a relatively rapid disease progression (supplementary material table VII). All the mutations previously mentioned were unknown and absent from 475 control sequences. The clinical profile of SALS patients with novel coding variations in the *FUS* gene is available in supplementary material table VIII.

One patient was found to have the p.G226S variant that was previously identified by Corrado et al. (2010)[251] in one control. No control participant of our cohort had the mutation, but the glycine is not very well conserved across the nine species (figure 3C). In contrast, the mutation p.R216C (c.646C>T) found in one sporadic case by Corrado et al.

(2010)[251] was present in one of our controls. The novel variant p.R383C (c.1147C>T) was the only mutation uniquely identified in a control participant.

Variations in the stretch of the ten glycines in exon 6 were found in both our SALS and control populations. Two SALS patients had one glycine insertion (p.G222_G223insG) while three control participants had one glycine deletion (p.G223del). Variations in this repeat stretch have previously been found (rs72550890). It still needs to be determined if variations in this glycine stretch may predispose or be associated to ALS.

A two-tailed Fisher exact test was conducted to evaluate the statistical significance of the frequencies of coding variations found in SALS versus the control participants. Considering that a total of 454 SALS patients were tested and 25 samples did have a coding variant (which represents 0.06% of our tested cohort), and that 450 control participants were tested and 18 samples did have a coding variant (which represents 0.04% of the control tested), the p-value is 0.29, and thus not significant.

2.2.7 Discussion

Four new variants were exclusively identified in five different SALS samples: one missense (p.P18S) in two individuals, one in frame deletion of six amino acids (p.G144_Y149del) in one person, one duplication causing a frameshift in one patient, and one nonsense substitution in one person. Only one novel missense was identified in a control participant. The six amino acids deletion, the frameshift mutation, and the nonsense substitution are all strong mutations which might predispose to ALS as no such mutation was identified in ours, or any other reported controls. Unfortunately no conclusions can be made with respect to the possible role of the p.P18S missense in SALS. Even if the p-value

is not statistically conclusive, three of the four variants found in SALS are predicted to have important effects on the protein. However, these data suggest that FUS mutations may be a rare cause of SALS. Parents were not available for any of these individuals, so we were not able to determine paternity or if the mutations were *de novo*.

SALS patients with a mutation in the C-terminal of FUS seem to have an earlier age of onset, as previously reported by Blair et al.[3] In contrast, patients with mutations in the first half of the protein had the typical age of onset for ALS. The number of mutated patients in our study is however too small to arrive at any conclusion, though the age of onset of our patients with the stronger mutations are consistent with this previous work, providing some support their having a role in disease.

The identification of new mutations will help to better understand the proportion of sporadic ALS cases with *FUS* mutations as well as to provide new variants that can be studied to better understand the pathological mechanism underlying this neurodegenerative condition.

2.2.8 Acknowledgments

We would like to thank the patients involved in this study, Mélanie Benard, Isabelle Thibault and Pierre Provencher for sample collection and organization, Cynthia Bourassa, Najib Nassani and Natalia Abian for technical support, and to acknowledge support from the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARS), the Canadian Institutes of Health Research, ALS Canada, the Association Française contre les Myopathies (AFM), and the French Group on MND.

2.2.9 Tables and Figures

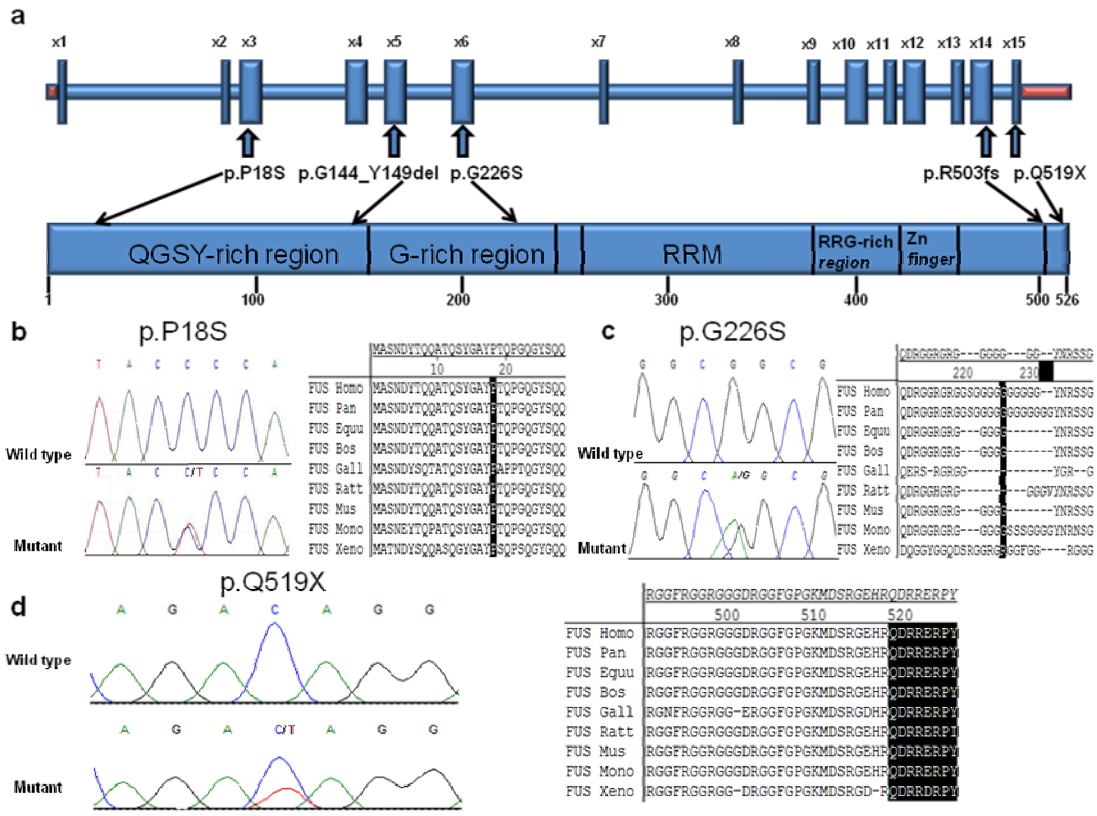
Table VI Description of coding genetic variations in *FUS* found in SALS patients and/or control participants

#	Localization	Coding DNA variant	Non-synonymous Variant	SALS (n=454)	Controls (n=450)	Status
1	x3	c.52C>T	p.P18S	2	0	new
2	x3	c.188A>G	p.N63S	3	8*	reported in SALS and controls
3	x5	c.430_447delGGA CAGCAGCAAAGCTAT	p.G144_Y149del	1	0	new
4	x5	c.475 A>T	p.N159Y	11	3	new
5	x5	c.491_495+1delGAGGTg	p.G174_G175del	3	2	reported in patients and controls
6	x6	c.646C>T	p.R216C	0	1	reported in one SALS
7	x6	c.666_667insGGC	p.G222_G223insG	2	0	rs72550890
8	x6	c.667_669delGGC	p.G223del	0	3	rs72550890
9	x6	c.676G>A	p.G226S	1	0	reported in one control
10	x11	c.1147C>T	p.R383C	0	1	new
11	x14	c.1506dupA	p.R502fsX15	1	0	new
12	x15	c.1555C>T	p.Q519X	1	0	new

#	Localization	Coding DNA variant	Synonymous Variant	SALS (n=454)	Controls (n=450)	Status
1	x1	c.6C>T	p.A2A	2	0	new
2	x3	c.147C>A	p.G49G	188	153	rs741810
3	x3	c.153 C>T	p.G51G	5	3	rs61733962
4	x4	c.222A>G	p.G74G	0	2	new
5	x4	c.269C>T	p.Y91Y	1	0	rs73530286
6	x4	c.287C>T	p.Y97Y	371	351	rs1052352
7	x5	c.504A>T	p.G168G	23	10	new
8	x5	c.510A>T	p.G170G	10	11	new
9	x6	c.684C>T	p.G228G	1	0	new
10	x11	c.1156C>A	p.R386R	1	0	rs61733965
11	x12	c.1173C>A	p.P391P	1	0	new
12	x14	c.1464C>T	p.G488G	1	0	Reported in controls and patients
13	x15	c.1566G>A	p.R522R	0	2	Reported in controls and patients

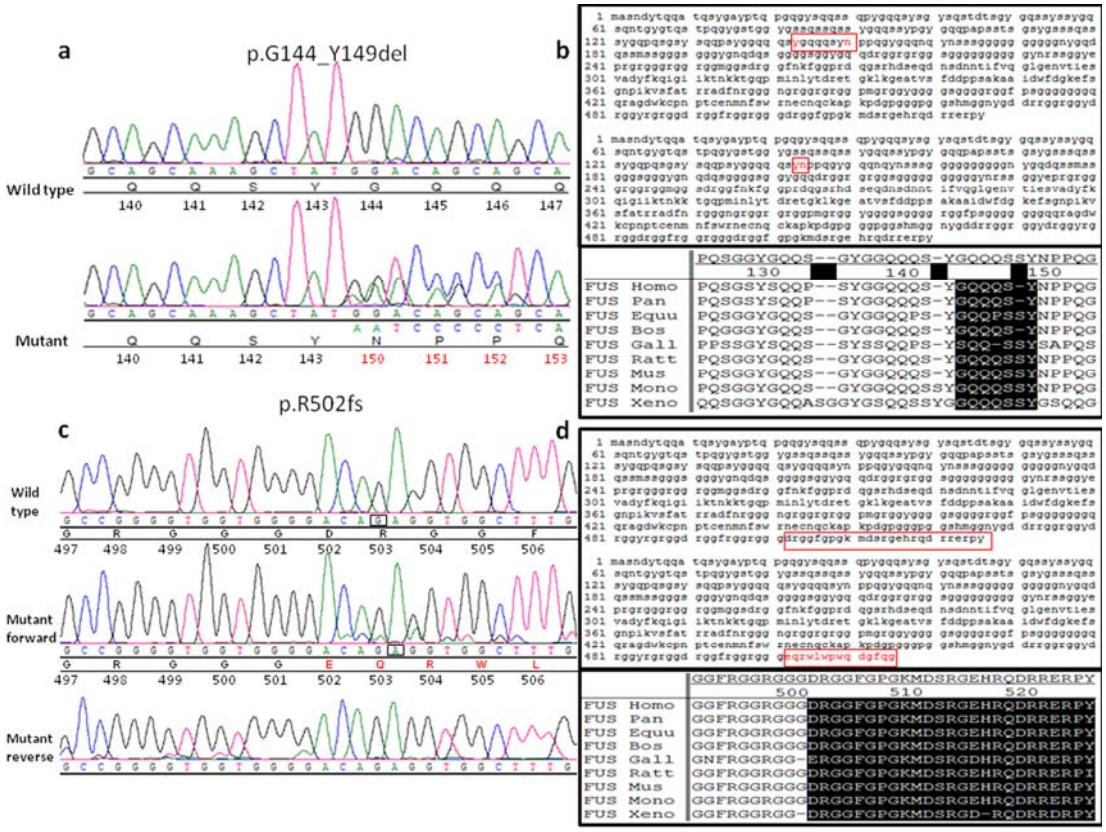
*Same control participants with N63S variant reported in Belzil et al., Neurology 2009.[250] When numbering the mRNA, +1 represents the A of the ATG. The start methionine is amino acid number 1.

Figure 3. Position of the P18S, G226S and Q519X mutations in *FUS* gene, sequence traces and across species conservation



(A) Schematic (not to scale) representation of the coding region of the *FUS* gene and motifs of the FUS protein with the localization of the coding mutations found exclusively in SALS cases. (B-C-D) Sequence traces for the non-synonymous variants found exclusively in SALS cases; patients are shown below wild type sequences. Amino acids conservation across nine species at specific substituted site is also shown using the Clustal W method.

Figure 4 Sequence trace and protein sequence of the G144_Y149del and R502fs mutations in *FUS*



(A) Chromatograms for the c.430_447delGGACAGCAGCAAAGCTAT variant. The wild type sequence is on top of the mutant sequence. The change in the protein sequence is also illustrated. (B) Complete protein sequence of the wild type 526 amino acids in *FUS* on top, followed by the mutant sequence below. The six amino acids deleted in frame are indicated in black between the preceding and following amino acids in red in the wild type sequence, and are indicated by a square. The preceding and following amino acids are still in red and marked by a square in the mutant protein sequence. The in frame deletion of these six amino acids results in a truncated peptide of 520 amino acids. The conservation of the deleted six amino acids across nine species is also shown using the Clustal W method. (C) Chromatograms for the c.1509dupA variant. The wild type sequence is on top of the mutant

sequence. The mutant sequence is shown in forward and reverse. The change in the protein sequence is also illustrated. The position of the known substitution from a glycine to an adenine (rs10684) at aa 503 (p.R503K) is marked by a square on the wild type and the mutant forward sequences. (D) Complete protein sequence of the wild type 526 amino acids in *FUS* on top, followed by the mutant sequence below. The first 501 amino acids of the wild type sequence are conserved in the mutant sequence; the last 25 amino acids of the protein in the wild type state are marked by a square. The last 14 new amino acids in the mutant state are in red and marked by a square. This mutant protein results in a truncated peptide of 515 amino acids. The conservation of the last 25 amino acids of the protein across nine species is also shown using the Clustal W method.

2.2.10 Supplemental material

Table VII Summary of amplicons sequenced and analyzed in *FUS* for SALS and control samples

	SALS	Controls	Total
Amplicons tested	5 448	5 400	10 848
Amplicons amplified	5 232	5 200	10 432
Percentage amplified	96.04%	96.30%	96.17%

The number of samples tested is based on the amplification of the twelve fragments needed to cover the entire coding region of the *FUS* gene in 454 SALS patients and 450 control participants.

Table VIII Clinical profile of SALS patients with novel coding variations in the *FUS* gene

Sample	Gender	Origin	Coding variant	Age of onset	Site of onset	Duration
R2592	M	France	p.P18S	36	Right inferior limb	Still alive in 1990, date of death unknown
X4981	M	France	p.P18S	74	Inferior limbs	3 years
X5055	M	France	p.G144_Y149del	57	Inferior limbs	56 months
X4904	M	France	p.G226S	62	Bulbar	5 months
R31291	M	Canadian	p.R502fs	49	Left upper limb	Onset 2005 still alive
R13291	M	French-Canadian	p.Q519X	20	Right upper limbs	1 year

Only information regarding SALS sample with mutations not found in our control population is listed here.

2.3 Identification of a *FUS* splicing mutation in a large family with amyotrophic lateral sclerosis

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2.3.1 Rationale

The previous study contributed to approximate at 1.3% the frequency of *FUS* mutations in sporadic ALS cases in the French and French-Canadian populations. The percentage of mutations found differed from other reports;[5, 7, 251, 252] it is slightly higher. Another concern was that our first report found only one mutation in one FALS sample after testing 80 patients, representing only 1% of the samples tested. This is significantly lower than what was previously reported in different populations,[4-6, 71, 103, 253, 254] but lower percentages have also been published.[7, 251, 255, 256]

Interestingly, higher *FUS* mutation proportions were found in FALS or SALS cases when testing more homogenous populations. For instance, we identified a higher proportion of mutations in sporadic cases because the tested cohort was only composed of French and French-Canadian samples. A group from the Netherlands reported *FUS* mutations in 7.7% of their FALS samples[4], while a German group found variants in about 7% of their FALS tested cohort.[253] This is definitely higher than what have been published so far. On the contrary, lower incidence of *FUS* mutations in FALS cases was found in Belgium, and this can be explained by the fact that the population is composed of individuals of different origins, including Dutch, French, German, Moroccan, Italian, Spanish and Turkish. The same type of heterogeneity is found in Canada since North America was colonized by different countries and was mainly populated by multicultural immigration. Because of this, it is difficult to categorize specifically the origins of inhabitants. Considering that our first tested FALS cohort was composed of European descents samples from different origins, the genetic heterogeneity of the cohort can explain the lower proportion of mutated FALS cases identified.

Considering this, we decided to better evaluate the proportion of *FUS* mutations in a larger FALS cohort still of European descents, and tested 154 additional individual FALS samples.

2.3.2 Contribution of authors

Belzil : Study design, data generation and analysis, statistical analysis, manuscript writing.

St-Onge : Data generation and analysis, manuscript revision.

Daoud : Manuscript revision.

Anne Desjarlais : Clinical information organization, manuscript revision.

Bouchard, Dupré, Camu : Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

2.3.3 Abstract

Background: Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease characterized by the degeneration of upper and lower motor neurons. Genetic studies have led, thus far, to the identification of twelve loci and nine genes for familial ALS (FALS). While the distribution and impact of *SOD1* mutations has been extensively examined for over a decade, the recently identified FALS associated *FUS* gene has been less studied. Therefore, we set out to screen our collection of FALS cases for *FUS* mutations. Methods: All 15 exons of *FUS* were amplified and sequenced in 154 unrelated FALS cases and 475 ethnically matched healthy individuals. Results: One substitution located in the acceptor splice site of intron 14 was identified in all affected members of a large family, causing the

skipping of the last 13 amino acids of the protein and the translation of 7 novel amino acids, resulting from the new transcription of a part of the 3'UTR. Conclusion: Our study identified a new splicing mutation in the highly conserved c-terminal of the FUS protein. Thus far most *FUS* mutations are missenses and our findings, combined with those of others, confirm the importance of the C-terminal portion of the protein, adding additional support for *FUS* mutations playing a critical role in ALS.

2.3.4 Short communication

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting the upper and lower motor neurons. Specifically, the neurons of the motor cortex, brain stem, and spinal cord are progressively involved, causing gradual spasticity and muscle weakness starting in the limbs in 75% of cases and in the bulbar region in 25% of cases. 90% of patients are believed to be sporadic (SALS), without any family history of the disease, while the other 10% of cases are familial (FALS), primarily segregating in an autosomal dominant manner.[249] SALS and FALS patients are clinically indistinguishable, except for the mean age of onset which is 56 years old for SALS, compared to an average of 46 years old for FALS.[25] The overall prevalence is 4-6/100,000 and the incidence is 1-2/100,000,[257] which make ALS the most common of motor neuron diseases.

Mutations in the copper superoxide dismutase 1 (*SOD1*) gene were first shown to be ALS causative over 15 years ago and they account for 15-20% of all FALS cases, representing a proportion of 1-2% of all ALS cases.[60] In the last two years, the identification of ALS causative mutations in the TAR-DNA binding protein (*TARDBP* encoding TDP-43) gene in both SALS and FALS cases[102] and in *FUS/TLS* encoding FUS,[6, 103] thus far mostly but not exclusively in FALS, opened a new era for the investigation of mechanisms underlying the disease. While *SOD1* mutations have been reported throughout

the full length of the protein, *TARDBP* and *FUS* mutations are mostly clustered to specific regions. For *TARDBP*, most of the mutations identified are in the glycine rich region encoded by exon 6, and for *FUS/TLS*, which also contains a glycine rich region, mutations are mostly in the extreme C-terminal part of the protein.[237] The aim of this study was to evaluate the proportion of *FUS* mutations in a portion of our FALS cohort and to see if any mutations identified would be clustered to the same region.

The 15 coding exons of *FUS* were amplified and sequenced in 154 unrelated FALS cases, and only one variant was identified. It is an unreported substitution of an adenine to a cytosine in the acceptor splice site of intron 14 (c.1542-2A>C). The variant was present in six samples of a large ALS family (figure 5) recruited in France: II:5, III:8, IV:1, IV:3, IV:4, and IV:6. Two of those individuals had an ALS phenotype (II:5, III:8), while the four unaffected mutation carriers are in the fourth generation and are younger than the average age of onset of the first ALS symptoms. By carrying the mutation, they confirm that their three affected fathers likely had the substitution. In addition, three mothers married to affected fathers (II:4, III:1, and III:7) were tested and were negative for the mutation. We prepared cDNA using total RNA from the immortalized lymphoblast cell lines of the two affected individuals with the adenine to cytosine substitution. These cDNAs were PCR amplified, and two products of 425 and 167 bp were observed on agarose gel (figure 6A), demonstrating that the mutant allele was not degradated by nonsense mediated mRNA decay. The heterozygous sequence trace was analyzed as well as the separate sequence of the two different alleles after a gel extraction of the two products (figure 6B). Sequencing showed that patients with the c.1542-2A>C change expressed a mutated allele missing the 40 pb coding sequence of exon 15 as well as the first 203 bp of the 3'UTR. More precisely, the sequence of the mutant mRNA brings together the last two bp of amino acid 514 located in exon 14 and the nucleotide at the c.*204 position in the 3'UTR, as an AG alternative acceptor site located at position c.*202_*203 is encountered (figure 6C); translation subsequently ends with a stop codon at position c.*297. Consequently, the

mutant protein undergoes a silent change at amino acid 514 (p.R514R) followed by 7 new amino acids (SMSRSGR) at the end of the protein. The adenine of the wild type acceptor site of intron 14 located at base pair 31,110,219 on chromosome 16 is highly conserved across species and is not listed as a known SNP. The Alternative Splice Site Predictor (ASSP) bioinformatics program attributed a score of 15.533 to the wild type AG acceptor splice site, and a score of 4.249 for the acceptor splice site with the cytosine substitution. The alternative AG acceptor splice site located in the 3'UTR at position c.*202_*203 had a score of 4.620 and is suggested to be responsible for this new isoform. Since this *FUS* mutation segregates in all affected individuals of the family as well as in the children of deceased affected members, and was not present in 475 control participants, we believe that it is likely responsible for the ALS phenotype observed in this family.

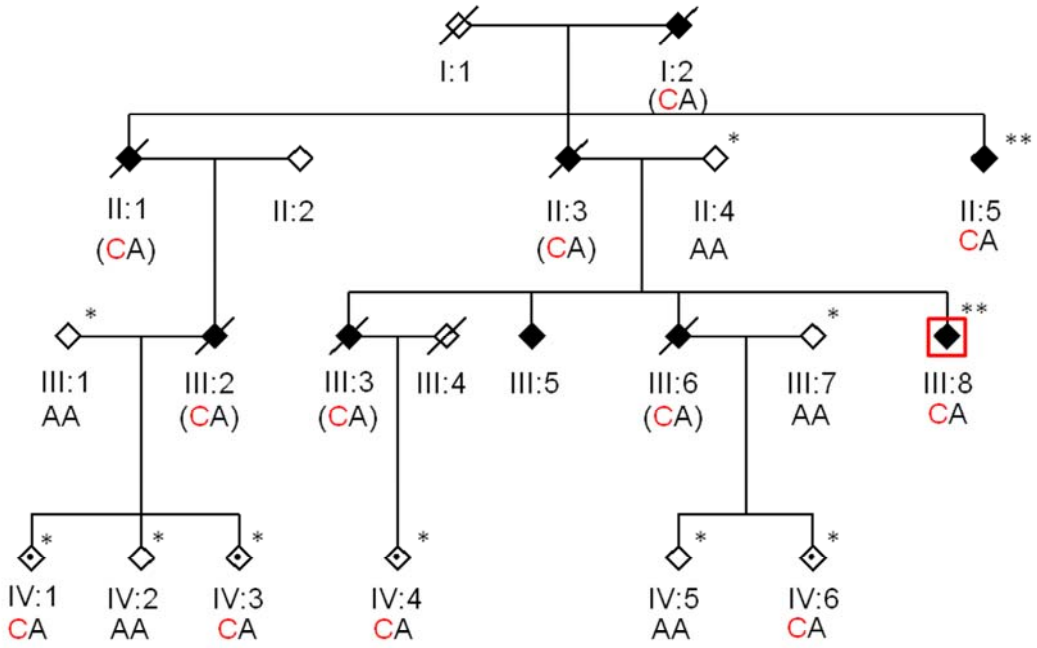
The substitution is located in the RGG-domain enriched in arginine-glycine-glycine motifs, where most other *FUS* mutations were reported. Importantly, truncated proteins were previously observed with the two other major ALS causative genes.[63, 113, 258] But more interestingly, this is the second splicing mutation reported in *FUS* as another one was recently shown to lead to the skipping of exon 14 after the substitution of the adenine located in the acceptor splice site of intron 13.[259] Unfortunately, no clinical information could be retrieved for the family presented in our study, so we were unable to compare the disease evolution caused by our splicing mutation in intron 14 with the splicing mutation in intron 13 reported by Dejesus-Hernandez et al. The splicing mutation identified here removes the last 13 amino acids that partly encode the c-terminal portion of FUS, in which a number of mutations have been associated with ALS. Our finding reinforces the notion that this portion plays a key role in the pathology of ALS, most likely by affecting the RNA binding domain it encodes for. In the future, such splicing defect causative mutations may become treatable through drugs or gene therapies aimed at correcting splicing, as explored for a number of disorders.[260-262]

2.3.5 Acknowledgments

VVB, HD and GAR are supported by the Canadian Institutes of Health Research. We would like to thank the patients involved in this study, Mélanie Benard, Isabelle Thibault and Pierre Provencher for sample collection and organization, Anne Noreau, Cynthia Bourassa, Sophie Massart, and Bertrand Boutié for technical support, and to acknowledge support from the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARS), the Association Française contre les Myopathies (AFM), and the French Group on MND.

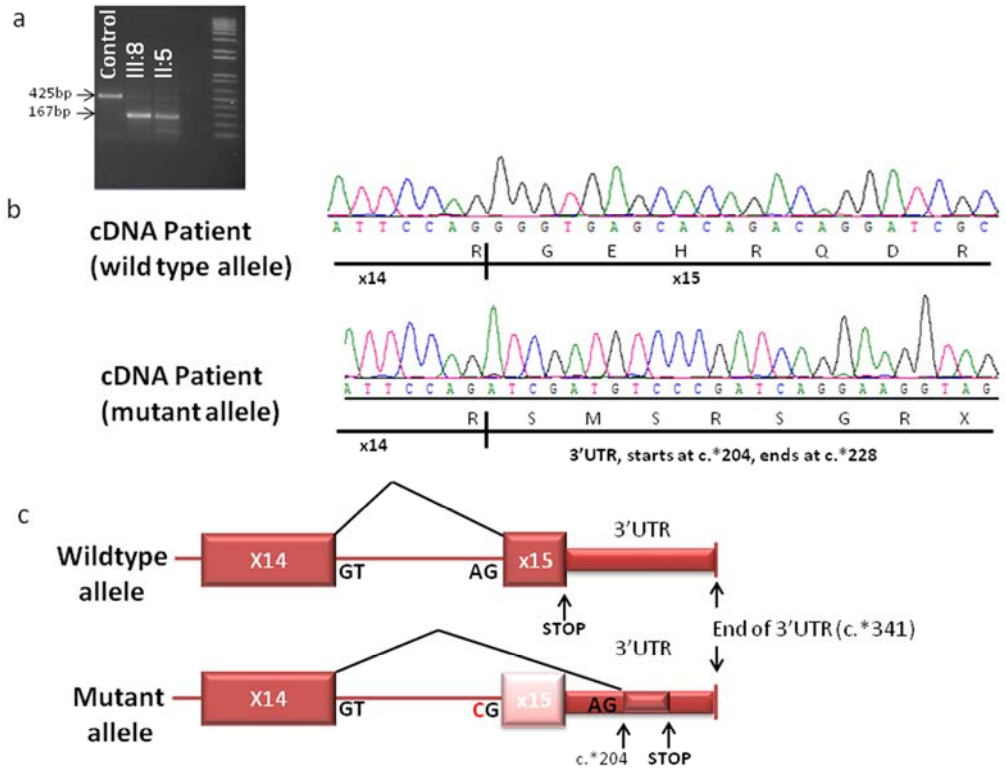
2.3.6 Tables and Figures

Figure 5 Pedigree of the family with the c.1542-2A>C variant in *FUS*



Black symbols represent affected individuals, while open symbols represent unaffected family members. Symbols with dots represent unaffected individuals carrying the substitution. Slashed symbols indicate deceased individuals. Red square around the individual III:8 symbol marked the sample sequenced in the primary screening and asterisks indicate family members for whom exon 15 of the *FUS* gene was sequenced in the secondary screening. Symbols with two asterisks represent individuals for whom cDNA was amplified. Genotypes at position c.1542-2 are indicated in black for the wildtype allele and in red for the mutant allele. Genotypes in parentheses are inferred.

Figure 6 Splicing mutation in *FUS*: agarose gel, chromatograms and schematic representation



(A) Agarose gel electrophoresis of *FUS* cDNA amplified in one unrelated control, and two affected family members (II:V, III:VIII), showing the wild type allele (425bp) and the mutant allele (167bp) for the fragment amplified using foward primer 5'-GAGGGGGACCAGGTGGCTCTCAC in exon 14 and reverse primer 5'-TCATTTGGCCTTCTCCCCGAACAC in 3'UTR. (B) cDNA sequence chromatogram of the wildtype and mutant alleles in one patient, confirming the skipping of exon 15 and the first 203bp of the 3'UTR. (C) Schematic (not to scale) representation of exons 14, 15, and 3'UTR of the *FUS* gene for the wildtype and mutant alleles. In the mutant allele, a part of the 3'UTR is translated which adds 7 new amino acids to the protein (SMSRSGR).

2.4. Novel *FUS* deletion in a patient with juvenile amyotrophic lateral sclerosis

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2.4.1 Rationale

Many years ago, juvenile amyotrophic lateral sclerosis (JALS) has been linked to the ALS2,[84] ALS4[90] and ALS5[95] loci and mutations have been identified respectively in the *ALSIN*,[86, 263] *SETX*[264-266] and *SPG11*[267] genes. More recently, ALS families with a few affected members developing the first symptoms before 25 years of age have been linked to the X chromosome and mutations have been found in the *UBQLN2* gene.[20] Noteworthy, the *ALSIN*, *SETX*, and *SPG11* genes have also been associated with at least one other phenotype. Precisely, mutations in *ALSIN* have been identified both in juvenile primary lateral sclerosis (PLS)[88, 263] and infantile onset ascending spastic paraplegia (IAHSP) cases,[268] mutations in the *SETX* gene were found in ataxia-ocular apraxia-2 patients (AOA2), and mutations in *SPG11* were first identified in individuals affected with hereditary spastic paraplegia-11 (HSP-11).[269] This suggests that ALS, PLS and HSP are certainly part of a disease continuum, as the same biological pathway must contribute in different ways to define one specific phenotype. Consequently, a better understanding of the pathological cascade involved in one disease might help to develop therapeutics for a larger spectrum of neurodegenerative diseases.

In 2009, mutations in *FUS* were identified in FALS cases by two groups.[6, 103] We noticed already that some mutated individuals in one of these reports started to develop ALS symptoms much earlier than the mean age of disease onset. Since then, many familial as well as sporadic *FUS* mutated cases have been identified with an unusual young age of onset. Specifically, in our first report, our group identified two sporadic patients with *FUS* missense mutations who started to experience ALS symptoms at 26 and 32 years of age. In addition, in our second report, we found two other mutated sporadic patients with a nonsense and a missense mutation, who respectively developed the disease at 20 and 36 years of age. A group from the US also reported a sporadic patient having a splice mutation with a disease onset at 20.[259] In addition, an Italian group reported an 11 year old girl with a

missense mutation in *FUS*. The affected little girl who developed ALS was characterized by a very rapid progression.[270] Three *FUS* mutated families with several members developing symptoms before the age of 30 years old have also been reported by a group from the US.[71]

Of interest, a group from the UK reported four *FUS* mutated sporadic patients with an age of onset ranging from 17 to 22 years old, also with a rapid disease progression.[271] This group reported the presence of basophilic inclusions in their patients, which were positive for FUS protein but negative for TDP-43 in glia and neuronal cytoplasm and nuclei. A US group also observed the same basophilic inclusions in spinal motor neurons but not in glial cells of two juvenile ALS patients aged 13 and 21. A *FUS* missense mutation was identified in the 13 years old little girl.[272] This distinct JALS phenotype with basophilic inclusions characterized by predominant lower motor neurons involvement has already been reported in the past.[273-275] These motor neuron deposits are particular to juvenile ALS and are usually not found in neurons of pure adult onset ALS patients, as no abnormal accumulation of FUS has been found.[272] Only two groups reported two adult-onset patients exhibiting features resembling JALS with basophilic inclusions.[276, 277] Basophilic inclusions not only contain aggregates of FUS, but also aggregates of intracellular organelles,[272] which explains the rapid progression experienced by patients and differentiate the pathology from what is observed in neurons of pure ALS cases.

While JALS associated with mutations in the *ALSIN*, *SETX*, and *SPG11* genes is characterized by a disease onset before the age of 25 and a slow progression, JALS patients with mutations in *FUS* generally develop the disease before 30 years of age, experience a very fast progression with a predominant involvement of lower motor neurons, and have FUS positive basophilic inclusions in their spinal neurons. The same phenotype has been

found in different populations, this type of JALS is thus not specific to a particular world region.

An 18 years old patient displaying an ALS phenotype with an extremely rapid progression was hospitalized at our Research Centre hospital. The assigned clinician contacted us for screening of the *FUS* gene, considering the particular phenotype of the patient and the similarity with other *FUS* mutated JALS cases in the literature. This case study is presented here.

2.4.2 Contribution of authors

Belzil : Study design, data generation and analysis, manuscript writing.

Langlais: Clinical evaluation, sample collection, manuscript writing and revision

Daoud : Manuscript revision.

Brais: Clinical evaluation, manuscript revision.

Dion & Rouleau : Experiment supervision, manuscript revision.

2.4.3 Abstract

Objective: Juvenile Amyotrophic Lateral Sclerosis (JALS) refers to a form of Amyotrophic Lateral Sclerosis (ALS) in which a progressive upper and lower motor neuron degeneration begins before the age of 25. It is generally associated with slow disease progression. During the last decade a number of genes have been reported to cause JALS. Mutations in the *ALSIN* gene causes JALS type 2 (ALS2), as well as juvenile primary lateral sclerosis

(JPLS) and infantile-onset ascending spastic paralysis (IAHSP). Mutations in the *SETX* gene can also sometimes lead to JALS. Conversely, mutations in *SOD1*, *TARDBP* and *FUS* typically cause pure ALS with an adult onset between 46 and 56 years of age, and usually rapid progression over a 3 to 5 year period. Recently, a few mutations in *FUS* have been associated with a juvenile onset of ALS characterized by a very rapid progression. Design/Subject: We sequenced all the coding exons of *SOD1*, *TARDBP* and *FUS* in one 18 years old patient experiencing a rapid degeneration of upper and lower motor neurons. Results: A novel one base pair deletion was detected in exon 14 of the *FUS* gene, leading to a frameshift and the integration of 33 new amino acids. The variant p.R495QfsX527 is located in the highly conserved, extreme c-terminal of the FUS protein, where most of the mutations in *FUS* have been identified. The variant was also identified in the unaffected 47 years old mother of the patient who remains asymptomatic. Conclusions: Our finding, along those from others, further confirms that *FUS* mutations can lead to an early onset rather malignant form of ALS. In addition, our data lends additional support to the notion that disruption of the conserved c-terminal of FUS is critical for developing ALS.

2.4.4 Background

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease affecting the upper and lower motor neurons. The mean age of symptom onset is 45 years old for cases with a previous family history, and 55 years old for sporadic cases.[25] Affected individuals generally have a pure ALS phenotype, with degeneration affecting mainly neurons of the motor cortex, brain stem, and spinal cord. Patients usually die from respiratory failure after a three to five years of disease progression, and no effective treatment currently exists. Approximately 10% of ALS patients have other family members affected with the disease[249]. Mutations in the *SOD1* gene explain approximately 2% of all ALS cases[60], while variants in *TARDBP* and *FUS* each explain about 1-3% of overall cases[106]. On rare occasions, the first symptoms of motor neuron degeneration start before

25 years of age, usually with slower disease progression compared to later onset typical ALS[278]. This juvenile type of ALS has been associated in the past with mutations in the *SETX* gene[91] or the *ALSIN* gene for ALS2[86]. Mutations in *ALSIN* are also known to cause juvenile primary lateral sclerosis (JPLS) and infantile-onset ascending spastic paralysis (IAHSP)[268]. Since the report of FALS cases with mutations in *FUS* in 2010[6, 103], a few groups identified *FUS* mutations in ALS patients with an age of onset before the age of 25 with an unusual and unexpected rapid progression[71, 104, 259, 271]. Here, we report an 18 years old male with a deletion in exon 14 of the *FUS* gene experiencing a very rapidly progressing disease.

2.4.5 Case presentation

The patient is a 19-year-old man with a past history of mild learning difficulties who had been working in commercial toxic waste recycling for less than a year. The patient first developed a painful right shoulder and arm weakness at the end of August 2009 which progressed rapidly, and in less than a month included neck weakness. At the beginning of December 2009, right arm muscle atrophy was noticed by members of his family, which prompted him to seek medical attention. A complete neurological exam and work-up was done including brain and spine MRI, repeated EMGs, toxicology screening, lumbar puncture, and biochemical blood tests. The general medical examination was normal. On neurological examination, he had a normal mental status. Examination of the cranial nerves showed tongue atrophy and fasciculations, bilateral facial weakness with right side worse than left, severe weakness and atrophy, and fasciculations of the sternocleidomastoids and trapezius with right side much worse than left. The remainder of the cranial nerves were normal. Motor examination showed atrophy and severe weakness of the right shoulder and upper arm including supraspinatus, infraspinatus, subscapularis, deltoid, pectoralis, and biceps. He had moderate weakness and atrophy of his right triceps, wrist and finger extensors and flexors, as well as interossei of the hands. There was normal bulk, tone and

strength of his left side and right leg. He was areflexic in the right arm, with mild hypereflexia in the left arm, knees and ankles, with equivocal plantar responses. The cerebellar exam was normal, with the exception of the right arm, which was not testable. The sensory exam was normal as well as his gait. The brain and spine MRI were normal. The lumbar puncture as well as the detailed biochemical tests and toxicology screen were normal. An EMG showed normal conduction velocities and normal needle exam of the left arm, shoulder and left leg. There was some denervation in muscles of the right leg and severe denervation in muscles of the right shoulder and arm. In March 2010, the patient was suspected of having juvenile onset ALS and was put on Riluzole. In May 2010, he developed dysphagia and dysarthria leading to anorexia and severe weight loss. In August 2010, a control neurological exam showed a 36 kg young man with diffuse severe muscle atrophy. Muscle weakness was asymmetric with a profound right upper limb weakness and, to a lesser degree, bilateral leg involvement. The patient rapidly progressed to respiratory failure requiring mechanical ventilation. Repeated neurological exams showed no cognitive or sensory symptoms or signs. Although follow-up visits were scheduled, the patient was lost to follow up. In summary, the patient mostly displayed a lower motor neuron phenotype, with the upper limbs and bulbar regions principally affected. Upper motor neurons seemed to be mildly affected.

When going back to the patient's clinical history, he was generally in good health for his first 17 years of life. He consulted for dysphasia at the age of six, was prescribed Ritalin between six and 16 years of age, but did not show any previous signs of paralysis, progressive weakness or neurological symptoms. However, he attended welding school when he was 16, and worked with recycling hazardous material at age 17.

While the patient had no apparent family history of motor neuron diseases, it is noteworthy that his mother was diagnosed with an Arnold-Chiari malformation at the age

of 13 years old; she actually experienced equilibrium problems and one cerebellar ataxia episode at that time. The mother's parents were not reported to have developed any neurodegenerative symptoms. Other family members including the patient's father and brother are still in good health.

2.4.6 Methods

DNA from the patient and his parents was extracted from peripheral blood using standard protocols. Primers for *SOD1*, *TARDBP* and *FUS* were designed using the ExonPrimer software from the UCSC human genome browser website (http://genome.ucsc.edu/cgi-bin/hgGateway). All 26 exons of the three genes were amplified for the patient for a total of 23 fragments, and the one fragment containing the mutation in the patient was amplified in his two parents. Amplification was performed by polymerase chain reactions (PCRs) using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per manufacturer's instructions. The PCR product contained a minimum of 50 bp from each of the flanking introns. Products were directly sequenced in forward and reverse at the Genome Quebec Innovation Centre (Montréal, Québec, Canada) using a 3730XL DNA analyzer (Applied Biosystems, Foster City, California, USA). Mutation surveyor software (version 3.10) was used for mutation detection analyses (SoftGenetics, Pennsylvania, USA). All exons of *FUS* were also amplified in 96 French-Canadian and 380 French control participants, and have already been published[104].

2.4.7 Results

A novel one base pair deletion of a guanine in exon 14 of the *FUS* gene (c.1484delG) (Figure 7A) was identified and is predicted to cause a shift in the reading

frame resulting in the inclusion of 33 new amino acids (p.R495QfsX527), which modifies the highly conserved c-terminal of the protein (Figure 7B) that is believed to play a crucial role in RNA processing[106]. The final mutated protein is predicted to have one more amino acids than the wild type, resulting in a probable change in the c-terminal function or interaction abilities. The variant was also identified in the 47 years old mother, who did not show any signs of motor neurons degeneration.

Interestingly, Elden et al. recently found that intermediate-length polyQ tracts (ranging between 24 and 33 repeats) in *ATXN2* confer an increased risk for developing ALS[126]. We decided to test the length of the repeat in *ATXN2* in the 18 years old patient and his parents, the hypothesis being that the affected juvenile ALS case developed the symptoms earlier than his mother because he inherited an intermediate-length polyQ tract from his father who does not carry the *FUS* mutation. We amplified the *ATXN2* CAG repeats by polymerase chain reaction. We determined the CAG repeat sizes by capillary electrophoresis by incorporating a VIC-labeled M13 universal primer into the PCR reaction. PCR products were then diluted (1:20) and mixed with LIZ-500 size standard (Applied Biosystems) and processed for size determination on an ABI 3730 DNA analyser. Repeat sizes were determined using the GeneMapper Software Version 4.0 (Applied Biosystems). All three individuals had both the $(CAG)_{21}$ and $(CAG)_{22}$ alleles, which are the most common in the general population. The experiment was repeated twice, yielding the same results.

2.4.8 Comments

A novel one base pair deletion was detected in exon 14 of the *FUS* gene, leading to a frameshift and the integration of 33 new amino acids. The variant c.1484delG (p.R495QfsX527) is located in the highly conserved, extreme c-terminal of the FUS

protein, where most of the mutations in *FUS* have been identified. The variant was also identified in the unaffected 47 years old mother of the patient, who remains well. Separate deletions were already reported in familial ALS cases one base pair upstream (c.1483delC), and one base pair downstream stream (c.1485delA), respectively producing p.R495EfsX527 and p.G497Afs527[71]. This confirms the importance of a conserved c-terminal for the normal functioning of the FUS protein. Additionally, the same group published a nonsense substitution at position c.1483C>T that was shown to produce a truncated protein (p.R495X)[71], further reinforcing the importance of this region. What is specifically interesting is the age of onset and disease duration associated with the p.R495EfsX527, p.G497Afs527 and the p.R495X reported mutations. In fact, the two affected members of the family with the p.R495EfsX527 mutation displayed a significant difference in terms of age of onset and duration, since one develop the first symptoms at 23 years of age and the disease progressed over a 48 month period, while the other developed ALS at 72 years old and died 12 months after symptom onset. On the other hand, the seven members of the family in whom the nonsense p.R495X mutation segregated had an early age of onset but varied in terms of disease duration. Precisely, five members developed ALS at age 14, 24, 27, 39 and 44, while two other members with the variant were still unaffected at age 57 and 61. Moreover, the p.G497Afs527 mutation segregated in three members of another family who experienced the first symptoms at age 13, 29 and 29. The disease progressed very rapidly, over a 12, 13 and 18 months period in those patients.

In addition, another group reported a sporadic ALS affected female with a *FUS* p.G466VfsX14 splice mutation who experienced the first symptoms at 20 years old; the disease progressed for a 22 months period[259]. Interestingly, our group also previously published one French-Canadian sporadic ALS case with a *FUS* p.Q519X mutation. This patient started to develop motor neuron symptoms at 20 years old, and the disease progressed rapidly over 12 month period[104], still underlying the fact that a mutation in that specific region gave rise to an early ALS onset often characterized by a rapid progression.

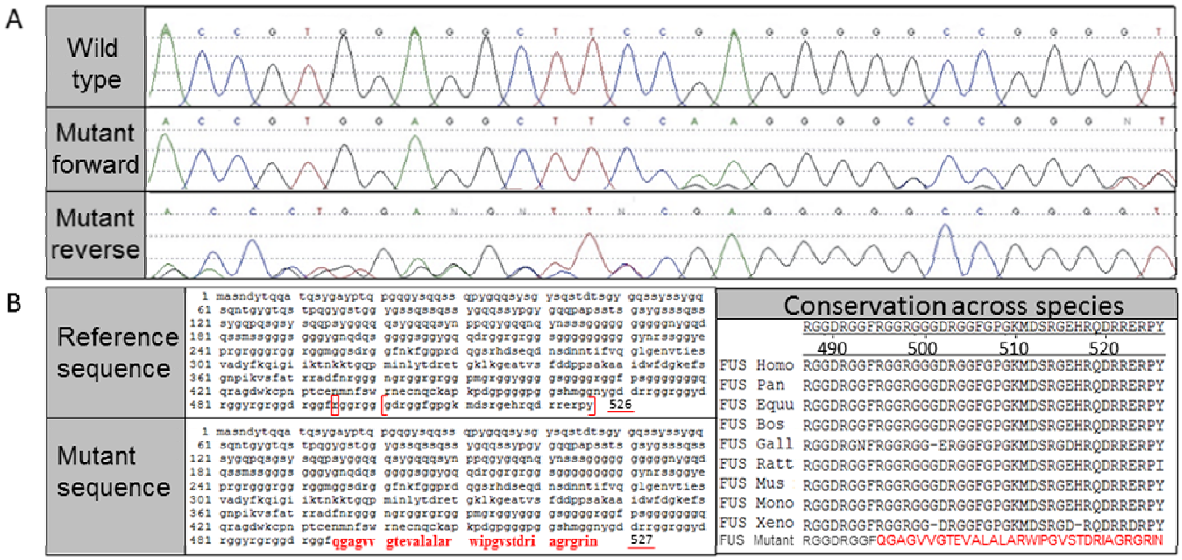
The example of the published FALS and SALS patients just described along with our reported juvenile case underscore the importance of the last 32 amino acids of the FUS protein, and demonstrate that despite an existing variability in terms of age of onset, it seems that patients carrying such mutations frequently develop ALS at an earlier age while the disease progresses more rapidly. However, other genetic modifiers might influence the age at which the first ALS symptoms will appear. It is interesting to note that an intermediate-length of the polyQ tracts in *ATXN2* is not an age of onset modifier in our juvenile patient. On the other hand, the patient was highly exposed two years before developing the first symptoms to welding material in an industry as well as to heavy metals, solvents, pesticides, and agricultural chemicals, all of which have been reported to be associated with ALS[179, 279, 280]. While detailed biochemical tests and toxicology screen were normal, unknown genetic variants already present in the patient leading to a certain vulnerability to toxic exposition, or mutations caused by such environmental exposition could explain why the patient developed the first signs of ALS while his mother remains well. However, it cannot be ruled out that other susceptibility variants in other genes explain the early onset. It could also be hypothesized that the mother carries a protective variant, preventing the early onset of the disease.

2.4.9 Acknowledgments

VVB and HD are supported by the Canadian Institutes of Health Research. GAR holds the Canada Research Chair and a Jeanne-et-J.-Louis-Levesque Chair for the Genetics of Brain Diseases. We would like to thank the patient and his parents who were involved in this study, Catherine André-Guimont and Annie Levert for technical support, as well as Anne Desjarlais and Catherine Boyer for sample collection and organization.

2.4.10 Tables and Figures

Figure 7 Chromatograms, protein sequence and amino acid conservation in JALS *FUS* mutated sample



(A) Chromatograms for the c.1484delG variant. The wild type sequence is on top, followed by the mutant sequence in forward and reverse. (B) Complete protein sequence of the wild type 526 amino acids of FUS on top, followed by the 527 amino acids mutant sequence below. The first amino acid affected by the nucleotide deletion is marked by a red rectangle on the reference sequence, and the highly conserved extreme c-terminal is between red brackets on the same sequence. The 33 new amino acid of the mutant sequence are in red in the protein sequence box and in the conservation across species box. The cluster analysis was performed using the Clustal W method, and show that the last 32 amino acid of the FUS protein are highly conserved across nine species. The closest homologues were retrieved by use of NCBI's BLAST program.

Chapter 3 : New genetic avenues for the *SOD1* gene

3.1 A mutation that creates a pseudoexon in *SOD1* causes familial ALS. Published in Annals of Human Genetics. November 2009;73(Pt 6):652-7.

3.2 No effect on *SOD1* splicing by *TARDBP* or *FUS* mutations. Published in Archives of Neurology. March 2011;68(3):395-6.

3.1 A mutation that creates a pseudoexon in *SOD1* causes familial ALS

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3.1.1 Rationale

Mutations in the *SOD1* gene have first been identified in 1993,[60] explaining about 20% of FALS and 3% of overall ALS cases.[65] Almost 19 years later, more than 168 mutations have been identified in this 154 amino acids gene.[281] There is no mutation cluster as variants, mostly missense mutations, are present in all five exons of the gene. Until the end of 2011, *SOD1* was the gene explaining the larger proportion of ALS cases. Considering that mutations are estimated to be found in 1:5 FALS patient, FALS samples in labs all over the world are screened systematically for *SOD1*coding mutations before being used for other ALS screening. But what about possible noncoding mutations? What if the *SOD1* mutation proportion has been underestimated?

While it was believed for a long time that causative mutations can only be located in the coding regions of genes, it is now well known that mutations in cis-acting regulatory sequences influence the expression of genes and are a significant cause of diseases. It is proven that humans are more polymorphic at functional regulatory sites than they are in coding regions.[282] Precisely, cis-regulatory mutations disturb a wide range of physiological, morphological, or neurological phenotypes and can increase or decrease the amount of transcribed products.[283] According the Human Gene Mutation Database (HGMD), about 2158 regulatory mutations actually cause human inherited disorders, which represent approximately 1.8% of all disease causing mutations (updated Sept 30th, 2011).[284] For instance, Mendelian disorders such as β-thalassemia, hemophilia and atherosclerosis are respectively caused by regulatory mutations in the beta-chain of *hemoglobin* (*HBB*), coagulation *Factor IX* and *low-density lipoprotein receptor* (*LDLR*) genes.[283] Although *trans*-acting determinants are more numerous and code, among other things, for components of the splicing machinery, *cis*-acting elements have a stronger influence on gene expression.[285] In addition, mutations in cis-regulatory elements can result in a misregulation of splicing or alternative splicing of the nearby genes.[286]

We present here a family with several members affected with ALS linked to chromosome 21. However, no *SOD1* mutation was identified. The same problem was encountered in the past by another lab when studying a group of individuals from Melanesia with α-thalassemia. The patients showed an important reduction in *α-globin* transcription despite the absence of known mutations.[287] A SNP segregating in all affected individuals was finally identified, this regulatory mutation creating a new GATA-1-binding site. This change caused the recruitment of an erythroid-specific transactivation complex, which interfered with normal transcription of the α-*globin* genes.[287] In the same way, considering this, we believed that a regulatory or splicing mutation in *SOD1* was carried by the affected members of this family which might cause the ALS phenotype. This hypothesis was explored in the next paper.

3.1.2 Contribution of authors

Valdmanis & Belzil : Study design, data generation and analysis, manuscript writing.

Lee, St-Onge & Hince : Technical support, manuscript revision.

Funalot, Couratier, Clavelou, Camu : Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Experiment supervision, manuscript revision.

3.1.3 Abstract

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease which targets motor neurons of the cortex, brainstem and spinal cord. About 5-10% of all

amyotrophic lateral sclerosis cases are familial (FALS), and 15-20% of FALS cases are caused by mutations in the zinc-copper superoxide dismutase gene (*SOD1*). We identified a large family from France with ten members affected with ALS. Linkage was established to the *SOD1* locus on chromosome 21 and genomic and cDNA sequencing was performed for the *SOD1* gene. This revealed an activated pseudoexon between exons 4 and 5 that was present in two tested members of the family. Translation of this 43 base pair exon results in the introduction of seven amino acids before a stop codon is present, leading to a prematurely truncated SOD1 protein product of 125 amino acids. Sequencing intron 4 in a patient revealed a heterozygous change 304 bp before exon 5 (c.358-304C>G), but only 5 bp after the cryptic exon, thus causing this alternative splice product. This mutation segregated in all affected individuals of the family. This adds an additional genetic mechanism for developing *SOD1*-linked ALS and is one which can be more readily targeted by gene therapy.

3.1.4 Introduction

Amyotrophic lateral sclerosis (ALS) is caused by the degeneration of motor neurons in the brainstem, spinal cord and motor cortex. Mutations in the copper-zinc superoxide dismutase (*SOD1*) gene account for 15-20% of familial ALS cases. The locus for *SOD1*-linked ALS on chromosome 21 was identified by linkage analysis in 1991 [288] followed by the identification of mutations in the *SOD1* gene two years later [60]. Following this discovery, the screening of the *SOD1* gene for mutations has been a standard procedure to determine which families have mutations in *SOD1*, and which families have another genetic cause. The use of non-*SOD1* families has led to the identification of loci on chromosome 18, 20, and 9 and in mutations in the *FUS*, *DCTN*, *ANG*, and *TARDBP* genes [6, 21, 89, 98, 101-103, 111, 148, 289]. Meanwhile, several groups have more extensively analyzed the *SOD1* locus on chromosome 21 to determine if additional genetic information could be extracted from the region. This includes the examination of nearby genes and SNPs

surrounding *SOD1* which could act as modifiers or susceptibility factors for the development of ALS. In addition, certain families display unconventional (i.e. non-dominant) inheritance patterns, and additional genetic factors within or near *SOD1* may influence penetrance in these families. Recessive pedigrees have been identified harbouring the D90A *SOD1* mutation and the compound heterozygous D90A/D96N mutations [66, 74].

We have identified a family with ALS from France that maps to chromosome 21 and has a heterozygous mutation in the middle of intron 4 of *SOD1* that leads to the inclusion of a cryptic exon in the *SOD1* mRNA and a prematurely truncated protein product.

3.1.5 Materials and Methods

Blood samples were collected from 23 individuals (five affected individuals) who signed a consent form which was approved by the ethics review board of the Centre Hospitalier Universitaire de Montreal. DNA from seven individuals (three affected, four unaffected) was sent for a 550-marker eight centiMorgan (cM) whole genome scan by DeCODE genetics (Reykjavik, Iceland). Genome scan results were analyzed using Genehunter v2.1 with an autosomal dominant mode of inheritance, a disease frequency of 1 in 10,000, 90% disease penetrance, equal allele frequencies and equal male to female recombination rates. Subsequent two-point analysis was performed using the MLINK program from the LINKMAP software package [290]. Polymerase-chain reactions were performed using 50 ng of DNA, and amplified on Perkin Elmer 9600 thermocyclers (Perkin Elmer Applied Biosystems, Foster City, CA) using the following protocol: DNA melting at 94°C for 5 minutes followed by 30 replication cycles (30 seconds at 72°C, 40 seconds at

55°C and 40 seconds at 72°C) and a 10 minute final extension step at 72°C. PCR products were sequenced at the Genome Quebec Centre for Innovation.

3.1.6 Results

DNA from five affected individuals in the family, two at-risk individuals and one control were sequenced for the five exons of *SOD1* using two different sets of non-overlapping primers. No coding mutations were detected, so seven members from the family were sent for a 550 microsatellite marker genome-wide scan (DeCODE genetics). The highest LOD score was present on chromosome 21, a 25 Mb (32.1 centiMorgan) interval from marker D21S1432 to D21S266 (Figure 8). This region contains 134 RefSeq genes including *SOD1*, found at Mb 31.95 on chromosome 21. Fine mapping of additional markers in all collected individuals in the family yielded a maximum LOD score of 2.84 at marker D21S263 (Table IV). There was only one other region – on chromsome 12 - in the genome scan with a LOD score above 1.0 (but below 1.5). By genotyping the complete family, we were able to exclude the chromosome 12 region and confirm that the family maps to chromosome 21. This led us to believe that a mutation may be present in a non-coding sequence or an intergenic region surrounding *SOD1*.

We obtained immortalized lymphoblast cell lines from two of the patients (provided by Genethon , Evry, France) in order to extract mRNA. The *SOD1* sequence was tested by cDNA analysis using two primer pairs that covered the *SOD1* mRNA. This led to the identification of two bands in a patient sample when the products using a forward primer in exon 4 (5'-GACTTGGGCAATGTGACTGCTGAC) and a reverse primer in exon 5 (5'-TGCCATACAGGGTTTTTATTCACA) were run on gel (Figure 9A). Analysis of the sequence trace resolved the two alleles in heterozygous state, which appeared at the junction of exons 4 and 5. This sequence consisted of 43 nucleotides which map to a region in intron 4 that is

744 base pairs after exon 4 and 308 base pairs before exon 5 (Figure 9B). Thus, the patients contain a gene product that skips from the end of exon 4 to a novel 43 base pair cryptic exon, before proceeding to exon 5 (r.357_358ins357-351_358-309 of RefSeq NM_000454). Importantly the novel exon contains a canonical AG/GT splice sequence flanking the exon (Figure 9B) and no expressed sequence tags from human or other species align to this intronic region. Translation of the protein product reveals that the novel exon leads to the addition of seven novel amino acids (QLKKLPK) after codon 118 before reaching a stop codon. By sequencing the region surrounding this pseudoexon, we were able to detect a heterozygous change (c.358-304C>G) which is only five base pairs after the end of the cryptic exon. This cytosine is at base pair 31,962,351 on chromosome 21 based on NCBI build 36.1, is not listed as a known SNP and is not in a region of extensive interspecies homology. This mutation segregated in all affected individuals, thus we hypothesize that it is the cause of the inclusion of the cryptic exon and the genetic cause of ALS for this family.

These results were confirmed in a bioinformatic manner by use of the Berkeley Drosophila Genome Project (BDGP) which examines splice site prediction in humans [291]. The wildtype sequence of *SOD1* intron 4 includes a potential acceptor splice site sequence of the cryptic exon 4b (probability of 0.70). The donor splice site corresponding to the cryptic exon was predicted with a score that increased from 0.43 for the wildtype 'C' allele to 0.99 for the mutated 'G' residue. By comparison, the probability of the correct donor and acceptor sequences at the end of exon 4 and the beginning of exon 5 were 0.99 and 0.97 respectively. These findings were validated by a second splice site program, NetGene2 [292] which also predicted that the mutation would create a novel donor site, with a confidence score of 0.63, at the location determined by cDNA analysis.

3.1.7 Discussion

The identification of a novel alternative exon for *SOD1* is surprising considering that this gene has been extensively studied since its discovery for ALS, and no alternate exons have been described. In addition, alternative splicing does not appear to be a prominent feature of *SOD1* despite one publication which examined alternative forms [293]. It may be possible that additional families with ALS have a *SOD1* mRNA product that includes this novel exon. However, this will remain a type of mutation which is difficult to detect given the amount of intronic sequence which could potentially bear a mutation and the fact that detection or confirmation of such a mutation is dependent on the availability of cell lines for cDNA analysis. The identification of the mutation in this family likely would have gone unnoticed, except that enough individuals were collected to convince us that a mutation truly was present in the *SOD1* gene. To date, all the mutations described for *SOD1* have been exonic amino acid mutations, small (<10bp) exonic deletions, or intronic changes near exons. Additional mutations have been reported in intron 4 including a heterozygous change nine base pairs after exon 4 leading to the addition of three amino acids to the SOD1 protein [294] and another change 11 base pairs before exon 5 which led to a premature truncation product [258]. While none of these caused the creation of a pseudoexon, they do suggest that the intron is prone to variation which can influence its proper splicing. The identification of a novel mode of action for *SOD1* mutagenesis could promote the discovery of additional mutations in a likewise manner, thus increasing the proportion of familial ALS which is accounted for by mutations in *SOD1*.

The identification that a relatively deep intronic variant in *SOD1* can nonetheless cause ALS begs the question: how can these intronic variants be detected in the future, particularly in the context of large-scale DNA diagnostics? In many cases, generation of lymphoblasts from peripheral blood and subsequent RNA extraction and cDNA analysis of *SOD1* is a lengthy procedure and is not practically feasible for each patient. However, in

cases of large families with ALS in which suggestive linkage to chromosome 21 can be established, this may be a necessary option. Intron 4 of *SOD1* where most of the intronic variants have been detected is relatively small at 1095 base pairs. Thus it is a more reasonable option to completely sequence this intron with two or three sets of primer pairs to search for intronic variants that influence proper splicing. The implementation of such a strategy needs to be weighed with the fact that the proportion of intronic variants is likely much less than exonic changes.

This intronic variant in *SOD1* that leads to ALS makes for a prime candidate to selectively silence the mutant copy of *SOD1* in this family. Over twenty reports of cryptic or pseudoexons have been documented [295]. Silencing of the allele by antisense morpholino oligonucleotides have been described for deep intronic variants in proprionic and methylmalonic acidemia [296]. While the number of individuals with mutations like this one in *SOD1* is likely rare, this mutation remains a potential target for allele-specific therapy.

3.1.8 Acknowledgments

We would like to thank this family for their participation. P.N.V received support from the Fonds de Recherche en Sante Quebec (FRSQ), V.V.B and G.A.R received support from the Canadian Institute of Health Research (CIHR). We are grateful to the Association Française contre les Myopathies and the Association pour la Recherche contre la Sclérose Latérale Amyotrophique for their financial support. We would also like to thank Isabelle Bachand for technical support. Guy A. Rouleau takes responsibility for the integrity of the data and the accuracy of the data analysis.

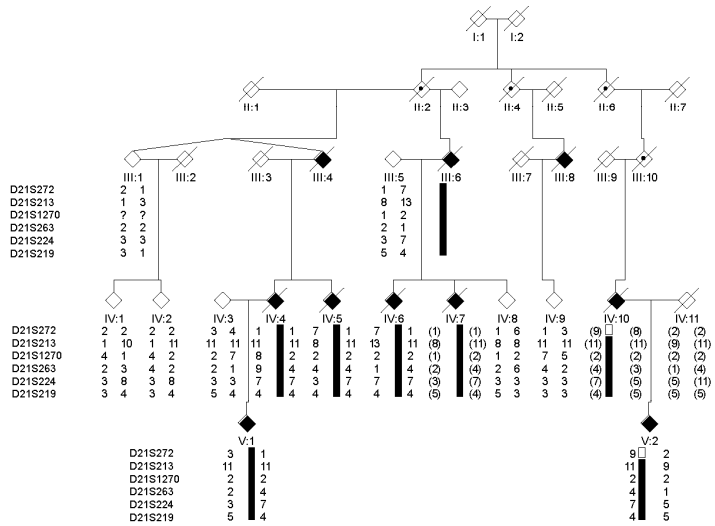
3.1.9 Tables and Figures

Table IX LOD scores for markers surrounding *SOD1* on chromosome 21

marker	Mb	θ = 0	θ = 0.01	θ = 0.05	θ = 0.1	θ = 0.2	θ = 0.3	θ = 0.4
D21S272	24.3	-1.62	-0.30	0.30	0.49	0.54	0.44	0.25
D21S213	30.1	2.03	2.00	1.87	1.70	1.31	0.86	0.4
D21S1270	30.6	1.40	1.38	1.28	1.15	0.84	0.53	0.24
D21S263	31.1	2.84	2.79	2.57	2.28	1.69	1.08	0.49
D21S224	32.8	1.28	1.27	1.17	1.03	0.68	0.33	0.08
D21S219	34.0	2.57	2.52	2.29	2.00	1.40	0.79	0.26
D21S266	41.6	-3.54	-0.85	-0.22	-0.02	0.10	0.10	0.06

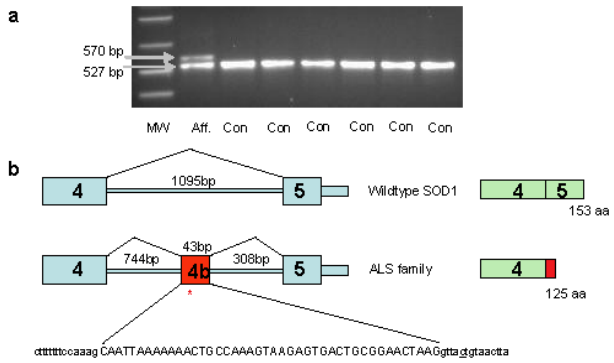
LOD scores are calculated at various recombination distances (θ) from each marker. Marker locations are listed in megabase pair (Mb) positions on chromosome 21. LOD scores from marker D21S266 are from the genome scan, the rest are from all genotyped individuals in the family.

Figure 8 Pedigree of the family with the haplotype on chromosome 21



Family members with affected status are filled in and obligate carriers are marked with a dot. The sex of individuals is masked and those in the bottom generation are omitted to maintain confidentiality. A diagonal line indicates a deceased individual. Genotypes in parentheses are inferred and those with a question mark were not typed. The vertical black bar indicates the haplotype on chromosome 21 which segregates among affected individuals.

Figure 9 Novel pseudoexon in *SOD1*: agarose gel and schematic representation



A) Sequence produce on an agarose gel indicating the wildtype and cryptic exon allele in the patient and only wildtype allele in the controls. B) A schematic of *SOD1* exons (thick boxes) and introns (lines) and the splicing which occurs normally in *SOD1* and in this examined family. Numbers refer to the size of each segment. An asterix marks the spot where a stop codon is present. The corresponding protein is listed to the right of the gene. The inserted pseudoexon sequence of the region between exon 4 and exon 5 is also displayed with exonic sequence in uppercase lettering and the c.358-304C>G variant, which is underlined 5 base pairs after exon 4b. The predicted premature stop codon is in bold.

3.2 No effect on *SOD1* splicing by *TARDBP* or *FUS* mutations

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3.2.1 Rationale

Approximately 94% of human genes are alternatively spliced, and it is estimated that about 50% of disease-causing mutations affect splicing. [297-299] Like mentioned in the previous section, mutations in both *cis*-acting elements and *trans*-acting components can disrupt gene splicing or regulation of alternative splicing. Specifically, *cis*-acting elements are required for accurate pre-mRNA processing, while *trans*-acting components are essential for splicing regulation. Splicing mutations can directly cause disorders, or contribute to the phenotype by influencing disease susceptibility or modulating disease severity.[286]

Recent reports in neurodegenerative disorders research underlined the contribution of RNA-binding protein in alternative splicing regulation and other RNA-processing events.[286] Precisely, there are a few examples of mutations in genes essential for splicing, and this is the case for ALS. TDP-43 is a *trans*-acting splicing regulator and a member of the hnRNP family. The protein encoded by *TARDBP* has been involved in the mechanism of cystic fibrosis after the demonstration of its binding to a repetitive (UG)*n* element in intron 8 of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which have been found to promote exon 9 skipping while decreasing the expression of the encoded protein.[300] TDP-43 is also known to play a role in FTD and ALS, as it is abnormally included in ubiquitinated protein aggregates localized in the cytoplasm of neurons and glial cells.[112, 301] Mutations in *TARDBP* as well as in *FUS*, another RNA-binding protein with similar functions,[237] have been reported in both ALS and FTD patients.[6, 101-103, 302, 303] This suggests that splicing abnormalities and other RNA-processing events might explain neurodegeneration.

The RNA targets of TDP-43 and FUS still need to be identified, and we hypothesized that *SOD1* was one of them. This hypothesis is tested in the work presented next.

3.2.2 Contribution of authors

Belzil : Study design, data generation and analysis, manuscript writing.

Daoud: Manuscript revision.

Dion & Rouleau : Experiment supervision, manuscript revision.

3.2.3 Abstract

There was no abstract included in the manuscript since it was published in a research letter format.

3.2.4 Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by a neuronal loss in the motor cortex, brain stem and spinal cord, that progresses over a three to five year period. Approximately 90% of cases are considered to be sporadic (SALS), while the other 10% of cases are familial (FALS)[304]. Extensive research has been conducted on the *SOD1* gene after mutations were identified more than seventeen years ago in 15-20% of FALS cases, as well as a small number of SALS cases in later reports[304]. In the last two years, mutations in *TARDBP*, which encodes TDP-43, and in *FUS* have been identified in both FALS and SALS cases[6, 101, 250]. Both genes encode for RNA/DNA binding proteins, are mainly localized in the nucleus, and are implicated in the regulation of RNA processing[305]. Specifically, TDP-43 and FUS have

been shown to be associated with other splicing factors and are believed to play a role in splicing regulation, as variation in their expression level influences the splicing of certain targets[305]. Interestingly, mutant TDP-43 proteins identified in ALS patients were recently reported to be more stable than wildtype TDP-43 and to display an enhanced interaction with FUS polypeptides[306]. Particularly, it was reported that the FUS protein interacts more predominantly with mutant TDP-43, which display an increased half-life by comparison to wild type TDP-43. The authors concluded on a note about the efforts needed to determine whether the increased association affects the RNA targets for TDP-43, FUS, or both[306]. Considering that abnormal RNA processing and splicing patterns are involved in neurodegenerative diseases[304], and that variation in the RNA splicing of *SOD1* can cause familial ALS by destabilizing the resulting protein[304], our aim was to assess if *TARDBP* and/or *FUS* ALS predisposing mutations which are known to lead to the accumulation of TDP-43 or FUS aggregations in the cytoplasm, may lead to aberrant *SOD1* RNA splicing events, and to determine if *SOD1* could be an RNA target for TDP-43, FUS, or both.

3.2.5 Methods

We studied total RNA prepared from immortalized lymphoblastoid cells of one healthy control individual, as well as seven different *TARDBP* (p.D169G,p.G287S, p.G348C, p.R361S, p.Y374X, p.A382T, p.N390D) and four different *FUS* (p.P18S, p.G174del, c.1542-2A>C, p.R521H,) mutated individuals. Protocols were approved by the ethics committee and the institutional review board of the University of Montreal. All patients gave written informed consent after which patient information and blood were collected. mutated individuals The second set of primers amplified a product of 528 bp (c.398-462), starting at the beginning of exon 4 and ending at the end of the 3'UTR.

3.2.6 Results

Agarose gel electrophoresis showed that only one product was amplified for each of the cDNA amplified (figure 10). In addition, no variation was found in the sequence traces of the *SOD1* cDNA products.

3.2.7 Comments

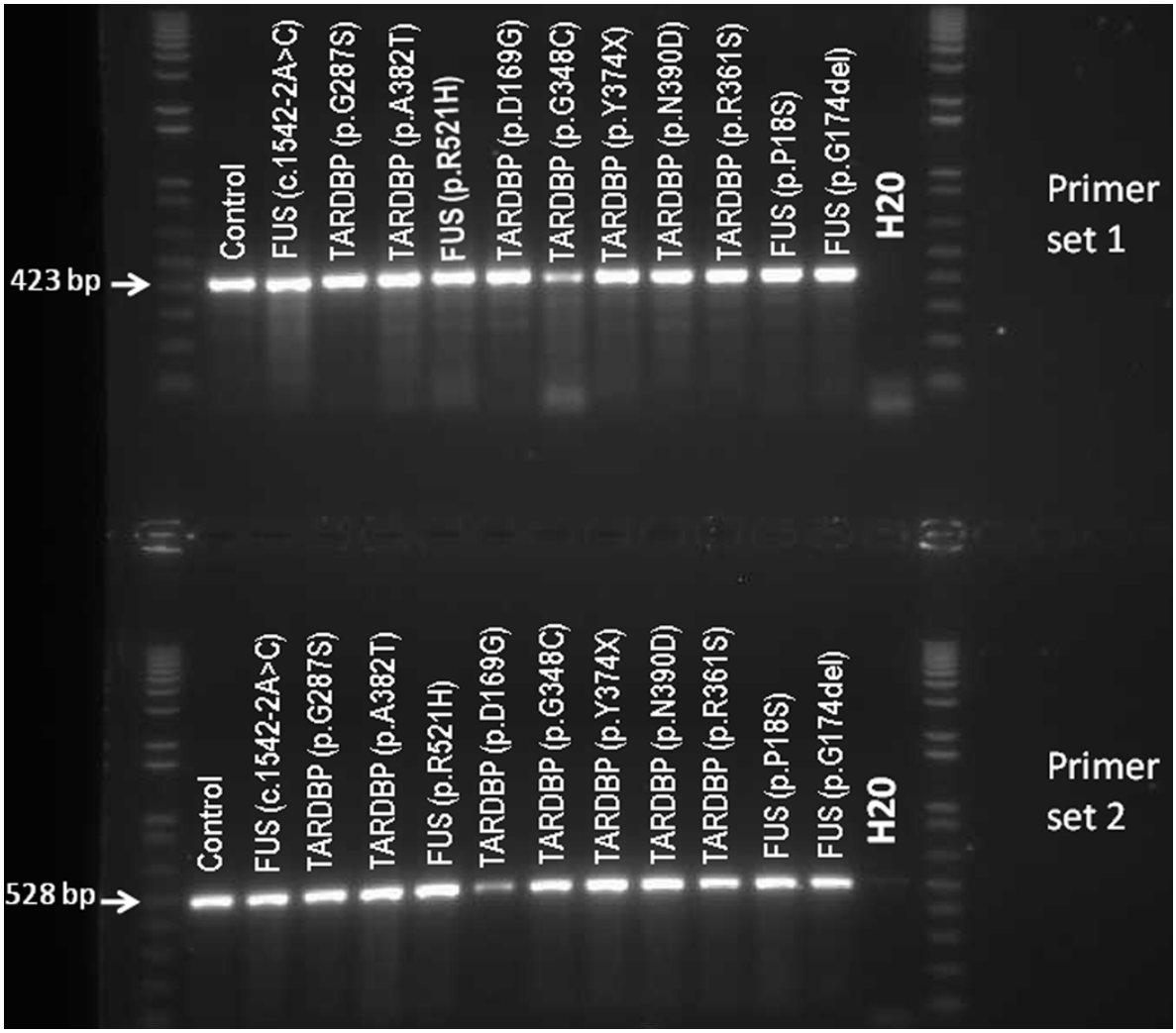
We can thus conclude that *TARDBP* or *FUS* ALS-predisposing mutations do not affect the splicing of *SOD1* and that, while it can't be excluded that there may be a common ALS pathogenic pathway, it appears that mutant *TARDBP* and *FUS* do not act by affecting the splicing of the most frequently mutated gene, *SOD1*, in ALS.

3.2.8 Acknowledgments

We would like to thank the patients involved in this study. VVB, HD and GAR are supported by the Canadian Institutes of Health Research. Guy A. Rouleau had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

3.2.9 Tables and Figures

Figure 10 Agarose gel electrophoresis of *SOD1* mRNA in *TARDBP* and *FUS* mutated samples



The first amplified product of 423 bp for the control and the eleven *TARDBP* or *FUS* mutants is shown on top. The second product of 528 bp is shown under the first product for the same samples.

Chapter 4 : Contribution of mutations in new candidate genes to ALS

4.1 Analysis of *OPTN* as a causative gene for amyotrophic lateral sclerosis. Published in Neurobiology of Aging. March 2011;32(3):355.

4.2 Genetic analysis of *SIGMAR1* as a cause of familial ALS with dementia. Submitted to European Journal of Human Genetics, December 27, 2011.

4.3 Analysis of the *SORT1* gene in familial cases of amyotrophic lateral sclerosis. Accepted in Neurobiology of Aging, January 20, 2012.

4.1 Analysis of *OPTN* as a causative gene for amyotrophic lateral sclerosis

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4.1.1 Rationale

It is well known that inherited disease mutations are not always expressed in the same way in all individuals based on the principles of penetrance, expressivity and influence of modifier genes. Precisely, penetrance measures the fraction of a population who carry a disease-causing allele and develop the disease phenotype, while expressivity evaluates the extent to which a genotype shows its phenotypic expression. The action of gene modifiers, on the other hand, can be responsible for differences in penetrance and expressivity parameters.[307] A good example is the divergence found in FALS pedigrees, where mutation carriers start to develop the disease at different ages, notice muscle weakness in different regions of their body, experience a different disease progression, and sometimes even develop another completely different neurodegenerative phenotype.

During the last four years, we witnessed exciting advancements in the genetics of ALS as novel mutations in several new genes have been identified. While these findings have been replicated in different populations for some of the genes, the implication of other was controversial and further reports were needed. One example is a publication from a Japanese group in 2010, identifying *OPTN* mutations in ALS patients.[121] Interestingly, the *OPTN* gene has been linked to two other phenotypes. However, contrary to the other ALS genes associated with various motor phenotypes, the three other conditions in which *OPTN* mutations were found are completely different from ALS. Specifically, *OPTN* mutations have been first involved in primary open-angle glaucoma (POAG) in 2002[122] and then associated with Paget's disease of bone (PDB) in 2010.[308] Based on our scepticism towards the *OPTN* involvement in ALS, we decided to screen for possible mutations a sub-group of our FALS cohort.

4.1.2 Contribution of authors

Belzil : Study design, data generation and analysis, manuscript writing.

Daoud: Manuscript revision.

Desjarlais: Clinical information organization, manuscript revision.

Bouchard, Dupré & Camu: Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

4.1.3 Abstract

Mutations in the *OPTN* gene are well known to be associated with the development of glaucoma. Recently, unique variations in the same gene have been reported in familial and sporadic Japanese cases of amyotrophic lateral sclerosis. We set out to evaluate the frequency of *OPTN* mutations in a sample of our FALS and SALS cohorts. All coding exons of the *OPTN* gene were amplified and sequenced in 95 unrelated FALS and 95 SALS cases of European descent. Two variants were newly identified in two individual FALS cases. Unique variations in the *OPTN* gene are rare in FALS cases and were not identified in any SALS patients, all of European descent.

4.1.4 Introduction

Amyotrophic lateral sclerosis (ALS) is a severe progressive adult onset neurodegenerative disease characterized by the death of motor neuron located in the motor cortex, brain stem, and ventral horn of the spinal cord. Muscle weakness and spasticity usually progress over a 3 to 5 year period, typically resulting in death from respiratory

failure. Three genes are considered to be definitely implicated in a small proportion of classical ALS: *SOD1, TARDBP,* and *FUS*.[309] However, overall, no genetic causative variations have been found for the majority of patients affected with ALS. On the other hand, mutations in the *OPTN* gene are known to predispose to glaucoma.[122] Surprisingly, a recent study of Japanese ALS patients identified two different variations in the *OPTN* gene in two recessively inherited familial ALS (FALS) cases as well as in one sporadic ALS (SALS) patient.[121] They also identified one mutation transmitted in an autosomal dominant way with incomplete penetrance in two individual FALS patients. In order to confirm those findings in our familial and sporadic ALS cohorts, we sequenced the entire open reading frame of the *OPTN* gene in 95 FALS and 95 SALS patients of European descent.

4.1.5 Results and Discussion

Three variants were found in the same three exons in which variants were previously identified in ALS cases. First, the p.K59N missense was identified in one FALS patient, but was also found in one control participant out of 190 controls tested. Second, a substitution from a guanine to an adenine was identified in the donor splice site located in intron 12 of one FALS case. The substitution was followed by the insertion of another adenine (c. 1242+1G>A_insA), which might cause a frameshift and the inclusion of 3 new amino acids before a stop codon is encountered (NIHX). The end product is predicted to be a truncated protein of 417 amino acids instead of the 577 amino acids protein found in the wildtype state. The substitution was not present in 190 matched controls. Third, previously unreported missense p.A481V variant was identified in one FALS patient, and was not found in 190 control participants.

It is interesting to note that the *OPTN* variants identified in our study are only from dominant FALS, while those reported in the Japanese families were found both in dominant

and recessive families. It is true that our FALS kindreds are primarily dominant, which might explain why no mutations were found in recessive FALS. However, no variants were found in the SALS cases, some of which may well represent recessive ALS cases. Considering that primary open angle glaucoma (POAG) is the most common type of glaucoma, that it is found in comorbidity with normal tension glaucoma (NTG) in 90% of the Japanese affected with POAG, that POAG with NTG is more frequent in Japanese than Caucasians,[310] and that we identified two times less variations in our FALS Caucasian cohort (2%) than in the original Japanese FALS report (4.4%), one could speculate that the variations identified are actually associated with a genetic predisposition to glaucoma instead of being ALS causative.

4.1.6 Supplementary material

Complete manuscript: long version submitted as supplementary material.

Abstract

Background: Mutations in the *OPTN* gene are well known to be associated with the development of glaucoma. Recently, unique variations in the same gene have been reported in familial and sporadic Japanese cases of amyotrophic lateral sclerosis, a severe degenerative disease affecting the neurons of the motor cortex, brain stem, and spinal cord. Therefore, we set out to evaluate the frequency of *OPTN* mutations in a sample of our FALS and SALS cohorts. Methods: All coding exons of the *OPTN* gene were amplified and sequenced in 95 unrelated FALS and 95 SALS cases of European descent. Results: One substitution followed by one base pair insertion which affect the donor splice site of intron 12, as well as one unreported missense in exon 14 were newly identified in two individual FALS cases. Conclusion: Unique variations in the *OPTN* gene are rare in FALS cases and were not identified in any SALS patients, all of European descent.

Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or maladie de Charcot, is a severe progressive adult onset neurodegenerative disease characterized by the death of motor neuron located in the motor cortex, brain stem, and ventral horn of the spinal cord. Muscle weakness and spasticity usually progress over a 3 to 5 year period, typically resulting in death from respiratory failure. About one affected individual out of ten have a family history of the disease (FALS). Twelve loci and nine genes have been so far identified in FALS cases. Three of these genes are considered to be

definitely implicated in classical ALS: *SOD1, TARDBP,* and *FUS*. Since the identification of mutations in the *SOD1* gene in 1993,[60] the proportion of ALS patients with *SOD1* mutations have been evaluated in many different populations, with variations in the *SOD1* gene explaining about 1-3% of cases[65]. More recently, mutations in the *TARDBP* gene encoding the TDP-43 protein and in the *FUS* genes have been identified in ALS, together representing approximately 5-10% of FALS cases.[309] Overall, no genetic causative variations have been found for the majority of patients affected with ALS.

Mutations in the *OPTN* gene[121] are known to predispose to glaucoma, usually in families with an autosomal dominant inheritance and an adult age of onset. Surprisingly, a recent study of Japanese ALS patients identified two different variations in the *OPTN* gene in two recessively inherited FALS cases as well as in one sporadic ALS (SALS) patient. They also identified one mutation transmitted in an autosomal dominant way with incomplete penetrance in two individual FALS patients. In order to confirm those findings in our familial and sporadic ALS cohorts, we sequenced the entire open reading frame in 190 unrelated cases of ALS of European descent. One previously unreported missense variant in exon 14 and one novel substitution followed by an insertion located in the donor splice site of intron 12 were identified in two individual with FALS. No mutations or splice site variations were identified in SALS.

Materials and Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Protocols were approved by the ethics committee on human experimentation of the Centre Hospitalier de l'Université de Montréal. All patients gave written informed consent after which patient information and blood were collected.

Subjects

All ALS patients participating in this study were recruited through clinics in France and Canada and independently ascertained by trained neurologists. DNA was extracted from peripheral blood using standard protocols. A total of 95 unrelated FALS and 95 SALS cases, all of European descent, were screened for the 13 coding exons of the *OPTN* gene. The three exons of the gene in which we identified variations were the same in which variants were previously identified in Japanese ALS patients;[121] we therefore sequenced exons 5, 12, and 14 in 190 controls of the same ethnic origin.

Gene Screening.

Using 13 sets of primers, the open reading frame of the *OPTN* gene was amplified in each sample. The PCR product contained a minimum of 50 bp from each of the flanking introns. Primers were designed using the ExonPrimer software from the UCSC human genome browser website (http://genome.ucsc.edu/cgi-bin/hgGateway). Amplification was conducted by polymerase chain reactions (PCRs) using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per manufacturer's instructions. PCR products were amplified with the same procedure of direct sequencing in patients and controls, and were sequenced at the Genome Quebec Innovation Center (Montréal, Québec, Canada) using a 3730XL DNA analyzer. Mutation surveyor software (version 3.10) was used for mutation detection analyses (SoftGenetics, Pennsylvania, USA).

Bioinformatics prediction programs.

Prediction scores of functional effects for non synonymous SNPs were obtained using Polyphen prediction[247] and SIFT bioinformatics[311] programs.

Results

The entire coding region of the *OPTN* gene was amplified and sequenced in 95 FALS and 95 SALS patients. Three variants were found in the same three exons in which variants were previously identified in ALS cases. First, the p.K59N missense was identified in one FALS patient, but was also found in one control participant out of 190 controls tested. The amino acid change is predicted to be possibly damaging by the Polyphen prediction program, and the SIFT bioinformatics program concluded that the change does affect the protein function. The control with the p.K59N variant was a female in her 80's at the time of collection, without any motor disease symptoms. Second, a substitution from a guanine to an adenine was identified in the donor splice site located in intron 12 of one FALS case. The substitution was followed by the insertion of another adenine (c. 1242+1G>A_insA), which might cause a frameshift and the inclusion of 3 new amino acids before a stop codon is encountered (NIHX). The end product is predicted to be a truncated protein of 417 amino acids instead of the 577 amino acids protein found in the wildtype state. The substitution was not present in 190 matched controls. Unfortunately, we were unable to prove the frameshift at the RNA level, considering that immortalized lymphoblast cell lines were not available for the patient, and DNA from other family members was not obtainable. Third, previously unreported missense p.A481V variant was identified in one FALS patient, and was not found in 190 control participants. Polyphen prediction program concluded that the change is possibly damaging, and SIFT bioinformatics program predicted that the amino acid change do affect the protein function. All the variants identified are listed in supplementary material table X.

Discussion

Three new *OPTN* variants were identified in three FALS cases, and nothing was found in the 95 SALS patients tested. The three exons of the gene where those three variants were identified were the same in which the first report identified variations. Two of the three variants, c.1242+1G>A_insA and p.A481V, were not found in 190 control participants. Since the p.K59N variant was identified in one control participant having no symptoms of motor neuron diseases, the change is believed to be benign. No clinical information regarding possible glaucoma was available for the patient or the control with the mutation, and the missense was not previously reported in any glaucoma patients. The donor site substitution in intron 12 followed by the adenine insertion was found in a family with autosomal dominant transmission. The subject's mother, two aunts, as well as her two brothers were also affected. Unfortunately no DNA samples were available from these or any other family members. The two parents of the FALS sample with the p.A481V missense both developed ALS, so cases of conjugal ALS. Common environment exposure could be an alternative explanation for the ALS in this family, as no other family members of the same or previous generations were affected. No information was available concerning the possibility of a consanguineous marriage.

It is interesting to note that the *OPTN* variants identified in our study are only from dominant FALS, while those reported in the Japanese families were found both in dominant and recessive families. It is true that our FALS kindreds are primarily dominant, which might explain why no mutations were found in recessive FALS. However, no variants were found in the SALS cases, some of which may well represent recessive ALS cases.

Primary open angle glaucoma (POAG) is the most common type of glaucoma, and is found in comorbidity with normal tension glaucoma (NTG) in 90% of the Japanese affected with POAG.[310] Considering that POAG with NTG is more frequent in Japanese than Caucasians, and that we identified two times less variations in our FALS Caucasian cohort (2%) than in the original Japanese FALS report (4.4%), one could speculate that the variations identified are actually associated with a genetic predisposition to glaucoma instead of being ALS causative.

Overall, our data suggest that *OPTN* mutations are at most a rare cause of FALS in subjects of European descent. Additional work is needed to clearly define the role of *OPTN* gene mutations in the ALS pathogenesis.

Acknowledgments

VVB, HD and GAR are supported by the Canadian Institutes of Health Research. We would like to thank the patients involved in this study, Mélanie Benard, Isabelle Thibault and Pierre Provencher for sample collection and organization, and to acknowledge support from the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARS), the Association Française contre les Myopathies (AFM), and the French Group on MND.

Table X Description of genetic variations in *OPTN* found in FALS/SALS patients and control participants

#	Location	Coding DNA variant	Non-synonymous Variant	FALS (n=95)	SALS (n=95)	Controls (n=190)	Status
1	x4	102G>A	T34T	32	31	n/a	Reported[312, 313]
2	x4	123 G>A	L41L	0	2	n/a	rs11591687
3	x5	177G>C	K59N	1	0	1	new
4	x5	293T>A	M98K	6	3	23	Reported[122, 313]
5	x12	1242+1G>A_insA	n/a	1	0	0	new
6	x13	1328delA	Q443fs	0	1	n/a	rs67884543
7	x14	1442C>T	A481V	1	0	0	new

When numbering the mRNA, +1 represents the A of the ATG. The start methionine is amino acid number 1.

4.2 Genetic analysis of *SIGMAR1* as a cause of familial ALS with dementia

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4.2.1 Rationale

ALS and FTD are two phenotypes clearly linked based on the fact that a few families have been identified with members affected with either one or both disorders[20, 21, 146-148] and that *TARDBP*, *FUS, VCP, CHMP2B* and *UBQLN2* mutations have been identified in both ALS and FTD patients.[6, 20, 101-103, 132, 133, 141, 145, 302, 314] While the *GRN* gene is not typically involved in ALS, mutations in this gene have been reported in FTD patients showing some motor neuron deficits.[315, 316] In addition, about 5% of patients with ALS develop FTD, and up to 50% of ALS cases do experience some cognitive impairment.[19] However, the exact frequency of FTD or cognitive impairment in ALS patients still needs to be clarified.

In 2006 and 2007, linkage to chromosome 9p has been established using large families from the UK, US, France and Canada, this way identifying the 9p13.3–21.3 ALS-FTD locus. [21, 147, 148] There was an overlap of 9 cM (8.8 Mb) between the three reported regions, precisely encompassing 36 genes. Concerted efforts to identify the causative gene in the region have been made considering the possibility of a major locus on this chromosome, but no mutations were identified after screening the entire region. In 2010, two groups conducted a genome-wide association study using samples from the UK and Finland and found a significant association to chromosome 9p21.[154, 155] This finding further emphasized the importance of the 9p13.3–21.3 locus. Also importantly, the ubiquitin/TDP-43 pathology has been observed and confirmed in some ALS/FTD families linked to the same locus.[150] Later in the same year, a variant in the 3'-untranslated region (UTR) of the *SIGMAR1* gene was reported in affected members of a large FTLD-MND pedigree.[317] Two additional 3'UTR variants were also identified in two other but independent FTLD families. While the gene is also located on the chromosome 9p, the UTR variants were mostly present in individuals affected with FTD, and its implication in the ALS phenotype was questionable.

Very recently, two groups identified *C9ORF72* expansions as responsible for the ALS-FTD phenotype linked to this chromosome 9p locus. [158, 159] More importantly, this variant was believed to explain 3% of sporadic FTD, 11.7% of familial FTD, 4.1% of sporadic ALS, and 23.5% of familial ALS.[158] The noncoding hexanucleotide repeat expansions located in intron 1 of the *C9ORF72* gene are the most common cause of familial ALS and familial FTD identified to date. This expanded region is now considered to be a major cause of ALS and FTD, and reinforce the previously discussed idea that *cis*-acting elements are essential for accurate pre-mRNA processing. Recently, a consanguineous family with members affected with JALS was reported to carry a *SIGMAR1* coding variant.[318] We present here the screening of the *SIGMAR1* gene that was conducted before the finding of the *C9ORF72* repeat expansions and before the report of the homozygous *SIGMAR1* variant in JALS, when we wanted to validate the *SIGMAR1* implication in the disease.

4.2.2 Contribution of authors

Belzil : Study design, data generation and analysis, manuscript writing.

Daoud: Manuscript revision.

Camu: Clinical evaluation, sample collection, manuscript revision.

Strong: Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

4.2.3 Abstract

Amyotrophic lateral sclerosis (ALS) is the most common of the motor neuron diseases (MND), while frontotemporal lobar degeneration (FTLD) is the second most common cause of early-onset dementia. Many ALS families also segregating FTLD have been reported, particularly over the last decade. Recently, mutations in *TARDBP*, *FUS/TLS* and *C9ORF72* have been identified in both ALS and FTLD patients, while mutations in *VCP,* a FTLD associated gene, have been found in ALS families. Distinct variants located in the 3'-untranslated region (UTR) of the *SIGMAR1* gene were previously reported in three unrelated FTLD or FTLD-MND families. We directly sequenced the coding and UTR regions of the *SIGMAR1* gene in a targeted cohort of 25 individual familial ALS cases of Caucasian origin with a history of cognitive impairments. This screening identified one variant in the 3'-UTR of the *SIGMAR1* gene in one ALS patient, but the same variant was also observed in one out of 380 control chromosomes. Subsequently, we screened the same samples for a *C9ORF72* repeat expansion: 52% of this cohort was found expanded, including the sample with the *SIGMAR1* 3'UTR variant. Consequently, coding and non-coding variants located in the 3'-UTR region of the *SIGMAR1* gene are not the cause of FTLD-MND in our cohort, and more than half of this targeted cohort is genetically explained by C9ORF72 repeat expansions.

4.2.4 Introduction

Amyotrophic lateral sclerosis (ALS) is the most common of the motor neuron diseases (MND), with an incidence of 1.2/100,000 person-years,[319] while frontotemporal lobar degeneration (FTLD) is the second most common cause of early-onset dementia with an incidence of 3.5/100,000 person-years.[320] Interestingly, up to 50% of ALS patients are deemed likely to develop some cognitive impairments.[19] In 2006 the TDP-43 protein was found to be an important constituent of aggregates in neurons of MND-FTLD patients,[112]

which suggested a common pathological pathway for the two conditions. Mutations in the *TARDBP* gene encoding the TDP-43 protein,[101, 302] and in the *FUS/TLS* gene encoding the FUS protein[6, 303] have been identified in both ALS and FTLD patients, while mutations in ALS cases have recently been reported in *VCP,*[133] in which mutations have been previously associated with FTLD,[132] Furthermore, neurons of ALS and FTLD patients both display a nuclear clearing and cytoplasmic sequestration of normal cellular TDP-43 or FUS proteins,[237] which are encoded by genes that, when mutated, are estimated to account for up to 5-10% of all ALS cases.[6, 101] Twelve families linked to a locus on chromosome 9p have been reported to have members with either MND or FTLD, while a few members display both phenotypes.[21, 147-150, 153] The causative pathogenic hexanucleotide repeat in the gene *C9ORF72* was recently identified by two different groups.[158, 159] One of these reports found that this repeat expansion explains approximately 3% of sporadic FTD, 11.7% of familial FTD, 4.1% of sporadic ALS, and 23.5% of familial ALS cases.[158] The second report shows that the repeat expansion is detectable in about 50% of familial and 20% of sporadic Finnish cases, in more than one third of familial cases of wider European ancestry, and in more than 29% of the Finnish FTD population among which 36% have a family history.[159] In addition, recently, a variant in the 3'-untranslated region (UTR) of the *SIGMAR1* gene, encoding a receptor protein playing an important role in various cellular functions, was reported to segregate in a FTLD-MND pedigree.[317] Two additional 3'-UTR variants were identified by the same group in two unrelated FTLD families thought to be linked to the chromosome 9p locus, but no segregation could be observed in these. Another group recently conducted homozygosity mapping in a large consanguineous family with six members affected with juvenile ALS. They identified a *SIGMAR1* missense mutation in all affected individuals.[321] Considering the finding of variants in the *SIGMAR1* gene and the recent publication of hexanucleotide repeat expansions in *C9ORF72* located in the same chromosomal region, we wanted to evaluate the potential contribution of *SIGMAR1* variants in ALS patients of European descents. We selected 25 individual familial ALS cases with a family history of cognitive impairments, and directly sequenced the entire

coding and UTR regions of the *SIGMAR1* gene. We also evaluated this specific targeted cohort for *C9ORF72* repeat expansions.

4.2.5 Materials and Methods

4.2.5.1 Standard Protocol Approvals, Registrations, and Patient Consents.

Protocols were approved by the ethics committee on human experimentation of the Centre Hospitalier de l'Université de Montréal. All patients gave written informed consent after which patient information and blood were collected.

4.2.5.2 Subjects

Clinical information from 260 familial ALS cases of Caucasian origin not mutated in *SOD1*, *TARDBP* or *FUS* was analyzed; 25 families were found to have a history of cognitive impairments. One affected member from each family was selected for sequencing the entire coding and UTR regions of the *SIGMAR1* gene (NM_005866.2). In addition, 190 control participants were sequenced for one fragment encompassing the first 396 bp of the 3'-UTR, in which one novel substitution was identified in one familial ALS patient. Moreover, the same patients were assessed for the presence of expanded hexanucleotide repeat in *C9ORF72* (NM_001256054.1).

4.2.5.3 Gene Screening.

Six sets of primers were used for each sample to amplify the open reading frame of the *SIGMAR1* gene (Table 1). The PCR products containing the exons included a minimum of 50 bp from each of the flanking introns. Primers were designed using the ExonPrimer

software from the UCSC human genome browser website (http://genome.ucsc.edu/cgi-bin/hgGateway). Amplification was conducted by polymerase chain reactions (PCRs) using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per manufacturer's instructions. PCR products were amplified with the same procedure of direct sequencing in patients and controls, and were sequenced at the Genome Quebec Innovation Center (Montréal, Québec, Canada) using a 3730XL DNA analyzer. Mutation surveyor software (version 3.10) was used for mutation detection analyses (SoftGenetics, Pennsylvania, USA).

4.2.5.4 Hexanucleotide repeat analysis

We performed a repeat-primed PCR assay using the FastStart PCR Master Mix (Roche, Indianapolis, Indiana, USA) using the reported optimized conditions.[158, 159] PCR products were analyzed on an ABI 3730 sequencer with GeneMapper software version 4.0 (Applied Biosystem, Foster City, California, USA).

4.2.6 Results and Discussion

Only one variant (c.672*+43G>T) located in the 3'UTR of the *SIGMAR1* gene was identified in one patient. The substituted guanine is well conserved through different species. Other affected family members were not available for testing, but the variant was not present in six other unaffected family members. However, after testing 190 matched controls, the variant was identified in one control, suggesting that the substitution is not causative of ALS in the family. No other unknown variants were identified in the 25 patients tested. Interestingly, after performing the repeat-primed method, we found that 13 patients actually carried a hexanucleotide repeat expansion in *C9ORF72*. This represents

52% of the total tested. The cause of the disease for the remaining 12 patients is still unexplained genetically.

The *SIGMAR1* gene was a positional candidate based on linkage in an Australian MND-FTLD family.[149] The c.672*51G>T 3'UTR substitution in the *SIGMAR1* gene was identified in the linked family after the sequencing of about 200 candidate genes in the chromosome 9p candidate region. The nonpolymorphic nucleotide change segregated with the disease haplotype of this Australian pedigree. The variant was not identified in 1,269 controls. In addition, c.672*26C>T and c.672*47G>A substitutions also located in the 3'-UTR of *SIGMAR1* were identified in different FTLD pedigrees of Australian and Polish origins respectively. These variants were also absent in matched controls. A modification in *SIGMAR1* gene expression was found in the Australian, while a small expression difference was observed for the other two families, each with only one patient actually carrying the variant. It was also shown that modulating the expression of *SIGMAR1* influences the cellular localization of TDP-43 and FUS, and it was suggested that the 3'UTR of *SIGMAR1* variant causes a pathogenic alteration of *TARDBP* and *FUS* expression levels.[317] Considering that the *C9ORF72* hexanucleotide repeat expansion was not reported when the *SIGMAR1* mutations were identified and that the genes are close to each other on chromosome 9p, it is possible that the *SIGMAR1* variants identified in the first report actually segregated with *C9ORF72* expansions. The finding of a 3'UTR variant in our specifically targeted cohort permitted to test and confirm this hypothesis.

Further studies in different populations will help to evaluate the contribution of the sigma nonopioid intracellular receptor 1 protein in MND and/or FTLD, and future studies need to address the reported effects on expression levels of the different proteins involved. However, based on our study, it can be concluded that coding mutations or UTR variants in *SIGMAR1* are not a cause of ALS in our cohort of Caucasian ALS families with a history of

cognitive impairments, but that repeat expansions in *C9ORF72* genetically explain a significant proportion of the same cohort.

4.2.7 Acknowledgments

VVB, HD and GAR are supported by the Canadian Institutes of Health Research. We would like to thank the patients involved in this study, and to acknowledge support from the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARS), the Association Française contre les Myopathies (AFM), and the French Group on MND. GAR holds the Canada Research Chair and a Jeanne-et-J.-Louis-Levesque Chair for the Genetics of Brain Diseases.

4.3 Analysis of the *SORT1* gene in familial amyotrophic lateral sclerosis

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4.3.1 Rationale

It is becoming more and more apparent that defective RNA metabolism contributes to the pathogenesis of several neurodegenerative diseases, including ALS.[106, 322, 323] Indeed, TDP-43, mutated in about 5% of familial ALS, regulates various aspects of RNA metabolism such as transcriptional repression, exon skipping and RNA splicing.[324] Also, it was demonstrated that the cytoplasmic redistribution of TDP-43, primarily a nuclear protein, seems to appear early in ALS.[325] Thus, the mislocalization of TDP-43 to the cytoplasm, its abnormal post-translational modifications and its aggregation are believed to adversely affect TDP-43 function and the regulation of its RNA targets. Since TDP-43 RNA targets are likely altered in FTD and ALS, an important aim in the field today is to identify those targets and determine whether these are altered in ALS and FTD. This could provide valuable insight into the mechanisms of TDP-43 toxicity and eventually help develop therapeutics for the two disorders.

On the other hand, mutations in *GRN*, the gene encoding progranulin (PGRN), are the second most common cause of inherited FTD after *C9ORF72* expansions.[326] *GRN* mutations lead to the degradation of mutant RNA and cause PGRN haploinsufficiency.[327] The neuropathological hallmarks in common between ALS and FTD suggest that TDP-43 and PGRN are mechanistically linked in the disease pathogenesis. However, the normal functions of TDP-43 and PGRN and how they contribute to neurodegeneration remain unclear. Nonetheless, it was recently discovered that depletion of TDP-43 in the mouse adult brain using antisense oligonucleotides modifies the expression levels of *FUS* and *GRN*, while altering the splicing of *SORT1*.[328] Precisely, it has been shown that the presence of TDP-43 is associated with the exclusion of exon 18 in *SORT1*.[328] Also, it was demonstrated that sortilin (SORT1) is a neuronal PGRN receptor that mediates PGRN uptake,[329] where sortilin is the principal neuronal binding site for PGRN.[329] Binding occurs via the c-terminus of PGRN, and this results in a rapid endocytosis of PGRN by sortilin. In

vivo, the absence of sortilin raises PGRN levels by 2.5- to 5-fold. Given that FTLD is caused by a 50% reduction of PGRN levels,[330] this change has clear pathophysiological consequences. All of these make *SORT1* an excellent candidate gene for ALS and a possible RNA target for TDP-43.

We attempted to demonstrate in the next study that *SORT1* splicing could be modified by mutated *TARDBP*. We also screened the *SORT1* gene for possible mutations in FALS cases. While our results are negative, they explored a critical hypothesis and helped further refine our understanding of the pathogenesis of ALS and FTD.

4.3.2 Contribution of authors

Belzil : Study design, data generation and analysis, manuscript writing.

André-Guimont: Technical support, data generation and analysis.

Atallah: Technical support

Daoud: Manuscript revision.

Dupré, Bouchard, Camu: Clinical evaluation, sample collection, manuscript revision.

Dion & Rouleau : Sample collection, experiment supervision, manuscript revision.

4.3.3 Abstract

Background: Substantial efforts have been deployed in the last decade to identify the genetic causes of amyotrophic lateral sclerosis (ALS), and we hypothesized here that

mutations in *SORT1* or aberrant *SORT1* splicing reduce progranulin level and promotes neurodegeneration. **Methods:** We sequenced the coding exons of *SORT1* in a cohort of 112 unrelated individuals with familial ALS. We also tested for aberrant *SORT1* splicing by RT-PCR using RNA samples from cell lines expressing six different ALS-associated *TARDBP* mutations. **Results**: We identified one unique missense and two unique silent mutations in our cohort. None are predicted to have functional effects. No aberrant *SORT1* splicing event was observed. **Conclusions:** *SORT1* mutations are not a common cause of familial ALS and the influence of *TARDBP* mutations on *SORT1* splicing still needs to be clarified.

4.3.4 Introduction

Recently, causative mutations were reported in *TARDBP*, [101, 102] encoding TDP-43, and *FUS* [6, 103] in both amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) cases; [303, 331] the latter is a condition commonly seen in comorbidity with ALS. Mutations in *GRN,* which encodes progranulin, lead to FTLD with TDP-43 inclusions (FTLD-TDP) as a result of progranulin haploinsufficiency. [332] It has also been recently found that depletion of TDP-43 in the mouse adult brain modifies the expression levels of *FUS* and *GRN*, and alters the splicing of *SORT1,* which encodes sortilin. [328] Specifically, it has been shown that the expression of TDP-43 leads to altered splicing of *SORT1* with exclusion of exon 18. [328] Sortilin has also recently been identified as a receptor that mediates progranulin uptake. [329] Thus, one can speculate that *TARDBP*, *GRN* and *SORT1* act in a common pathological pathway. To test this notion, we screened the *SORT1* coding sequence for mutations and assessed if *TARDBP* ALS predisposing mutations led to aberrant *SORT1* RNA splicing, which would confirm that *SORT1* pre-mRNA is a target of TDP-43.

4.3.5 Methods

A total of 112 unrelated FALS cases, all of European descent and negative for mutations in *SOD1*, *TARDBP* or *FUS* and without a *C9ORF72* repeat expansion, were screened for the 20 coding exons of the *SORT1* gene and its alternative exon 1. We also studied total RNA prepared from immortalized lymphoblastoid cells of six different *TARDBP* mutated samples (p.D169G, p.G287S, p.G348C, p.Y374X, p.A382T, p.N390D) as well as two healthy control individuals. The amplifications were performed using one set of primer pairs that covered exons 17, 18 and 19 of *SORT1* mRNA, encompassing the splice region reported to be altered after TDP-43 depletion.

4.3.6 Results

Sequence analysis identified six novel variants in six unrelated FALS cases. One p.A773T (c.2317G>A) missense mutation was found in one patient, but no additional family members were available for testing to confirm segregation. The variant was also predicted to be benign or tolerated by Polyphen and SIFT bioinformatics programs. [247, 248] A p.V104V silent mutation was found in one patient but no other familial DNA was available for testing. An intronic substitution of an adenine to a guanine was identified at position c.832+46, possibly creating a new donor splice site. However, no alternative isoform or aberrant splice site event were predicted by the Alternative Splice Site Predictor (ASSP) or Berkeley Drosophila Genome Project softwares. [333, 334] These three variants were nevertheless absent from 380 control chromosomes. Also, a p.K302E missense mutation was identified in one patient, but was also found in five control participants. Two additional variants were identified in two individual FALS cases, a p.I544V missense mutation and a p.Y600Y silent mutation, but both were not cosegregating with the disease. We then amplified the cDNA of six different *TARDBP* mutated samples and two controls to test for inclusion or exclusion of exon 18 in the *SORT1* RNA sequence. We were expecting to

amplify two products: one of 181 bp for control cDNA (excluding exon 18) and one of 287 bp for *TARDBP* mutated samples (including exon 18). Only one product of 287 bp was amplified in all samples, and exon 18 was confirmed to be included in all products by sequencing cDNAs of patients and controls.

4.3.7 Discussion

While the role of *SORT1* in the TDP-43 pathogenic pathway has been proposed, [328] our findings suggest that mutations in the gene do not directly predispose to ALS. One limitation of our study was that it was restricted to the screening of FALS samples and that only a few of these had a positive family history of dementia. Other mutation screens will be necessary to further evaluate the possible contribution of *SORT1* mutations to ALS and FTLD. A single *SORT1* isoform was amplified from cells bearing ALS-predisposing *TARDBP* mutations and from control cells. Interestingly, in all cases, the *SORT1* mRNA contained exon 18, despite the fact that these cells are known to express TDP-43.[101] Therefore, we were unable to confirm that *SORT1* is a pre-RNA target of TDP-43, both in normal cells and in cells from patients with ALS predisposing *TARDBP* mutations. Nor did we find any effects of mutant TDP-43 on exon 18 splicing in *SORT1*. However, because we extracted cDNA from lymphoblastoid cells, it is possible that there are cell specific differences in *SORT1* splicing. Possibly TDP-43 expression is necessary for the exclusion of *SORT1* exon 18 only in neuronal cells; brain tissues were unfortunately unavailable from *TARDBP* mutated samples. Other methods will need to be used to confirm if *SORT1* is an RNA target of TDP-43.

4.3.8 Supplementary Material

Complete manuscript: long version submitted as supplementary material.

Abstract

Background: Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease, which affects upper and lower motor neurons. Substantial efforts have been deployed in the last decade to identify the genetic causes of ALS, and significant progress has been made, such as the identification of mutations in the *TARDBP* gene. Interestingly, depletion of TDP-43, the protein encoded by *TARDBP*, in mouse adult brain modifies the expression of *FUS* and *GRN* which encodes progranulin, and alters the splicing of *SORT1,* which encodes sortilin, a progranulin receptor. Specifically, the presence of TDP-43 is associated with the exclusion of exon 18 in *SORT1*. We hypothesized that mutations in *SORT1* or aberrant *SORT1* splicing reduces progranulin level and promotes neurodegeneration. In addition, we assessed if *TARDBP* ALS predisposing mutations lead to aberrant *SORT1* RNA splicing, which might provide insights into disease pathogenesis. **Methods:** We sequenced the coding exons of *SORT1* in a cohort of 112 unrelated individuals with familial ALS; among these, 23 had a positive family history of dementia. We also tested for aberrant *SORT1* splicing by RT-PCR using RNA samples from cell lines expressing six different ALS-associated *TARDBP* mutations. **Results**: We identified one unique missense and two unique silent mutations in our cohort. None are predicted to have functional effects. No aberrant *SORT1* splicing event was observed and exon 18 was retained despite the presence of TDP-43. **Conclusions:** *SORT1* mutations are not a common cause of familial ALS and the influence of *TARDBP* mutations on *SORT1* splicing still needs to be clarified.

Introduction

Amyotrophic lateral sclerosis (ALS) is a disease that affects approximately two new individuals per 100,000 each year.[257] Because motor neurons located in their cortex, brain stem and spinal cord degenerate, ALS patients generally experience progressive muscle weakness and spasticity of upper and lower limbs, as well as in the bulbar region. This progressive degeneration typically evolves over a three to five year period resulting in death from respiratory failure. The first symptoms are generally reported in the mid 50's and 90% of cases do not have a prior family history of motor neuron diseases.[309] The identification of ALS causative genes began with the identification of *SOD1* mutations in ~15-20% of familial ALS (FALS) and ~2% of all cases. More recently, causative mutations were reported in *TARDBP*, [101, 102] encoding TDP-43, and *FUS*; [6, 103] these genes together explain ~5% of all cases. The role of the proteins encoded by those three genes is only partially understood and their pathological pathways are the subject of much research. Interestingly mutations were identified in *TARDBP* and *FUS* both in ALS and frontotemporal lobar degeneration (FTLD) cases; [303, 331] the latter is a condition commonly seen in comorbidity with ALS. In fact, it is estimated that up to 50% of ALS patients may develop some sort of cognitive impairments, [19] reinforcing the belief that a common pathological pathway is involved in the development of the two phenotypes. Furthermore, two groups recently reported hexanucleotide repeat expansions in the *C9ORF72* gene [158, 159] in chromosome 9p association in ALS-FTLD families. This repeat explained ~11% of familial FTLD, ~24% of familial ALS, and 1/3 of European descents familial ALS cases.

Mutations in *GRN* are a major cause of FTLD, explaining the disease in 10% of the overall FTLD population and more than 20% of patients with a family history of dementia.[326] Mutations in *GRN,* which encodes progranulin, lead to FTLD with TDP-43 inclusions (FTLD-TDP) as a result of progranulin haploinsufficiency. [332] It has also been recently found that depletion of TDP-43 in the mouse adult brain using antisense

oligonucleotides modifies the expression levels of *FUS* and *GRN*, and alters the splicing of *SORT1,* which encodes sortilin. [328] Specifically, it has been shown that the expression of TDP-43 leads to altered splicing of *SORT1* with exclusion of exon 18. [328] Sortilin has also recently been identified as a receptor that mediates progranulin uptake. [329] Thus, one can speculate that *TARDBP*, *GRN* and *SORT1* act in a common pathological pathway. To test this notion we screened the *SORT1* coding sequence for mutations. We also assessed if *TARDBP* ALS predisposing mutations led to aberrant *SORT1* RNA splicing, which would confirm that *SORT1* pre-mRNA is a target of TDP-43.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Protocols were approved by the ethics committee on human experimentation of the Centre Hospitalier de l'Université de Montréal. All patients gave written informed consent after which patient information and blood were collected.

Subjects

All ALS patients participating in this study were recruited through clinics in France and Canada and independently ascertained by trained neurologists. DNA was extracted from peripheral blood using standard protocols. A total of 112 unrelated FALS cases, all of European descent and negative for mutations in *SOD1*, *TARDBP* or *FUS* and without a *C9ORF72* repeat expansion, were screened for the 20 coding exons of the *SORT1* gene. In addition, the alternative exon 1 included in alternative isoforms was screened.

Gene Screening

Using 19 sets of primers, the open reading frame of the *SORT1* gene was amplified in each sample. The PCR products contained a minimum of 50 bp from each of the flanking introns. Primers were designed using the ExonPrimer software from the UCSC human genome browser website (http://genome.ucsc.edu/cgi-bin/hgGateway). Primer sequence and amplification conditions are available upon request. Amplification was conducted by polymerase chain reactions (PCRs) using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per manufacturer's instructions. PCR products were amplified with a procedure of direct sequencing in patients, and were sequenced at the Genome Quebec Innovation Center (Montréal, Québec, Canada) using a 3730XL DNA analyzer. Mutation surveyor software (version 3.10) was used for mutation detection analyses (SoftGenetics, Pennsylvania, USA).

Bioinformatics prediction programs

Scores predicting the functional effects for nonsynonymous SNPs were obtained using Polyphen prediction[335] and SIFT[248] bioinformatics programs. Splice site score predictions were obtained using the Alternative Splice Site Predictor (ASSP)[334] and Berkeley Drosophila Genome Project bioinformatics programs.[333]

Splicing study

We studied total RNA prepared from immortalized lymphoblastoid cells of six different *TARDBP* mutated samples (p.D169G, p.G287S, p.G348C, p.Y374X, p.A382T, p.N390D) as well as two healthy control individuals. RNA was extracted using standard conditions. Reverse transcription polymerase chain reactions (RT-PCRs) were conducted to prepared cDNA using the M-MLV RT enzyme (Invitrogen, Carlsbad, California, USA) as

per manufacturer's instructions. The amplifications were performed using one set of primer pairs that covered exons 17, 18 and 19 of *SORT1* mRNA, encompassing the splice region reported to be altered after TDP-43 depletion. The primers were predicted to amplify a region of 287 bp if exon 18 is included or a product of 179 bp if the same exon is excluded.

Results

Sequence analysis identified six novel variants in six unrelated FALS cases. One p.A773T (c.2317G>A) missense mutation was found in one patient, but no additional family members were available for testing to confirm segregation. The variant was also predicted to be benign or tolerated by Polyphen and SIFT bioinformatics programs. [247, 248] A p.V104V silent mutation was found in one patient but no other familial DNA was available for testing. An intronic substitution of an adenine to a guanine was identified at position c.832+46, possibly creating a new donor splice site. However, no alternative isoform or aberrant splice site event were predicted by the Alternative Splice Site Predictor (ASSP) or Berkeley Drosophila Genome Project softwares. [333, 334] These three variants were nevertheless absent from 380 control chromosomes. Also, a p.K302E missense mutation was identified in one patient, but was also found in five control participants. Two additional variants were identified in two individual FALS cases, a p.I544V missense mutation and a p.Y600Y silent mutation, but both were not cosegregating with the disease. All the variants identified are listed in table 1.

We then amplified the cDNA of six different *TARDBP* mutated samples and two controls to test for inclusion or exclusion of exon 18 in the *SORT1* RNA sequence. We were expecting to amplify two products: one of 181 bp for control cDNA (excluding exon 18) and one of 287 bp for *TARDBP* mutated samples (including exon 18). Only one product

of 287 bp was amplified in all samples (figure 1A), and exon 18 was confirmed to be included in all products by sequencing cDNAs of patients and controls (figure 1B).

Discussion

Three unique *SORT1* variants were identified in three of 112 individual with FALS. However, these variants are unlikely to be causative of ALS or ALS with dementia as they are predicted to have only a minor or no effect on the protein. While the role of *SORT1* in the TDP-43 pathogenic pathway has been proposed, [328] our findings suggest that mutations in the gene do not directly predispose to ALS. One limitation of our study was that it was restricted to the screening of FALS samples and that only a few of these had a positive family history of dementia. Other mutation screens will be necessary to further evaluate the possible contribution of *SORT1* mutations to ALS and FTLD.

A single *SORT1* isoform was amplified from cells bearing ALS-predisposing *TARDBP* mutations and from control cells. Interestingly, in all cases, the *SORT1* mRNA contained exon 18, despite the fact that these cells are known to express TDP-43.[101] Therefore, we were unable to confirm that *SORT1* is a pre-RNA target of TDP-43, both in normal cells and in cells from patients with ALS predisposing *TARDBP* mutations. Nor did we find any effects of mutant TDP-43 on exon 18 splicing in *SORT1*. However, because we extracted cDNA from lymphoblastoid cells, it is possible that there are cell specific differences in *SORT1* splicing. Possibly TDP-43 expression is necessary for the exclusion of *SORT1* exon 18 only in neuronal cells; brain tissues were unfortunately unavailable from *TARDBP* mutated samples. Other methods will need to be used to confirm if *SORT1* is an RNA target of TDP-43.

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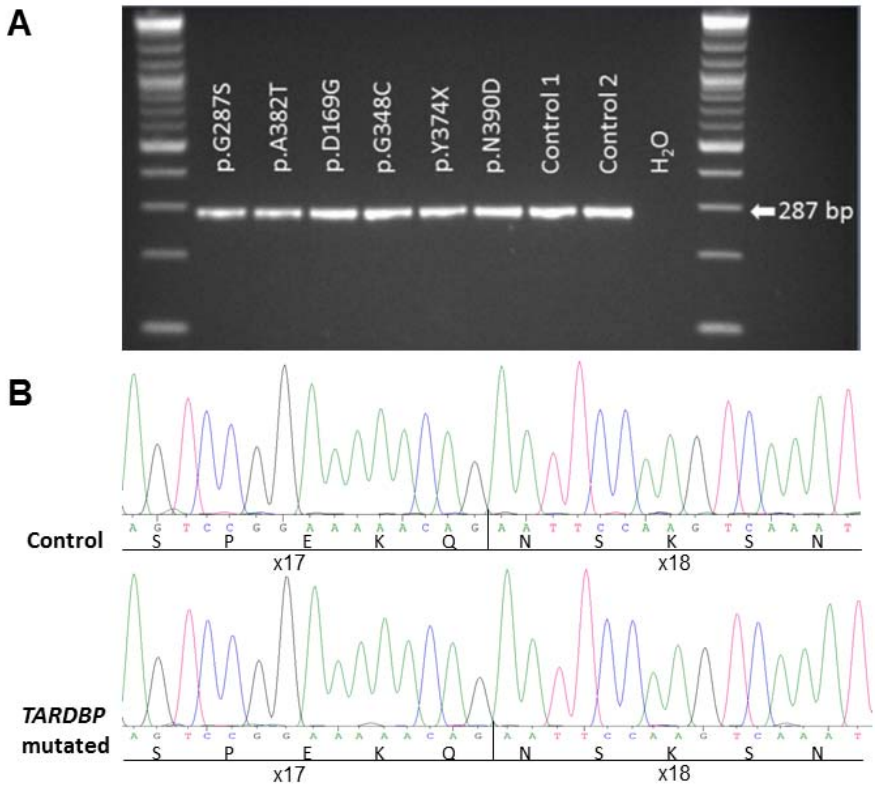
4.3.9 Tables and Figures

Table XI Variants identified in the *SORT1*gene for FALS samples

Variant SORT1	Localization	Coding DNA Variant (nucleotide)	Coding DNA Variant (protein)	FALS (*n*=112)	Controls (n=190)	dbSNP
1	x1	c.12 C>G	p.P4P	62	n/a	rs72646553
2	5'UTR & x1 alt	n/a	n/a	147	n/a	rs2140924
3	5'UTR & x1 alt	n/a	n/a	2	n/a	rs116767369
4	x2	c.312 G>A	p.V104V	1	0	n/a
5	x5	c.594 T>C	p.F198F	163	n/a	rs11142
6	x5	c.597 A>G	p.R199R	13	n/a	rs72646560
7	x7	c.832+46A>G	n/a	1	0	n/a
8	x8	c.904 A>G	p.K302E	1	5	n/a
9	x9	c.969 A>C	p.T323T	75	n/a	rs2228604
10	x11	c.1330 G>C	p.E444Q	5	n/a	rs72646568
11	x13	c.1630 A>G	p.I544V	1	in family controls	n/a
12	x14	c.1800C>T	p.Y600Y	1	in family controls	n/a
13	x18	c.2317G>A	p.A773T	1	0	n/a
14	3'UTR	n/a	n/a	68	n/a	rs5776974
15	3'UTR	n/a	n/a	36	n/a	rs464218

Figure 11 Agarose gel electrophoresis of *SORT1* mRNA and chromatograms



(A) *SORT1* RNA was amplified in six *TARDBP* mutated samples and two controls using 5'- CAAATGCCAGGGTGGGGTAAATCCAGTTCG-3' forward primer and 5'-at the end of exon 17 and the beginning of exon 19. Only one product of 287 bp was amplified in all samples, which includes exon 18. (B) The amplified RNA product sequence trace of one control participant is shown on top, followed by the sequence trace of one mutated *TARDBP* sample. All sequences were the same. This shows the exon 17-18 boundary, confirming that exon 18 was included in all amplified products.

Chapter 5 : Discussion and conclusions

5.1 Discussion

Substantial efforts have been deployed during the last decade to better understand the defective biological pathway in ALS. These efforts were guided by the sudden emergence in the last four years of new mutated genes in affected patients, ALS researchers now considering new possible avenues to the disease.

Genomic mutations can definitely modify the tertiary structure of the encoded protein and consequently alter its ability to interact with still uncharacterized targets. It is also possible that a change in the protein structure results either in a gain of a new function or a complete loss of its initial function. For instance, evidence supports that mutated *SOD1* is not characterized by a loss of its dismutase activity, but rather by one or several toxic gain-of-functions.[309] On the other hand, mutations in certain genes might alter their own expression and/or modify directly or indirectly the expression of their RNA targets. Precisely, mutations in *TARDBP* or *FUS*, two DNA/RNA binding proteins, are believed to influence the expression of their still unidentified main targets. Considering this, it can be suggested that the same ALS phenotype might result from two different pathological pathways: one caused by oxidative stress to neuronal cells involving *SOD1*, a pathway that has been extensively studied, and another caused by an RNA pathology involving *TARDBP* and/or FUS, a pathway that still need to be understood. Precisely, it was demonstrated that patients with *SOD1* mutations display a distinct metabolic profile in the cerebrospinal fluid (CSF) when compared with patients without *SOD1* mutations, reinforcing the idea that two different pathways are involved.[336] In addition, cases with *SOD1* mutations did not show any TDP-43 pathology, also suggesting that degeneration may result from a different mechanism.[337] Conversely, it is possible that mutant *SOD1* contributes to the RNA

pathology through an adjacent pathway implicated in RNA processing. Specifically, G93A-SOD1 mice have been reported to compete with the RNA stabilizer HuR for binding to the 3'UTR of *VEGF*.[109] Mutant *SOD1* was actually found to impair the post-transcriptional processing of *VEGF* mRNA and decrease its expression. [81] *VEGF* encodes for a protein having an important neuroprotective role in the nervous system. Geneticists were prompted to screen for *VEGF* mutations in ALS patients after the report that *VEGF* depletion in mice produces a motor neuron phenotype[338] and that *VEGF* influences motor neuron degeneration in ALS mice and humans.[339] While the first report found an at-risk associated allele including *VEGF*, following studies were unable to replicate the results.[340-343] Some reports however suggested a possible gender-dependant association.[344, 345] Deletion of the hypoxia-response element (HRE) in the promoter region of *VEGF* in G93A-SOD1 mice demonstrated a susceptibility to persistent paralysis after spinal cord ischemia. Treating the mice with *VEGF* isoform A actually protected them against ischemic motor neuron death. [339] This, combined with *VEGF* association with *SOD1*, suggests that long-term treatment with *VEGF* might delay the onset of ALS symptoms or slow down its progression.

Moreover, one of the main interests in the field is intracellular aggregates resulting from abnormal protein misfolding after genomic mutations. *SOD1*, *TDP-43* or *FUS* dysfunctional aggregates have been repeatedly observed in ALS patients and are used to categorize distinct proteinopathies of the disease. It is possible that these protein aggregates do not directly cause ALS but do so indirectly by preventing other proteins trapped in these aggregates to perform their function. These aggregations are known to cause endoplasmic reticulum (ER) stress then consequently mitochondrial dysfunction and excessive reactive oxygen species (ROS) production. In turn, oxidative stress and increased ROS induce ER stress, protein misfolding and a cell stress response named the unfolded protein response,[346] this way leading to a neurotoxic cycle. The cell's pro-survival pathway and vitagene system are responsible for the anti-oxidant and anti-apoptotic activities contributing to reduce oxidative stress. The inability of these systems to effectively perform their role is believed

to accelerate the aging process and eventually lead to neurodegeneration. The failure to rescue neurons can be explained by the antioxidants being overwhelmed by the cell toxicity and/or by a reduced expression or action of these vitagenes.[347] It can be speculated that a therapeutic approach targeting the vitagene system and increasing its antioxidant action might decelerate neurodegeneration in ALS patients.

Another interest in ALS research is the role of environmental influences in the emergence of sporadic ALS. Precisely, oxidative stress can be induced by epigenetic factors as a result of environmental toxicity.[174] Oxidative damage to lipids, proteins and nucleic acids has been extensively observed in spinal cords and other tissues of ALS patients[348] along with impairment of the DNA repair activity.[349] It is now firmly believed that oxidative stress play a central role in the ALS etiology, but its place in the disease biological cascade needs to be clearly defined. What precisely provokes oxidative stress and how it specifically contributes to neuronal death in ALS is still unclear. Several experiments gave rise to conflicting results concerning the association of several environmental factors with the increased risk to develop ALS. It is possible that certain genomic predispositions present in targeted individuals are activated by being exposed to an associated environmental factor. It is also probable that some exposed individuals do not develop the disease because they are genetically unsusceptible or are actually exposed after an undetermined critical age. Future studies will definitely help to better understand the implications and contributions of these factors in the pathogenesis of ALS as well as their role in other neurodegenerative diseases.

No gene is known to be uniquely mutated in ALS patients (Table I). The fact that patients affected with different phenotypes are mutated in the same gene suggests a common pathway to the pathogenesis of several neurodegenerative diseases. The most obvious example is the comorbidity of ALS with FTD. Up to 50% of ALS patients

experience some impairments of frontotemporal functions,[350] and about half of FTD patients eventually develop motor neuron symptoms.[351] In turn, family members of ALS patients have an increased risk to develop either FTD or Parkinson's disease (PD).[23] Of interest, TDP-43 aggregates have been identified in both ALS and FTD patients.[112] Noteworthy, 60% of genes known to be mutated in ALS patients have also been found mutated in patients affected with FTD or other types of cognitive impairment. Interestingly, the biological changes caused by oxidative stress and observed in neuronal cells of ALS patients are also present in brain and peripheral tissues of individuals affected with other degenerative diseases, including Alzheimer's disease (AD), PD, and Huntington's disease (HD).[348] In addition, neurological disorders such as AD, PD, ALS, HD, and Friedreich ataxia (FRDA) have all been associated with the production of unusual aggregated proteins, these diseases commonly belonging to the "protein conformational diseases" class.[352] Moreover, repeat expansions in *C9ORF72* were not only present in ALS and/or FTD patients, but also in AD cases.[353] Since the finding of those expansions is relatively new, it is possible that additional *C9ORF72* variations will eventually be identified in patients with other neurodegenerative phenotypes. Also, the finding of *C9ORF72* expansions in ALS and FTD cases put these diseases in the same growing class of noncoding repeat expansion disorders, which might also guide research on treatment. Expansions in this class of diseases are thought to cause either a perturbation of the target gene expression or splicing, or the release of abnormal amounts of toxic RNA, which disrupts normal cellular pathways.[159] Specifically, RNA generated from pathogenic repeat expansions have been demonstrated to disrupt transcription after sequestering wildtype RNA and proteins involved in transcription regulation.[354] Indeed, the role of RNA metabolism has been shown to clearly play a role in ALS after being associated to mutations in *TARDBP* and *FUS*.[106]

Of interest, several observations were reported concerning both ALS and PD patients, suggesting that the two phenotypes formally believed to be independent actually

have a lot in common biologically. First, the incidence of ALS/PD was communally reported to be 100 times higher in Chamorro people of the Guam Island after being extensively exposed to BMAA produced across the cyanobacterial order.[355, 356] Interestingly, this important syndrome has now disappeared on the Guam Island, probably because of a drastic change in the Chamorro people's diet.[357] Similarly, an ALS/parkinsonism-dementia complex (PDC) was identified in the region of Kii, where the clinical and neuropathological manifestations of patients were similar to the syndrome reported in Guam.[358] Another common finding is an increased level of iron and selenium observed in the brains of both ALS and PD patients.[173] In addition, OPTN aggregates have been identified in both ALS and PD patients, and mutations in *FUS*, *ANG* and *TARDBP* known to cause ALS were also identified in patients affected with either PD or Parkinsonism (Table I). It was also recently demonstrated that an excess of rare variants in *ANG* are significantly associated with ALS and PD,[359] a gene previously reported to be mutated not only in ALS but also in FTD and Parkinsonism patients. It is also interesting to mention that family members of ALS patients have an increased risk to develop PD,[23] and that they are some reports of family members of FALS patients with both ALS and/or Parkinson's disease[360] or patients with ALS and Parkinsonism.[361] ALS, PD and FTD are consistently found in comorbidity, and must share a common biological pathway. Research on treatment must indeed combined results obtained from the three different fields to succeed and eventually treat the patients effectively.

Another interesting observation is the phenotypic variability found in ALS families. Some family members of FALS patients may experience different phenotype, age of onset, place of symptoms onset, or disease duration, and this even if they carry the same genomic mutation. This fact is well illustrated by the inherited A382T *TARDBP* mutation identified in Sardinian ALS patients.[68] Another example is the report of A382P *TARDBP* mutation previously reported in ALS[362] but also found in a patient experiencing sensory and motor symptoms.[363] Another case of a juvenile ALS patient experiencing a rapid progression and carrying a *FUS* mutation with the same mutation carried by his unaffected mother was

described in this work.[186] Considering that ALS shares a lot genetically and biologically with other neurodegenerative phenotypes and that exposure to the same environmental toxin can lead to two different phenotypes just like mutations in the same gene can lead to different neurological diseases, we will certainly learn much from research conducted on other diseases and this will surely guide research on treatment discovery. In fact, research on ALS treatment has not been successful up to now. The only known treatment is Riluzole, which has a modest effect on survival and is expected to prolong life of only two to three months.[2]

Furthermore, the accumulation of misfolded proteins inside and outside neurons and glial cells in ALS prompted the suggestion that ALS may be a prion-like pathology. Precisely, this comes from the fact that protein aggregates play an essential role in ALS initiation and progression by acquiring toxic properties through increased hydrophobicity and sequestration of vital cellular constituents within the aggregates. This degenerates into ROS production, proteasome inhibition, and dysfunction of other pathways.[12] On the other hand, prion diseases have been demonstrated to also consist of a misfolded form of the normal prion protein that accumulates into aggregates, recruiting the wildtype form of the protein and inducing its conformation change.[364] This misfolded protein is then released out of the cell, which eventually induces conformation changes of normal prion proteins in the neighboring cells. This type of spreading and propagation has been previously reported in other neurodegenerative diseases.[12] It was demonstrated that aggregated forms of either SOD1 or TDP-43 proteins can seed misfolding into a more important amount of the corresponding wild-type protein in vivo.[365, 366] Misfolded forms of both SOD1 and TDP-43 in culture cells prompted the misfolding and successive aggregation of the normal proteins.[366-369] Significantly, induced aggregation of endogenous SOD1 was demonstrated to continue after removing the misfolded seeds.[367, 368] These results suggest that the new aggregates can act as templates for the subsequent misfolding of wildtype SOD1. This type of spreading is consistent with a self-perpetuating cyclic reaction similar to the one induced

by infectious prion aggregates, which explains why some groups proposed that a prion-like pathology defines ALS. Considering this, current research on prion treatment should not be disregard to elaborate therapeutics for ALS.

One last field of research that is promising is the contribution of hormones to the initiation and /or progression of ALS. Precisely, the hypothalamic-pituitary-adrenal (HPA) axis regulates itself by biofeedback and plays a major role in homeostasis by regulating the body's reaction to stress and other vital processes including the activation of sympathetic nervous system and immune response as well as the regulation of many inflammatory genes.[370] The latter is specifically of interest, considering the role of inflammation in ALS.[371] Overproduction of glucocorticoid such as cortisol, one of the end products of the HPA axis, is associated with a significant disruption of cellular functioning leading to widespread physiological dysfunction and aging.[372] Considering that the majority of patients experience the first ALS symptoms at a time when most individuals retire from work, it is tempting to suggest that an increase in cortisol level following such stressful life event may influence other biological systems and initiate the disease. Precisely, just like depressed individuals who are found with a persistent increased cortisol level,[373] ALS patients experience a dysregulation of adrenal activity characterized by a loss of the cortisol circadian rhythm.[374] Consequently, ALS patients have an increased cortisol level in the evening compared to controls whose cortisol level is at its lowest. This dysregulation of cortisol rhythm is also revealed by the lack of cortisol variation following a stress reaction. ALS patients do not show any physiological increase of cortisol after a mild stressor.[374] Considering that depression is more prevalent in war veterans, the association between high cortisol level in veterans and the development of ALS should be explored in the future. Opposing results were reported by other groups. One example is the transgenic overexpression in mice of the corticotropin releasing hormone (CRH), a modulator of the HPA axis and a precursor of cortisol. The resulting increased in cortisol expression has been demonstrated to provide a protective effect on neuronal cells.[375] In the same way, elevated cortisol level induces the release of glucose, which is known to increase neuronal

cells survival and branching through exogenous galanin when released in sufficient amounts.[376] In addition, estrogen administration has been shown to elevate cortisol level.[377] Estrogen is known to interact with *VEGF*,[378] has been proven to be an effective neuroprotective agent and its use has been suggested to attenuate motoneuron death in ALS.[379] Precisely, considering that the incidence of sporadic ALS is higher in men than women, it was demonstrated that the female advantage results from the protective attribute of estrogen. The female advantage actually disappears with increased age because of a drop in estrogen level during menopause.[380] Another opposing proposition about gender disparity in terms of incidence is the possible role played by free testosterone which is known to be significantly reduced in ALS.[381] These controversial results about cortisol along with the influence of other hormones in ALS still need to be clarified, but they might underline new therapeutic avenues to consider.

Combined efforts from different fields to understand ALS are crucial. Among those, mutation reports are clearly essential to define the pathological pathway involved in ALS, and each mutation finding along with all mutated gene identifications add crucial pieces to the puzzle, bringing us closer to a complete understanding of the disease. Considering what we know today, it can be speculated that *SOD1*, *VEGF* and *ALSIN* are part of the same pathway, which could be influenced by the endocrine system. This is based on the fact that *SOD1* binds to *VEGF*,[109] that estrogen interacts with *VEGF*,[378] and that *ALSIN* binds uniquely to mutant *SOD1*.[382] On the other hand, *TARDBP*, *FUS* and *SETX* all have a DNA/RNA recognition domain in their c-terminal region and are thus all involved in RNA processing. Like suggested earlier, these two different pathways involving different genes can be closely interrelated (Figure 12A).

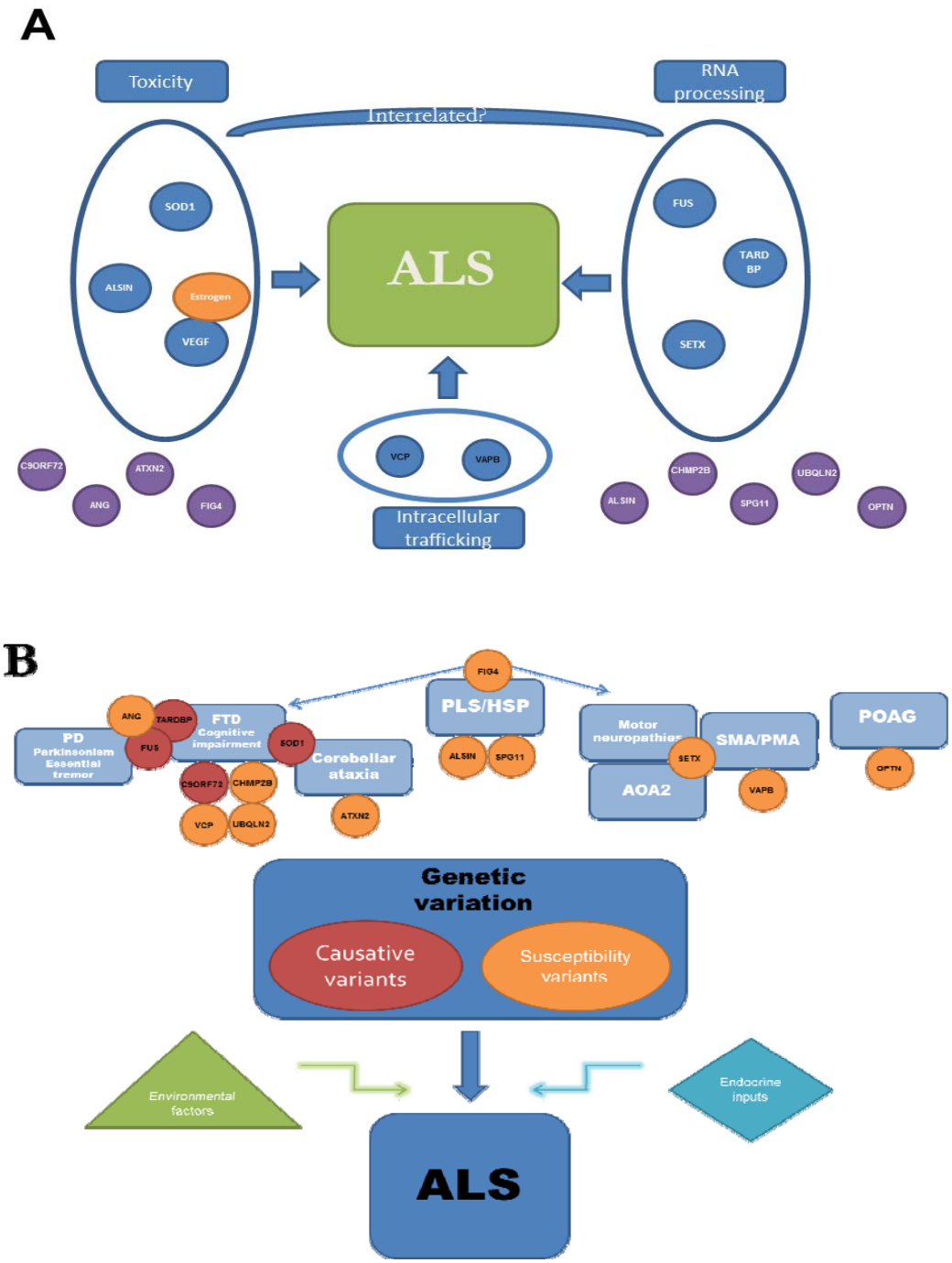
The genetic results obtained from this Ph.D project definitely permitted a better understanding of the role of certain mutated genes in ALS and ruled out the direct implication of others. New mutations were identified, and the proportion of patients of European descents that could be genetically explained by variations in the identified

causative genes have also been assessed, which permitted all together a better evaluation and understanding of these genes contribution to the ALS pathology. Precisely, it was demonstrated in chapter 2 that mutations in *FUS* are found in about 1.3% of the FALS and 1.3% of the SALS tested cohorts of European origin (FALS: 3/235; SALS: 8/595) representing the same percentage of the total tested cohort. This is slightly more than what is reported to date for sporadic cases, but definitely less than what was expected for familial cases. The fact that the tested cohort is part of a heterogeneous population can explain why so few genetic variations were identified. It is predicted that the screening for expansions in the *C9ORF72* gene will genetically explain a major proportion of this previously tested cohort. Genetic screening for *FUS* mutations also permitted the identification of a new causative variant in a juvenile ALS patient and his still unaffected mother, discovery that contributed to reinforce the idea that environmental factors may play an important role in disease onset. Chapter 3 permitted to demonstrate that the percentage of mutation in *SOD1* is probably underestimated, and that patients pretended to be *SOD1*negative can possibly carry an intronic variation influencing the splicing of the gene. Also, it was demonstrated that *SOD1* is not an RNA target of *TARDPB* or *FUS*, as mutations in these two genes do not influence *SOD1* splicing. This discovery reinforces the idea that *SOD1* is part of an independent pathway. Finally, chapter 4 demonstrated that mutations in *OPTN*, *SIGMAR1* and *SORT1* all considered by the field as candidate genes for ALS, are not a direct cause of the disease in patients of European origin. It can however be speculated that these genes play an indirect role in the pathology.

The aim of genetic screening in ALS is first to identify the genes implicated in the pathological pathway or pathways involved in order to understand the role of each player in the disease, and second to offer genetic diagnosis for patients. What has been described throughout this work and what is known so far in the genetics of ALS is summarized in Figure 12B. The final outcome of this work is to target the most upstream players of the pathway in therapeutics to eventually obtain more efficient treatments. Identifying all mutated genes in ALS will also permit the implementation of personalized genetic

treatments. While being able to decelerate disease progression would be a major breakthrough in the ALS field, the ultimate goal would be to stop and even prevent the degeneration of motor neurons. Precisely, a complete understanding of the pathological pathway or pathways will lead to the development of appropriate treatments for ALS patients mutated in different genes. Genetic diagnosis will be essential to provide appropriate targeted preventive treatments to patients. Considering that so many neurodegenerative diseases possibly share some attributes in the pathological pathway involved, such findings would be beneficial for different research fields and ultimately for a wider spectrum of patients.

Figure 12 Genes and factors contributing to ALS and other associated phenotypes



5.2 Conclusions and future perspectives

After reviewing the literature and describing the results obtained, it was demonstrated in this work that mutations in genes such as *SOD1*, *TARDBP*, and *FUS* definitely cause ALS. The implication of *OPTN*, *SIGMAR1* and *SORT1* is not as conclusive. Consequently, the production of truncated or dysfunctional SOD1, TDP-43 and FUS proteins is believed to cause oxidative stress to neuronal cells, the phenomenon being so widespread in the nervous system that the anti-oxidant mechanisms of neurons are unable to act efficiently and rescue the cells. The biological consequences of the *C9ORF72* expansions still need to be determined, but when better understood it will definitely fill an important portion of the puzzle. While the genetic implication of the four causative genes has definitely been demonstrated, the role of others is still questionable. The contribution of the other eleven genes in the ALS pathology cannot be dismissed, since a few patients do carry mutations and changes at the protein level have been biologically demonstrated. However, since mutations in those genes are so rare, it is possible that they actually act as a disease modifier instead of playing a direct causative role in ALS. It is also possible that the genes identified are part of different interacting pathways, and not part of a single biological cascade.

The identification of ALS loci using large families marked an era of ALS genetic research. Three ALS/FTD and fifteen ALS loci for a total of eighteen regions have been identified, and mutations have been found in fifteen different genes. It can be speculated that the three remaining loci are actually associated to mutations in genes already identified. Specifically, the associated locus on chromosome 20p13 might actually be linked to mutations in *VAPB* on chromosome 20q13.32, and the published locus on chromosome 9q21-22 might actually be linked to *C9ORF72* expansions on chromosome 9p21-22 or *VCP* mutations on chromosome 9p13.3. Concerning the ALS3 locus on chromosome 18q21, conflicting results might have arisen due to variations in linkage results, probably

explained by inconsistence in clinical assessments. Still, this hypothesis needs to be demonstrated. Now that most if not all ALS loci are elucidated, ALS geneticists turn to exome and genome sequencing to identify the remaining players of the pathological pathway or pathways involved. The finding of additional genes will definitely help to clearly define and understand the role of each contributor in the pathology, and to adequately target the best therapeutic approach. While a major portion of familial ALS have been elucidated, sporadic patients who are principally affected by the disease still need to be interpreted and the role of environmental or hormonal influences should not be underestimated. Precisely, 25-35% of familial cases and less than 75% of sporadic patients still need to be genetically explained.[383] The eventual development of biomarkers will play an important role in eliminating other diagnostic possibilities and detecting ALS earlier.[14], Knowing that the penetrance is extremely high in autosomal dominant families, the identification of biomarkers will permit to treat mutation carrier before they experience the first symptoms, when effective therapeutics will be available.[384]

The sudden explosion of genetic results during the last ten years in the field permitted the emergence of more precise and better directed hypotheses about the disease etiology. This is an exciting time for ALS researchers, and I strongly believe that an effective treatment to slow down the disease progression will be soon described. I sincerely hope for all the patients and family members affected directly or indirectly by the disease that ALS will be, one day in a near future, effectively cured and prevented.

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Electronic resources

Alternative Splice Site Predictor (ASSP): http://es.embnet.org/~mwang/index.html

Berkeley Drosophila Genome Project (BDGP): http://www.fruitfly.org/

Human Gene Mutation Database (HGMD): http://www.hgmd.org

National Center of Biotechnology Information (NCBI): www.ncbi.nlm.nih.gov

Polyphen: http://genetics.bwh.harvard.edu/pph2/index.shtml

Sorting Intolerant From Tolerant (SIFT): http://sift.jcvi.org/

University of California at Santa Cruz (UCSC): www.genome.ucsc.edu