Neurobiology of Aging 43 (2016) 180.e1–180.e5

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

TBK1 is associated with ALS and ALS-FTD in Sardinian patients

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ARTICLE INFO

Article history:
Received 18 October 2015
Received in revised form 25 March 2016
Accepted 28 March 2016
Available online 9 April 2016

Keywords:
Amyotrophic lateral sclerosis
Genetics
Sporadic
TBK1

ABSTRACT

Recently, mutations in the *TANK-binding kinase 1* (*TBK1*) gene were identified as a cause for amyotrophic lateral sclerosis (ALS) with or without comorbid frontotemporal dementia. We have assessed the frequency and clinical characteristics of TBK1 mutations in a cohort of ALS patients of Sardinian ancestry. Whole-exome sequencing was performed on Hiseq2000 platform (Illumina). Genome analysis Toolkit was used to align and to code variants according to Human Genome (UCSC hg19). Mutation was confirmed with Sanger sequence. In our screening of 186 Sardinian ALS cases, we found 3 (1.6%) patients carrying 3 distinct novel genetic variants: a nonsynonymous SNV c.1150C>T leading to a p.Arg384Thr change in exon 9; a nonsynonymous SNV c.1331G>A causes a p.Arg444Gln change in exon 11; and a frameshift deletion c.2070delG (p.Met690fs) at the exon 20 of the gene leading to a stop at 693 codon. The latter patients also carried missense mutation c.98C>T of the *SQSTM1* gene causing a substitution of an arginine with a valine at the position 33 (p.Arg33Val). All variants were found to be deleterious according to in silico predictions. All cases were apparently sporadic and one of them showed frontotemporal dementia associated to ALS. These mutations were not found in 2 cohorts of 6780 ethnic-matched controls. We have found that *TBK1* mutations account for 1.6% of Sardinian ALS cases. Our data support the notion that *TBK1* is a novel ALS gene, providing important evidence complementary to the first descriptions.

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http://dx.doi.org/10.1016/j.neurobiolaging.2016.03.028

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a degenerative disorder of the central nervous system involving the motor system, namely spinal, bulbar and cortical motor neurons, and, in a subset of cases, frontal cognitive function (frontotemporal dementia, [FTD]).

Most cases of ALS appear sporadically in the community, whereas 10%–15% of patients report a family history for ALS, FTD, or both. To date, the genetic etiology of two-thirds of familial cases and about 11% of sporadic ALS cases has been determined with mutations in *C9ORF72, SOD1, TARDBP*, and *FUS* being the most common (Renton et al., 2014).

Recently, mutations in the *TANK-binding kinase 1* (*TBK1*) gene were identified as a cause for ALS using exome sequencing techniques in a large series of ALS patients (Cirulli et al., 2015). Loss of function and missense mutations and in-frame deletions of *TBK1* were subsequently detected in patients with ALS and ALS-FTD (Freischmidt et al., 2015) and in patients with pure FTD (Pottier et al., 2015).

The aim of this article is to report the frequency and the clinical characteristics of *TBK1* mutations in a cohort of ALS patients of Sardinian ancestry.

2. Methods

2.1. Patients

Whole-exome sequencing was performed in 190 ALS patients and 84 healthy controls of Sardinian ancestry. Cases were collected through the SARDINIALS consortium, a collaborative group involving the neurological departments of Sardinia, and the ITALSGEN consortium, which involves 20 ALS centers throughout Italy.

Controls were identified by the Neurology Department of the University of Cagliari. Both cases and controls had to be Sardinian for at least 2 generations to be included in the study.

After the identification of the 3 novel variants of TBK1, which were not present in the controls who underwent exome sequencing, we wanted to confirm the absence of these variants in a larger series of controls. Therefore, we Sanger sequenced the 3 variants in a further series of 94 novel healthy controls recruited by the Neurology Department of the University of Cagliari. To further evaluate the frequency of the identified *TBK1* variants in the general Sardinian population, we queried whole genome sequence data generated for a cohort of the 6602 subjects enrolled in the SardiNIA study (Pilia et al., 2006; Sidore et al., 2015)

2.2. Genetic analysis

DNA was enriched using either Nextera or Truseq Exome target enrichment technology according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Whole-exome sequencing was performed on Hiseq2000 platform (Illumina) according to producer's protocols. Each sample generated approximately 8.0Gb of sequences. Genome analysis Toolkit (http://www.brodinstitute.org/gatk/) was used to align and to code variants according to Human Genome (UCSC hg19) following best practices (McKenna et al., 2010).

Annovar software (Wang et al., 2010) was used for functional annotation of *TBK1* genetic region against different public databases (human sequence reference Hg19; ESP6500 build October 2014; db SNP138; ExAc release 02; Clinical Variant database, release 29th September, 2014). Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org/) were used to assess the functional effect of the missense mutations on TBK1 protein.

Sanger sequence confirmation was performed using polymerase chain reaction custom primers and BigDye termination v.1.1 (Life Technologies) technologies according to standard protocol. Sequencing products were separated using 3130 Genetic Analyzer (Life Technologies), and sequences were analyzed using SeqScape v2.6.

We screened the exome sequence data for other ALS-related genes for mutations in those patients who carried the mutations of the *TBK1* gene. A list of genes is reported in Supplementary Data. Also, *C9ORF72* GGGGCC repeats were searched for using standard procedures (Chiò et al., 2012); a number of repeats $\geq$30 was considered pathological.

2.3. Standard protocol approvals and patient consents

The study was approved by the ethical committees of the recruiting centers. All patients and controls signed a written informed consent. Databases were treated according to the Italian regulations for privacy.

3. Results

In our screening of 186 Sardinian ALS cases, we found 3 patients carrying 3 distinct genetic variants in *TBK1* (Table 1, Fig. 1). None of these mutations have been previously reported in the literature. The mutations were not found in the 6780 ethnic-matched controls. The first is a nonsynonymous SNV c.1150C>T leading to a p.Arg384Thr change in exon 9. The second is a nonsynonymous SNV c.1331G>A causes a p.Arg444Gln change in exon 11. The third is a frameshift deletion c.2070delG (p.Met690fs) at the exon 20 of the gene leading to a stop at 693 codon. The patients carrying the frameshift deletion also had the missense mutation c.98C>T of the *SQSTM1* gene causing a substitution of an arginine with a valine at the position 33 (p.Arg33Val). The other patients did not carry mutations in any other ALS-related genes. In silico predictions using SIFT, PolyPhen2, and MutationTaster show both missense variants to be deleterious (p.Arg384Thr: SIFT score 0.02, PolyPhen2 score 1.0, and MutationTaster score 101; p.Arg444Gln: SIFT score 0.02, PolyPhen2 score 0.99, and MutationTaster score 43).

Table 1
Mutations of *TBK1* gene detected in Sardinian ALS cases

Sample	Coordinates[a]	Variant[b]	Protein change	Protein domain	ExAC frequency[c]
SLA2009-200	Chr12: 64878240	c.C1150T	p.Arg384Thr	Ubiquitin-like	Not present
SLA2011-515	Chr12: 64879788	c.G1331A	p.Arg444Gln	CCD1	0.00004156
SLA2010-513	Chr12: 64891750	c.2070delG	p.Met690fs	CCD2, OPTN binding site	Not present

Key: ALS, amyotrophic lateral sclerosis; OPTN, optineurin.
[a] Coordinates based on Hg19.
[b] Variant position based on NM_013254.
[c] ExAC frequency based on www.exac.broadinstitute.org (accessed on 30/07/2015).

A frameshift insertion c.1330dupC (p.I1443fs) in the exon 11 of the *TBK1* gene has been found both in an ALS case and in a control.

3.1. Case description

3.1.1. Case 1

The patient presented a rapidly progressive lower limb weakness with frequent falls. The weakness extended to upper limbs within 2 months. At the time of diagnosis, 6 months after the clinical onset, she had both upper and lower motor signs and dysarthria. Electromyography (EMG) showed acute and chronic denervation, and motor-evoked potentials were consistent with corticospinal pathways involvement. Cognition was normal. She was diagnosed with definite ALS according to the El Escorial revised criteria (Brooks et al., 2000). She underwent noninvasive ventilation 4 months later and died 19 months after symptom onset because of respiratory failure. Her father had Parkinson's disease, and her son has a cervical dystonia. She carried the p.Arg384Thr missense mutation of the *TBK1* gene. DNA was not available to test for the presence of the mutation in the father or son.

3.1.2. Case 2

At 72 years of age, the patient developed left hand weakness, rapidly spreading to involve the right hand and lower limbs. His family members reported narrowing of interests, distractibility, poor short-term memory, and difficulties in recognizing other persons before the onset of his motor symptoms. Neuropsychological examination confirmed the presence of frontotemporal cognitive

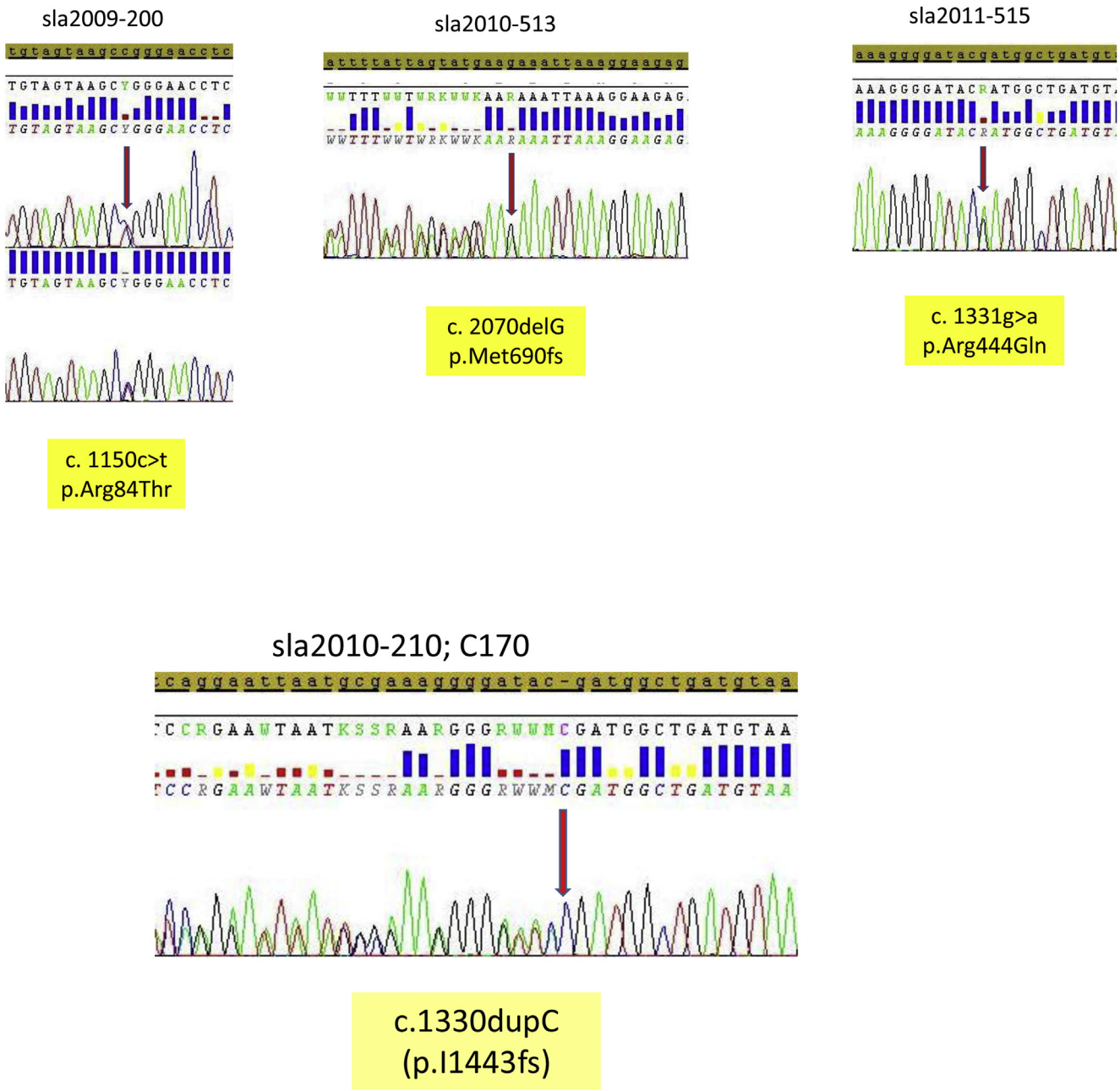

Fig. 1. Sanger sequencing traces of the three identified variants of TBK1 gene.

disturbances. He had brisk reflexes, bilateral Hoffmann sign, and hypophonia. EMG showed diffuse active and chronic denervation, and he was diagnosed as definite ALS with behavioral FTD. His motor signs and cognitive dysfunction worsened in the next few months, and he died 10 months after motor symptom onset. There was no family history of ALS or other neurological disorders. He carried the p.Arg444Gln missense mutation of the *TBK1* gene.

3.1.3. Case 3

This 66-year-old woman presented with progressive difficulties in walking and frequent falls because of foot drop. At diagnosis, she was weak in both lower limbs and had bilateral hand muscle wasting. Reflexes were brisk. She was cognitively normal. EMG revealed generalized active and chronic denervation. In the following years, she manifested progressive upper and lower limb weakness, mild dysphagia, and dysarthria. She is still alive, 104 months after ALS onset, and uses noninvasive ventilation (14 h/d). She did not have family history of ALS or other neurological disorders. She carries both a frameshift deletion p.Met690fs at exon 20 of *TBK1* and a p.Arg33Val missense mutation of *SQSTM1*.

4. Discussion

TBK1 has been recently reported as a novel gene related to both ALS and FTD (Cirulli et al., 2015; Freischmidt et al., 2015; Pottier et al., 2015). We found that mutations of this gene are also present in the isolated population of Sardinia, where they have been detected in 1.6% of cases. This is similar to the frequency of *TARDBP* mutations in the Italian ALS population (Chiò et al., 2012). These mutations were not detected in the 2 cohorts of Sardinian controls, made up of a total of 6780 Sardinian subjects. All mutated cases were apparently sporadic, meaning that it was not possible to demonstrate segregation of the mutation with disease within a family. The apparent sporadic nature of these cases supports the hypothesis that *TBK1* mutations have reduced penetrance. In addition, the p.Arg444Gln missense mutation is reported in the ExAC database to have an allele frequency of 0.00004156, an observation that may also point to reduced penetrance. In light of this, mutational screening of additional cohorts is required to definitively prove the pathogenicity of this variant.

Mutations of the *TBK1* gene have been so far reported in patients with ALS, ALS-FTD, and FTD of Caucasian ancestry (Cirulli et al., 2015; Freischmidt et al., 2015; Pottier et al., 2015). Our data also show the phenotype associated with *TBK1* mutations to be heterogeneous, including cognitive impairment, and a variable clinical course ranging from 10 months to more than 8 years. *TBK1* gene duplications are also involved in normal tension and open angle glaucoma with a mechanism involving the binding of TBK1 protein with optineurin (*OPTN*).

One of our Sardinian ALS patients carried a frameshift deletion p.Met690fs of *TBK1* and a p.Arg33Val missense mutation of the *SQSTM1* gene. This second mutation has been previously reported in 1 familial ALS and 2 sporadic ALS patients (Fecto et al., 2011) and is located in the Src homology 2–binding domain of the SQSTM1 protein. TBK1 phosphorylates p62/SQSTM1 at serine 403, increasing the affinity between p62 and polyubiquitin chain, thereby allowing efficient targeting of polyubiquitinated proteins to autophagosomes (Matsumoto et al., 2011). Although this mutation has not being found in our Sardinian controls, it is reported with a frequency of 0.001207 in ExAC, making it difficult to interpret its pathogenicity. However, interestingly enough, it has been described that TBK1 coordinates assembly and function of the autophagic machinery by phosphorylating the autophagic adaptor p62 (SQSTM1) on Ser403, a residue essential for its role in autophagic clearance (Matsumoto et al., 2011; Pilli et al., 2012).

We have found *TBK1* mutations in 1.6% of Sardinian ALS cases. Our data thus support the hypothesis that *TBK1* is a novel ALS gene.

Disclosure statement

This work was supported in part by the Intramural Research Programs of the US National Institutes of Health (NIH), National Institute on Aging (Z01-AG000949-02). The work was also supported by the Agenzia Italiana per la Ricerca sulla SLA (ARISLA) (SARDINIALS grant), Packard Centre for ALS Research at Johns Hopkins, Fondazione Vialli e Mauro Onlus, European Community's Health Seventh Framework Programme (FP7/2007-2013) under grant agreement #259867, the Joint Programme–Neurodegenerative Disease Research (Sophia Project, granted by the Italian Health Ministry, and Strength Project, granted by the Italian Ministry of University and Research), the Fondazione Mario e Anna Magnetto, and the Associazione Piemontese per l'Assistenza alla Sclerosi Laterale Amiotrfica (APASLA).

Acknowledgements

Other members of the ITALSGEN consortium are: Francesco O. Logullo (Ancona); Isabella Simone (Bari), Giancarlo Logroscino (Bari and Tricase, LE); Fabrizio Salvi, Ilaria Bartolomei (Bologna); Margherita Capasso (Chieti); Claudia Caponnetto, Paolo Mandich, Gianluigi Mancardi, Paola Origone (Genova); Francesca L. Conforti (Mangone, CNR); Giuseppe Vita, Sonia Messina, Massimo Russo (Messina University and Messina NEMO Sud Center); Gabriele Mora, Kalliopi Marinou, Riccardo Sideri (Milan, Maugeri Foundation); Christian Lunetta (Milan, NEMO Center); Silvana Penco, Lorena Mosca (Milan, Niguarda Ca' Granda Hospital); Giuseppe Lauria Pinter (Milan, Besta Neurological Institute), Massimo Corbo (Milan, Casa di Cura del Policlinico); Nilo Riva, Paola Carrera, (Milan, San Raffaele Hospital); Paolo Volanti (Mistretta, Maugeri Foundation); Lucio Tremolizzo, Carlo Ferrarese (Milano, Bicocca); Nicola Fini, Antonio Fasano (Modena); Maria Rosaria Monsurrò, Gioacchino Tedeschi, Francesca Trojsi, Giovanni Piccirillo, Viviana Cristillo (Napoli); Letizia Mazzini, Sandra D'Alfonso, Anna Bersano, Lucia Corrado, Alessandra Bagarotti (Novara); Vincenzo La Bella, Rossella Spataro, Tiziana Colletti (Palermo); Mario Sabatelli, Marcella Zollino, Amelia Conte, Marco Luigetti, Serena Lattante, Giuseppe Marangi (Rome, Catholic University of Sacred Heart); Marialuisa Santarelli (Rome, San Filippo Neri Hospital); Antonio Petrucci (Rome, San Camillo Forlanini Hospital); Fabio Giannini, Stefania Battistini, Claudia Ricci, Michele Benigni (Siena); Gabriella Restagno, Federico Casale, Giuseppe Marrali, Giuseppe Fuda, Irene Ossola, Stefania Cammarosano, Antonio Ilardi, Davide Bertuzzo (Torino); Raffaella Tanel (Trento); Fabrizio Pisano (Veruno, Maugeri Foundation). Other members of the SARDINIALS consortium are: Emanuela Costantino, Carla Pani, Roberta Puddu, Carla Caredda, Valeria Piras, Stefania Tranquilli, Stefania Cuccu, Daniela Corongiu, Maurizio Melis, Antonio Milia, Stefania Cuccu e Stefania Tranquilli (Cagliari), Angelo Pirisi, Leslie D. Parish (Sassari), Enzo Ortu (Ozieri).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.03.028.

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