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Coexistence of variants in *TBK1* and in other ALS-related genes elucidates an oligogenic model of pathogenesis in sporadic ALS

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ABSTRACT

Variants in tank-binding kinase 1 (*TBK1*) are responsible for a significant proportion of amyotrophic lateral sclerosis (ALS) cases. In the present study, we analyzed variants in *TBK1* extracted by targeted sequencing of 32 genes in a group of 406 Italian patients with ALS. We identified 7 different *TBK1* variants in 7 sporadic cases, resulting in a frequency of 1.7%. Three patients had missense variants (p.R357Q, p.R358H, and p.R724C), one patient had a small deletion (p.E618del), and 3 had truncating variants (p.Y482*, p.R229*, and p.N681*). Notably, we found that 4 patients had an additional variant in ALS-related genes: 2 in *OPTN* and 2 in the 3′UTR region of *FUS*. By studying an independent group of 7 *TBK1*-mutated patients previously reported, we found another variant in the 3′UTR region of *FUS* in one patient. The presence of a second variant in *TBK1* variant carriers is an interesting finding that needs to be investigated in larger cohorts of patients. These findings suggest that *TBK1* belongs to the category of genes conferring a significantly increased risk but not sufficient to cause disease.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is caused by a complex interaction of genetic variants with environmental and stochastic factors. Genetic variants can increase the risk of developing the disease or modify some clinical characteristics, such as the age at onset and the survival. The genetic contribution is relevant in familial cases, which are approximately 10% of the total cases, but it is significant also in sporadic ALS. Several ALS-related genes have been identified (Chia et al., 2018), and the number is increasing also because of the advent of new-generation sequencing technologies.

A new risk gene, identified in 2015, is tank-binding kinase 1 (*TBK1*, OMIM: 604834) (Cirulli et al., 2015; Freischmidt et al., 2015). A number of cohorts with different geographic origins have been screened with *TBK1* variant frequency of 0.5%–9% (Borghero et al., 2016; de Majo et al., 2018; Dols-Icardo et al., 2018; Kim et al., 2017; Müller et al., 2018; Pozzi et al., 2017; Shu et al., 2016; Tsai et al., 2016; Williams et al., 2015) in patients with pure ALS and of 3.5%–4.5% in ALS cases with frontotemporal dementia (FTD) (de Majo et al., 2018; Gijselinck et al., 2015; Le Ber et al., 2015; van der Zee et al., 2017). Most cases have likely pathogenic truncating variants, whereas the significance of missense variants remains unclear.

In the present work, we screened *TBK1* variants in a cohort of 406 Italian patients with ALS by next-generation sequencing and analyzed their role in primary fibroblast cultures. We further

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investigated whether *TBK1* variants occurred in association with additional variants in 32 ALS-related genes.

2. Materials and methods

A cohort of 406 unrelated Italian patients (32 familial and 374 sporadic) admitted to NEMO Clinical Center of Rome was analyzed in this study. ALS diagnosis was made according to El Escorial criteria (Brooks et al., 2000; Ludolph et al., 2015). The presence of familiarity and cognitive impairment was deeply investigated to determine the exact contribution of TBK1 in dementia. Blood samples were obtained after signing a written informed consent. The Ethics Committee of our Institution approved the study. Genomic DNAs were extracted from leukocytes using Wizard Genomic DNA Purification Kit (Promega). A panel of 32 genes (previously described in the study by Marangi et al., 2017) was analyzed by next-generation sequencing technologies in all patients. A HaloPlex Custom kit (Agilent Technologies, USA) was used to prepare libraries, and an Ion Torrent PGM machine (ThermoFisher Scientific, USA) was used to perform the run. Read alignment and first part of the quality control analysis (i.e., sequencing run metrics and read quality scores and alignment statistics) was performed with the default pipeline included in the Torrent Suite software (ThermoFisher Scientific, USA). An average coverage of at least 200 × (usually between 400× and 600×) was obtained for each sample, with at least 95% of target bases covered at 20×. Ion Reporter cloud computing (ThermoFisher Cloud, www.thermofisher.com/it/en/home/cloud.html) was used for variant calling and variant quality control evaluation with a custom pipeline for the detection of constitutional variants in target genes (including coding regions, splice sites, and 5′ and 3′UTRs). Variant annotation was performed with the Annovar software (annovar.openbioinformatics.org) (Yang and Wang, 2015). Variant filtering was based on the following criteria:

(1) Variants with a minor allele frequency >1% in non-Finnish European population (according to the gnomAD database, gnomad.broadinstitute.org) were discarded;
(2) Intronic, intragenic, and synonymous variants with no predicted effect on splicing were discarded (apart from canonical splice sites, dbscSNV 1.1 and SPIDEX 1.0 scores) (Jian et al., 2014; Xiong et al., 2015);
(3) Read alignments in regions around detected variants were visually inspected with the Integrative Genomics Viewer tool (software.broadinstitute.org/software/igv/) for an initial evaluation of possible misalignments and false-positive results.

Genetic variants in *TBK1* gene have been extracted from the pool of data and confirmed by Sanger sequencing. Repeat-primed polymerase chain reaction was used to screen all the patients for the *C9orf72* hexanucleotide expansion, according to a previous report (Renton et al., 2011).

Skin biopsies and primary fibroblast cultures were performed as previously described (Sabatelli et al., 2015). Primary fibroblasts from patients with ALS carrying the Y482* and N681* *TBK1* variants and healthy control were grown in Dulbecco's modified Eagle medium with GlutaMAX I (GIBCO) (DMEM with glutamine, sodium pyruvate, and 4.5 g/L glucose) supplemented with 15% fetal calf serum (EuroClone) and antibiotic/antimycotic (Sigma) according to manufacturer's instruction. Nonsense-mediated decay (NMD) inhibition was achieved with cycloheximide (Sigma) at 100 μg/mL, and cells were harvested at 3 different time points (0 hours, 2 hours, 4 hours) after compound administration. For each variant, 3 independent experiments were performed in duplicate (Pozzi et al., 2017). Total RNA was extracted from fibroblasts with Trizol (Invitrogen), reverse transcribed into cDNA with Oligo(dT)15 Primer using ImProm-II Reverse Transcriptase kit (Promega), and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using GoTaq qPCR Master Mix (Promega) kit. Sybr green–based quantitative RT-PCR analysis on total TBK1 transcripts has been carried out with primers between exons 12 and 14.

Primary fibroblasts were lysed on a nutator for 40 minutes at 4 °C in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% NP40%, 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Roche). Protein lysates were clarified by centrifugation at 13,000 × *g* for 10 minutes at 4 °C. 30–60 μg of total protein per sample was subjected to Western blotting on PDVF membranes (Millipore) and probed with the indicated antibodies. Detection was performed with a standard enhanced chemiluminescence method. Rabbit anti-TBK1 (1:1000; #3013, Cell Signaling) was used for the Western blot analysis. Rabbit anti-GAPDH (1:10.000; 2118S, Cell Signaling) was used as loading control.

3. Results

3.1. Genetic variants in TBK1 were found in 1.7% of Italian patients with ALS

We identified 7 different *TBK1* variants in 7/406 Italian patients with ALS (1.7%). Three patients had missense variants (exon9:c.1070 G>A:p.R357Q; exon9:c.1073 G>A:p.R358H; exon21:c.2170 C>T:p.R724 C), one patient had a small deletion (exon17:c.1852_1854delGAA:p.E618del), and 3 had truncating variants (exon 13:c.1445_1446delAT:p.Y482*, exon6:c.684dupT:p.R229*; exon19:c.2040dupT:p.N681*) (Table 1). These variants were absent in 196 controls in whom the same panel of genes was sequenced.

Clinical and demographic features of mutated *TBK1* patients are summarized in Table 2. All of them had a sporadic form of the disease and came from different regions of Italy. The age of onset

Table 1
TBK1 variants identified in our patients

Patient	TBK1 variant (cDNA and protein change, NM_013254)	Genomic coordinates (hg19)	Population frequencies (gnomAD)		Protein domain	Additional variants
			European (non-Finnish)	All		
1	c.1852_1854delGAA:p.E618del	chr12:64890820–64890822	NA	NA	CCD1	
2	c.2040dupT:p.N681*	chr12:64891508–64891508	NA	NA	CCD2	*OPTN* p.E408 A
3	c.1445_1446delAT:p.Y482*	chr12:64883823–64883824	NA	NA	CCD1	*FUS* c.*59G>A
4	c.2170 C>T:p.R724C	chr12:64895141–64895141	0.00001759	0.00005970	CCD2	*FUS* c.*1998T>C
5	c.1070 G>A:p.R357Q	chr12:64878160–64878160	0.00004404	0.00001991	ULD	
6	c.1073 G>A:p.R358H	chr12:64878163–64878163	0.00003101	0.00006371	ULD	*OPTN* p.Q314 L
7	c.684dupT:p.R229*	chr12:64868153–64868153	NA	NA	KD	

Key: CCD, coiled-coil domain; ULD, ubiquitin-like domain; KD, kinase domain; TBK1, tank-binding kinase 1.
For each variant, genomic coordinates, cDNA and protein change, and functional domains affected are reported. The frequency of the variants in the general population has been checked on gnomAD browser. Additional variants found in other genes are also indicated.

Table 2
Clinical features of our patients carrying *TBK1* variants

Patient	Age at onset	Site of onset	Disease duration (in months)	FTD
1	53	Bulbar	36 (Tracheostomy[a])	No
2	59	Upper limb	40 (Deceased)	Yes
3	49	Upper limb	35 (Tracheostomy[b])	No
4	63	Bulbar	20 (Deceased)	No
5	66	Upper limb	76 (Deceased)	No
6	41	Upper limb	8 (Alive)	No
7	50	Bulbar	20 (Alive)	No

Age at onset, site of onset, disease duration, and the presence of frontotemporal dementia are indicated.
Key: FTD, frontotemporal dementia; TBK1, tank-binding kinase 1.
[a] Alive after 1 y since tracheostomy.
[b] Alive after 9 y since tracheostomy.

ranged from 40 to 66 years. Three patients had a Flail arm phenotype, 2 had a predominant upper motor neuron form and 2 had a classic ALS. In one patient, ALS was associated with signs of overt FTD. Time from onset to death or tracheostomy ranged from 20 to 40 months.

3.2. TBK1 *variants led to a reduction of mRNA and protein*

To assess the potential effect of the variants, we obtained primary fibroblasts from 5 mutated patients and we performed transcript and protein analysis. In silico analysis of the Y482* and N681* *TBK1* variants predicted the insertion of a premature termination codon and degradation of the aberrant transcript through NMD. In agreement with this hypothesis, quantitative RT-PCR analysis of *TBK1* transcripts from patient-derived primary fibroblasts showed a ~50% reduction in total mRNA levels of the Y482* and N681*, respectively (Fig. 1A and B). Coherently, immunoblot analysis confirmed a corresponding decrease in protein levels (Fig. 2; Supplementary Figure). Inhibition of NMD with cycloheximide restored normal levels, comparable to healthy control, of total *TBK1* transcripts for the Y482* and N681* *TBK1* variants (Fig. 1A and B). These results support the hypothesis that the Y482* and N681* genomic variants cause pre-mRNA misprocessing leading to *TBK1* haploinsufficiency through NMD and should be considered as loss of function (*LoF*) variants. *TBK1* level was significantly reduced in all patients carrying *TBK1* variants (Fig. 2; Supplementary Figure).

3.3. *Additional variants in ALS-related genes*

Four patients had additional variants in ALS-related genes (Table 1). In particular, 2 patients carried concomitant missense variants in *OPTN* (c.1223 A>C:p.E408 A and c.491 A>Y:p.Q314 L) and 2 patients had a *FUS* variant in the 3′UTR region (NM_004960.3:c.*59G>A and c.*1998T>C). To further assess the presence of additional genetic variants in *TBK1*-mutated patients, we analyzed an independent cohort of 7 Italian sporadic patients with *TBK1* variants previously reported (Pozzi et al., 2017) and we found a 3′UTR *FUS* variant in one case (c.*816delG). None of the patients carrying the *TBK1* variants had a *C9orf72* expansion.

4. Discussion

In the present study, we found 7 distinct *TBK1* variants in 7 patients with ALS, resulting in a frequency of 1.7% in a cohort of 406 Italian patients with ALS. Three patients had novel truncating variants (p.Y482*, p.R229*, and p.N681*). These variants have been neither reported in our control group nor in control databases and are likely to cause a loss of protein function because they are expected to generate truncated proteins with altered biological activity. Accordingly, *TBK1* expression in primary fibroblast cultures from p.Y482*, p.N681* patients was significantly decreased. One patient had a deletion of a single amino acid (E618del), which is a novel variant too. Primary fibroblasts cultures from the E618del-mutated patient showed a reduction of *TBK1* analogous to that of truncating variants. These data suggest that the pathogenic mechanism of these 4 novel variants is based on haploinsufficiency, through NMD.

Three patients had missense variants: p.R357Q, p.R358H, and p.R724C. The p.R357Q variant has been consistently reported in patients with ALS and has been demonstrated to impair TBK1 catalytic activity (de Majo et al., 2018; Freischmidt et al., 2015; Pozzi et al., 2017). The p.R357Q variant is present in 5/251094 control alleles (gnomAD browser, gnomad.broadinstitute.org; Lek et al., 2016) and it is predicted to be benign by PolyPhen-2 (genetics.bwh.harvard.edu/pph2) and deleterious by SIFT (sift.bii.a-star.edu.sg). In fibroblasts from our patient with the p.R357Q variant, TBK1 protein level was slightly reduced, suggesting that this variant may lead to an instable protein, as described in p.K401E and p.E696K (Pottier et al., 2015). The p.R358H has been recently reported in 2 first-degree relatives with ALS (de Majo et al., 2018). It was not present in our controls but it was reported in 18/282510 control alleles in the gnomAD database (in 12/24936 Africans, 2/35376 Latinos, and in 4/128972 Europeans alleles, respectively). It is predicted to be damaging by PolyPhen-2 and tolerated by SIFT. Both p.R357Q and p.R358H are in the ubiquitin-like domain and may impair the recruitment of ubiquitinated proteins. The missense variant p.R724C was not found in our controls but was reported in 15/251244 alleles (13/18382 in East Asian population, 2/113680 in

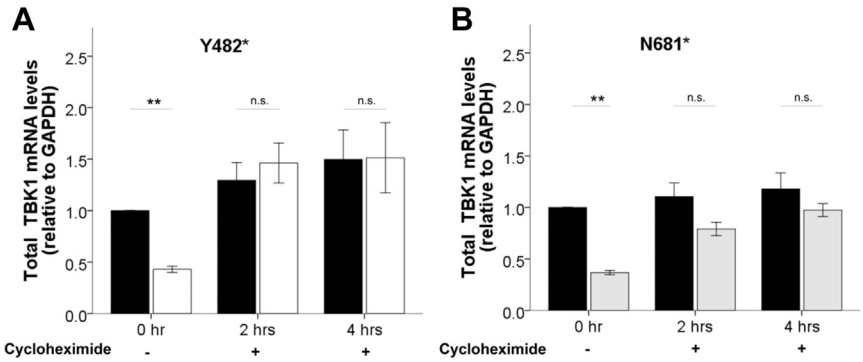

Fig. 1. In vitro functional analysis of the 2 *LoF TBK1* variants in patient-derived fibroblasts. Quantitative RT-PCR analysis of TBK1 mRNA levels extracted from Y482* (A), N681* (B) TBK1 and control primary fibroblasts. Total RNA was extracted before (0 hours) and after (2 and 4 hours) cycloheximide administration. Results are expressed as mean ± SE of 3 different experiments performed in triplicate. **: $p < 0.005$ (*t*-test). Abbreviations: LoF, loss of function; TBK1, tank-binding kinase 1.

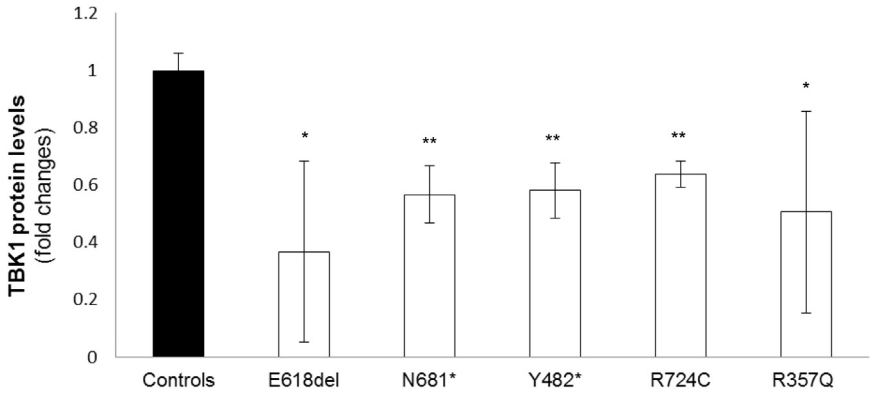

Fig. 2. Quantification of immunoblot analysis of TBK1 protein levels in controls and *TBK1* patients' primary fibroblasts. GAPDH serves as a loading control. (mean ± SE from 3 independent experiments). **: $p < 0.005$; *: $p < 0.05$ (*t*-test). Abbreviation: TBK1, tank-binding kinase 1.

Europeans) (gnomAD browser) and in 2/2117 control subjects (Verheijen et al., 2018), respectively. It is predicted to be probably damaging by PolyPhen-2 and deleterious by SIFT. Western blot on primary fibroblast from the carrier of the p.R724C variant showed a slight reduction of *TBK1*. Although the pathogenicity of p.R724C variant is unclear, its localization in the OPTN binding site, encompassing amino acids 601–729, suggests that it may impair the interaction between the 2 proteins.

TBK1 variants have been identified in familial and in sporadic ALS cases, similarly to what has been observed in other ALS-related genes. Notably, all the 17 Italian patients with *TBK1* variants described to date, including ours, have a sporadic disease. One plausible explanation for the sporadic occurrence is that *TBK1* belongs to the category of genes conferring a significantly increased risk but not sufficient to cause the disease. Based on the oligogenic model, additional genetic variants with small/moderate effect size might contribute to disease onset (Lattante et al., 2012, 2018; van Blitterswijk et al., 2012, 2013). Several *TBK1* cases have been reported to harbor a second variant in ALS-related genes (Table 3). Interestingly, 4 of our 7 patients showed a second variant in a different ALS gene. The identification of missense variants in *OPTN* in 2 patients is of particular interest as TBK1 physically interacts with OPTN and phosphorylates the serine 177, regulating the formation of a protein complex involved in vesicle trafficking and autophagosome dynamics. One of our patients with concomitant *OPTN* and *TBK1* variants had an overt FTD and showed an upper motor neuron phenotype with a striking bulbospinal spasticity. Notably, the only one patient with a double variant in *OPTN* and *TBK1* described in the literature had a pure FTD phenotype (Pottier et al., 2015). The identification of variants in the 3′UTR region of *FUS* gene in 3 patients suggests a possible link between FUS and TBK1.

Table 3
List of variants in *TBK1* reported to occur in concomitance with variants in other ALS-associated genes

TBK1 variants	Additional genetic variants	Reference
p.Y185*	*FUS* p.R524G	Freischmidt et al. (2015)
p.E643del	*C9orf72* expansion	Gijselinck et al. (2015)
p.R117*	*OPTN* p.G538Efs*27	Pottier et al. (2015)
p.M690 fs	*SQSTM1* p.R33V	Borghero et al. (2016)
p.M184 V	*C9orf72 expansion*	van der Zee et al. (2017)
p.G244 V	*C9orf72 expansion*	van der Zee et al. (2017)
p.Y394D	*TARDBP* p.M337V	de Majo et al. (2018)
p.R358H	*FUS* p.R521C	de Majo et al. (2018)
p.N254Kfs*4	*SQSTM1* p.P392L	Dols-Icardo et al. (2018)
p.Y185*	*DCTN1* p.I195L, *FUS* p.R524G	Müller et al. (2018)

Key: ALS, amyotrophic lateral sclerosis; TBK1, tank-binding kinase 1.

Previous genetic and biochemical studies consistently indicated that variants in the 3′UTR region of *FUS* play a role in the pathogenesis of ALS (Dini Modigliani et al., 2014; Morgan et al., 2017; Sabatelli et al., 2013). Because autophagy is critical to managing the burden of misfolded and toxic proteins, it is not unexpected that perturbed autophagy induced by *TBK1* variants exacerbate the consequences of overexpression of wild-type FUS. Furthermore, there is evidence that autophagy regulates stress granules dynamics which is known to be impaired by mutant FUS (Monahan et al., 2016), indicating a possible intersection between TBK1 and FUS functions.

Our results confirm that *TBK1* is a major ALS-related gene and that *LoF* is a consistent mechanism of 3 novel variants. In our study, we identified a second variant in ALS-related genes in one-third of the *TBK1*-mutated patients, *C9ORF72*. Although the number of our patients is limited, our data suggest the hypothesis that *LoF* variants are likely to work as partner through an oligogenic model. Further studies of larger cohorts are needed to confirm these results and will elucidate whether variants in *OPTN* and in the 3′UTR region of *FUS* are preferential accomplices of *TBK1*.

Disclosure

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2019.03.010.

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