TBK1 mutations in Italian patients with amyotrophic lateral sclerosis: genetic and functional characterization

Laura Pozzi,[1] Fabiola Valenza,[2] Lorena Mosca,[3]Andrea Dal Mas,[2] Teuta Domi,[1] Alessandro Romano,[1] Claudia Tarlarini,[3] Yuri Matteo Falzone,[1,4] Lucio Tremolizzo,[5] Gianni Sorarù,[6] Federica Cerri,[1,4] Raffaella Fazio,[4] Federica Agosta,[7] Massimo Filippi,[7,8] Giancarlo Comi,[4,8] Maurizio Ferrari,[8,9]Angelo Quattrini,[1] Christian Lunetta,[10] Silvana Penco,[3] Dario Bonamomi,[2] Paola Carrera,[9] Nilo Riva[1,4]

[1] Neuropathology Unit, Institute of Experimental Neurology (INSPE), Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

[2] Molecular Neurobiology Laboratory, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

[3] Medical Genetic Unit, Department of Laboratory Medicine, Niguarda Hospital, Milan, Italy.

[4] Department of Neurology, Institute of Experimental Neurology (INSPE), Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

[5] Neurology Unit, "San Gerardo" Hospital and University of Milano-Bicocca, Monza, Italy.

[6] Department of Neurosciences, Neuromuscular Center, University of Padova, Padua, Italy.

[7] Neuroimaging Research Unit, Department of Neurology, Institute of Experimental Neurology (INSPE), Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy.

[8] Università Vita e Salute San Raffaele, Milan, Italy.

[9] NEuroMuscular Omnicentre (NEMO), Fondazione Serena Onlus, Milan, Italy.

[10] Division of Genetics and Cell Biology, Unit of Genomics for human disease diagnosis, San Raffaele Scientific Institute, Milan, Italy

Supplementary Information

1 Genetic analysis and in-silico predictions..

Genetic analysis was performed on genomic DNA extracted from peripheral blood using NucleoSpin Blood L Extraction Kit (Macherey-Nagel) following the manufacturer's instructions. For all the patients, *TBK1* whole coding region and exon junctions were analysed by directly sequencing PCR products on both strands with a Sanger protocol, using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). PCR and sequencing primers are listed in Suppl. Table1. PCR and sequencing reactions were purified with Ampure (Agencourt, Beckman-Coulter)

and Big Dye XTerminator (Applied Biosystems), respectively, on a liquid handling system (Biomeck FX, Beckman-Coulter), according to protocols developed in the laboratory. Dye terminator reaction sequences were loaded on a 3730 AB Genetic Analyzer (Appled Biosystems). Called sequences were aligned to the *TBK1* reference sequence (NCBI Entrez Gene ID 29110; NM_013254.3) with the Sequencer 5.0 Software (Gene Codes). Gene variants were evaluated by their absence or frequency in the public NCBI genome database: dbSNP (https://www.ncbi.nlm.nih.gov/SNP/) Exome Aggregation Consortium Sequencing Project (ExAc; http://exac.broadinstitute.org) and Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/). Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://www.mutationtaster.org/) were used to assess the functional effect of missense variants. Human Splicing Finder (http://www.umd.be/HSF/, Marseille, France), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/, Lyngby, Denmark), Splice Site Prediction by Neural Network Site (http://www.fruitfly.org/seq_tools/splice.html, Berkley, CA, USA), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) and Gene Splicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml) were used to evaluate the potential effects on gene splicing. The Disease Specific database (http://alsod.iop.kcl.ac.uk/), the Human Gene Mutation Database Professional 2016.4 (https://portal.biobase-international.com/hgmd/pro/start.php) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) have been interrogated to check for previously reported gene variants.

2. Structure-based analysis of TBK1 mutations

The crystal structure of the C-terminally truncated human TBK1 (PDB code: 4IW0; residues 1-657; [1]) was used to assess the impact of missense mutations on protein. The effects of mutations on TBK1 structure and thermodynamic stability (ΔΔG), were predicted by Rosetta Backrub server[2] and FoldX.[3] The electrostatic surface potentials were calculated using the Adaptive Poisson-Boltzmann Solver (APBS) software [4].

3. Transcript analysis

RNA was extracted from peripheral blood cells using the PAXgene Blood RNA Kit (Quiagen). First-strand cDNA was synthesized from total RNA with random hexamer primers using the GeneAmp RNA PCR Core Kit (Applied Biosystems), then amplified using FastStart Taq DNA Polymerase (Roche) with primers spanning the coding region of the TBK1 transcript between exons 3-6, 9-11 and 13-17 (Suppl. Table 2). The amplified fragments were first separated on 2% agarose

gel electrophoresis, then Sanger sequenced in order to confirm the predicted alteration of the normal splicing of the transcript caused by the variants.

4. Expression plasmids

Human cDNA clones were obtained from the MGI library collection. Standard PCR-cloning was used to add a FLAG-tag sequence to the 5'-end of TBK1 cDNA (clone BC034950), and to the 3'-end of either Optineurin (clone BC013876) or IRF3 (clone BC071721) cDNAs. Tagged fragments were inserted in the CMV expression vector pN1-EGFP (Clontech) to replace EGFP. GeneArt Mutagenesis kit (Thermo Scientific) was used to introduce the p.L59Ffs*16, p.D118N, p.R357Q and p.I397T TBK1 variants into the FLAG-tagged wild-type expression plasmid. All constructs were verified by sequencing.

5. Cell culture and transfection

Primary fibroblasts from ALS patient carrying the c.358+5G>A TBK1 variant and healthy control were grown in Dulbecco's modified Eagle's medium with Glutamax I (GIBCO) (DMEM with glutamine, sodium pyruvate, pyridoxine and 4.5g/l glucose) supplemented with 15% fetal calf serum (Euro Clone) and antibiotic/antimycotic (Sigma) according to manufacturer's instruction. NMD inhibition was achieved with cycloheximide (Sigma) at 100 µg/mL and cells were harvested at 3 different time points (0 hr, 2 hrs, 4 hrs) after compound administration.

HEK293T (precisely, modified AD-293 cell line) and NSC-34 cells were maintained in DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% Penicillin/Streptomycin. HEK cells were transfected with a total of 8µg of DNA and 16µl of Lipofectamine 2000 (Thermo Scientific). NSC-34 cells were transfected with 200ng of DNA and 1µl of Lipofectamine 2000 and maintained in DMEM 1% FBS. 12 hrs after transfection, cells were treated with retinoic acid (5µM) for additional 36 hrs to induce neuronal differentiation.

6. Quantitative real-time polymerase chain reaction (qRT-PCR)

For splicing analysis on the c.358+5G>A TBK1 variant, total RNA was extracted from fibroblasts with Trizol (Invitrogen), reverse transcribed into cDNA with random hexamers using SuperScript III First Strand synthesis kit (Invitrogen) and analyzed by RT-PCR. Syber green-based quantitative RT-PCR analysis on total TBK1 transcripts has been carried out with primers between exons 12-14 (Suppl. Table 2). Semiquantitative analysis of TBK1 splicing isoforms has been carried out with primers spanning exons 2-3 and 5 (Suppl. Table 2). The resulting amplified products were solved

on 2% agarose gel and the intensity of the bands quantified with ImageJ Software (National Institutes of Health; freely available at http://rsb.info.nih.gov/ij/).

7. Immunofluorescence analysis

Differentiated NSC-34 cells cultured on glass coverslips were fixed with 4% PFA diluted in PBS and containing 4% sucrose for 20 min at RT and then incubated with primary anti-FLAG antibody followed by Alexa488-conjugated anti-mouse secondary antibody. Both primary and secondary antibodies were diluted in PBS containing 1% BSA (Jackson ImmunoResearch) and 0.1% Tx-100. Samples were briefly stained with DAPI (Sigma) prior to mounting with PermaFluor (Thermo Scientific).

8. Biochemical procedures

AD293 (HEK) cells transfected with Lipofectamine 2000 for 36 hrs were lysed on a nutator for 40 min at 4°C in modified RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 10mM EDTA, 1% NP40, 0.5% Sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Roche). Protein lysates were clarified by centrifugation at 13,000 xg for 10 min at 4°C. 500-600µg of total protein lysate were incubated overnight at 4°C with 2µg of anti-FLAG antibody followed by incubation with protein A/G beads (Santa Cruz Biotechnology) for 2 hrs at 4°C to immunoprecipitate FLAG-tagged TBK1 variants. After washing in 0.5% NP40 lysis buffer the beads were resuspended in sample buffer. Precipitates were subjected to western blotting on PDVF membranes (Millipore) and probed with the indicated antibodies. Western blot for total cell lysates (inputs) was performed with 30-60µg of total protein per sample. Detection was performed with a standard enhanced chemiluminescence method.

9. Antibodies

Alexa488-conjugated anti-mouse secondary antibody (1:1000; A21202, Invitrogen) and mouse anti-FLAG antibody (1:300; M2, #F1804, Sigma), for immunofluorescence analysis.

Mouse anti-FLAG (1:1000; M2, #F1804, Sigma), mouse anti-HA (1:1000; #16B12; Covance), rabbit anti-TBK1 (1:1000; #3013, Cell Signaling) and rabbit anti-pIRF3 (Ser396) (1:1000; #4947, Cell Signaling), for western blot analysis. Rabbit anti-GAPDH (1:5000; 2118S, Cell Signaling) was used as a loading control.

Supplementary Table 1: Primer sequences for the TBK1 gene

exon 2	5’-CACTTCATGTCAGTAACAGA-3’
	5’-AGTGTCAACATGCATCTTAG-3’
exon 3	5’-CTGTGCCTAAAAGATGCATG-3’
	5’-AGGTTCTATTTCTTGACTCG-3’
exon 4	5’-CAAAGTGCCTTCATCATTG-3’
	5’-CATTGCATAAAGGTCTTCAG-3’
exon 5	5’-TTTGGGTGACTATATCAATG-3’
	5’-ATAGTGAATTGGCTTGACCA-3’
exon 6	5’-CAGATTGGGAAAGTGAAGT-3’
	5’-CAGGTAGCTTAAAATATCAG-3’
exon 7	5’-CTAGTAGATAATTGTAGGTGC-3’
	5’-GCACATGCTTTGACAATATCT-3’
exon 8	5’-CAGTTCCTTTGATTTGCTG-3’
	5’-GGTGAAGCTGAGGCATCT-3’
exon 9	5’-TGGATGTTGTTGCACTCAT-3’
	5’-ACAAGGTCTCACTATGTTG-3’
exon 10	5’-CTACATTACAGATTTTGCCAC-3’
	5’-CCTAAGTCATATAGACATAC-3’
exon 11	5’-CTCTCCATTGAGATAGTGC-3’
	5’-AGCACCTAACCTAACTAGT-3’
exon 12	5’-GAACTGTAGTACTGCAGTA-3’
	5’-TGCCAAGGATAACTAAGTC-3’
exon 13	5’-AATGGTTATGATGAAAGCTG-3’
	5’-TCTAATAGTGCCAGCAAAG-3’
exon 14+15	5’-TGTGGTCCAGACTTTAGAC-3’
	5’-CTACCCTTACAGATATACCA-3’
exon 16	5’-TGAGCCACAAATCTATGACA-3’
	5’-CTACATATTGTAACAATCCTG-3’
exon 17+18	5’-GCCACAACAATCATTATAGGA-3’
	5’-CCTTGTCAATCACTATCAGT-3’
exon 19+20	5’-ACACTTGATGTCAGATCTG-3’
	5’-GACCCTTTACCACTGCTG-3’
exon 21	5’-TAATACCATGCTGTTCCAG-3’
	5’-CAAGTGACTATCTATAAAGGC-3’

Supplementary Table 2: Primer sequences for the TBK1 transcript

exon 2/3 Fw	5’-GAAGACATAAGAAAACTGGTG-3’
exon 5/6 Rev	5’-CATATCAGGGTGCAAATATTC-3’
exon 8/9 Fw	5’-CATAGCTATAATACTGCTAC-3’
exon 11/12 Rev	5’-TTAATTCAATCAGCCATCGT-3’
exon 13/14 Fw	5’-GTTGAGACTTTCCAGTTCTC-3’
exon 17 Rev	5’-CATCTGTAAAGTGCGTCATAG-3’
hsTBK1 e12 Fw	5’-GACAGAAGTTGTGATCACATTGGATTTCTG-3’
hsTBK1 e13-14t Rev	5’-TGTTCCCTGAGAACTGGAAAGTCTC-3’
hsTBK1 e2/3 Fw	5’-CGTGGAAGACATAAGAAAACTGGTG-3’
hsTBK1 e5 Rev	5’-CTCTCGTAGATGATTCATTCCACCC-3’

Supplementary Table 3: In-silico splicing predictions

Variant	HSF (wt-mut)	NetGene2 (wt-mut)	NNS (wt-mut)	MES (wt-mut)	GS (wt-mut)	Predicted change*
c.358+5G>A	84.4 – 72.19	0.82 - 0	0.72 - 0	7.43 – 2.05	=	at ds, 5 bps upstream -61%
p.Asp118Asn	=	=	0.72 - 0.86	=	=	at ds, 7 bps downstream +6.9%
p.Ile397Thr	87.28 – 87.42	=	0.66 - 0.58	7.7 – 7.6	0 – 1.2	at as, 1 bp upstream -4.2%
c.1644-5_1644-2delAATA	70.79 – 78.57	0 - 0.14	0 - 0.97	0.5 – 3.53	=	at as, 2 bps downstream +239%

Legend: HSF: Human Splicing Finder; NNS: Neural Network Site; MES: Max Ent Scan; GS: Gene Splicer; ds: donor splice site; as: acceptor splice site. =: unchanged *combined

Supplementary Table 4: Review of the literature of TBK1 variants in the ALS-FTD spectrum reported so far

Protein change	cDNA	Type of variant	Exon	Clinical presentation	Sex	Age of onset (years)	Ref.
LoF							
Lys29ArgfsX15	c.86delA	frameshift	2	FTD	M	73	[5]
Lys30_Glu76del	c.228+1G>A	in-frame deletion	3	-	-	-	[5]
Thr79del	c.235_237delACA	in-frame deletion	4	ALS-FTD	M	56	[5]
Val97PhefsX2	c.288delT	frameshift	4	ALS	M	62	[5]
Thr77TrpfsX4	c.358+2T>C	frameshift	4	fALS	M	35	[6]
Arg117X	c.349C>T	non-sense	4	FTD	M	68	[7]
				FTD	M	67	[5]
Arg127X	c.379C>T	non-sense	5	ALS	F	70	[5]
Thr156ArgfsX6	c.467_468delCA	frameshift	5	ALS-FTD	-	-	[8]
Asp167del	c.499_501delGAT	in-frame deletion	5	ALS	M	60	[9]
Tyr185X	c.555T>A	non-sense	6	fALS*	F	47	[6]
				fALS *	F	41	[6]
Gly272_Thr331del	c.992+1G>T	splice site	8	FTD	M	48	[9]
Gly272_Thr331del	c.992+4_992+7delAGTA	splice site	8	FTD	M	48	[5]
Thr320GlnfsX40	c.958delA	frameshift	8	fALS	M	60	[6]
Ser398ProfsX11	c.1191delT	frameshift	10	ALS	M	59	[9]
Leu399fs	c.1197delC	frameshift	10	fALS	M	62	[10]
Ala417X	c.1340+1G>A	non-sense	11	fALS	F	65	[6]
				sALS	F	62	[6]
				sALS	M	62	[6]
				sALS	M	65	[6]
				FTD	F	68	[5]
Arg440X	c.1318C>T	non-sense	11	fALS *	M	47	[6]
				ALS-FTD*	M	58	[6]
				ALS*	M	73	[6]

Arg444X	c.1330C>T	non-sense	11	ALS-FTD	F	57	[11]
Arg444X	c.1331G>A	non-sense	11	ALS-FTD	M	72	[12]
Trp445X	c.1335G>A	non-sense	11	FTD	F	78	[5]
Ile450LysfsX15	c.1343_1346delAATT	frameshift	12	fALS*	F	57	[6]
				fALS*	F	77	[6]
				fALS*	M	51	[6]
Thr462LysfsX3	c.1385_1388delCAGA	frameshift	12	ALS	M	74	[5]
Ile472Serfs*8	c.1414delA	frameshift	12	ALS	M	53	[13]
Val479GlufsX4	c.1434_1435delTG	frameshift	12	fALS	F	65	[6]
Tyr482X	c.1146T>G	non-sense	13	ALS-FTD	-	-	[8]
Ser518LeufsX32	c.1551_1552insTT	frameshift	14	ALS	F	64	[9]
Glu643del	c.1927_1929delGAA	frameshift	18	ALS-FTD	F	62	[9]
				FTD	F	64	[9]
				FTD	M	70	[9]
				FTD	F	69	[9]
				ALS	M	63	[9]
				ALS	M	51	[9]
Glu643del	c.1928_1930delAAG	frameshift	18	sALS	M	-	[6]
				sALS	M	-	[6]
				D*	F	71	[9]
				D*	F	86	[9]
				ALS*	M	69	[9]
				FTD*	M	69	[9]
				D*	M	70	[9]
				FTD*	F	61	[9]
				D*	F	63	[9]
				ALS-FTD*	F	62	[9]
				D*	F	73	[9]
Gln655X	c.1963C>T	non-sense	19	ALS-FTD	-	-	[8]

IVS18-2A>G	c.1960-2A>G	splice site	19	ALS-FTD	-	-	[8]
Met690fs	c.2070delG	frameshift	20	ALS	F	66	[12]
690-713del	c.2138+2T>c	frameshift	20	fALS*	F	63	[6]
				-*	F	52	[6]
				fALS	M	64	[6]
Functional missense variants							
Gln2X	c.4C>T	missense	2	FTD	F	56	[9]
Thr4Ala	c.10A>G	missense	2	FTD	-	-	[8]
Gly26Glu	c.77G>A	missense	2	ALS	-	-	[8]
Arg47His	c.140G>A	missense	3	fALS	M	-	[6]
Leu62Pro	c.185T>C	missense	3	sALS	M	47	[14]
Ile73Val	c.217A>G	missense	3	-	-	-	[9]
Leu94Ser	c.281T>C	missense	4	ALS	M	44	[5]
Tyr105Cys	c.314A>G	missense	4	sALS	-	-	[6]
Gly121Asp	c.362G>A	missense	5	ALS	M	34	[5]
Arg143Cys	c.427C>T	missense	5	ALS	-	-	[8]
Arg229Ser	c.687G>T	missense	6	ALS	M	47	[5]
Gly244Val	c.731G>T	missense	7	ALS-FTD	F	41	[5]
Ile246Thr	c.737T>C	missense	7	ALS	F	57	[5]
Lys291Glu	c.871A>G	missense	8	FTD	M	52	[9]
Ile305Thr	c.914T>C	missense	8	sALS	-	-	[6]
Leu306Ile	c.916C>A	missense	8	ALS-FTD	F	70	[7]
Arg308Gln	c.923G>A	missense	8	sALS	M	40	[6]
Thr320Ile	c.959C>T	missense	8	ALS	-	-	[8]
Ile334Thr	c.1001T>C	missense	9	sALS	F	51	[14]
Arg357Gln	c.1070G>A	missense	9	fALS	M	61	[6]
Lys401Glu	c.1201A>G	missense	10	FTD-AD	F	80	[7]
Ile418Val	c.1252A>G	missense	11	FTD	F	53	[5]
Met559Arg	c.1676T>G	missense	15	fALS	M	60	[6]

Ala571Val	c.1712C>T	missense	15	sALS	F	66	[6]
Met598Val	c.1792A>G	missense	17	sALS	F	62	[6]
Met662Thr	c.1985T>C	missense	19	ALS-FTD	-	-	[8]
Glu696Lys	c.2086G>A	missense	20	fALS	M	65	[6]
				sALS	F	61	[6]
				FTD-AD	F	78	[7]
Missense variants of not defined pathogenicity							
Ile43Val	c.127A>G	missense	3	sALS	M	45	[11]
Asn63Ser	c.188A>G	missense	3	-	-	-	[5]
Met184Val	c.550A>G	missense	6	-	-	-	[5]
Val265Ala	c.794T>C	missense	7	-	-	-	[5]
Arg271Leu	c.812G>T	missense	7	FTD	M	80	[9]
Gly294Asp	c.881G>A	missense	8	sALS	-	47	[11]
His322Tyr	c.964C>T	missense	8	sALS	M	64	[9]
Arg384Thr	c.1150C>T	missense	9	ALS	F	-	[12]
Arg384Trp	c.1150C>T	missense	9	ALS	M	40	[13]
Ile353Val	c.1057A>G	missense	9	-	-	-	[5]
Ile393Met	c.1179A>G	missense	9	-	-	-	[5]
Ile397Thr	c.1190T>C	missense	10	-	-	-	[5]
Tyr406Cys	c.1217A>G	missense	10	-	-	-	[5]
Ile475Thr	c.1424T>C	missense	12	ALS	F	55	[13]
Glu476Lys	c.1426G>A	missense	12	ALS	F	56	[13]
Ile515Thr	c.1544T>C	missense	14	ALS	F	59	[9]
Ala535Thr	c.1603G>A	missense	14	FTD	M	52	[9]
His538Tyr	c.1612C>T	missense	14	-	-	-	[5]
Lys570Arg	c.1709A>G	missense	15	-	-	-	[5]
Arg573Gly	c.1717C>G	missense	15	-	-	-	[5]
Glu653Gln	c.1975G>C	missense	19	-	-	-	[5]

Legend: *******members of a family harboring the same TBK1 variant; -, not reported data; Ref: reference

Supplementary Figure Legend 1

Supplementary Figure 1. Effects of missense variants on human TBK1 structure. (A) Three dimensional model of the human TBK1 dimer. The symmetry axis is indicated by a solid line. One monomer is shown uncolored the second monomer is colored with kinase domain, ubiquitin-like domain and coiled-coil domain shown in blue, yellow and green, respectively. The p.Asp118Asn (D118N) variant is located in an alpha-helix forming the kinase domain of human TBK1; while the p. Ile397Thr (I397T) lays in the protein linker connecting the ubiquitin-like domain to coiled-coil domain. The mutated residues are highlighted in red. (B) Upper panels: close view of the structure around residue 118 in wild type (blue) and mutated (orange) TBK1 and histogram of free energy changes (ΔΔG) due to p.Asp118Asn. The p.Asp118Asn variant determines major changes in the conformation of TBK1 catalytic domain lowering the global stability of the protein (ΔΔG: 3.98 ± 0.27 kcal/mol). Lower panels: electrostatic surface potential of TBK1 wild type and p.Asp118Asn mutant: the mutation drastically affects electrostatic surface potential of the entire domain. (C) Upper panels; close view of the structure around residue 397 in wild type (gray) and mutated (magenta) TBK1 and histogram of free energy changes due to p.Ile397Thr mutation. Lower panels; electrostatic surface potential of TBK1 wild type and p.Ile397Thr mutant. The p.Ile397Thr slightly affects the structure and global stability (ΔΔG: 1.51 ± 0.18 kcal/mol) of the protein and does not changes electrostatic surface potential. Electrostatic surface potentials in B and C were colored according to charge with blue denoting positive charge (+10 kT/e-) and red, negative charge (–10 kT/e-).

References:

1. Larabi A, Devos JM, Ng SL, et al. Crystal structure and mechanism of activation of TANK-binding kinase 1. *Cell Rep* 2013;3(3):734-46. doi: 10.1016/j.celrep.2013.01.034
2. Lauck F, Smith CA, Friedland GF, et al. RosettaBackrub--a web server for flexible backbone protein structure modeling and design. *Nucleic acids research* 2010;38(Web Server issue):W569-75. doi: 10.1093/nar/gkq369
3. Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. *Journal of molecular biology* 2002;320(2):369-87. doi: 10.1016/S0022-2836(02)00442-4
4. Baker NA, Sept D, Joseph S, et al. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proceedings of the National Academy of Sciences of the United States of America* 2001;98(18):10037-41. doi: 10.1073/pnas.181342398
5. van der Zee J, Gijselinck I, Van Mossevelde S, et al. TBK1 Mutation Spectrum in an Extended European Patient Cohort with Frontotemporal Dementia and Amyotrophic Lateral Sclerosis. *Human mutation* 2016 doi: 10.1002/humu.23161
6. Freischmidt A, Wieland T, Richter B, et al. Haploinsufficiency of TBK1 causes familial ALS and fronto-temporal dementia. *Nat Neurosci* 2015;18(5):631-6. doi: 10.1038/nn.4000
7. Pottier C, Bieniek KF, Finch N, et al. Whole-genome sequencing reveals important role for TBK1 and OPTN mutations in frontotemporal lobar degeneration without motor neuron disease. *Acta Neuropathol* 2015;130(1):77-92. doi: 10.1007/s00401-015-1436-x
8. Le Ber I, De Septenville A, Millecamps S, et al. TBK1 mutation frequencies in French frontotemporal dementia and amyotrophic lateral sclerosis cohorts. *Neurobiology of aging* 2015;36(11):3116 e5-8. doi: 10.1016/j.neurobiolaging.2015.08.009
9. Gijselinck I, Van Mossevelde S, van der Zee J, et al. Loss of TBK1 is a frequent cause of frontotemporal dementia in a Belgian cohort. *Neurology* 2015;85(24):2116-25. doi: 10.1212/WNL.0000000000002220
10. Williams KL, McCann EP, Fifita JA, et al. Novel TBK1 truncating mutation in a familial amyotrophic lateral sclerosis patient of Chinese origin. *Neurobiology of aging* 2015;36(12):3334 e1-5. doi: 10.1016/j.neurobiolaging.2015.08.013
11. Tsai PC, Liu YC, Lin KP, et al. Mutational analysis of TBK1 in Taiwanese patients with amyotrophic lateral sclerosis. *Neurobiology of aging* 2016;40:191 e11-6. doi: 10.1016/j.neurobiolaging.2015.12.022
12. Borghero G, Pugliatti M, Marrosu F, et al. TBK1 is associated with ALS and ALS-FTD in Sardinian patients. *Neurobiology of aging* 2016;43:180 e1-5. doi: 10.1016/j.neurobiolaging.2016.03.028
13. Kim YE, Oh KW, Noh MY, et al. Genetic and functional analysis of TBK1 variants in Korean patients with sporadic amyotrophic lateral sclerosis. *Neurobiology of aging* 2017;50:170 e1-70 e6. doi: 10.1016/j.neurobiolaging.2016.11.003
14. Shu S, Li XL, Liu Q, et al. Screening of the TBK1 gene in familial and sporadic amyotrophic lateral sclerosis patients of Chinese origin. *Amyotroph Lateral Scler Frontotemporal Degener* 2016;17(7-8):605-07. doi: 10.1080/21678421.2016.1183681