Loss of TBK1 is a frequent cause of frontotemporal dementia in a Belgian cohort

ABSTRACT

Objective: To assess the genetic contribution of TBK1, a gene implicated in amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and FTD-ALS, in Belgian FTD and ALS patient cohorts containing a significant part of genetically unresolved patients.

Methods: We sequenced TBK1 in a hospital-based cohort of 482 unrelated patients with FTD and FTD-ALS and 147 patients with ALS and an extended Belgian FTD-ALS family DR158. We followed up mutation carriers by segregation studies, transcript and protein expression analysis, and immunohistochemistry.

Results: We identified 11 patients carrying a loss-of-function (LOF) mutation resulting in an overall mutation frequency of 1.7% (11/629), 1.1% in patients with FTD (5/460), 3.4% in patients with ALS (5/147), and 4.5% in patients with FTD-ALS (1/22). We found 1 LOF mutation, p.Glu643del, in 6 unrelated patients segregating with disease in family DR158. Of 2 mutation carriers, brain and spinal cord was characterized by TDP-43-positive pathology. The LOF mutations including the p.Glu643del mutation led to loss of transcript or protein in blood and brain.

Conclusions: TBK1 LOF mutations are the third most frequent cause of clinical FTD in the Belgian clinically based patient cohort, after C9orf72 and GRN, and the second most common cause of clinical ALS after C9orf72. These findings reinforce that FTD and ALS belong to the same disease continuum. Neurology® 2015;85:2116-2125

GLOSSARY

ALS = amyotrophic lateral sclerosis; bvFTD = behavioral variant frontotemporal dementia; FTD = frontotemporal dementia; FTLD = frontotemporal lobar degeneration; KD = kinase domain; LOF = loss of function; NCI = neuronal cytoplasmic inclusions; Ser172 = serine 172.

Frontotemporal lobar degeneration (FTLD) is a heterogeneous neurodegenerative disorder associated with amyotrophic lateral sclerosis (ALS) in approximately 10%-15% of patients with frontotemporal dementia (FTD). Evidence that common disease pathways are involved in FTD and ALS stems from the observation of families and individual patients in which both diseases occur (FTD-ALS), and the TDP-43 inclusions in both patient groups.

Nearly 50% of FTD cases and 10% of ALS cases aggregate in families, suggesting a strong genetic component. The most convincing genetic evidence for a common pathomechanism is provided by the repeat expansion mutations in C9orf72 in patients with FTD, ALS, and FTD-ALS. Recently, TBK1 loss-of-function (LOF) mutations were identified in patients with ALS6,7 and FTLD-TDP.8

In the Belgian FTD cohort, mutations in known genes accounted for 30% of familial FTD and 75% of familial FTD-ALS, with several families with autosomal dominant inheritance.
remaining genetically unresolved.\textsuperscript{5–9} Here, we investigated the genetic role of \textit{TBK1} in a Belgian cohort of 629 patients with FTD, FTD-ALS, and ALS.

**METHODS** Subjects. Our study population consisted of 482 patients with FTD, 22 of whom had concomitant ALS (FTD-ALS), and 147 patients with ALS ascertained in Belgium through an ongoing multicenter collaboration of neurology departments and memory clinics partnering in the Belgian Neurology (BELNEU) consortium. Additional patients were included who had initially been referred to the Diagnostic Service Facility for medical genetic testing. Patients were diagnosed using a standard protocol and established clinical criteria.\textsuperscript{10–12} Postmortem neuropathologic analysis confirmed diagnosis in 25 patients with FTD, 3 patients with FTD-ALS, and 6 patients with ALS. A positive familial history, i.e., at least one first-degree relative with a FTD-ALS spectrum disease, was recorded in 132 patients with FTD (28.7%), 4 patients with FTD-ALS (36.4%), and 18 patients with ALS (12.2%). Of these familial index patients, 93 cases of FTD (70.5%), 1 case of FTD-ALS (25.0%) and 7 cases of ALS (38.9%) were not explained by mutations in the known FTLD and ALS genes (\textit{MAPT}, \textit{GRN}, \textit{CHSYF72}, \textit{VCP}, \textit{CHMP2B}, \textit{FUS}, \textit{TARDBP}, \textit{SOD1}), in the AD genes (\textit{PSEN1}, \textit{PSEN2}, \textit{APP}), or in the prion gene (\textit{PRNP}). One patient with unexplained FTD-ALS was the index patient of a 4-generation family (family DR158), of which we collected genomic DNA and generated lymphoblast cell lines of 38 individuals including 4 patients in generation III (figure 1 and table 1). A Belgian control cohort of 1,044 persons free of personal and familial history of neurodegenerative or psychiatric diseases and with a Mini-Mental State Examination score $>26$ was also analyzed.

Standard protocol approvals, registrations, and patient consents. All participants provided written informed consent for participation in clinical, pathologic, and genetic studies. Clinical study protocols and informed consent forms were approved by the local medical ethics committees of the collaborating clinical centers. Genetics study protocol and informed consent forms were approved by the medical ethics committees of the University Hospital Antwerp and the University of Antwerp.

Experimental procedures. Nineteen coding \textit{TBK1} exons were amplified in multiplex PCR reactions using MASTR technology (http://www.multiplicom.com) and sequenced on a MiSeq platform (Illumina; San Diego, CA) and 1 exon was analyzed by Sanger sequencing. Identified variants were validated and relatives of mutation carriers were analyzed using Sanger sequencing. \textit{TBK1} transcripts were measured in lymphoblast cells of 8 mutation carriers and frontal cortex of 2 carriers using real-time PCR amplification of a \textit{TBK1} amplicon, quantified against 2 housekeeping genes. We sequenced real-time PCR products to establish the transcribed alleles based on the coding mutation or the coding polymorphism n7486100. We used western blotting of protein lysates of lymphoblast cells and brain with a monoclonal antibody against TBK1 and quantified against GAPDH. We performed neuropathologic analysis of brain and spinal cord of 2 \textit{TBK1} mutation carriers.

Appendix e-1 on the Neurology\textsuperscript{®} Web site at Neurology.org contains further technical details.

**RESULTS** \textit{TBK1} mutation analysis and the effect on transcript and protein expression. We screened the coding region of \textit{TBK1} and identified 2 frameshift, 1 nonsense, 1 splice site mutation, and 2 single amino acid deletions in 11 unrelated index patients (table 1, table e-1, and figure e-1). Further, we identified 5 missense mutations in 5 patients. Copy number variation analysis of all \textit{TBK1} exons did not reveal exonic or whole gene deletions or duplications.

We predicted that the nonsense and frameshift mutations resulted in a premature termination codon leading to mRNA degradation by nonsense-mediated decay. cDNA sequencing of \textit{TBK1} in lymphoblast cells of the Ser398Profs*11 carrier, and in brain of the p.Ser518Leufs*32 carrier, did not identify the mutant allele, suggesting a complete loss of the mutant transcript. Quantification of \textit{TBK1} transcripts demonstrated a highly reduced gene expression that was restored to normal levels using an inhibitor of protein synthesis (figure 2). Western blot analysis demonstrated near 50% reduction of protein expression in lymphoblast cells and in brain (figure 3).

In DR189, a mutation affecting the intron 8 splice donor site resulted in in-frame skipping of exon 8 in lymphoblast cells and brain. In addition, use of a cryptic splice site in exon 8 resulted in an out-of-frame transcript (figure 2). Quantitative PCR analysis showed 30% reduced expression in lymphoblast cells and 50% reduced expression in brain (figure 2). Western blot analysis detected only the normalized protein band with 45% reduced expression in lymphoblast cells and 34% in brain lysates, suggesting that no stable protein was produced from the mutant transcripts (figure 3).

In lymphoblast cells of patients carrying the in-frame deletion mutations, p.Asp167del and p.Glu643del (n = 5), \textit{TBK1} transcript expression was not reduced. Also, cDNA sequencing showed the presence of both alleles. However, we observed a significantly reduced protein level of 44% in the p.Glu643del carriers (p = 0.0017). In the p.Asp167del carrier, protein expression was not altered (figure 3).

The 5 missense mutations were scattered over the 4 functional domains (table e-1 and figure e-1). Three missense mutations—p.Arg271Leu, p.His322Tyr, and p.Ile515Thr—were absent from 1,044 control individuals. We observed p.Ala535Thr in 1 male control (age at inclusion 63 years), and p.Lys291Glu in a male control and a female control (age at inclusion 67 and 69 years). Two mutations, p.Arg271Leu and p.Ala535Thr, were predicted neutral, while the other 3 mutations received variable effects using 3 prediction algorithms. Missense mutation carriers did not show reduced transcript and protein expression levels (figure e-2).

**DR158 family, segregation, and sharing of p.Glu643del.** Six unrelated index patients carried the p.Glu643del mutation (table 1), among them index patient III-8 of autosomal dominant FTD-ALS family DR158,
containing 13 patients (table 1 and figure 1). Ten patients of family DR158 were diagnosed with a dementia syndrome, i.e., FTD (n = 4) or unspecified dementia (n = 6), while 2 patients had a diagnosis of ALS. The mean onset age was 69.1 ± 7.7 years, with a mean disease duration of 6.4 ± 3.9 years. The 5 patients with FTD or FTD-ALS presented with bvFTD (table e-2). Remarkably, memory loss and disorientation were present relatively early in the disease in most of the p.Glu643del mutation carriers (table e-2). However, most of these patients also developed early behavioral problems. One patient with ALS (III-1) and 1 patient with FTD-ALS (III-8) presented with ALS with spinal onset (table e-2), both showing signs of motor neuron disease on EMG. Analysis of
all family members indicated that the disease segregated with the p.Glu643del mutation on a disease haplotype of minimal 7.5 Mb (figure 1). Seven mutation carriers were without symptoms at age 70 years, including obligate carrier II-5 and 2 children of II-5 who, at their last clinical evaluation, were 79, 81, and 82 years of age, respectively (figure 1). Given the frequent occurrence of psychiatric disease in family DR158, it cannot be excluded that symptoms of FTD were not recognized (table e-2).

| Table 1 Clinical characteristics of patients of family DR158 and TBK1 mutation carriers |
|-----------------|----------------|---------------|--------------|-----------------|-----------------|
| Patient ID      | Sex | AAO, y | AAD, y | DD, y | Clinical diagnosis | Family history | Predicted protein* |
| Family DR158    |     |        |        |        |                  |               |                 |
| II-1            | F   | 71     | 81     | 10    | D               | F              | p.Glu643del     |
| II-2            | F   | 86     | 90     | 4     | D               | F              | p.Glu643del     |
| III-1           | M   | 69     | 72     | 3     | ALS             | F              | p.Glu643del     |
| III-2           | M   | 69     | 75     | 6     | FTD             | F              | p.Glu643del     |
| III-3           | M   | 70     | 73     | 3     | D               | F              | p.Glu643del     |
| III-4           | M   | 63     | —      | >3    | FTD             | F              | NA             |
| III-5           | F   | 66     | 71     | 6     | FTD             | F              | NA             |
| III-6           | F   | 61     | 74     | 13    | FTD             | F              | p.Glu643del     |
| III-7           | F   | 63     | 69     | 6     | D               | F              | p.Glu643del     |
| III-8*          | F   | 62     | 74     | 11    | FTD-ALS         | F              | p.Glu643del     |
| III-9           | F   | 73     | 84     | 11    | D               | F              | p.Glu643del     |
| III-10          | F   | 82     | 86     | 4     | D               | F              | NA             |
| III-11          | M   | 63     | 64     | 1     | ALS             | F              | NA             |
| Unrelated index patients |     |        |        |        |                  |               |                 |
| DR1120          | F   | 56     | 60     | 4     | FTD             | U              | p.Glu2*        |
| DR1127          | M   | 60     | 61     | 1     | ALS             | S              | p.Asp167del    |
| DR189           | M   | 48     | 50     | 2     | FTLD*           | F              | p.Gly272Thr331del |
| DR1123          | M   | 59     | —      | >5    | ALS             | U              | p.Ser398Profs*11 |
| DR1124          | F   | 64     | 64     | <1    | ALS*            | U              | p.Ser518Leufs*32 |
| DR158           | F   | 62     | 74     | 11    | FTD-ALS         | F              | p.Glu643del     |
| DR467           | F   | 64     | —      | >9    | FTD             | F              | p.Glu643del     |
| DR1121          | M   | 70     | —      | >6    | FTD             | F              | p.Glu643del     |
| DR1122          | F   | 69     | —      | >7    | FTD             | S              | p.Glu643del     |
| DR1044          | M   | 63     | 66     | 3     | ALS             | S              | p.Glu643del     |
| DR663           | M   | 41     | 41     | —     | ALS             | F              | p.Glu643del     |
| DR1192          | M   | 80     | 87     | 7     | FTD             | F              | p.Arg271Leu    |
| DR609           | M   | 52     | 62     | 10    | FTD             | F              | p.Ala535Thr    |
| DR1133          | M   | 60     | 64     | 4     | FTD             | F              | p.Lys291Ile    |
| DR1134          | M   | 64     | 66     | 2     | ALS             | S              | p.His322Tyr    |
| DR1135          | F   | 59     | —      | >10   | ALS             | U              | p.Ile515Thr    |

Abbreviations: AAD = age at death; AAO = age at onset; ALS = amyotrophic lateral sclerosis; D = dementia; DD = disease duration; F = familial; FTD = frontotemporal dementia; FTLD = frontotemporal lobar degeneration; NA = not analyzed; S = sporadic; U = unknown.

*p Protein numbering according to NP_037386.1 isoform.

b Index patient of family DR15B.
c TDP-43-positive pathology in brain or spinal cord.

Carrier II-5 and one of her children with a current age of 81 years had repeated depression. Carrier II-3 died at age 68 years and was diagnosed with schizophrenia. One of the asymptomatic children of patient II-1 had alcohol abuse and has a current age of 70 years. Among the other 5 carriers of the p.Glu643del mutation and their relatives, we identified 2 distinct disease haplotypes that differed from that in DR158 (figure 1 and table e-3). In family DR663, ALS patient II-1 and II-2 carried, besides the TBK1 mutation, a C9orf72 repeat.
expansion, as did the asymptomatic sib II-4 without the TBK1 mutation, age 63 years (figure 1).

**Genotype–phenotype correlations.** The mean onset age in the 11 patients carrying a LOF mutation (table 1) was 59.6 ± 8.6 years. In 4 p.Glu643del carriers, excluding the 2 patients carrying a C9orf72 repeat expansion, we calculated a mean onset age of 66.3 ± 3.9 years, which was significantly later ($p = 0.04$) than in the 5 patients with another LOF mutation, with a mean onset age of 57.4 ± 6.0 years. When we included all affected carriers of family DR158, the
significance became stronger ($p = 0.004$). Also, the mean disease duration of 8.3 ± 1.8 years was significantly longer ($p = 0.016$) than the mean disease duration of 2.6 ± 2.2 years in the patients with another LOF mutation with an increasing effect if all DR158 patients were included ($p = 0.002$). Four of 5 FTD mutation carriers and the patient with FTD-ALS had behavioral variant FTD (bvFTD) (table e-2). The 3 patients with FTD with a p.Glu643del mutation expressed extrapyramidal symptoms, while the patients with FTD with another TBK1 mutation did not (table e-2). In 1 patient with bvFTD (DR1120), muscular fasciculations were observed in 1 limb (table e-2). The proband of DR158 presented with spinal onset ALS; in the 3 other documented patients with ALS (DR663, DR1123, DR1124), the site of onset was bulbar (table e-2). Of 3 TBK1 mutation carriers with ALS or FTD-ALS (DR158.III-8, DR1123, DR1124), we had EMG reports, while of 3 other patients with ALS (DR663, DR1044, DR1127), no detailed information was available. Based on a clinical examination, there was no cognitive evidence for FTD in the majority of ALS cases. However, in ALS patient DR1124 memory deficits and the impression of behavioral disinhibition were reported (table e-2), but we did not have sufficient information to determine if the criteria for possible FTLD were fulfilled.

Neuropathologic brain examination of FTD patient DR189 (p.Gly272_Thr331del) showed mild neuronal loss of the frontal cortices. We found a moderate amount of TDP-43 and p62 neuronal cytoplasmic inclusions (NCI) and short dystrophic neurites.
but no intraneuronal intranuclear inclusions (figure 4, A, B, D, and E) compatible with a TDP type B proteinopathy. In ALS patient DR1124 (p.Ser518Leufs*32), pathologic signs of lower and upper motor neuron disease were apparent. The TDP-43 positive NCI were mainly restricted to the hypoglossal nucleus and the ventral horn neurons of cervical and thoraco-lumbar spinal cord (figure 4, C, F). Staining with TBK1 showed variable cytoplasmic neuronal immunoreactivity. The Nissl substance of the neurons in the ventral horn of the spinal cord was stained. We did not observe differences between patients and controls (figure 4, G–L).

**DISCUSSION**

The overall TBK1 LOF mutation frequency is 1.7% (11/629), with 1.1% (5/460) in FTD, 4.5% (1/22) in FTD-ALS, and 3.4% (5/147) in ALS. In patients with FTD, TBK1 LOF mutations explained 3.2% of the unexplained familial FTD cases and were the third most common genetic cause after C9orf72 and GRN mutations (figure e-3), consistent with the findings in a FTLD-TDP
cohort. In the ALS cohort, *TBK1* mutations were the second most common after *C9orf72* mutations (figure e-3).

All described LOF mutations, except 1, are different from those in the other published reports. The p.Glu643del mutation that was present in 61/629 (1%) unrelated Belgian patients was also found in 2/1,010 German patients with sporadic ALS. We showed segregation of the p.Glu643del mutation with disease. The presence of 3 different disease haplotypes of at least 3 Mb among the p.Glu643del carriers might be explained by a mutation hotspot due to a repetitive (GAA)$_3$ sequence encoding 3 Glu residues (codons 641–643) or because of an extreme distant common founder. Expression studies in lymphoblast cells and in brain of 3 LOF mutation carriers confirmed the loss of transcript and of protein leading to cells and in brain of 3 LOF mutation carriers common founder. Expression studies in lymphoblast TBK1 to the active state. In other loop and hence might hamper the conversion of p.Asp167del mutation is located in this activation genic. Since at least one of the used prediction some missense mutations were found to be patho-

sent in a significant part of sporadic and late-onset patients is not uncommon in adult-onset neurodegenerative diseases because of reduced penetrance, similar to what is described for *C9orf72* mutations and to a lesser extent for *GRN* mutations. A relatively high de novo mutation rate may be another explanation, supported by the observation that the p.Glu643del mutation occurred on 3 different haplotype backgrounds. Also, as proposed by others, more than 1 gene might be involved in some patients, as observed in family DR663 segregating both a *TBK1* mutation and a *C9orf72* repeat expansion of only 59 repeat units. This patient had later onset age of 63 years, corroborating our recent findings that patients with a short repeat expansion (45–80 units) have a later age at onset compared to patients with a longer expansion. Further, the disease penetrance in p.Glu643del mutation is highly variable, with 7 carriers of family DR158 still unaffected at age ≥70 years, with 2 at age 81 and 82 years, nearly 2 SDs beyond the mean onset age. The 7 unaffected carriers were not homozygous for the minor protective allele of *TMEM106B* rs1990622. The observed TDP-43 type B pathology in brain or spinal cord could be distinguished from that in patients with a *C9orf72* mutation by the absence of p62-positive inclusions containing dipeptide repeats. This pathology was previously reported in 1 patient with FTD-ALS while another study reported FTLD-TDP type A in 3 mutation carriers.

The observation that *TBK1* mutations were present in a significant part of sporadic and late-onset patients is not uncommon in adult-onset neurodegenerative diseases because of reduced penetrance, similar to what is described for *C9orf72* mutations and to a lesser extent for *GRN* mutations. A relatively high de novo mutation rate may be another explanation, supported by the observation that the p.Glu643del mutation occurred on 3 different haplotype backgrounds. Also, as proposed by others, more than 1 gene might be involved in some patients, as observed in family DR663 segregating both a *TBK1* mutation and a *C9orf72* mutation.

*TBK1* is an important serine/threonine kinase of the IKK family phosphorylating a wide range of substrates involved in several cellular processes, including innate immune response/inflammation, autophagy, and cell proliferation. Substrates of *TBK1* include optineurin (*OPTN*), another gene with LOF mutations in ALS, and p62, a major component of pathologic depositions and showing mutations in FTLD and ALS. p62 and OPTN are 2
autophagic adapters controlling protein degradation by selective autophagy.31,32 Moreover, TBK1 targets the VPS37C protein of the endosomal sorting complex required for transport-I,33 thereby regulating the vesicular retroviral budding system, which is also involved in neurodegeneration, e.g., CHMP2B.34 A dysfunctional vesicular transport system might in turn induce autophagy defects.35 As other FTD/ALS genes, including VCP,36 are also involved in autophagy, and since mutations in OPTN and TBK1 are also involved in glaucoma,37,38 our findings emphasize the major role of autophagic defects in neurodegeneration.39 Also, in the antiviral innate immune response both TBK1 and OPTN are involved through the NF-KB complex pathway.40

In this study, we demonstrated that LOF mutations in TBK1 are associated with FTD-ALS spectrum disorders in a Belgian clinical patient cohort, can segregate in families according to an autosomal dominant pattern, and are present in a significant part of sporadic cases. The identification of TBK1 emphasizes the convergence of FTD and ALS in 1 continuum and will accelerate effective drug development.

AUTHOR CONTRIBUTIONS
Ilse Gijselinck: literature search, figures, study design, genetic data collection, data analysis, data interpretation, writing. Sara Van Moseveelde: patient samples collection, clinical data collection, data analysis, data interpretation, writing. Julie van der Zee: patient data collection, genetic data collection, data analysis, data interpretation. Anne Sieben: patient samples collection, clinical data collection, neuropathology data collection, figures, data analysis, data interpretation, writing. Stephanie Philipijn: genetic data collection, data analysis. Baro Heeman: literature search, experimental data collection, data analysis, data interpretation. Sebastiaan Engelenborgh: patient samples collection, clinical data collection, data analysis, data interpretation. Mathieu Vandenbergh: patient samples collection, clinical data collection, data analysis, data interpretation. Greet De Barts: literature search, figures, protein structure data collection, data analysis, data interpretation. Veerle Bäumer: data collection, data analysis, data interpretation. Ivy Cuyp: genetic data collection, data analysis. Marleen Van den Broeck: genetic data collection, data analysis, data interpretation. Karin Peerens: patient samples collection, genealogy data collection, data analysis. Maria Matthijssens: patient samples collection, genealogy data collection, data analysis. Frederic Rousseau: data analysis, data interpretation. Rik Vandenbergh: patient samples collection, clinical data collection, data analysis, data interpretation. Peter De Jonghe: patient samples collection, clinical data collection, data analysis, data interpretation. Patrick Cruts: study design, genetic data collection, data analysis, data interpretation. Patrick De Vleeschauwer: genealogy data collection, data analysis, data interpretation. Luc De Mey: patient samples collection, clinical data collection, data analysis, data interpretation. Peter P. De Jonghe: patient samples collection, clinical data collection, data analysis, data interpretation. Marc Cruts: literature search, study design, data interpretation, writing, supervision. Ilse Gijselinck: literature search, figures, study design, genetic data collection, genealogy data collection, data analysis, data interpretation, writing, study supervision.

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