Neurobiology of Aging xxx (2017) 1.e1–1.e6

Contents lists available at ScienceDirect

Neurobiology of Aging

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

Investigating *CCNF* mutations in a Taiwanese cohort with amyotrophic lateral sclerosis

Pei-Chien Tsai [a,b,c], Yi-Chu Liao [a,b], Po-Lin Chen [d], Yuh-Cherng Guo [e,f], Ying-Hao Chen [a], Kang-Yang Jih [a], Kon-Ping Lin [a,b], Bing-Wen Soong [a,b,c], Ching-Paio Tsai [a,g,h,*], Yi-Chung Lee [a,b,c,**]

[a] *Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan*
[b] *Department of Neurology, National Yang-Ming University School of Medicine, Taipei, Taiwan*
[c] *Brain Research Center, National Yang-Ming University, Taipei, Taiwan*
[d] *Neurological Institute, Taichung Veterans General Hospital, Taichung, Taiwan*
[e] *Neuroscience Laboratory, Department of Neurology, China Medical University Hospital, Taichung, Taiwan*
[f] *School of Medicine, Medical College, China Medical University, Taichung, Taiwan*
[g] *Taipei Beito Health Management Hospital, Taipei, Taiwan*
[h] *Department of Biotechnology, Asia University, Taichung, Taiwan*

ARTICLE INFO

Article history:
Received 11 May 2017
Received in revised form 8 August 2017
Accepted 30 September 2017

Keywords:
Amyotrophic lateral sclerosis
ALS
CCNF
Cyclin F

ABSTRACT

Mutations in the cyclin F gene (*CCNF*) have been recently identified in a small number of patients with amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia, and their role in patients with ALS in Taiwan remains elusive. The aim of this study was to elucidate the frequency and spectrum of *CCNF* mutations in a Taiwanese ALS cohort of Han Chinese origin. Mutational analyses of the *CCNF* gene were performed using Sanger sequencing in a cohort of 255 unrelated patients with ALS. Among these patients, the genetic diagnoses of 204 patients remained unclear after mutations in *SOD1*, *C9ORF72*, *TARDBP*, *FUS*, *ATXN2*, *OPTN*, *VCP*, *UBQLN2*, *SQSTM1*, *PFN1*, *HNRNPA1*, *HNRNPA2B1*, *MATR3*, *CHCHD10*, *TUBA4A*, and *TKB1* had been investigated. Two novel heterozygous missense mutations in *CCNF*, p.S222P (c.664T>C) and p.S532R (c.1596C>T), were identified; 1 in each patient with apparently sporadic ALS. In vitro functional study demonstrated that both mutations result in a general and cyclin F-mediated ubiquitin-proteasome pathway dysfunction. The frequency of *CCNF* mutations in ALS patients in Taiwan is, therefore, approximately 0.8% (2/255). These findings expand the mutational spectrum of *CCNF* and also emphasize the pathogenic role of *CCNF* mutations in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by a rapidly progressive degeneration of motor neurons in the brain and spinal cord, resulting in progressive weakness and atrophy, bulbar palsy, and consequent respiratory failure with a short survival, typically within 3–5 years of symptom onset (Testa et al., 2004). Although the exact cause of ALS in most patients remains unclear, the genetic contributions to ALS pathogenesis have clearly demonstrated that approximately 10% of patients inherit ALS from their parents. To date, more than 50 genes have been implicated in ALS pathogenesis, and mutations in at least 10 of these genes have been unequivocally demonstrated to cause familial ALS (Peters et al., 2015; Taylor et al., 2016). Moreover, only *SOD1*, *FUS*, *TARDBP*, *C9ORF72*, *VCP*, and *TBK1* account for a significant number of patients with ALS, indicating a high degree of genetic heterogeneity of ALS (Peters et al., 2015; Renton et al., 2014; Sreedharan and Brown, 2013). However, the roles of several newly identified causal genes of ALS, such as *CCNF* (Williams et al., 2016), are yet to be completely understood because of the lack of adequate relevant studies.

The *CCNF* gene encodes cyclin F, a protein of 786 amino acids, comprising 3 functional modules: a pseudocatalytic module containing the F-box domain and a nuclear localization signal, a substrate recruitment module containing 2 cyclin domains, and a regulatory module containing an nuclear localization signal, and a short amino acid sequence enriched in proline, glutamic acid, serine, and threonine, referred to as PEST (D'Angiolella et al., 2013).

* Corresponding author at: Department of Neurology, Taipei Veterans General Hospital, #201, Sec.2, Shih-Pai Road, Peitou District, Taipei 11217 (ROC), Taiwan. Tel.: 886 2 28712121 3300; fax: 886 2 28727578.
** Corresponding author at: Department of Neurology, Taipei Veterans General Hospital, #201, Sec. 2, Shih-Pai Road, Peitou District, Taipei 11217 (ROC), Taiwan. Tel.: 886 2 28712121 3790; fax: 886 2 28727577.
E-mail addresses: cptsai@vghtpe.gov.tw (C.-P. Tsai), ycli@vghtpe.gov.tw (Y.-C. Lee).

0197-4580/$ – see front matter © 2017 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.neurobiolaging.2017.09.031

Cyclin F is the founding member of the family of F-box proteins, which are the substrate recognition subunits of Skp1-Cul1-F-box (SCF) E3 ubiquitin ligase complexes and mediate the ubiquitination and proteasome-dependent degradation of eukaryotic proteins (Bai et al., 1996). Cyclin F is crucial for genome stability; it regulates deoxyribonucleotide triphosphate levels (D'Angiolella et al., 2012), centrosome duplication (D'Angiolella et al., 2010), and spindle assembly (Emanuele et al., 2011) and also suppress DNA rereplication (Walter et al., 2016) by targeting and modulating the levels of the $SCF^{cyclin\ F}$ substrates, including ribonucleoside-diphosphate reductase subunit M2 (RRM2), centriolar coiled-coil protein of 110 kDa, nucleolar- and spindle-associated protein, and cell division cycle 6 protein. A recent study revealed that cyclin F interacts with B-Myb to regulate DNA damage-induced checkpoint signaling (Klein et al., 2015).

Recently, *CCNF* mutations were identified in patients with familial or sporadic ALS and/or frontotemporal dementia (FTD) (Williams et al., 2016). The p.S621G mutation was identified in a large family of British ancestry with ALS/FTD through linkage analysis and exome sequencing. Further screening of *CCNF* mutations in the ALS/FTD cohorts of different populations in Europe, North America, Japan, and Australia led to the identification of 5 and 19 different mutations in patients with familial and sporadic ALS, respectively, suggesting that *CCNF* mutations are present in ALS cohorts of diverse ethnicities. A replication study on 611 patients with sporadic ALS and 1424 controls demonstrated a significant enrichment of *CCNF* rare variants in Australian patients with sporadic ALS. Functional analyses of *CCNF* mutations in neuronal cells revealed abnormal ubiquitination and accumulation of poly-ubiquitinated proteins, indicating the pathogenic role of *CCNF* mutations in ALS.

To further understand the role of *CCNF* mutations in ALS, we screened 255 unrelated Taiwanese patients with ALS for *CCNF* mutations. In addition, in vitro studies were conducted to assess the functional effects of the mutant gene products.

2. Methods

2.1. Patients

Two hundred fifty-five unrelated individuals with ALS were enrolled into this study. All participants were of Han Chinese descent and recruited from the Neurology Clinics of Taipei Veterans General Hospital with the diagnosis of probable or definite ALS based on the revised EL Escorial criteria (Brooks et al., 2000). Among the 255 ALS patients, 17 have a *SOD1* mutation, 12 have a *TARDBP* mutation, 10 have a *C9ORF72* GGGGCC hexanucleotide expansion, 7 have a *FUS* mutation, 2 have an *ATXN2* intermediate-length CAG repeat expansion (32 and 33 repeats), and another 3 carry a single mutation in *OPTN*, *MATR3*, or *TBK1* each. The genetic diagnoses of the remaining 204 patients are still unclear after screening for mutations in *SOD1*, *C9ORF72*, *TARDBP*, *FUS*, *ATXN2*, *OPTN*, *VCP*, *UBQLN2*, *SQSTM1*, *PFN1*, *HNRNPA1*, *HNRNPA2B1*, *MATR3*, *CHCHD10*, *TUBA4A*, and *TBK1* (Lin et al., 2015; Soong et al., 2014; Tsai et al., 2016). The mean age at onset of the cohort was 53.2 years (range 21–89). Thirty-nine patients (15.3%) had a positive family history of ALS (FALS), and 216 (84.7%) were apparently sporadic cases. Fifty patients (19.6 %) were of bulbar-onset and 205 (80.4 %) of spinal-onset ALS. Family history was considered positive if the patient had at least 1 affected relative within 3 generations. Peripheral blood samples were collected from the patients after obtaining written informed consents. The protocols for this study were approved by the Institutional Review Board of Taipei Veterans General Hospital.

2.2. Mutation analyses

Genomic DNA was extracted from peripheral white blood cells. Mutation analyses of the coding exons and their flanking regions of *CCNF* were performed by polymerase chain reaction amplification and Sanger sequencing with the intronic primers using the Big Dye 3.1 dideoxy terminator method (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Amplicon sequences were compared with the reference *CCNF* coding sequence (NM_001761.2). The sequence variations were validated by sequencing both sense and antisense strands of the amplicons. The putative pathogenic variants were first discriminated by their absence in the 997 healthy Taiwanese control exomes in the Taiwan Biobank database (https://taiwanview.twbiobank.org.tw/index), and then also in the 500 neurologically healthy individuals of Han Chinese origin recruited at our hospital. The genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org) was also queried for these variants. In silico predictions of the pathogenicity of the mutations were conducted using Combined Annotation Dependent Depletion (CADD) (http://cadd.gs. washington.edu) (Kircher et al., 2014), MutationTaster (http://www.mutationtaster.org) (Schwarz et al., 2014), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) (Adzhubei et al., 2010), and SIFT (http://sift.jcvi.org) (Kumar et al., 2009). Evolutionary conservation of the mutation sites was analyzed by aligning amino acid sequences using the UniProt Web site (http://www.uniprot.org) (The UniProt Consortium, 2014).

2.3. In vitro studies to verify the functional significance of the CCNF variants

2.3.1. Expression plasmids, cell culture, and transfection

The entire coding region of human *CCNF* (NM_001761.2) was cloned into pFlag-CMV5a (Sigma-Aldrich, St. Louis, MO, USA) to generate wild-type (WT) cyclin F expression construct. The cDNA clone of the ribonucleoside-diphosphate reductase subunit M2 gene (*RRM2*; BC001886) was purchased from transOMIC (Huntsville, AL, USA). Myc-tagged RRM2 expression vector was constructed by subcloning a full-length coding sequence of *RRM2* into pcDNA3.1/myc-His (Invitrogen, Grand Island, NY, USA). The GFP^{u} reporter construct is a fusion of the CL1 degron, a 16 amino acid constitutive degradation signal (Bence et al., 2005), with the carboxyl terminus of green fluorescence protein (GFP) in EGFP-C1 plasmid backbone (Clontech, Palo Alto, CA, USA). The *CCNF* mutations, including c.664T>C (p.Ser222Pro) and c.1596C>G (p.Ser532Arg), were separately introduced into the WT cyclin F expression plasmid using a QuikChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA).

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were transfected using Lipofectamine 2000 (Invitrogen) in the following transfection experiments.

2.3.2. RRM2 degradation assay

Degradation of RRM2 was demonstrated to be processed by $SCF^{cyclin\ F}$ complexes via its interaction with cyclin F (D'Angiolella et al., 2012). For analysis of $SCF^{cyclin\ F}$-mediated RRM2 degradation, HEK293 cells were cotransfected with Myc-tagged RRM2 and WT or mutant *CCNF* constructs or empty vector. At 48 hours after transfection, cells were lysed with radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktails (Merck Millipore, Billerica, MA, USA), and the relative abundance of RRM2 was determined by Western blotting.