Neurobiology of Aging xxx (2012) 1–10

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

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

A novel functional low-density lipoprotein receptor-related protein 6 gene alternative splice variant is associated with Alzheimer's disease

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ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 1 November 2012
Accepted 12 November 2012

Keywords:
Alternative splicing
Alzheimer's disease
LRP6
Wnt/β-catenin signaling

ABSTRACT

We previously found that single nucleotide polymorphisms in the low-density lipoprotein receptor-related protein 6 (*LRP6*) gene are associated with Alzheimer's disease (AD). Here, we studied the post-transcriptional metabolism of the *LRP6* message scanning sequentially the 23 *LRP6* exons in human tissues and found a novel *LRP6* isoform that completely skips exon 3 (*LRP6*Δ3) in all tissues examined and was also conserved in mice. Expression levels of the LRP6 isoforms were determined in 47 cortical brain messenger (m)RNA samples including 22 AD cases, 11 control subjects, and 14 individuals with other neurological disorders. *LRP6*Δ3 mRNA levels were significantly augmented in AD brains compared with controls (1.6-fold; $p = 0.037$) or other pathological samples (2-fold; $p = 0.007$). Functional analysis in Wnt/β-catenin signaling assays revealed that skipping of exon 3 reduced significantly the signaling activity of the *LRP6* coreceptor. We conclude that the *LRP6*Δ3 isoform is a novel splice variant, which shows diminished Wnt/β-catenin signaling activity and might have a functional role in individuals with AD.

1. Introduction

Alzheimer's disease (AD) is the most common neurological disorder and it is clinically characterized by a progressive loss of memory and other cognitive functions. The neuropathologic hallmarks of AD are the presence of extracellular β-amyloid plaques, derived from the proteolytic processing of the amyloid precursor protein, and the intracellular accumulation of neurofibrillary tangles composed of the hyperphosphorylated tau protein (Hardy and Selkoe, 2002). Sustained loss of function of Wnt/β-catenin signaling components has been proposed to underlie the onset and progression of the pathologic hallmarks in AD brains (Caricasole et al., 2003; De Ferrari and Inestrosa, 2000; De Ferrari and Moon, 2006; Grilli et al., 2003; Moon et al., 2004; Mudher and Lovestone, 2002). Indeed, while Wnt signaling components β-catenin and glycogen synthase kinase-3b (GSK3b) form multiprotein complexes with familial AD-linked presenilin proteins (Kang et al., 2002; Takashima et al., 1998b; Zhou et al., 1997), which reduces β-catenin levels in AD individuals bearing presenilin mutations (Zhang et al., 1998), active GSK3β accumulates in vivo in AD brains (Pei et al., 1999), where it has a key role in tau hyperphosphorylation (Hanger et al., 1992; Li and Paudel, 2006; Lovestone et al., 1994; Lucas et al., 2001; Sato et al., 2002). Likewise, Wnt signaling seems to have a prominent role in amyloid precursor protein processing (Mudher et al., 2001; Phiel et al., 2003; Sun et al., 2002), and in the neurotoxicity of self-aggregated β-amyloid peptide (Alvarez et al., 2002, 2004; Caricasole et al., 2004; De Ferrari et al., 2003; Takashima et al., 1998a).

More recently, Wnt/β-catenin signaling has also been shown to be essential in neurogenesis (Lie et al., 2005), synapse formation, and axonal remodeling (Purro et al., 2008; Takeichi and Abe, 2005), and involved in excitatory synaptic transmission (Ahmad-Annuar et al., 2006; Avila et al., 2010; Beaumont et al., 2007; Cerpa et al., 2008; Chen et al., 2006), processes which are affected in AD, adding support to the notion that Wnt signaling, as a whole cascade, is a functional candidate for genetic studies aimed to understand AD

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http://dx.doi.org/10.1016/j.neurobiolaging.2012.11.004

etiology. In this regard, several candidate gene association studies have examined common genetic variations among Wnt signaling components in AD including the *GSK3β* gene in chromosome 3 (Mateo et al., 2006; Russ et al., 2001), Dishevelled 1 in chromosome 1 (Russ et al., 2002), and α-T-catenin in the long arm of chromosome 10 (Blomqvist et al., 2004; Busby et al., 2004; Ertekin-Taner et al., 2003). Nevertheless, it is not clear whether these genes represent risk for AD.

We previously reported on the association of common single nucleotide polymorphisms (SNPs) within the Wnt coreceptor, the *LRP6* gene, and late-onset AD in a large family-based sample ascertained by the National Institute of Mental Health–National Institute of Aging ASPs (affected sibling pairs) Genetics Initiative, and in a multi-center case-control series (De Ferrari et al., 2007). Our association results are in agreement with genome-wide linkage studies that defined a broad susceptibility region for late-onset AD on chromosome 12, and which was observed in individuals segregated according to apolipoprotein protein E-ε4 carrier status. Haplotype tagging SNPs with a set of 7 allelic variants of the *LRP6* identified a putative risk haplotype, which included a highly conserved coding sequence SNP in exon 14 (14e, rs2302685; Ile1062→Val) and a synonymous SNP in exon 18 (18e, rs1012672). Interestingly, though the coding sequence SNP in exon 14 (Ile1062→Val), which had decreased efficacy for activating a Wnt/β-catenin signaling, reached significance only in the family-based sample and in apolipoprotein protein E-ε4 noncarriers, the synonymous *LRP6-18e* variant was found markedly overrepresented in late-onset AD individuals throughout our study (De Ferrari et al., 2007). Therefore, in order to examine whether the synonymous *LRP6-18e* is in linkage with other unidentified functional variants and considering that common genetic variation has been shown to be related to altered messenger (m)RNA metabolism (International HapMap Consortium et al., 2007; Myers et al., 2007; Sabeti et al., 2007; Stranger et al., 2007; Webster et al., 2009), here we examined the posttranscriptional metabolism of the *LRP6* message in human and mice tissue and determined its level in cortical brain samples derived from normal and pathologic brains.

2. Methods

2.1. Human tissue and brain mRNA samples

We used the FirstChoice Human Total RNA Survey Panel (cat# AM6000, Life Technologies) and the FirstChoice Human Brain Reference Total RNA (cat# AM6050, Life Technologies) to identify *LRP6* splicing variants. Likewise, 47 brain mRNA samples from the middle temporal cortex of neuropatholologically diagnosed individuals were obtained from the Department of Pathology, University of Washington School of Medicine (Seattle, WA, USA) (Hu et al., 2000), following the guidelines of the local ethics committee donor program. These anonymized samples included 22 AD cases, 11 nonpathologic controls, and 14 individuals suffering from other neurological disorders (OD), whose neuropathologic diagnoses and postmortem intervals have been described earlier (Hu et al., 2000) (see also Supplementary Table 1).

2.2. Mouse mRNA isolation

Mouse tissues from the B6SJL strain (The Jackson Laboratory) were obtained at different developmental stages (day of birth [postnatal day; P]0, P20, and P150). Following recommended guidelines from the Bioethical Committee of the Universidad Andres Bello, mice (P20 and P150) were euthanized by CO_2 inhalation and perfused gently with 30 mL ice cold phosphate buffered saline through the left ventricle. The P0 mice were first anesthetized by hypothermia and then decapitated. Fresh mouse tissues were then dissected (within 10 minutes), snap frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA was isolated by homogenization of selected tissues with TRIzol (Invitrogen) and purified RNA was resuspended in RNase-free water and stored at −80 °C. The integrity of RNA was evaluated by 1% agarose gels with GelRed Nucleic Acid Gel Stain (Biotium Inc) and the purity was determined by optical density 260 nm/OD 280 nm ratio.

2.3. Synthesis of cDNA

Two micrograms of total RNA in a 20 μL reaction volume was reverse transcribed with the Affinity Script cDNA Synthesis Kit (Stratagene); briefly, 10 μL of first strand master mix, 300 ng/μL OligodT primer, 100 ng/μL random primers, and 1 μL Affinity Script RT/RNase Block enzyme mixture. The reaction was incubated at 25 °C for 5 minutes, 42 °C for 45 minutes followed by heat inactivation of the enzyme. The cDNA was stored at −20 °C until use.

2.4. RT-PCR and sequencing

Primer pairs were designed to amplify approximately 500 base pairs (bp) of the *LRP6* transcript (IDT-DNA Inc, Foster City, CA, USA) (Supplementary Table 2) using Primer3 software (http://frodo.wi.mit.edu/). Polymerase chain reaction (PCR) was performed using the FastStart PCR Master Mix (Roche, South San Francisco, CA, USA), with 40 ng cDNA and 0.2 μM of each *LRP6* specific primer. PCR conditions were as follows: 5 minutes at 95 °C, followed by 33 cycles at 95 °C (30 seconds), 58 °C (30 seconds), 72 °C (30 seconds), and final extension of 72 °C (5 minutes). PCR products were separated on 1% agarose gels, visualized under ultraviolet light with GelRed Nucleic Acid Gel Stain (Biotium Inc, Hayward, CA, USA), and isolated using the DNA Gel Extraction Kit (Stratagene). Amplification products were sequenced using the BigDye Terminator chemistry and run on an ABI3100 DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA).

2.5. Real-time qPCR

Primers pairs for quantitative PCR (qPCR) were designed to have an annealing temperature of approximately 60 °C to give amplicons approximately 100–200 bp in length (Supplementary Table 2). Primers were checked using Primer Blast and showed no significant sequence homology other than its intended target. Transcript levels of selected genes were measured by qPCR using the Brillant II SYBR Green Master Mix (Applied Biosystems) and analyzed on Mx3000p (Stratagene) equipment. Each reaction was run in triplicate in a reaction volume of 20 μL. All reactions contained an equimolar concentration of each primer (200–400 nM), 40 ng of cDNA, and diethylpyrocarbonate (DEPC)-treated water. The reaction protocol starts with a 10-minute template denaturation step at 95 °C, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds, and 72 °C for 20 seconds. The SYBR Green assay also included a melting curve at the end of the cycling protocol, with continuous fluorescence measurement from 70 °C to 95 °C. Nontemplate controls were also run in triplicate for each primer master mix. Baseline and threshold for cycle quantitative values were automatically determined for all reactions using the MxPro software version 4.10 (Stratagene).

2.6. Genotyping of LRP6 SNPs

Coding SNPs rs2302685 in exon 14 (14e; Ile-1062 → Val) and rs1012672 in exon 18 (18e; Cys-1270 → Cys), and the intronic SNP