Gene 511 (2012) 227–234

Contents lists available at SciVerse ScienceDirect

Gene

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

Molecular analysis of *SLC25A13* gene in human peripheral blood lymphocytes: Marked transcript diversity, and the feasibility of cDNA cloning as a diagnostic tool for citrin deficiency

Zhan-Hui Zhang, Wei-Xia Lin, Mei Deng, Xin-Jing Zhao, Yuan-Zong Song *

Department of Pediatrics, The First Affiliated Hospital, Jinan University, Guangzhou 510630, China

ARTICLE INFO

Article history:
Accepted 19 September 2012
Available online 26 September 2012

Keywords:
SLC25A13
Citrin deficiency
Peripheral blood lymphocytes
Alternative splice variant
cDNA cloning

ABSTRACT

Human *SLC25A13* gene encodes citrin, the liver-type aspartate–glutamate carrier isoform 2, and *SLC25A13* mutations lead to citrin deficiency (CD). The definitive diagnosis of CD relies on *SLC25A13* analysis, but conventional DNA analysis could not identify all *SLC25A13* mutations. We investigated transcriptional features of *SLC25A13* gene in peripheral blood lymphocytes (PBLs) from CD patients and healthy volunteers. *SLC25A13* mutations were explored by PCR/LA-PCR, PCR-RFLP and direct sequencing. *SLC25A13* cDNA was amplified by RT-PCR, cloned and then sequenced. All diagnoses of the CD patients were confirmed, including a heterozygote of *g*.2T>C and an unknown mutation yielding an aberrant transcript *r*.16_212dup. Twenty-eight alternative splice variants (ASVs) were identified from normal *SLC25A13* alleles. Among them, *r*.213_328del took account for 53.7%, the normal transcript *r*.=, 16.6%, and the remaining 26 novel ASVs, collectively 29.3%, of all cDNA clones. Moreover, similar ASVs, all reflecting corresponsive mutations, were detected from the mutated alleles. These results indicated that the normal *SLC25A13* transcript could be cloned, and the abundance of the ASV *r*.213_328del predicted the existence of a constructively novel protein isoform for this gene in human PBLs. And, the 26 novel ASVs, along with the novel aberrant transcript *r*.16_212dup and the SNP *g*.2T>C, enriched the transcript/variation spectrum of *SLC25A13* gene in human beings. The findings in this paper, for the first time, uncovered the marked transcript diversity of *SLC25A13* gene in human PBLs, and suggested that cDNA cloning analysis of this gene in human PBLs might be a feasible tool for CD molecular diagnosis.

1. Introduction

The human gene *SLC25A13* locates at chromosome 7q21.3 and encodes a 3.4 kb transcript with a predicted ORF of 2025 bp (Kobayashi et al., 1999). The protein encoded by *SLC25A13*, which has been designated as citrin, acts as a liver-type calcium-binding/stimulated aspartate–glutamate carrier that plays important roles in the metabolic pathways of aerobic glycolysis, gluconeogenesis, urea cycle, and synthesis of proteins and nucleotides (Kobayashi and Saheki, 2003; Palmieri et al., 2001; Saheki and Kobayashi, 2005; Saheki et al., 2004, 2006). *SLC25A13* mutations lead to citrin deficiency (CD)(Kobayashi et al., 2012; Saheki et al., 2011), which has been well recognized as a worldwide panethnic disease entity (Ben-Shalom et al., 2002; Dimmock et al., 2009; Fiermonte et al., 2011; Ohura et al., 2007; Song et al., 2009a, 2011; Tabata et al., 2008; Tazawa et al., 2004; Takaya et al., 2005; Thong et al., 2010; Yeh et al., 2006) that encompasses different clinical phenotypes including adult-onset citrullinemia type II (CTLN2) (Komatsu et al., 2008; Saheki et al., 2002), neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD)(Saheki and Kobayashi, 2002; Saheki et al., 2002; Yamaguchi et al., 2002), and failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD), a post-NICCD state before CTLN2 onset which was proposed very recently (Kobayashi et al., 2012; Song et al., 2009b, 2011).

SLC25A13 mutation analysis could provide reliable evidences for CD definitive analysis. However, routine DNA analytic approaches, such as PCR, PCR-RFLP and direct sequencing, could not identify all *SLC25A13* mutations (Lu et al., 2005; Tokuhara et al., 2007). For example, approximately 15% of compound heterozygotes or homozygotes carrying *SLC25A13* mutations in both alleles cannot be accurately identified with genomic analysis (Tokuhara et al., 2007). Furthermore, these methods mainly detect point mutations or small deletions, and are invalid for large intragenic duplications and deletions.

Abbreviations: CD, citrin deficiency; cDNA, complementary Deoxyribonucleic acid; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; LSM, lymphocyte separation medium; EDTA, ethylene diamine tetraacetic acid; LA-PCR, Long and Accurate-PCR; RFLP, restriction fragment length polymorphism; RACE, rapid amplification of cDNA ends; PBLs, peripheral blood lymphocytes; ASVs, alternative splice variants; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon.

* Corresponding author.
E-mail address: songyuanzong@vip.tom.com (Y.-Z. Song).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.09.049

Citrin protein analysis by means of Western blot has also been tried to facilitate CD definitive diagnosis (Dimmock et al., 2007; Fu et al., 2011; Tokuhara et al., 2007). The citrin antibodies in this approach were designed to detect specific antigen changes in citrin protein, but citrin changes in some CD patients might take place outside of the specific positions for the antibodies, and moreover, single amino-acid changes within citrin due to *SLC25A13* missense mutations might not be detected by Western blot, and in such cases, it is not so reliable to analyze citrin alterations just using this tool.

There have been a few reports on abnormal *SLC25A13* mRNA analysis that helped to establish definitive diagnosis of CD patients. However, these studies were limited to fragmental cDNA but not full-length encoding sequence (Kobayashi et al., 1999; Tabata et al., 2008), and the sample sources were cultured fibroblasts or autopsied/biopsied liver specimens (Dimmock et al., 2007; Kobayashi et al., 1999; Tabata et al., 2008), which were not only invasive and hence less-feasible, but also time- and cost-consuming. Our recent findings (Lin et al., 2012) have suggested that human peripheral blood lymphocytes (PBLs) could be taken as a feasible specimen source for *SLC25A13* transcriptional analysis. However, the transcriptional features of *SLC25A13* gene remain far from being completely elucidated in this less-harmful more-feasible human specimen, and the significance of *SLC25A13* transcript analysis using PBLs also needs to be evaluated for molecular diagnosis of CD patients. By means of molecular cloning of *SLC25A13* cDNA, we herein further investigated the *SLC25A13* transcriptional products in PBLs of healthy volunteers and citrin-deficient patients.

2. Materials and methods

2.1. Subjects and ethics

The research subjects enrolled in this study included 4 families with NICCD patients, 7 healthy volunteers for *SLC25A13* cDNA cloning, and 100 control individuals for variation *g*.2T>C screening. This research was carried out with informed consent from the subjects or their parents, and has been approved by the Committee for Medical Ethics, the First Affiliated Hospital, Jinan University in China, adhering to the World Medical Association Declaration of Helsinki (WMADH 2008).

2.2. Mutation analysis

The genomic DNA of blood was extracted with genomic DNA extraction kit (Simgen) in accordance with instructions. Four high-frequency mutations of *SLC25A13* gene, including *g*.851_854del, *g*.1638_1660dup, IVS6+5G>A and IVS16ins3kb, were screened by means of PCR, LA-PCR and PCR-RFLP procedures. If only one or none of the mutations was identified, all the 18 exons and their flanking sequences of *SLC25A13* gene would be analyzed to identify the possible novel mutation by direct sequencing method, as described previously (Lin et al., 2012; Song et al., 2009a, 2011).

Table 1
Sequences and positions for the primers used for nest PCR amplification of *SLC25A13* cDNA.

Primers	Sequences (5′ to 3′)	Positions in mRNA/cDNA
RAS2	AACGCACGCTGCCTGGCCGTATC	c.8_30 (Ex 1)
RACEA1	CCACCTTCACAAATTCATGCGCC	c.3092_3114 (Ex 18)
RAS3	GCCGCCGGGACTAGAAGTGAGC	c.49_70 (Ex 1)
Ex18R	TGCTTCATTCCCAGGAGGGA	c.2220_2239 (Ex 18)
UP-dT	CGGCAGTGGTATCAACGCAGAGTAC$(T)_{18}$	3′ end of the first strand cDNA
UPF	TGGATTTGGAAAGCATGGTCTAC	New Ex 17 in abnormal mRNA due to mutation XIX

2.3. PCR-RFLP procedure for g.2T>C screening in control individuals

A PCR-RFLP procedure was established to screen for the variation *g*.2T>C in 100 normal individuals. The nucleotide sequences of forward (a) and reverse (b) primers for PCR amplification were 5′-ggcgtgccagcaatatttgc-3′ and 5′-caaagtttccgctgcgaggt-3′, respectively. The restriction enzyme in RFLP analysis was NIa III (New England Biolabs). For frequency calculation of the variation, the number of mutated alleles detected in all 100 control samples was divided by 200, and then the quotient was amplified by 100%.

2.4. PBL isolation and RNA extraction

Two milliliters EDTA anticoagulant peripheral blood was centrifuged over lymphocyte separation medium (LSM, MP) immediately after blood sampling. PBLs were collected according to the manufacturer's instructions and then homogenized immediately in RNAiso Plus (TaKaRa) to extract total RNA following the manufacturer's protocol. Total RNA concentration and quality were verified with spectrophotometer (Bio-Rad) and electrophoresis of a 1.2% agarose gel.

2.5. Reverse transcription (RT) and nest PCR

cDNAs were synthesized from 2 μg total RNA in the presence of primer oligo-$(dT)_{18}$ or UP-dT and 200 U MMLV reverse transcriptase (Promega) following the manufacturer's protocol (Lin et al., 2012). Three primers (RAS2, RACEA1 and RAS3; Table 1) were designed based on human *SLC25A13* mRNA sequence (GenBank ID: AF118838), and the primer Ex18R was cited from literature (Tabata et al., 2008). The positions and sequences of the primers were illustrated and shown in Fig. 1 and Table 1, respectively.

To amplify cDNA from normal and mutated *SLC25A13* alleles except mutation XIX, the first PCR was carried out in a 50 μL volume containing 10 μL of 5× PrimeSTAR® Buffer (Mg^{2+} plus) (TaKaRa), 4 μL of dNTP (10 mM), 1 μL of each primer RAS2 and RACEA1 (20 μM, Table 1), 32.5 μL of PCR-grade water, 0.5 μL of PrimeSTAR® HS DNA Polymerase (2.5 U/μL, TaKaRa) and 1 μL of cDNA. After initial denaturation at 94 °C for 3 min, 20 cycles of DNA amplification were performed (98 °C for 10 s, 60 °C for 15 s, 72 °C for 4 min), followed by terminal extension at 72 °C for 7 min. Subsequently, 1 μL of first

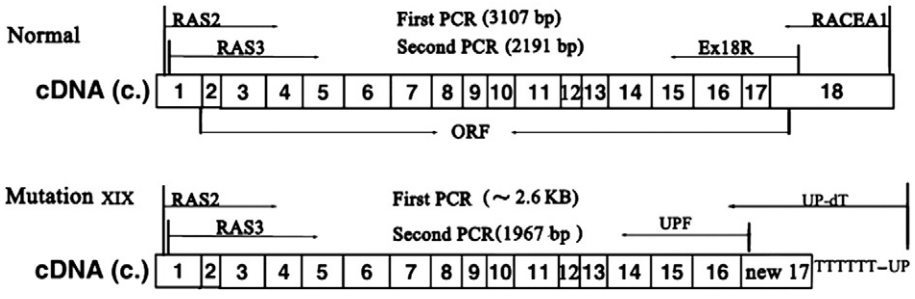

Fig. 1. Schematic diagram of the primer positions for Nest PCR.