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Loss of Leucyl-tRNA synthetase b leads to ILFS1-like symptoms in zebrafish

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ABSTRACT

Leucyl-tRNA synthetase (LARS) is a kind of aminoacyl-tRNA synthetases (aaRSs), which is important for protein synthesis. Following the discovery of three clinical cases which carry *LARS* mutations, it has been designated as the infantile liver failure syndrome type 1 (ILFS1) gene. ILFS1 is a kind of infantile hepatopathy, which is difficult to diagnose and manage. As the mechanism underlying this disease is poorly understood and LARS is conserved among vertebrates, we obtained zebrafish *larsb*^{cq68} mutant via CRISPR/Cas9 technology to investigate the role of *larsb* in vivo. In mutant, the proliferation ability of liver was drastically decreased at later stages accompanied with severe DNA damage. Further studies demonstrated that the mTORC1 signaling was hyperactivated in *larsb*^{cq68} mutant. Inhibition of mTORC1 signaling pathway by Rapamycin or mTORC1 morpholino can partially rescue the liver failure of the mutants. These data revealed that *larsb* mutation caused ILFS1-like phenotype in zebrafish, and indicated this mutant may serve as a potential model for ILFS1. Furthermore, we demonstrated that rapamycin treatment can partially rescue the liver defect in mutants, thus providing a practicable therapeutic plan for ILFS1.

1. Introduction

Considering the important roles of liver in sustaining life, liver related diseases are always a global research hotspot. Among them, infantile hepatopathy is a category of liver diseases which affect about 1/2500 newborns [1]. The diagnosis and management of these diseases are difficult, therefore result in a high lethality [2]. While various nosogenesis are responsible for these diseases, such as heredity, poisoned, infections and so on [3]. So, there is an urgent need to unravel the mechanisms underlying different kinds of infantile hepatopathy.

In human, *LARS* mutation leads to infant liver failure syndromes type 1 (ILFS1), which is a new kind of infantile hepatopathy [4,5]. Since the first discovery of this disease in 2012, three independent cases (11 patients in total) have been reported by now. The predicted morbidity is about 1/1,000,000, but it should be much higher as *LARS* mutation isn't assessed as a marker for infantile hepatopathy. These patients range from 20 months to 35 years old, and the infant's symptoms include acute liver failure in the first few months associated with anemia, microcephaly, seizures and failure to thrive [4–6]. As it is difficult to diagnose, there is no therapeutic plan for this unique disease, and the mechanism underlying remains unclear.

Leucyl-tRNA synthetase is a kind of aminoacyl-tRNA synthetases (aaRSs). aaRSs charge amino acids onto aminoacyl-tRNA, then the charged aminoacyl-tRNA can transfer the amino acid into a growing peptide via ribosome [7]. Recently, however, other researchers found that other than transforming amino acid, aminoacyl-tRNA synthetases also have noncanonical roles in biological processes. For example, Threonyl-tRNA synthetase takes part in vascular patterning regulation [8]; histidyl-tRNA synthetase is involved in vascular development [9]; tyrosyl-tRNA synthetase facilitates macrophages remove cell corpses [10]. And two studies in yeast and cell lines reported that leucyl-tRNA synthetase is an upstream sensor mTORC1 signaling pathway [11,12]. So, despite its

Abbreviations: ILFS1, infantile liver failure syndromes type 1; *LARS*, leucyl-tRNA synthetase; *larsb*, leucyl-tRNA synthetase b; aaRSs, aminoacyl-tRNA synthetases; mTORC1, mechanistic target of rapamycin complex 1; hpf, hours post fertilization; WISH, whole mount in situ hybridization; WT, wild-type.

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canonical function, leucyl-tRNA synthetase also have non-canonical function in amino acid induced mTORC1 signaling pathway. The mechanistic target of rapamycin complex 1 (mTORC1) controls cell growth and metabolism in eukaryotes, and it is critical for many fundamental cell processes, such as protein synthesis, cell motility, autophagy and so on [13,14]. Deregulated mTORC1 signaling is involved in many diseases like cancers, diabetes and seizures [15–17].

Zebrafish has developed into an important model organism over the last decades. Although it has disadvantages compare to mouse, zebrafish has its irreplaceable advantages, such as easy to manipulate, transparent at early developmental stages and feasible for large genetic screen [18]. Besides, recent conservation analysis shows that humans and zebrafish share 82% of disease-related targets and numerous drug metabolism pathways [19].

To explore the role of leucyl-tRNA synthetase in vivo, we obtained *larsb* (zebrafish homologous gene of *LARS*) loss of function mutant via CRISPR/Cas9 technology. Our *larsb*^{cq68} mutants exhibit similar phenotype with ILFS1, and we strikingly found that mTORC1 signaling is hyperactivated in this mutant. While rapamycin, a specific mTORC1 inhibitor, can partially rescue the liver failure of *larsb*^{cq68} mutant, and mTORC1 morphants showed the same result. Taken together, we conclude mTORC1 hyperactivation is responsible for ILFS1 and rapamycin treatment should be a practicable way for the therapy of this disease.

2. Materials and methods

2.1. Zebrafish strains

Zebrafish AB genetic background *Tg(lfabp: Dendra2-NTR)*, *Tg(lfabp: DsRed)* transgenic fish [20] and *larsb*^{cq68} were raised and maintained under standard laboratory conditions according to institutional animal care and use committee protocols. To inhibit pigmentation, embryos were treated with 0.003% 1-Phenyl-2-thiourea (PTU; Sigma, USA) from 24 hpf.

2.2. CRISPR/Cas9 -targeted mutagenesis

The CRISPR/Cas9 was performed as previously described [21], *larsb* target site is shown in Fig. 2A. The primer used to identify genotype were as follows: forward primer, 5′-CCGGTTGCCATG-GAATGAAC-3’; reverse primer, 5′-AGCTGAACTTTTTACCTGATC-TACA-3’.

2.3. In situ hybridization

Whole mount in situ hybridization was performed as previously described [20] using the following probes: *larsb*, *fabp10*, *cp*. The primer used to amplify *larsb* probe were as follows: forward primer, 5′-CATCCTTGGCTGTGCGTTGA-3′ and reverse primer, 5′-TTCCCGA-TACTCTGACGTCT-3’; *fabp10* and *cp* probe were synthesized as previously described [22,23]. The images were taken by SteREO Discovery V20 microscope (Carl Zeiss, Germany).

2.4. Whole mount antibody staining

Whole mount antibody staining was performed as previously described [24] using antibodies against DsRed (1:500; Santa Cruz, USA), Dendra2 (1:500; Evrogen, Russia), γH2AX (1:500; GeneTex, USA), Phospho-S6 Ribosomal Protein (Ser235/236) (1:200, Cell Signaling, USA) and Phospho-4E-BP1 (Thr37/46) (1:500; Cell Signaling, USA). The images were taken by LSM780 confocal microscope (Carl Zeiss, Germany).

2.5. EdU cell proliferation assay

Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, USA) was applied for EdU cell proliferation assay. EdU mixtures (0.2 mM Edu and 2% DMSO) were injected into embryos at 83 hpf. After incubation at 28.5 °C for 40 min, the embryos were fixed with 4% PFA at room temperature for 2 h or overnight, then EdU reaction was performed according to the manufacturer's instructions. After EdU reaction, whole mount antibody staining was performed as previously described.

2.6. Microinjection of synthetic mRNAs

Total RNA was isolated using Trizol reagent (Invitrogen, USA), then reverse transcribed to cDNA using Omniscript reverse transcription kit (QIAGEN, Germany). Zebrafish *larsb* and human *LARS* coding sequences were amplified using the following primers: *larsb* forward primer, 5′-ATGACGGAACGCAAAGGAAC-3′ and *larsb* reverse primer, 5′- CTAAACCAGGTAGATTAACG -3’; *LARS* forward primer, 5′-TCGTGGATTTGCACAGTCGG-3′ and *LARS* reverse primer, 5′-AGGTCCAGCCTAAGGTTCTG-3’. Then the coding sequences were sub-cloned into the PCS2+ vector. Capped mRNAs were synthesized by mMESSAGE mMACHINE® Kit (Ambion, USA), and injected into 1-cell stage embryos.

2.7. Morpholinos

The mTORC1 morpholino and control morpholino were designed as previously described [25] and purchased (Gene Tools, USA). Each embryo was injected with 2 ng morpholino at 1-cell stage.

2.8. Rapamycin treatment

To inhibit mTORC1 pathway, embryos were treated with 5 μM rapamycin (Sangon Biotech, China) in PTU egg water from 60 hpf to 96 hpf, or 0.2% DMSO as control.

3. Results

3.1. Larsb is conserved among vertebrates

To assess the conservational value of Larsb among vertebrates, we aligned the leucyl-tRNA synthetase amino acid sequence of *Homo sapiens*, *Mus musculus* and *Danio rerio* (Fig. 1A). The leucyl-tRNA synthetase of these three species have 1176, 1178, 1176 amino acids, respectively. According to the alignment, zebrafish Larsb share 80% identities compared with human LARS. This ratio is higher than ppox (52%) and tpp1 (67%), which are mutated genes of zebrafish models for variegate porphyria and CLN2 disease [26,27]. This indicated that leucyl-tRNA synthetase is highly homologous in vertebrates. We then examined the expression pattern of *larsb* by whole mount in situ hybridization (WISH). *larsb* expressed at 1-cell stage (Fig. 1B), which meant *larsb* was a maternal gene. At bud (Fig. 1C) and 24 hpf (Fig. 1D), it was broadly expressed. While at 48 hpf (Fig. 1E) and 72 hpf (Fig. 1F), its expression focused at liver, head and intestine (Fig. 1E’ and 1F′). Since ILFS1 patients also exhibited liver and head symptoms [5], we speculated that *larsb* mutation may recapitulate ILFS1's phenotype in zebrafish.

3.2. Construction of larsb loss of function mutant and phenotypic identification

To obtain *larsb* mutant, we designed the CRISPR/Cas9 target site at the exon 9 of *larsb* genome locus as shown in Fig. 2A. Using the

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