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Short communication

Genome editing of pufferfish saxitoxin- and tetrodotoxin-binding protein type 2 in *Takifugu rubripes*

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ABSTRACT

The pufferfish saxitoxin- and tetrodotoxin-binding protein 2 (PSTBP2), which is involved in toxin accumulation, was knocked out in *Takifugu rubripes* embryos by using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 genome-editing technology. Treating the embryos with one of two single-guide RNA (sgRNA) resulted in mutation rates of 57.1% and 62.5%, respectively, as estimated using a heteroduplex mobility assay at 3 days postfertilization. Both sgRNAs might induced frameshift mutations that knocked out the *T. rubripes* PSTBP2.

The marine pufferfish *Takifugu rubripes* is well known for its potent toxicity, due to its accumulation of tetrodotoxin (TTX). TTX is produced by marine bacteria, and is thought to bio-accumulate in fish via the food web (Noguchi and Arakawa, 2008). Several toxic pufferfish possess pufferfish saxitoxin- (STX) and TTX-binding proteins (PSTBPs), which are implicated in the accumulation of toxin in fishes (Hashiguchi et al., 2015; Tatsuno et al., 2013; Yotsu-Yamashita et al., 2001, 2010, 2013, 2018). PSTBPs may play an important role in toxification of pufferfish, and are therefore likely to be advantageous for survival in certain ecosystems (Itoi et al., 2014).

PSTBPs are derived from duplicated tributyltin-binding protein (TBT-bp) genes (Hashiguchi et al., 2015; Oba et al., 2007, 2011), which are alpha 1-acid glycoprotein-like lipocalin proteins. Alpha 1-acid glycoprotein-like lipocalin proteins is a major mammalian acute phase protein that binds to low-molecular-weight basic lipophilic drugs, and is involved in the inflammatory response (Fournier et al., 2000; Gutiérrez et al., 2000). Similarly, PSTBPs may bind and transport low-molecular-weight basic chemical TTX or STX. PSTBPs have an affinity for STX and TTX and are involved in the accumulation and/or excretion of these toxins (Yotsu-Yamashita et al., 2001, 2002, 2018). In a previous study, we constructed recombinant *T. rubripes* PSTBPs (rTrub.PSTBP1 and rTrub.PSTBP2), and confirmed that rTrub.PSTBP2 binds to tributyltin and TTX *in vitro* (Satone et al., 2017). However, the mechanism of toxin accumulation and transportation *in vivo*, and the role of PSTBP, remains to be elucidated.

The role of PSTBP in toxification could be clarified by examining PSTBP knockouts *in vivo*. In recent years, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system-based RNA-guided endonucleases have rapidly accepted as a simple and efficient tool for targeted genome editing in a wide range of taxa, including fish (Ansai and Kinoshita, 2014; Chang et al., 2013; Edvardsen et al., 2014; Wiedenheft et al., 2012). As with zinc-finger nucleases and transcription activator-like effector nucleases, this system can efficiently induce site-specific DNA double-stranded breaks, resulting in targeted gene disruption through indels (insertions or deletions), or targeted gene integration by homologous recombination. In particular, frameshift mutations, which affect the reading frame during translation, can knock out gene expression or cause gene dysfunction.

We aimed to study the function of PSTBP2 (Trub.PSTBP2) by editing the PSTBP*2* gene in *T. rubripes* via the CRISPR/Cas9 system. To do so, we designed two target sequences of Trub.PSTBP2 gene from sequences in the first exon of Trub.PSTBP2 gene. The Trub.PSTBP2 genome sequences were obtained from Fugu Genome Assembly v5 (http://rohsdb.

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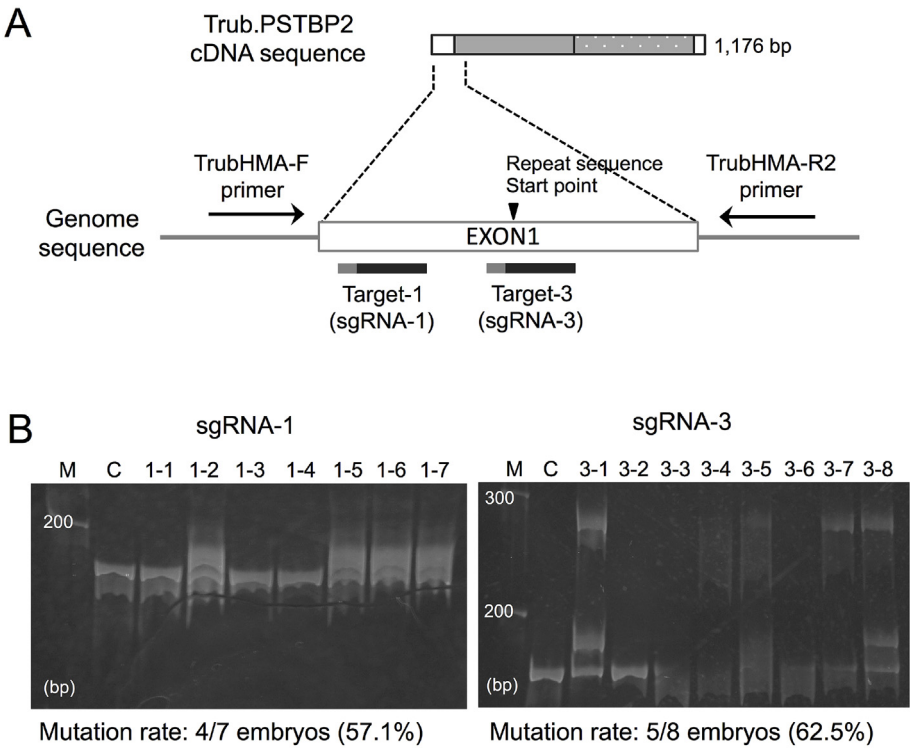

Fig. 1. Genome editing results on Trub.PSTBP2 gene (and/or Trub.TBT-bp2 gene) using the CRISPR/Cas9 System. (A) A schematic of the target sites on the Trub.PSTBP2 gene (accession no. AB901456) used for the CRISPR/Cas9 system. Gray boxes in the cDNA sequence indicate repeat regions. (B) Heteroduplex mobility assay (HMA) results for on-target mutations in embryos injected with each sgRNA. The HMA results for embryos injected with sgRNA-1 are shown on the left, and those for embryos injected with sgRNA-3 are shown on the right. Lane M was treated with a DNA marker of the size shown on the figure. Lane C is a control sample injected only with Yamamoto's Ringer's solution. Lanes denoted 1-1 through 1–7 and 3–1 through 3–8 correspond to embryo numbers. The mutation rate (number of mutated embryos/number of injected embryos) is shown at the bottom of each figure. (C) HMA results for off-target analysis. Results for the *raver2* gene are shown on the left, and those for the *tmem120b* gene are shown on the right. Lane C is a control sample injected only with Yamamoto's Ringer's solution. Lanes 1-1 through 1–7 and 3–1 through 3–8 correspond to embryos that were injected with sgRNA-1 and sgRNA-3, respectively (the same embryos as in Fig. B).

cmb.usc.edu/GBshape/cgi-bin/hgGateway?db=fr3) by referenced with that cDNA sequences (accession no. AB901456), and the exon/intron sites were inferred from Spidey analysis (http://www.ncbi.nlm.nih.gov/spidey/). Two target sequences (Target-1 and Target-3) on the first exon of Trub.PSTBP2 gene (Fig. 1A) were selected in upper of the repeat sequence, and for their low off-target risk. However, we could not avoid on target to TBT-bp2 gene (Target-1: AB901442 and AB901439, Target-3: AB901442) which component of PSTBP2 and is highly homologous gene (Hashiguchi et al., 2015). We could obtain the Trub.PSTBP2 gene specific mutant by more detail genotyping in wide region. Therefore, in this study, we confirmed the mutation of these target sequences regardless Trub.PSTBP2 gene or Trub.TBT-bp2 gene. In other off-target, our estimates of off-target risk were confirmed by a blast search of the Fugu Genome database (http://rohsdb.cmb.usc.edu/GBshape/cgi-bin/hgGateway?db=fr3), and by analyzing *T. rubripes* data (taxid: 31033) from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Single-guide RNAs (sgRNAs) for each target sequence, which we termed sgRNA-1 and sgRNA-3, respectively, were prepared in accordance with published methods (Ansai and Kinoshita, 2014). In brief, an oligonucleotide pair was annealed and then ligated into the pDR274 vector (Plasmid 42250, Addgene, MA USA) with *Bsa*I-HF (New England Biolabs, MA, USA). The sgRNAs were synthesized using the MAXIscript SP6/T7 Kit (Ambion, TX, USA) after digestion with *Dra*I, and purified by ammonium acetate precipitation. The annealed oligonucleotide sequences are as follows (underlined letters indicate a cohesive sequence with the vector): sgRNA-1 sense segment, 5′-TAGGATCAGCAGAAGAACCACTCC; sgRNA-1 anti-sense segment, 5′-AAACGGAGTGGTTCTTCTGCTGAT; sgRNA-3 sense segment, 5′- TAGGTTGTGACACTCTTCTGGAGC; and sgRNA-3 anti-sense segment, 5′- AAACGCTCCAGAAGAGTGTCACAA.

DNA-cleavage activity in the sgRNAs was confirmed by *in vitro* assays before use (results not shown). Template DNA fragments, including the sgRNA target sites, were amplified with Phusion DNA polymerase (New England Biolabs) from previously constructed Trub.PSTBP2 expression vectors (unpublished), and were purified using the Wizard SV Gel and PCR Clean-up System (Promega, WI, USA). One micromolar template DNA fragments were incubated for 1 h at 37 °C with 20 nM Cas9 nuclease, *Streptococcus pyogenes* (New England Biolabs), and 20 nM sgRNA in 1 × Cas9 Reaction Buffer (New England Biolabs). The reaction was stopped by raising the temperature to 70 °C for 10 min, and the reaction products were subjected to agarose gel electrophoresis for confirming cleaved template fragment.

The pCS2 + hSpCas9 vector (Plasmid 51815, Addgene) was used for Cas9 mRNA expression. This vector was linearized by *Not*I (TaKaRa, Shiga, Japan) digestion, and purified using the Wizard SV Gel and PCR Clean-up System (Promega). Capped RNA was synthesized using the mMessage mMachine SP6 Kit (Ambion) and purified using the RNeasy