DNA Microarray Analysis on the Genes Differentially Expressed in the Liver of the Pufferfish, *Takifugu rubripes*, Following an Intramuscular Administration of Tetrodotoxin

Takuya Matsumoto 1,†,‡, Holger Feroudj 1,†, Ryosuke Kikuchi 1, Yuriko Kawana 2, Hidehiro Kondo 2, Ikuo Hirono 2, Toshiaki Mochizuki 3, Yuji Nagashima 4, Gen Kaneko 1, Hideki Ushio 1, Masaaki Kodama 1 and Shugo Watabe 1,5,*

1 Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan; E-Mails: takuya62@pu-hiroshima.ac.jp (T.M.); Holger.Feroudj@gmail.com (H.F.); ryosuke-1129@hotmail.co.jp (R.K.); agkaneko@mail.ecc.u-tokyo.ac.jp (G.K.); aushio@mail.ecc.u-tokyo.ac.jp (H.U.); akodama1943@yahoo.co.jp (M.K.)

2 Laboratory of Genome Science, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato, Tokyo 108-8477, Japan; E-Mails: qqq69dododo@yahoo.co.jp (Y.K.); h-kondo@kaiyodai.ac.jp (H.K.); hirono@kaiyodai.ac.jp (I.H.)

3 Kawaku Company Limited, Shimonoseki, Yamaguchi 750-0093, Japan; E-Mail: mochizuki@kawaku.com

4 Department of Food Science and Technology, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato, Tokyo 108-8477, Japan; E-Mail: yujicd@kaiyodai.ac.jp

5 School of Marine Biosciences, Kitasato University, Minami, Sagamihara, Kanagawa 252-0373, Japan

† These authors contributed equally to this work.

‡ Current address: Faculty of Life and Environmental Science, Prefectural University of Hiroshima, Nanatsuka, Shobara, Hiroshima 727-0023, Japan.

* Author to whom correspondence should be addressed; E-Mail: swatabe@kitasato-u.ac.jp; Tel.: +81-42-778-9094; Fax: +81-42-778-5010.

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Abstract: Pufferfish accumulate tetrodotoxin (TTX) mainly in the liver and ovary. This study aims at investigating the effect of TTX accumulation in the liver of cultured specimens of torafugu Takifugu rubripes on the hepatic gene expression by microarray analysis on Day 5 after the intramuscular administration of 0.25 mg TTX/kg body weight into the caudal muscle. TTX was detected in the liver, skin and ovary in the TTX-administered individuals. The total amount of TTX accumulated in the body was 67 ± 8% of the administered dose on Day 5. Compared with the buffer-administered control group, a total of 59 genes were significantly upregulated more than two-fold in the TTX-administered group, including those encoding chymotrypsin-like elastase family member 2A, transmembrane protein 168 and Rho GTP-activating protein 29. In contrast, a total of 427 genes were downregulated by TTX administration, including those encoding elongation factor G2, R-spondin-3, nuclear receptor activator 2 and fatty acyl-CoA hydrolase precursor. In conclusion, our results demonstrate that the intramuscular administration of TTX changes the expression of hepatic genes involved in various signaling pathways.

Keywords: tetrodotoxin; pufferfish Takifugu rubripes; microarray analysis; gene expression; intramuscular administration; accumulation; toxification

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin, which binds to voltage-gated sodium channels with a very high affinity, and it is generally accepted that TTX is accumulated at high levels in specific tissues, such as the liver, ovary and skin, of Takifugu pufferfish [1,2]. The hypothesis that pufferfish themselves are unable to synthesize TTX is now widely accepted, which is mainly supported by the fact that cultured specimens are non-toxic, but become toxic by feeding TTX-containing artificial diets [3–7]. TTX was also found in various wild marine animals, such as worms, annelids, snails, starfish and crabs [8]. In addition, TTX-producing marine bacteria were isolated from pufferfish, xanthid crab and red calcareous alga, as well as from marine environments [9–14]. These results support that TTX is an exogenous substance for TTX-bearing organisms, and the toxification occurs via the food chain, bacterial parasitism or symbiosis [15,16].

We investigated the in vivo pharmacokinetics of TTX in cultured specimens of torafugu T. rubripes given a single intravenous and gastrointestinal administration [17,18]. TTX was well introduced into the systemic circulation from the gastrointestinal tract by a saturable mechanism and rapidly taken up into the liver. In addition, we developed tissue models of in vitro accumulation/uptake of TTX in the liver, revealing the involvement of the carrier-mediated transport system in the TTX uptake mechanism of torafugu T. rubripes [19–21]. These results strongly indicate that pufferfish have a special function to actively accumulate TTX in the liver at high concentrations.

We previously investigated the genes related to the accumulation of TTX in the liver by comparing mRNA expression patterns in the wild marine pufferfish, T. chrysops and T. niphobles, which have different concentrations of TTX in the liver using mRNA arbitrarily-primed (RAP) RT-PCR [22].
Briefly, RAP RT-PCR provided a 383-bp cDNA fragment, and its transcripts were higher in toxicity than non-toxic pufferfish liver. Its deduced amino acid sequence was similar to those of fibrinogen-like proteins reported for other vertebrates. The cDNA fragment of 383 bp was composed of at least three fibrinogen-like protein (flp) genes (flps), flp-1, flp-2 and flp-3, in the liver of *T. chrysops* and *T. niphobles* containing high concentrations of TTX, and the relative mRNA levels of these genes showed a linear correlation with TTX levels in the liver of the two species. The gene encoding flp-1 in the liver of *T. niphobles* located in scaffold 628 of the Fugu Genome Database, and the amino acid sequence in a C-terminal region of flp-3 in *T. chrysops* liver was homologous to hepcidin precursors of the spotted green pufferfish, *Tetraodon nigroviridis*, European sea bass *Dicentrarchus labrax*, mouse and human. In addition, we also examined the hepatic gene expression profile in cultured torafugu by suppression subtractive hybridization (SSH) at 12 h after the intramuscular administration of 0.50 mg TTX/kg body weight into the caudal muscle [23]. The intramuscular administration of TTX increased the transcripts encoding acute-phase response proteins, such as hepcidin, complement C3, serotransferrin, apolipoprotein A-1 and high temperature adaptation protein Wap65-2 in the liver at 12 h after administration. Very recently, we performed DNA microarray analysis with total RNAs from toxic and non-toxic wild pufferfish [24]. The mRNA levels of 1,108 transcripts were more than two-fold higher in toxic than in nontoxic specimens, and the expression levels of nine genes were upregulated more than 10-fold in toxic ones. It was noted that proteins encoded by these genes are related to vitamin D metabolism and immunity. It is unclear, however, whether the transcripts of these genes are involved in TTX disposition and how they function in pufferfish. Thus, the biological and physiological significance of TTX in pufferfish remains unknown.

In this study, we performed DNA microarray analysis on the liver of marine pufferfish *T. rubripes* at five days after the intramuscular administration of 0.25 mg TTX/kg body weight into the caudal muscle to investigate the effect of TTX accumulation into the liver on the hepatic gene expression and to identify the genes possibly related to TTX accumulation in the liver. This study would answer questions beyond just the transcriptomic changes that may help drive TTX accumulation in the liver and also contribute to better understanding how the transcriptomic response can limit the toxicity of TTX to pufferfish.

2. Experimental Section

2.1. Materials

Experimental marine pufferfish *T. rubripes* specimens (18 months old, approximately 1 kg body weight), cultured by the flow-through aquaculture system that efficiently utilized the underground seawater from the Kannon Tunnel at the Aquaculture Station, Kawaku Co., Ltd. in Shimonoseki, Yamaguchi Prefecture, Japan, were used in the present study. The temperature of the seawater was constant at around 20 °C throughout the year, and fish were fed arbitrarily with commercial diets. TTX used in the administration study was purified from the ovary of wild pufferfish *T. rubripes* collected at the coastal area of the Genkai-Nada Sea in Japan by a combination of ultrafiltration and a series of column chromatographic separations, as reported previously [21]. Crystalline TTX (Wako Pure
2.2. TTX Administration and Sample Preparation

The administration experiments were carried out at Kawaku Aquaculture Station in September, 2010. TTX was dissolved in modified Hank’s balanced salt solution buffer (160 mM NaCl, 5.4 mM KCl, 0.34 mM NaHPO₄, 0.44 mM KH₂PO₄, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, adjusted to pH 7.4 with NaOH solution), and five pufferfish specimens (0.99 ± 0.06 kg body weight) received an intramuscular injection of 0.25 mg TTX/500 µL/kg body weight into the caudal muscle and were maintained in a 1000-L circular culture tank using the flow-through aquaculture system for 5 days at 20 °C (the TTX-administration group, city, state, country). On Day 5 after administration, these fish were removed from the circular culture tank, and their tissues were dissected. The liver was cut into 5-mm pieces, immediately stored in RNAlater solution (Applied Biosystems, Foster City, CA, USA) and stored at −80 °C until use. For the control group, five pufferfish specimens (1.02 ± 0.06 kg body weight) were given an intramuscular injection of the buffer (500 µL/kg body weight) that did not contain TTX, and the liver samples were prepared as described above. The remaining liver samples and other tissues (ovary, skin, and muscle) were stored at −20 °C for TTX determination by LC-FLD.

2.3. TTX Extraction and Quantification

TTX was extracted from the tissue samples with 0.1% acetic acid by heating in a boiling water bath for 10 min after ultrasonication for 1 min according to the standard assay procedures for TTX [25]. TTX quantification was performed by LC-FLD analysis according to the methods of Nagashima et al. [26] and Shoji et al. [27] with some modifications. Briefly, the analytical column was a Wakopak Navi C30-5 (4.6 mm i.d. × 250 mm, 5 mm particle size, Wako Pure Chemical Industries, Osaka, Japan) and maintained at 25 °C. The mobile phase consisted of 5 mM sodium heptanesulfonate in 10 mM ammonium formate (pH 5.0) containing 1 vol% acetonitrile and was eluted at a flow rate of 1.0 mL/min. The eluates were heated at 105 °C with 4 N NaOH (flow rate 1.0 mL/min) in a Teflon tubing (0.5 mm i.d. × 20 m). The reaction products were cooled by flowing through the stainless tube (0.46 mm i.d. × 0.3 m) kept in ice-cold water and detected by an FP-2020 fluorescence detector (JASCO, Tokyo, Japan) with excitation at 365 nm and emission at 510 nm.

2.4. RNA Extraction for Microarray Analysis

Total RNAs were extracted from the liver samples of each group using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), as described in the manufacturer’s instructions. Total RNA concentrations were measured using a NanoPhotometer (IMPLEN, Munich, Germany), and the quality of total RNA was analyzed by agarose gel electrophoresis.
2.5. Preparation of Fluorescently-Labeled cRNA and Microarray Analysis

A custom 44k oligonucleotide microarray was designed using the Agilent eArray application (Agilent Technologies, Santa Clara, CA, USA) [28] based on the predicted cDNA data (FUGU version 4) of the genome assembly [29]. A 700-ng aliquot of total RNAs extracted from the liver sample each of TTX-administered and control groups was mixed with One-Color Spike-Mix (Agilent Technologies), reverse-transcribed and labeled with Cy3 using the Quick Amp Labeling Kit (Agilent Technologies). Cy3-labeled cRNA was purified using the RNeasy Mini Kit (Qiagen) and fragmented using the Gene Expression Hybridization Kit (Agilent Technologies). The samples were mixed with an equal volume of hybridization buffer and transferred on the microarray slide glass, which was subsequently incubated at 65 °C for 17 h. After hybridization, the microarray glass slides were washed with gene expression wash Buffer 1 at room temperature for 1 min and rinsed with gene expression wash Buffer 2 at 37 °C for 1 min. The slide glasses were then dried with nitrogen gas and scanned immediately using a GenePix4000B scanner (Axon Instruments, Foster City, CA, USA). The scanning image files were converted into expression data using Feature extraction software version 10.7.3 (Agilent Technologies) [30]. Microarray data analysis was performed using GeneSpring GX software version 11.0 (Agilent Technologies) [31]. The raw expression values were normalized, and gene expression ratios were calculated by normalizing the TTX-administered versus control group. Differentially expressed genes in the TTX-administered group were selected based on a >2.0-fold change.

2.6. Quantitative Real-Time PCR

The differential expression of chymotrypsin-like elastase family member 2A (cela2a), which was the highest upregulated gene in the TTX-administered group, was further validated by quantitative real-time PCR. Briefly, total RNAs were extracted from the liver samples by the above protocol and treated with DNase I (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were constructed using oligo-dT20 primers and SuperscriptTM III reverse transcriptase (Invitrogen), as described in the manufacturer’s instructions. Real-time PCR was performed in a 20-µL reaction mixture containing 2 µL of cDNA (1:20 dilution), 10 µL of SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 0.4 µL of ROX reference dye (Takara Bio), 0.8 µL of 10 µM gene-specific forward primer and 0.8 µL of 10 µM gene-specific reverse primer on an ABI7300 Real-Time PCR System (Applied Biosystems). Reactions were as follows: 95 °C for 30 s; then 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The relative fold change of the cela2a mRNA expression level was determined by the comparative delta threshold cycle (ΔCt) method for relative quantification based on the beta actin 1 mRNA (Accession Number U37499.1) expression level. The gene-specific primers were designed using Primer Express software version 3.0 (Applied Biosystems) [32], and the sequences are as follows: 5'-CTCTTCCAGCAGCATCTTCTT-3' (forward) and 5'-GACGTCGCACTTCAATGCT-3' (reverse) for beta actin 1 (Accession No. U37499.1) and 5'-GGCACCACACCTGACAT-3' (forward) and 5'-GGCTGGGAACAGATGGA ATG-3' (reverse) for cela2a (Ensemble FUGU ID, ENSTRUT00000045544).
2.7. Data Analysis and Statistics

Data from the quantitative real-time PCR are expressed as the mean ± standard error (SE), and the Student’s t-test was used to analyze the significance of differences among the means at the level of $p < 0.05$.

3. Results

3.1. TTX Determination

TTX in the tissues of pufferfish *T. rubripes* on Day 5 after administration was analyzed by LC-FLD. TTX was detected only in the tissues from the TTX-administered group, but not from the control group (<0.15 µg TTX/g tissue). The concentration and total amounts of TTX in the tissues are summarized in Table 1. The concentration was highest in the liver followed by the skin and ovary, whereas TTX in the muscle was not at the detectable level. The ratio of the total amount of TTX accumulated to that administered was quite high (about 70%).

| No. | Sex | Body weight (kg) | Dose (µg) | TTX concentration (µg/g) | Total amount (µg) | Accumulation (% of dose) |
|-----|-----|-----------------|----------|--------------------------|------------------|------------------------|
|     |     |                 |          | Liver | Skin | Ovary | Muscle | (µg) | (µg) |                        |
| 1   | F   | 1.14            | 285      | 0.48 | 0.30 | 5.43 | N.D.  | 145 | 51 |                        |
| 2   | F   | 0.84            | 210      | 0.68 | 0.97 | 5.30 | N.D.  | 162 | 77 |                        |
| 3   | F   | 1.00            | 250      | 1.12 | 0.62 | 4.97 | N.D.  | 206 | 82 |                        |
| 4   | F   | 1.10            | 275      | 0.54 | 0.33 | 4.51 | N.D.  | 124 | 45 |                        |
| 5   | M   | 0.86            | 215      | 1.35 | 0.79 | N.D.  | N.D.  | 176 | 82 |                        |
|     |     |                 |          | Mean ± SE | 247 ± 150.84 | 0.17 | 0.60 | N.D.  | 163 ± 14 | 67 ± 8 |

1 N.D.: not detected (<0.15 µg TTX/g tissue).

3.2. Gene Expression Analysis

To identify the differentially expressed genes between the TTX-administered pufferfish *T. rubripes* liver and control, the cDNA microarray analysis was performed using the Agilent eArray platform. A total of 59 genes were found to be upregulated more than two-fold with a $p$-value <0.05 in the TTX-administered group, as shown in Figure 1. The genes upregulated three-fold and more were extracted and are listed in Table 2. The highest upregulated gene was chymotrypsin-like elastase family 2A, and its fold change (FC) value was 37.6. The upregulated genes were assigned to have major molecular functions of the putative translated proteins based on their gene ontology information and the Gene Ontology Database [33] As shown in Figure 2, genes involved in enzyme and cofactors (metabolism) and transcription accounted for 27.1% and 15.3%, respectively, whereas those involved in receptor activity, protein binding and metal ion binding accounted for 13.6%, 10.2% and 8.5%, respectively.
**Figure 1.** Fold change analysis of 486 genes differentially expressed in the liver of *Takifugu rubripes*. The histogram shows the fold change values for 427 downregulated genes (left) and 59 upregulated genes (right) in the liver of *T. rubripes* on Day 5 after the intramuscular administration of TTX.

**Figure 2.** Functional classification of 59 genes significantly upregulated in the liver of *Takifugu rubripes* in the TTX-administered group.
| Ensemble ID            | Gene name              | Predicted description                                      | Functional classification               | FC      |
|------------------------|------------------------|------------------------------------------------------------|-----------------------------------------|---------|
| ENSTRUT00000045544     | Cela2a                 | Chymotrypsin-like elastase family member 2A                 | Enzyme, cofactor                        | 37.6    |
| ENSTRUT00000041722     | Tmem168                | Transmembrane protein 168                                  | Transport vesicle                       | 20.0    |
| ENSTRUT0000006023      | Arghp29                | Rho GTPase-activating protein 29                           | Enzyme, cofactor                        | 12.1    |
| ENSTRUT0000034782      | Kenma1                 | T. rubripes calcium channel alpha-1 subunit homolog (AF026198.1) | Ion channel activity                    | 10.4    |
| ENSTRUT0000004724      | Chma9                  | T. rubripes nicotinic acetylcholine receptor alpha 9d subunit (AY299471.1) | Ion channel activity                    | 8.1     |
| ENSTRUT0000038610      | Csmd1                  | CUB and sushi domain-containing protein 1                    | Protein binding                         | 7.0     |
| ENSTRUT0000036792      | Serinc4                | Serine incorporator 4                                       | Transporter activity                    | 6.8     |
| ENSTRUT0000026094      | C14orf135              | Pecanex-like protein C14orf135                              | Unknown                                 | 6.6     |
| ENSTRUT0000046072      | Lrp2                   | Low density lipoprotein receptor-related protein 2          | Receptor activity                       | 5.4     |
| ENSTRUT0000046718      | Col27a1                | Collagen alpha-1(XXVII) chain A-like                        | Structural molecule                     | 5.2     |
| ENSTRUT0000039397      | Khhl32                 | Kelch-like protein 32                                       | Protein binding                         | 4.5     |
| ENSTRUT00000017057     | Dmxb1                  | Diencephalon/mesencephalon homeobox protein 1                | Transcription factor                    | 4.3     |
| ENSTRUT0000023088      | Clk2                   | Dual specificity protein kinase CLK2                        | Enzyme, cofactor                        | 3.9     |
| ENSTRUT0000045827      | Myo3b                  | Myosin-IIIb                                                | Enzyme, cofactor                        | 3.9     |
| ENSTRUT0000025143      | Megf11                 | Multiple epidermal growth factor-like domains protein 11    | Metal ion binding                       | 3.7     |
| ENSTRUT0000035399      | Slle4a1                | Choline transporter-like protein 1                          | Transporter activity                    | 3.7     |
| ENSTRUT0000017798      | Cc4                    | Carbonic anhydrase 4                                        | Enzyme, cofactor                        | 3.5     |
| ENSTRUT0000006962      | OR4563-2               | T. rubripes odorant receptor (DQ306241.1)                   | Receptor activity                       | 3.5     |
| ENSTRUT0000024316      | Pitpm2                 | Membrane-associated phosphatidylinositol transfer protein 2 | Metal ion binding                       | 3.4     |
| ENSTRUT0000030952      | Ptfafr                 | Platelet-activating factor receptor                         | Receptor activity                       | 3.4     |
| ENSTRUT0000038903      | Hepacam2               | HEPACAM family member 2                                     | Unknown                                 | 3.4     |
| ENSTRUT0000043658      | Gpr22                  | Probable G-protein coupled receptor 22                      | Receptor activity                       | 3.3     |
| ENSTRUT0000031306      | Ptpn2                  | Tyrosine-protein phosphatase non-receptor type 2            | Enzyme, cofactor                        | 3.3     |
| ENSTRUT0000019629      | Eps15I1                | Epidermal growth factor receptor substrate 15-like 1        | Receptor activity                       | 3.2     |
| ENSTRUT0000036590      | Zdhhc8                 | Membrane-associated DHHC8 zinc finger protein (NM_001078596.1) | Enzyme, cofactor                        | 3.2     |
| ENSTRUT0000031045      | Rheb                   | GTP-binding protein Rheb                                    | Enzyme, cofactor                        | 3.2     |
| ENSTRUT0000047583      | Agap2                  | ArfGAP with GTPase domain, ankyrin repeat and PH domain 2  | Enzyme, cofactor                        | 3.0     |
| ENSTRUT0000017824      | Ppargc1a               | Peroxisome proliferator activated receptor gamma coactivator 1 alpha (DQ157766.1) | Receptor activity                       | 3.0     |
| ENSTRUT0000027961      | Pfkp                   | 6-phosphofructokinase type C                                | Enzyme, cofactor                        | 3.0     |
| ENSTRUT0000029743      | E2F2                   | Transcription factor E2F2                                   | Unknown                                 | 3.0     |

\(^1\) FC is the average fold change of the TTX-administered (n = 5) compared to the buffer-administered control group (n = 5).
In contrast, a total of 427 genes were found to be downregulated by TTX administration, as shown in Figure 1. The genes downregulated three-fold and more were extracted and are listed in Table 3. The highest downregulated gene in the TTX-administered group was elongation factor G2, and its FC value was $-13.0$. As shown in Figure 3, gene ontology classification of the downregulated genes showed that those involved in protein binding and enzyme and cofactors (metabolism) accounted for 20.8% and 13.1%, respectively, whereas those involved in the transcription factor, receptor activity and transporter activity accounted for 15.9, 10.1 and 6.6%, respectively.

To confirm the validity of the microarray data, real-time PCR was performed for the highest upregulated gene, chymotrypsin-like elastase family member 2A (Figure 4). There was a significant difference in the mRNA expression level between the TTX-administered (27.22 ± 4.18) and control group (1.00 ± 0.77) ($p = 0.0019$). This result is well correlated with the data of microarray analysis, FC 37.6 (Table 2).

**Figure 3.** Functional classification of 427 genes significantly downregulated in the liver of *Takifugu rubripes* in the TTX-administered group.
Table 3. List of genes downregulated in the liver of the TTX-administered pufferfish *Takifugu rubripes* group compared to those in the buffer-administered control group (FC $^1 > 3.0$).

| Ensemble ID                  | Gene name                  | Predicted description                                                                 | Functional classification | FC  |
|------------------------------|----------------------------|---------------------------------------------------------------------------------------|---------------------------|-----|
| ENSTRUT00000047556           | Fus2                       | Elongation factor G 2                                                                  | Transcription factor      | −3.0|
| ENSTRUT00000009260           | Rspos3                     | R-spondin-3                                                                           | Transduction              | −7.8|
| ENSTRUT00000029878           | Ncoa2                      | Nuclear receptor coactivator 2                                                         | Transcription factor      | −7.3|
| ENSTRUT00000005082           | Sasb                       | Fatty acyl-CoA hydrolase precursor, medium chain                                        | Enzyme, cofactor          | −6.2|
| ENSTRUT00000042623           | Kcnj3                      | G protein-activated inward rectifier potassium channel 1                               | Ion channel activity      | −5.9|
| ENSTRUT00000029012           | Galnt1                      | Polypeptide N-acetylgalactosaminyltransferase 1                                         | Enzyme, cofactor          | −5.7|
| ENSTRUT00000034755           | Stk11p                      | Serine/threonine kinase 11-interacting protein                                          | Protein binding           | −5.5|
| ENSTRUT00000007314           | Unc5d                       | Netrin receptor UNC5D                                                                  | Receptor activity         | −5.3|
| ENSTRUT00000024559           | Dnmt3a                      | DNA (cytosine-5)-methyltransferase 3A                                                   | Transcription factor      | −4.7|
| ENSTRUT000000028271           | finTRIM                     | Fish virus induced TRIM protein                                                        | Metal ion binding         | −4.7|
| ENSTRUT00000021561           | Synpo2                      | Synaptopodin-2                                                                        | Protein binding           | −4.4|
| ENSTRUT00000035155           | Klhl8                       | Kelch-like protein 8                                                                  | Protein binding           | −4.4|
| ENSTRUT00000013283           | Lnx2                       | Ligand of Numb protein X2                                                              | Metal ion binding         | −4.3|
| ENSTRUT00000005282           | Sox5                        | *T. rubripes* transcription factor SOX5 (AY277973.1)                                   | Transcription factor      | −4.1|
| ENSTRUT00000004950           | Egr3                       | Early growth response protein 3                                                        | Transcription factor      | −4.0|
| ENSTRUT00000001876           | Nme1                       | Nucleoside diphosphate kinase A                                                        | Transcription factor      | −4.0|
| ENSTRUT00000037748           | Cadm2                      | Cell adhesion molecule 2                                                               | Protein binding           | −3.9|
| ENSTRUT00000029148           | Cln3                       | Battenin                                                                               | Enzyme, cofactor          | −3.8|
| ENSTRUT00000018412           | Hecd3                      | Probable E3 ubiquitin-protein ligase HECTD3                                             | Protein binding           | −3.7|
| ENSTRUT00000010231           | Wipi2                      | WD repeat domain phosphoinositide-interacting protein 2                                | Enzyme, cofactor          | −3.7|
| ENSTRUT00000038056           | Angpt2                      | Angiopoietin-2                                                                        | Receptor activity         | −3.7|
| ENSTRUT00000011781           | Ecad1                      | Putative F-type lectin                                                                | Sugar binding             | −3.6|
| ENSTRUT00000025271           | Tyro3                       | Tyrosine-protein kinase receptor TYRO3                                                 | Transduction              | −3.6|
| ENSTRUT00000036834           | Pcolce                      | *T. rubripes* procollagen C-endopeptidase enhancer 1 (AF016494.1)                     | Protein binding           | −3.4|
| ENSTRUT00000009581           | Rims1                      | Regulating synaptic membrane exocytosis protein 1                                      | Protein binding           | −3.4|
| ENSTRUT00000013940           | Pdlim5                      | PDZ and LIM domain protein 5                                                           | Protein binding           | −3.4|
| ENSTRUT00000041778           | Foxa3                      | *T. rubripes* folkhead transcription factor FoxA3 (AB604763.1)                         | Transcription factor      | −3.3|
| ENSTRUT00000026385           | Fam70a                      | Protein FAM70A                                                                        | Unknown                   | −3.3|
| ENSTRUT00000003229           | Arhgef26                    | Rho guanine nucleotide exchange factor 26                                               | Transcription factor      | −3.2|
| ENSTRUT00000007982           | Kif2c                       | Kinesin-like protein KIF2C                                                             | Protein binding           | −3.2|
| ENSTRUT00000013282           | Lnx2                       | Ligand of Numb protein X2                                                              | Protein binding           | −3.2|
| ENSTRUT00000005850           | Crybb2                      | Beta-crystallin A2                                                                    | Protein binding           | −3.2|
| ENSTRUT00000003860           | Edaradd                     | Ectodysplasin-A receptor-associated adapter protein                                    | Protein binding           | −3.1|
| ENSTRUT00000015819           | Pbxbp1                      | Pre-B-cell leukemia transcription factor-interacting protein 1                          | Transcription factor      | −3.1|
| ENSTRUT00000011222           | Serpinh1                    | Serpin H1                                                                              | Protein binding           | −3.1|
### Table 3. Cont.

| Ensemble ID                  | Gene name | Predicted description                                      | Functional classification       | FC   |
|------------------------------|-----------|------------------------------------------------------------|--------------------------------|------|
| ENSTRUT00000020096           | Atp2b3    | Plasma membrane calcium-transporting ATPase 3              | Transporter activity            | −3.1 |
| ENSTRUT00000009874           | Plxdc1    | Plexin domain-containing protein 1                         | Unknown                         | −3.1 |
| ENSTRUT00000047512           | Suox      | Sulfite oxidase, mitochondrial                              | Enzyme, cofactor               | −3.1 |
| ENSTRUT00000020028           | finTRIM   | Fish virus induced TRIM protein                             | Metal ion binding               | −3.0 |
| ENSTRUT00000041535           | Pxn       | Paxillin                                                   | Protein binding                 | −3.0 |
| ENSTRUT00000033313           | Nox5      | *T. rubripes* NADPH oxidase 5 (BR000279.1)                | Enzyme, cofactor               | −3.0 |
| ENSTRUT00000027204           | Pacs2     | Phosphofurin acidic cluster sorting protein 2              | Unknown                         | −3.0 |
| ENSTRUT00000044222           | Tgfbap1   | Transforming growth factor-beta receptor-associated protein 1 | Protein binding               | −3.0 |
| ENSTRUT00000027017           | Hnrmpk    | Heterogeneous nuclear ribonucleoprotein K                 | Transcription factor            | −3.0 |
| ENSTRUT00000043644           | Hsp90b1   | Heat shock protein 90kDa beta member 1                     | Protein binding                 | −3.0 |
| ENSTRUT00000043157           | Tle3      | *T. rubripes* transducin-like enhancer protein 3 (AB236415.1) | Transcription factor            | −3.0 |
| ENSTRUT00000022207           | Vwa1      | Von Willebrand factor A domain-containing protein 1        | Unknown                         | −3.0 |
| ENSTRUT00000026592           | Tacr3     | Neuromedin-K receptor                                      | Receptor activity               | −3.0 |
| ENSTRUT00000028610           | Dach1     | Dachshund homolog 1                                        | Protein binding                 | −3.0 |
| ENSTRUT00000011629           | Gas213    | GAS2-like protein 3                                        | Protein binding                 | −3.0 |
| ENSTRUT00000018505           | Epha2     | Ephrin type-A receptor 4 precursor                         | Receptor activity               | −3.0 |

1 FC is the average fold change of the TTX-administered (n = 5) compared to buffer-administered control group (n = 5).

**Figure 4.** Real-Time PCR of the gene encoding chymotrypsin-like elastase family member 2A in the liver of *Takifugu rubripes* from both TTX- and buffer-administered groups. Each value represents the mean ± SE of three individuals, each performed in duplicate.
4. Discussion

In this study, we performed a single intramuscular administration of TTX to cultured marine pufferfish specimens of *T. rubripes* and DNA microarray gene expression analysis on Day 5 after the administration to identify genes possibly related to TTX accumulation in the liver.

TTX was detected in the liver, skin and ovary, but not in the muscle and testis of the pufferfish specimens in the TTX-administered group. The amount of TTX was highest in the liver and skin. The skin accumulated 28 ± 5% of the administered dose at the same level as that of the liver (28 ± 6%) on Day 5. We previously reported that the liver and skin of cultured pufferfish specimens of *T. rubripes* (940–1120 g body weight) accumulated up to 63 ± 5% and 9 ± 3% of the administered dose of 0.25 mg TTX/kg body weight at 60 min after intravascular administration, respectively [17]. In this connection, the examination of the tissue distribution of 3H-labeled TTX in cultured *T. rubripes* (90 and 110 g body weight) revealed that the total radioactivity was distributed mainly in the skin (45.1% and 54.1%, respectively), muscle (7.4% and 8.0%, respectively) and liver (19.0% and 15.7%, respectively) on Day 6 after intraperitoneal administration of 3H-labeled TTX [34]. In addition, Honda *et al.* [5] performed the feeding experiments, in which zero-year- and one-year-old pufferfish specimens of cultured *T. rubripes* were reared for 60 days with various types of TTX-containing diets, and revealed that the test fish accumulated a small amount of TTX (less than 3 MU (mouse unit)/g in most cases) mainly in the skin and liver at low doses (0.1 MU/g body weight/day) and a large amount (up to 57 MU) mostly in the liver and ovary at higher doses (0.2–1.0 MU/g body weight/day). Moreover, Ikeda *et al.* [35] examined the transfer profile of intramuscularly administered 50 MU of TTX to the cultured young immature pufferfish *T. rubripes* (approximately four-months-old, 13.2 ± 3.4 g body weight). They reported that TTX tends to be transferred to the skin from the other tissues, such as the liver and circulating blood, and that the total amount of TTX remaining in the entire body at 72–168 h after administration was approximately 60%–80%. These results suggest that TTX was transferred to skin tissues regardless of the administration routes and would be released from the skin tissues to excrete excess TTX or as a biologic defense substance against predators [36–38].

Lee *et al.* [22] previously reported three fibrinogen-like protein genes expressed in toxic liver of two different pufferfish, akamefugu *T. chrysops* and kusafugu *T. niphobles*. However, the expressions of these genes were not observed in this study. Little is known about the timing of expression of these genes after the toxification of pufferfish liver. The other possibility is that these genes were tremendously expressed, and their transcripts were too highly labeled with Cy3 to be measured by microarray analysis. Further investigations are required about the relationship between the hepatic toxicity and the expression mechanism to understand the functions of these genes.

Matsumoto *et al.* [23] examined the hepatic gene expression profile of cultured *T. rubripes* at 12 h after intramuscular administration of TTX by suppression subtractive hybridization and found that upregulated genes encoded acute-phase response proteins, including hepcidin, complement components, serotransferrin, apolipoprotein A-1, high temperature adaptation protein Wap65-2, fibrinogen beta chain and 70 kDa heat-shock protein 4, in the liver. In this study, the increased expression of these genes were not detected, suggesting that these proteins subsided within five days after intramuscular administration of TTX.
Feroudj et al. [24] performed DNA microarray analysis with total RNAs from toxic and non-toxic wild pufferfish, demonstrating that 1108 transcripts were more than two-fold higher in toxic than nontoxic specimens. The expression levels of nine genes were upregulated more than 10-fold in toxic and proteins encoded by these genes were related to vitamin D metabolism and immunity.

Yotsu-Yamashita et al. [39] reported liver-specific expression of pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) in the marine pufferfish, T. pardalis. In addition, Tatsuno et al. [40] found four genes (Tr1–Tr4) encoding PSTBP homologs from the publicly available Fugu genome database and revealed the constitutive expression of two distinct isoforms (Tr1 and Tr3) in the liver of cultured non-toxic T. rubripes specimens, declining in their toxin-triggered gene expression. In this study, the expression change of genes encoding PSTBP homologs was hardly observed. PSTBP and its homologs may have functions to bind toxic substances other than TTX, when TTX is absent.

In the TTX-administered group, 59 and 427 genes were significantly upregulated and downregulated, respectively, in comparison with the buffer-administered control group (two-fold change, \( p < 0.05 \)). The highest upregulated gene was chymotrypsin-like elastase family 2A (Cela2a), known as elastase-2A, with 37.6 FC. The validity of this value was confirmed by real-time PCR analysis, indicating a good quantitative performance of the microarray analysis. A homologous gene encoding elastase 2A was first cloned from the human pancreas [41]. Further details about human pancreatic elastase has recently been revealed through the human gene project, and the expression of human elastase 2A gene encoding “neutrophil elastase” has been found to be regulated by hematopoietic transcription factors, such as AML1, C/EBP\(\alpha\), PU.1 and c-Myb transcription factors [42,43]. However, there is still limited information on secretion in pancreatic juice [44]. Although T. rubripes Cela2a is estimated to correspond to elastase 2A in hepatopancreatic juice, which digests elastic and fibrous proteins, little information is available on the gene expression and regulation mechanism of T. rubripes Cela2a. One possibility is that the hepatopancreatic digestion is activated during enterohepatic metabolism of TTX in the pufferfish liver.

This study demonstrated the upregulation of the sodium channel beta-2 subunit gene (Scn2b, FC value of 4.0) by TTX administration. The sodium channel beta-2 subunit modulates the kinetics of channel gating, as well as the stabilization and location of TTX-sensitive voltage-gated sodium channels [45–47]. Pertin et al. [48] reported a marked upregulation of the beta-2 subunit in the spared nerve injury model of rat. Lopez-Santiago et al. [49] also reported that beta-2 subunit modulates mRNA and protein expression of TTX-sensitive voltage-gated sodium channels. These findings suggest that TTX accumulation in pufferfish liver affects the expression and composition of voltage-gated sodium channels.

Dysferlin gene (Dysf, an FC value of 4.1) was also upregulated in the liver of the TTX-administered group. Dysferlin is a ubiquitously expressed transmembrane protein involved in Ca\(^{2+}\)-mediated plasma membrane repair, vesicle fusion and Ca\(^{2+}\) homeostasis in skeletal muscle, regulating cell adhesion in human monocytes [50–52]. Oulhen et al. [53] suggested that dysferlin is essential for endocytosis oogenesis and embryogenesis in the sea star, Patiria miniata. TTX accumulation may damage plasma membranes, and thus, the upregulation of dysferlin found in this study may be related to the upregulation of the sodium channel beta-2 subunit gene, because channel proteins fuse with the plasma membrane.
Kitamura et al. [54] investigated gene expression changes in the cerebral cortical cells from E18 rat embryos by DNA microarray analysis in the presence and absence of TTX. They identified genes involved in the postsynaptic scaffold, regulation of actin dynamics, synaptic vesicle exocytosis and regulation of G-protein signaling as those downregulated in the presence of TTX and upregulated in the absence of TTX. In the present study, Rho GTase-activating protein 29 gene (Arhgap29) was upregulated with 12.1 FC on Day 5 after TTX administration. Arhgap29 is a negative regulator of the Rho GTase signaling pathway, which controls cytoskeletal rearrangement in human and other organisms [55–57]. This study also demonstrated the upregulation of the probable G-protein-coupled receptor 22 gene (GPR22, 3.3 FC), the GTP-binding protein Rheb gene (Rheb, 3.2 FC), Arf GAP with GTase domain ankyrin repeat and the PH domain 2 gene (Agap2, 3.0 FC). Recently, Adams et al. [58] have found that the GPR22 gene is selectively expressed in the brain and heart of human and rodents, suggesting a possible role of GPR22 protein in the regulation of cardiac contraction. However, natural ligands for this receptor remain to be understood, and its function in other animal tissues is also unclear. Rheb protein is a molecular switch in many cellular processes, such as cell volume increase, cell cycle progression, neuronal axon regeneration, autophagy regulation, nutritional deprivation, cellular stress resistance and cellular energy control [59,60]. On the other hand, genes encoding the G-protein-activated inward rectifier potassium channel 1 gene (Kcnj3, FC value of −5.9) and the Rho guanine nucleotide exchange factor 26 gene (Arhgef26, FC value of −3.2) were downregulated in the liver of the TTX-administered group. As is well known, these genes are related to a G-protein-coupled receptor signal transduction system, suggesting that receptors and signaling pathways involved in cellular response to TTX may exist in pufferfish liver to reduce the toxic effect and to accumulate TTX.

It was demonstrated in the present study that the intramuscular administration of TTX influences the hepatic gene expression involved in gene transcription, the signaling pathway via receptors and channels and metabolic pathways. It has been reported that genes related to immunity and acute-phase responses were found to be upregulated in cultured T. rubripes on the intraperitoneal injection of TTX by SSH [23], in wild T. chrysops and T. niphobles by RAP RT-PCR [22] and in wild T. rubripes microarray analysis [24]. It is noted that samples were taken on Day 5 after TTX administration in the present study, differing from those taken at 12 h after administration of TTX for SSH, although both investigations adopted cultured T. rubripes. It may be important to have a G-protein-related signaling pathway for shifting acute-phase to steady-state metabolic responses. Alternatively, health conditions of pufferfish may cause varied gene expression patterns relating accumulation of TTX. Further investigation is needed to understand the biological significance of TTX in pufferfish.

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Author Contributions
Ta.M designed the study. S.W. arranged and oversaw the project. Ta.M., H.F., R.K., To.M., M.K., and Y.N. planned and performed the TTX administration test and TTX analysis. Ta.M., H.F., Y.K., H.K., and I.H. performed DNA microarray analysis. Ta.M. performed real-time PCR analysis. Ta.M., H.F., G.K., and H.U. undertook the data analysis. Ta.M. and S.W. wrote the manuscript with support from all authors.

Conflicts of Interest
The authors declare no conflict of interest.

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