The Impact of Exogenous Tetrodotoxin on the piRNAs and mRNAs Profiles of Gonads in Pufferfish *Takifugu Flavidus*

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Research Article

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Abstract

Tetrodotoxin (TTX) is a deadly neurotoxin and usually accumulates in large amounts in the ovaries but is non-toxic or low toxic in the testis of pufferfish. The molecular mechanism underlying sexual dimorphism of TTX accumulation in gonads is complex and unclear. Piwi/piRNA complexes are essential for germline specification, gametogenesis, and gonadal development, they also demonstrate sexual dimorphism in teleosts. Hence, the present study investigated the expression of piRNAs and mRNAs by transcriptomics in cultured pufferfish *Takifugu flavidus* after intramuscular administration of exogenous TTX. The results showed 80 piRNAs were down-regulated and 223 genes were up-regulated in the ovary after TTX administration. By contrast, 286 piRNAs were down-regulated and 445 genes were up-regulated after TTX administration in testis. Functional and pathway analyses indicated that the TTX up-regulated genes were enriched in the Wnt, ErbB and GnRH signaling pathways in ovary, while were enriched in the oocyte meiosis, estrogenesis biosynthesis and cell apoptosis-related pathways in testis. Interestingly, these genes were also the potential target genes of TTX downregulated piRNAs. *Amhr2* and *cyp19a* were also identified as sex-related genes, involved in TTX administration. These results showed a certain level of consistency with the enrichment pathway analysis, which indicated that the TTX could affect the expression of sex-related genes, and work as an inhibitor for the testicular meiosis, but as a promoting factor for ovary development through piRNAs in pufferfish. In addition, TUNEL staining showed that significant apoptosis was detected in the TTX treated testis, and the role of the cell apoptotic pathway was further confirmed. Overall, this research will contribute to an understanding of the piwi function in TTX sexual dimorphism accumulation in pufferfish and provide a basis for further studies.

Introduction

Tetrodotoxin (TTX), one of the most potent neurotoxins which is specific to voltage-gated sodium channels (VGSCs) on excitable membranes of muscle and nerve tissues, was long believed to occur exclusively in TTX-bearing pufferfish, mainly the genus Takifugu[1, 2]. TTX is produced primarily by marine bacteria, and accumulated extremely high in pufferfish through the food chain with tissue-specific and seasonal change[3–5]. It is suggested that TTX may function as a chemical defense against predators and as pheromone during spawing[6, 7].

The accumulation of TTX in pufferfish has been studied extensively [1, 2, 8–11]. Interestingly, the ovaries of pufferfish accumulate large amounts of tetrodotoxin, whereas the testes accumulate few or none, especially the ovarian toxicity was extremely high during the maturation period, indicating that the accumulated TTX showed sex difference in the toxicity of tissues[3–5]. Previous studies had found that tissue-specific distribution and the amount of TTX in the mature pufferfish *Takifugu niphobles* were sex-dependent, female gonads and male liver showed the highest concentrations of the toxin followed by male skin[12]. The larvae of *Takifugu* pufferfish were protected by maternal TTX which had accumulated in the eggs during their development in the ovary[13, 14]. In addition, studies have shown that TTX as a toxin or pheromone accumulated in the ovary protects pufferfish larvae from predators [6, 13, 14]. Although TTX was accumulation demonstrates sexual dimorphism gonads of pufferfish, the molecular

mechanism underlying and the relationship between TTX accumulation and steroidogenesis/reproduction is complex and unclear.

To gain insights into sexual dimorphism of TTX accumulation in gonads, steroidogenesis/reproduction-related genes and small non-coding RNAs were chosen to further investigate the molecular mechanisms. Piwi-interacting RNAs (piRNAs) are single-stranded, 26-32 nt long small RNAs that play a role function *via* the formation of RNA-protein complexes through interactions with piwi proteins[15, 16]. In animals, previous studies revealed that piRNAs are limited expressed in a few tissues, such as tumors[17–19], brain and nervous tissue[18, 20–22], whereas generally abundant in gonads of invertebrates[23–27] and vertebrates[18, 28–33], including zebrafish (*Danio rerio*)[25, 34], tilapia (*Oreochromis niloticus*)[35], Japanese flounder (*Paralichthys olivaceus*)[36], tongue soles (*Cynoglossus semilaevis*)[37] and other teleosts. Previous studies showed that maternal Piwi /piRNA is required for the fertility and normal gonad morphology of female, but not male, progeny in *Drosophila*[38]. piRNAs derived from the W chromosome are expressed more abundantly in the ovary than in the testis of silk worm[39]. The piRNAs play a role in targeting the transcripts of active TEs and maintaining the methylation pattern of maternal genomic DNA, and proving that ovaries and testes piRNAs are two different classes of piRNAs with different functions and that their expression appears to be regulated by distinct transcription factors in human[40]. Above all, at least a large number of piRNA show sexual dimorphism in the testis and ovaries. In addition, putative piRNAs were found abundant in the gonads of *Takifugu rubripes*[41], but the function and connection between tetrodotoxin and piRNAs in the gonads of pufferfish have not been investigated.

In the present study, we investigated the expression changes of piRNAs and mRNAs in both ovary and testis of *Takifugu flavidus* on the transcriptomic level after the intramuscular injection with TTX. The up and down-regulated piRNAs and genes were identified, function and pathway of these piRNAs and genes were also analyzed. At last, apoptosis detection was performed in the TTX treated gonads. These data would provide us some clues to understand the specific effects of TTX accumulated and function in the gonads of pufferfish.

Results

Exogenous TTX accumulated in both ovary and testis of artificially breeding pufferfish

A TTX treatment was performed in 6 months old of *Takifugu flavidus*, after TTX administration, the TTX concentration of the TTX treated group is significantly higher than the control group in kidney, cholecyst, skin, liver, heart and muscle tissue by LC-MS/MS (Liquid Chromatography with tandem mass spectrometry) methods (Figure 1A).

To further research the TTX accumulation effect in gonads of *Takifugu flavidus*, histological analysis was performed to identify the sex and gonadal features in 6 months old of *Takifugu flavidus*. Twelve gonads were dissected from each of the control and TTX treated groups. Five ovaries and seven testes

were confirmed in the control group, six ovaries and six testes in the TTX-treated group through the gonadal sections histological observation. All of these gonads are immature, the ovarian cavity is obvious, oogonia and oocytes were observed in the ovary (Figure 1B-a, b). In the testis, the spermatogonia were observed but without spermatocytes or spermatids (Figure 1B-c, d). Immunohistochemical observations with anti-TTX antibody suggested that TTX was localized at the somatic cells around the oocytes in the control ovary with intense signals (Figure 1B-e, f). However, just a few TTX signals were detected in the control testis (Figure 1B-g, h). After TTX administration, the total TTX signals in the TTX-treated gonads were significantly higher than the control gonads (Figure 1B-i, j, k, l &1C).

piRNA and mRNA sequencing and novel piRNA identification

Total RNA of gonads (testis or ovary) was isolated and sequenced for piRNA and mRNA analysis from the TTX-treated and control groups of *Takifugu flavidus*. Overall, 94.91% of con_O (control ovary group), 94.91% of con_T (control testis group), 98.09% of TTX_O (TTX-treated ovary group) and 94.32% of TTX_T (TTX-treated testis group) of the clean reads had scored at the Q30 level in mRNA sequencing (Table 1). Of all the clean reads, 85.06% (con_O), 86.10% (con_T), 86.04% (TTX_O) and 85.63% (TTX_T) were matched onto the fugu reference genome sequence (*fTakRub1.2*).

For piRNA sequencing, the peak value of all groups was mainly concentrated at 28 bp (Figure s1-A) with the character of 1U-bias of the molecule (Figure s1-B), which corresponded with the characteristic length and structure bias of piRNAs. The numbers of unique piRNAs for the species were calculated as follows: 507,004 in the control ovary group, 530,323 came from the TTX-treated testis group; 346,106 came from the control testis group while 472,764 in the TTX-treated ovary group, the total unique piRNA is 1,247,865 (Table 1 and Additional file 1). In contrast, more than 288,864 unique piRNAs display less than 10 reads in all groups. These results indicate that expression varied significantly among different piRNA families. Multiple piRNAs with at least 10 piRNAs located on the same chromosome and no more than 5000 nt apart are defined as a piRNA cluster. A total of 5,457 piRNA Clusters were isolated. These piRNA clusters exist on each chromosome and are unevenly distributed on each chromosome (Figure s2-B). Based on the source of each piRNA sequence in the piRNA cluster and the starting position of the cluster on the chromosome, these piRNAs are sequentially aligned with transposon sequences and gene sequences. The piRNA clusters are significantly enriched with non-coding sequences (65.53%) and repetitions. Sequences (including transposon) (33.29%), of which 1.18% comes from the gene sequences, including the identity with the gene sequences and the reverse complement of the gene sequences (Figure s2-A). The region consistent with the gene sequence may be the generation region of the piRNA clusters, and the gene sequence that is reversed complementary to the piRNA cluster may be the target site of the piRNA clusters.

TTX affecting the piRNA/mRNAs profiles in the ovary

In the piRNA and mRNA transcriptomes, the differential expression in the ovaries of the control group and the TTX treatment group were analyzed. TTX administration resulted in 23 piRNAs and 223 genes were up-regulated, meanwhile, 80 piRNAs and 126 genes were down-regulated in the ovaries (Figure 2-A, B and Additional file 2).

To investigate the reliability of differential expression data in the piRNA and mRNA transcriptomes. Eight piRNAs (*uniq_1163081*, *uniq_3850196*, *uniq_556841*, *uniq_1137493*, *uniq_1958827*, *uniq_1013268*, *uniq_479370* and *uniq_1713020*) and six genes (*amhr2*, anti-Mullerian hormone receptor type 2; *cacna1c*, calcium voltage-gated channel subunit alpha1 C; *myc*, MYC proto-oncogene bHLH transcription factor a; *ppard*, peroxisome proliferator activated receptor delta; *LOC101078894*, alcohol dehydrogenase 1-like and *LOC101069468*, low-density lipoprotein receptor-related protein 5-like) were screened for further verification by qRT-PCR. The piRNA and mRNA expression change between the TTX-treated group and control group in the qRT-PCR results are consistent with the transcriptome data indicating that the transcriptome data is reliable (Figure 2-C, D and Additional file 3).

Studies have been suggested that Piwi/piRNA complexes are important in gene silencing, cell differentiation and gonadal development regulation in animals. Similar to the method of miRNA and other small RNAs to predict target genes, we obtained differential piRNAs between TTX-treated and control ovary for target gene prediction and enrich the target genes with KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene ontology) analysis. KEGG and GO analysis were also used for the prediction target gene of TTX down-regulated piRNA in ovaries. As revealed in Figure 3-A and Additional file 4, MAPK signaling pathway, Rap1 signaling pathway, cAMP signaling pathway, Axon guidance, Calcium signaling pathway, and Thyroid hormone signaling pathway were enriched. The KEGG and GO analysis in TTX up-regulated mRNA in the ovaries showed that Tight junction, Wnt signaling pathway, ErbB signaling pathway, GnRH signaling pathway, and Retinol metabolism were enriched (Figure 3-B and Additional file 4).

TTX up-regulated pathways in the ovary were compared with the piRNA transcriptome data, the oocyte meiosis, progesterone-mediated oocyte maturation, and steroid hormone biosynthesis in TTX-up-regulation genes were selected for mapping with the TTX-down-regulation piRNA. The results showed that *uniq_1958827* predicated target gene including calcium voltage-gated channel subunit alpha1(*cacna1c*); *uniq_1013268* predicated target gene including alcohol dehydrogenase 1-like (*LOC101078894*); *uniq_479370* predicated target gene including low-density lipoprotein receptor-related protein 5 (*LOC101069468*); *uniq_1713020* predicated target gene including peroxisome proliferator-activated receptor delta (*ppard*). *uniq_671648* predicated target gene including anti-Mullerian hormone receptor type 2 (*amhr2*). The gene *cacna1c* was involved in the GnRH signaling pathway, the gene *LOC101078894* was involved in Retinol metabolism; both *LOC101069468* and *ppard* were involved in the Wnt signaling pathway; The gene amhr2 was involved in the TGFβ signaling pathway (Figure 3-C and Additional file 3). In addition, the *uniq_671648-amhr2* mRNA pairing structure was shown in Figure 3-D.

TTX affecting the piRNA/mRNAs profiles in testis

In the piRNA and RNA transcriptome, the differential expression of the control group and the TTX treatment group testes were analyzed. In the testis, TTX up-regulated the expression of 224 piRNAs and down-regulated the expression of 286 piRNAs (Figure 4-A and Additional file 5). Only the *ddah2* (dimethylarginine dimethylaminohydrolase 2) and *ing4* (inhibitor of growth family member 4) in the testis were significantly down-regulated. However, 445 genes were up-regulated (Figure 4-B and Additional file 5).

To investigate the reliability of differential expression data in the piRNA and RNA transcriptomes, nine piRNAs (*uniq_2692448*, *uniq_904526*, *uniq_1754588*, *uniq_1320158, uniq_1010678*, *uniq_1323470*, *uniq_2869791*, *uniq_1678838* and *uniq_766456*) in the testis were selected for further verification by qRT-PCR. For the differential expressed genes, the *hsd17b1*(hydroxysteroid 17-beta dehydrogenase 1), *ccnb1*(cyclin B1), *aurka* (aurora kinase A), *fbxo43* (F-box protein 43), *LOC101065671*(cyclin N-terminal domain-containing protein 2-like), *pygl*(glycogen phosphorylase L), *LOC101080011*(growth arrest and DNA damage-inducible protein GADD45 beta) and the TTX down-regulated two gene *ing4* (inhibitor of growth family member 4) and *ddah2* (dimethylarginine dimethylaminohydrolase 2) were selected. The qRT-qPCR results confirmed the good correlation between the piRNA expression level and piRNA sequencing. Besides, the expression changes between the TTX-treated group and control group in the qPCR results are consistent with the transcriptome data (Figure 4-C, D and Additional file 3), showed the piRNA/mRNA transcriptome data were reliable.

KEGG and GO analysis were used for prediction potential target genes of TTX down-regulated piRNAs in testis. Results showed as in Figure 5-A and Additional file 4. Focal adhesion, Wnt signaling pathway, Calcium signaling pathway, Apoptosis, Autophagy, GnRH signaling pathway, Thyroid hormone signaling pathway, Estrogen signaling pathway and oocyte meiosis were enriched. KEGG and GO analysis showed that the enrichment of TTX up-regulated gene in the testis are mainly in cell apoptosis-related pathways, such as cell cycle, tight junction, PPAR signaling pathway, p53 signaling pathway, Ferroptosis and Necroptosis. Besides, oocyte reproduction-related pathways were also enriched, such as oocyte meiosis, progesterone-mediated oocyte maturation and Steroid hormone biosynthesis (Figure 5-B and Additional file 4). Compare the differential expression piRNAs potential target gene enrichment with the differential expression gene enrichment. Both piRNA and mRNA showed that after TTX injection, the gene expression changes in the testis develop toward the "ovarian" pattern and rises the apoptosis pathway (p53, Ferroptosis and Necroptosis).

TTX up-regulated pathways in testis were compared with the piRNA transcriptome data, the oocyte meiosis, progesterone-mediated oocyte maturation, steroid hormone biosynthesis, p53 signaling pathway, Ferroptosis, Necroptosis and cellular senescence in TTX up-regulated genes were selected for mapping with the TTX-downregulated piRNAs. The results showed that *uniq_1010678* predicated target genes including cyclin N-terminal domain-containing protein 2-like (*LOC101065671*); *uniq_1138005* predicated target genes including G2/mitotic-specific cyclin-B2-like (*LOC101076205*); *uniq_1678838* predicated target genes including aurora kinase (*aurka*); *uniq_2509649* and *uniq_1115741* predicated target genes including mitotic arrest deficient 2 like 1(*mad2l1*). *uniq_1504863* and *uniq_2344671* predicated target

genes including cyclin B1 (*ccnb1*). *uniq_988037* predicated target genes including cell division cycle protein 20 homolog (*LOC101063487*); uniq_1323470 predicated target genes including F-box protein 43(*fbxo43*); *uniq_766456* predicated target genes including hydroxysteroid 17-beta dehydrogenase 1(*hsd17b1*); *uniq_554482* predicated target genes including cytochrome P450 19A1-like (*cyp19a*). *uniq_1713020* predicated target gene including growth arrest and DNA damage-inducible protein GADD45 beta (*LOC101080011*); *uniq_2869791* and *uniq_1253913* predicated target gene including glycogen phosphorylase L (*pygl*); acyl-CoA synthetase long chain family member 5 (*acsl5*) maybe the potential target gene of *uniq_1938531*. The genes *LOC101075671*, *LOC101076205*, *mad2l1*, *ccnb1*, *LOC101063487* and *fbxo43* were involved in the oocyte meiosis or progesterone-mediated oocyte maturation pathway, the genes *hsd17b1* and *cyp19a* were in the Steroid hormone biosynthesis, the gene *LOC101080011* was in the p53 signaling pathway, the gene *pygl* was in Necroptosis and *acsl5* was in Ferroptosis (Figure 5-C). In addition, the *uniq_554482-cyp19a*/ *ccnb1* RNA pairing structures were shown in Figure 5-D.

Exogenous tetrodotoxin induced the cell apoptosis of testis

As we have known that pufferfish testis usually accumulates few tetrodotoxins, the excessive exogenous tetrodotoxin may be harmful to testis. Besides, the piRNA and mRNA transcriptomes data showed that cell apoptosis-related genes were up-regulated in testis and related piRNA were down-regulated. These results indicated that abundant exogenous tetrodotoxin will induce the apoptosis of testis. TUNEL assay (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) was used for identifying apoptotic cells *in situ*. As shown in Figure 6-A, most nuclei were stained as a discernible brown in the TTX-treated testis but few signals were found in the control testis. The TTX-treated and control ovary were both found the signals at the somatic cells, very few signals were found in the oocytes. In addition, the area of the positive signals was statistics and analysis, the results showed that no significant difference of cell apoptosis between the TTX-treated and control ovary, but the signals of cell apoptosis in TTX-treated testis is significantly higher than that in the control testis (Figure 6-B), and the cell apoptosis induced by exogenous tetrodotoxin administration.

Discussion

In this study, the exogenous tetrodotoxin was administration to the artificially cultured *Takifugu flavids*. TTX level elevation was observed at the testis and other tissues after eight hours. The piRNA and transcriptome sequence results showed that TTX down-regulated piRNAs and up-regulated genes in testis were biased toward the ovarian expression pattern, it is reflected by piRNAs and genes enriched in Wnt signaling pathway, Estrogen biosynthesis signaling pathway and oocyte meiosis pathway. Additionally, the cell apoptosis pathway was also enriched and confirmed by the TUNEL assay in testis.

To our knowledge, the present study is first time investigated the piRNA and transcriptome expression changes in *Takifugu flavids*' separate sex. As the ovary can accumulate abundant TTX while the testis has just been detected little TTX in the control group, we speculate a high level of tetrodotoxin will hamper the development of testis. Processes involving sex determination and reproduction were

complicated and regulated by various internal and/or external factors, particularly in pufferfish which show sexual dimorphism of TTX accumulation in gonads. With limited molecular data available for *Takifugu* to know about the exact internal mechanism or role of tetrodotoxin in the development of gonads. The accumulation and tissue-specific distribution of tetrodotoxin in pufferfish, mainly in the genus *Takifugu* has been widely investigated from the viewpoint of the TTX-resistance VGSCs expression and distribution[2, 10, 42]. Evidence also showed that tetrodotoxin is usually significantly at a high level during the maturation/spawning period than in the ordinary period, exogenous tetrodotoxin administration also showed consistent results[1, 4, 5, 9, 14]. But the tetrodotoxin in the developing stage of pufferfish, especially for the gonads marginally been investigated. The present study was performed to elucidate the possible TTX impact on the ovary and testis of juvenile pufferfish development stage through piRNA and mRNA transcriptome.

The piRNA and mRNA profile change of both ovary and testis were analyzed from the view of transcriptomics, total of 1,247,865 unique piRNAs was identified from four groups, but unique piRNAs from every group is different. The most interesting is the total number of uniq piRNA is increase after exogenous TTX administration in testis, but decrease after exogenous TTX administration in the ovary. it is also showed that the number of DEpiRNA (differentially expressed piRNA) which is affected by TTX in the testis (510) is more than that in the ovary (103), this indicated TTX has a bigger influence on the testis. The increased piRNAs in testis silence more transposons or genes to resist/reduce the toxicity of TTX. In addition, TUNEL analysis showed that exogenous tetrodotoxin induced the cell apoptosis of testis, but there is no significant effect on the apoptosis of the ovary.

Despite fewer genes were down-regulated by the TTX in the testis, many genes involved in the oocyte meiosis, reproduction and apoptosis-related gene/pathway were remarkably up-regulated in the testis, showed the “ovarian” developmental tendency and cell apoptosis tendency. These results of KEGG pathway analysis in differential expression gene/piRNA in TTX treated testis revealed gametogenesis and reproduction-related pathways, including the Wnt signaling pathway, MAPK signaling pathway, oocyte meiosis pathway. Especially, the up-regulated oocyte meiosis pathway was found in the TTX-treated testis. The B-type cyclins take important roles in oocyte meiosis [43]. Cyclin B1(*ccnb1*) and Cyclin B2 like (*ccnb2L*) are essential for the is critically required for the proliferation of gonocytes [44], both of them were upregulated by the TTX. Combined with the predication of differential expression piRNA target gene, piRNA *uniq_1504863*, *uniq_1302131*, *uniq_2344671* may target them. Another interesting upregulation in the TTX-treated testis pathway was steroid hormone biosynthesis. Steroidogenesis is critical for gamete maturation in teleosts, more essentially oogenesis [45], the c*yp19a* is the key gene encoding cytochrome P450 aromatase, which is responsible for estrogen production in the ovary [46–48], differential expression piRNA *uniq_554482* predicted target also including *cyp19a*. In addition, related steroidogenesis gene (*nr5a1*), growth factor (*LOC101078012* or *gsdf*), germ cell-related marker (*piwil1*; *nanos2* or *LOC101063734*) and meiosis related gene (*scyp3*, *LOC101063989* or *cyp26a1*) were non-significant down-regulated by TTX. TTX also up-regulated the apoptosis-related pathway and genes in the testis, the p53 signaling pathway, Necroptosis and Ferroptosis pathway. The p53 signaling pathway has been widely accepted that it is involved in the regulation of cell cycle arrest, senescence and

apoptosis[49–51]. Both Necroptosis and Ferroptosis were pathways discovered form of regulated cell death[51–53]. Interestingly, these down-regulated piRNA-predicted target mRNAs were involved in the upregulated genes in the testis, showed the impact of TTX on gene expression may through the role of piRNA in the testis.

Studies have shown that the components of the Wnt signaling pathway affect reproductive functions, including the regulation of follicular maturation and the production of steroids in the ovaries. Both GnRH signaling pathways and Retinol metabolism are related to meiosis, indicating that a certain increase TTX in the ovary contributes to ovarian meiosis and development. Similarly, the Wnt signaling pathway, GnRH signaling pathway, TGFβ signaling pathway and Retinol metabolism related genes were up-regulated after TTX administration. The Wnt and GnRH signaling pathways are required normal fertility and reproductive cycles[54–57]. The Retinol metabolism pathway plays a vital role in germ cell development and meiosis for gonads[58–62]. The TGFβ signaling pathway regulates growth, division and proliferation in the ovary[63, 64], one of the TGFβ signaling pathway *amhr2* was up-regulated after TTX administration, the *amhr2* was associated with sex determination in *Takifugu rubripes*, and showed regulation of germ cell proliferation [63–66]. Combined with the differential analysis base on piRNA sequencing, the up-regulated gene involved in the TTX down-regulated piRNA in the ovary, that indicating TTX may impact the gene expression of the ovary through the regulation of piRNA and showed a positive effect. To our surprise, a female pathway gene cyp19a/cyp19a1a was significantly elevated in TTX administration testis. As we known, knockout of male pathway genes or overexpression of female pathway genes often result in the upregulation of cyp19a/cyp19a1a and increase estrogen level, promoting ovary development. The opposite is also true[67]. Excessive estrogen also induce initiation of cell apoptotic pathways by Stabilizing Schlafen-12 Protein Turnover[68]. These results mightbe explanation, al least a part of reason for the testicular significant cell apoptosis.

Above all, TTX accumulation in gonads of pufferfish showed sexual dimorphism. TTX may work as a promoting factor to pufferfish ovary development and ovarian meiosis but as an inhibitor to pufferfish testis development, too much TTX will induce the apoptosis of testis. The metabolism of TTX in males and the molecular mechanism of testicular resistance to TTX accumulation still require further investigation.

Conclusion

In conclusion, the present research highlights the differentially expressed piRNAs and genes after TTX administration in gonads. The differentially expressed piRNAs potential regulated genes and the differentially expressed gene were partly overlapping. The regulatory role of TTX may through piRNA on genes related to reproduction support the notion that piRNAs play crucial roles in reproduction and the tetrodotoxin novel function in pufferfish reproduction. A certain level of consistency with the enrichment pathway analysis, which indicated that the TTX could affect the expression of sex-related genes, and work as an inhibitor for the testicular meiosis, but as a promoting factor for ovary development through piRNAs in pufferfish. TTX administration also induces significant apoptosis in testis, further confirmed

that excessive TTX is harmful for testicular development. Overall, this research will contribute to an understanding of the piwi function in TTX sexual dimorphism accumulation in pufferfish and provide a basis for further studies.

Materials And Methods

Animals and chemical

Cultured marine pufferfish *Takifugu. flavidus* Juveniles (6-month-old, 100.0 ± 10.0 g body weight, 100 individuals) purchased from Shanghai Aquatics Research Institution were used as the experimental fish. Crystalline TTX from (Supelco, USA) was used in the administration experiment and as a standard for the liquid-chromatography-tandem mass spectrometry (LC-MS/MS) analysis. All other chemicals were of reagent grade. All other chemicals were of reagent grade (Sinopharm, China). The methods for *Takifugu. flavidus* research was carried out following the relevant guidelines and regulations (following the ARRIVE guidelines[69]). The protocols were approved by the academic ethics committee of Shanghai Ocean University.

TTX treatment and sample preparation

The administration experiment was performed at the laboratory of the shanghai ocean university. The tetrodotoxin was directly dissolved in 0.1% acetic acid to 1 mg/ml, then diluted with 0.7% saline solution to 0.25 ug/ul. The test fish received an intramuscular administration of 0.25 ug TTX/g body weight into the caudal muscle as described previously and were maintained in an 80-L oxygenated plastic tank of aerated artificial seawater at 20 ℃[70]. At 8 hours after administration, each group of 12 fish were randomly collected, ice-cold anesthetized, and dissected. The gonads of each were immediately separated into two parts, 1/4 gonad was stored in 4% PFA for histological observation; 3/4 gonads were stored at -80 ℃ until RNA extraction.

TTX extraction and quantification

TTX in the tissue samples from pufferfish was extracted with 0.1% acetic acid as reported previously [71, 72]. The TTX assay from tissues was determined by the LC-MS/MS analysis according to the method used previously [71, 73, 74].

Histological observation, TTX immunofluorescence

For histological observation and immunofluorescence analyses, the gonads of *Takifugu flavidus* were dissected, fixed in 4% PFA (Paraformaldehyde) for 12 hours at 4℃, dehydrated, embedded in paraffin, and sectioned at 5 μm. Hematoxylin-eosin staining and immunofluorescence staining were performed as described previously[75, 76]. The antibody against TTX (tetrodotoxin) was purchase from CD (Creative diagnostics, USA) and was diluted at 1:200 to use, Hoechst 33258 (Sigma, USA; 1:1000) was used to stain the nucleic acid. The TTX signals ratio of gonads was statistics the three different areas of one

sample under TTX immunofluorescence by using ImageJ (NIH, USA) and Graph Prism8.0 (San Diego, USA).

TUNEL analysis

The sections of gonads were used for apoptosis analysis by TUNEL staining, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted by a colorimetric TUNEL Apoptosis Assay Kit (Beyotime, China) according to the manufacturer's instructions. Briefly, The sections of gonads were deparaffinized twice in dimethyl benzene and then graded in a series of ethanol, after repair with proteinase K (2 mg/mL) and then moved the slides into the 0.3% H_2O_2 in methanol for 20 min at RT (room temperature). Washed 3 times with 1×PBS (Phosphate Buffered Saline). That was followed by incubation with TdT enzyme solution for 60 min at 37°C. The reaction was terminated by incubation in a stop/wash buffer for 10 min at 37°C. Then used the Streptavidin-HRP (Streptavidin-Horseradish Peroxidase) for coloration. Incubated the sections in the Streptavidin-HRP working solution for 30 min at RT, following with the color reagent incubation for 3 min, and then restaining by hematoxylin for 3 min, hydrated and finally sealed and documented as digital images on a Nikon Eclipse 80i microscope (Nikon, Japan). The apoptosis signal ratio of gonads was statistics the three different areas of one sample under TUNEL staining by using ImageJ (NIH, USA) and Graph Prism8.0 (San Diego, USA) as described before.

piRNA/RNA library construction and sequencing

For piRNA and RNA transcriptome analysis, including library construction, sequencing, and bioinformatic analysis were performed by Novegene Company (Beijing, China). Briefly, total RNAs from every group (four fish gonads mixed to one sample) were extracted using RNAiso Plus (Takara, Japan). The quality of RNA was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and agarose gel electrophoresis.

For piRNA, Small RNA libraries were constructed and sequenced using TruSeq Small RNA Donor Prep Kits (Illumina, USA) by the Novegene Company (Beijing, China), sequenced using the basic reads were converted into sequence data (also called raw data/reads) by base calling. Low-quality reads were filtered out, and the reads with 5' primer contaminants and poly (A) regions were removed. Reads without a 3' adapter and insert tag, reads shorter than 24 nt, and longer than 32 nt were filtered out of the raw data to obtain clean reads.

For piRNA primary analysis, the length distribution of the clean sequences in the reference genome was determined (closely related species *Takifugu rubripes, fTakRub1.2*, PRJNA543527). Non-coding RNAs of 26-32 nt were annotated as piRNAs. The known piRNAs were identified by aligning against the piRNABank (http://pirnabank.ibab.ac.in), and the remaining unannotated reads were characterized by piRNA predictor [77]. The novel piRNAs reads were analyzed using Piano to predict novel piRNAs (http://ento.njau.edu.cn/Piano.html).

Previous studies showed that by using the structure and sequence characteristics of transposon-piRNA interactions, Piano demonstrated excellent predictive performance for piRNAs. Differentially expressed piRNAs were identified with the threshold of |log2 fold change|>1 and *p*value<0.05. The *p*-value was calculated using the DEG algorithm[78]. In the R package with the Audic-Claverie statistic without biological replicates. The targets of differentially expressed piRNAs were predicted using the MiRanda software[79]for animals, with the following parameters: S ≥ 150; ΔG ≤ −30 kcal/mol and strict 5' seed pairing. Differentially expressed piRNA-target genes were classified according to the official annotation of the Gene Ontology (GO) Consortium, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was performed using phyper, a function of R[80, 81]. False discovery rate (FDR) was used to determine the threshold of the P-value and GO or KEGG terms (FDR≤0.01) were considered significantly enriched.

For mRNA, Total RNA libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA) according to the manufacturer's instructions, sequenced on an Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated. For mRNA primary analysis, the assembly was performed with clean reads (about 6 GB in every group) to produce the transcripts which were mapped to the *fTakRub1.2* genome for bioinformatic analysis. Differential expression profiles between control and TTX-treated samples were calculated using RSEM SEM [RNA sequencing (RNAseq) by expectation maximization][82]. The transcripts were classified according to the official annotation of the Gene Ontology (GO) Consortium, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was performed using phyper, a function of R[80, 81]. Statistical analysis of differentially expressed genes (DEGs) was performed using |log2 fold change|>1 and *p*value<0.05 as the threshold to judge the significance of gene expression difference. False discovery rate (FDR) and *p*-value were used to determine the threshold of GO and KEGG terms. (*p*-value≤0.05) were considered significantly enriched.

Validation of DEG results of piRNA/mRNA

Gonadal piRNA and mRNA expression were assayed using real qRT-PCR. For mRNA, total RNA was extracted using RNAiso Plus (TaKaRa, Japan) following the protocol provided by the manufacturer. cDNAs were synthesized using HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme, China). Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (Yeason, China) was used for quantitative qRT-PCR assays. The gene names and the primers are listed in Additional file 6. *gadph* was used as an internal control[83, 84].

For piRNA, we validated the differential expression of four randomly-selected piRNAs (*uniq_2692448*, *uniq_1288101*, *uniq_1694274* and *uniq_1744355*), four RNA-seq DEG related pathway piRNA (*uniq_1958827*, *uniq_1013268*, *uniq_479370*, *uniq_1713020*) in the ovary and four randomly-selected piRNAs (*uniq_2692448*, *uniq_904526*, *uniq_1754588* and *uniq_1320158*) five RNA-seq DEG related pathway piRNA (*uniq_1010678*, *uniq_1678838*, *uniq_1323470*, *uniq_766456* and *uniq_2869791*) in the testis and with qRT-PCR. we used 1μg total RNA above as a template for the synthesis of the first-strand cDNA with a Mir-X miRNA First-Strand Synthesis (Takara, Japan) following the manufacturer's instructions. we measured the expression of the above piRNAs on a CFX96 Real-Time PCR System (Bio-

rad, USA) with a miScript SYBR Green PCR Kit (Takara, Japan), following the manufacturer's instructions, the primers for piRNA qPCR were listed in Additonal file 6. We used *U6* as an internal reference gene to control for differences among samples. Each sample was run in triple times and the relative quanti□cation of piRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [85].

Declarations

Consent for publication

Not applicable.

Ethics approval and consent to participate

Shanghai Aquatics Research Institute (shanghai, China) provided Cultured marine pufferfish *Takifugu. flavidus* juveniles for research. The methods for *Takifugu. flavidus* research was carried out following the relevant guidelines and regulations The methods for *Takifugu. flavidus* research was carried out following the relevant guidelines and regulations (following the ARRIVE guidelines). The protocols were approved by the academic ethics committee of Shanghai Ocean University.

Availability of data and materials

All sequencing raw data have been deposited in the Sequence Read Archive (SRA) database under Bioproject number PRJNA769942 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA769942).
the *Takifugu rubripes* genome reference *fTakRub1.2* is available from:
https://www.ncbi.nlm.nih.gov/bioproject/PRJEB31988.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The authors declare no conflict of interest. Xue He conceived and designed the project. Hexing Wu carried out the computational analysis and expression profiling. Xue He performed the TTX treatment,

Hematoxylin-eosin staining, immunofluorescence staining, TUNEL staining, qRT-PCR analysis.and related experiments. Yaping Ye contributed to TTX extraction and quantification by LC-MS/MS analysis. Xue He wrote the manuscript. Baolong Bao and Xiaoling Gong edited the manuscript. All authors read and approved the final manuscript.

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Tables

Table1 Summary of piRNA/mRNA transcriptome data for *Takifugu flavidus* between TTX-treated and control gonads.

Sample group		con_O	TTX_O	con_T	TTX_T
piRNA-seq	clean bases	0.983G	0.668G	1.032G	0.841G
	total reads	17695205	11741016	17391118	13693446
	uniq reads	2915273	2660367	2515290	3116492
	error rate (%)	0.02	0.02	0.02	0.02
	Q20 (%)	99.62	99.64	99.55	99.59
	Q30 (%)	98.86	98.94	98.75	98.79
	GC content (%)	48.9200	49.3300	48.8600	49.1900
	uniq piRNA	507004	472764	335106	530323
	numbers of piRNA (reads ≥500)	723	365	403	345
	piRNA cluster	5396	5385	5297	5418
mRNA-seq	raw reads	20935814	23188308	23438065	20223260
	clean reads	20420252	22290909	22827658	19650444
	total mapped	34740218 (85.06%)	38356040 (86.04%)	39307194 (86.10%)	33651476 (85.63%)
	clean bases	6.13G	6.69G	6.85G	5.90G
	error rate (%)	0.02	0.02	0.02	0.02
	Q20 (%)	98.28	98.2	98.04	98.09
	Q30 (%)	94.91	94.91	94.32	94.62
	GC content (%)	52.31	53.58	50.89	52.91

con_O: control ovary group; TTX_O: TTX-treated ovary group; con_T: control testis group; TTX_T: TTX-treated testis group;

Figures

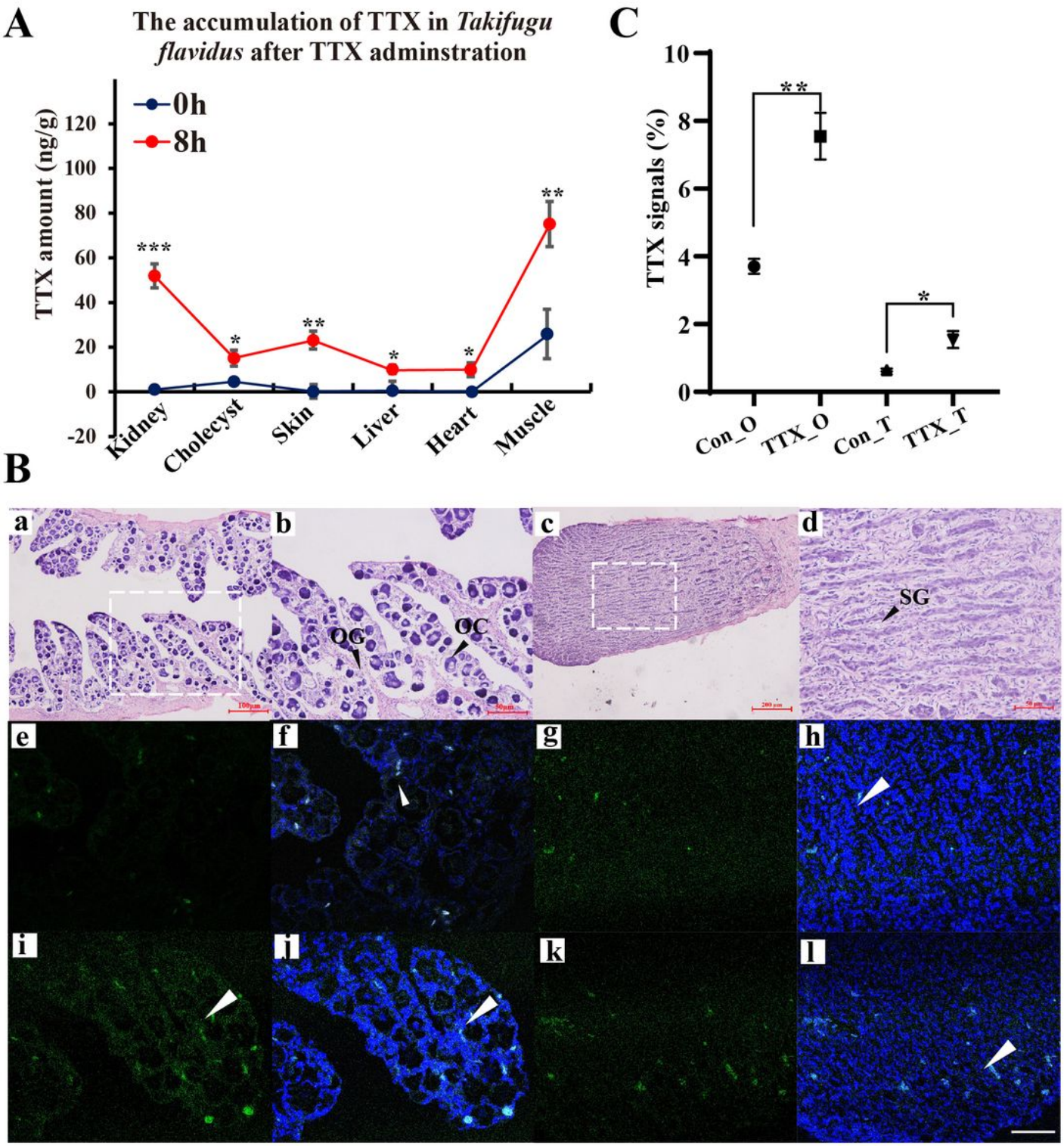

Figure 1

Exogenous TTX accumulated in artificially cultured pufferfish. A, the TTX amount of Takifugu flavidus tissues after intramuscular injection of TTX. B, hematoxylin-eosin staining and TTX fluorescence immunohistochemistry of Takifugu flavidus gonads after intramuscular injection of TTX. An ovary is under the developmental stage of meiosis (a), b, the magnify of a; c and d, the ovary is under the developmental stage of meiosis, d is the magnify of c□e, f, g, h are the control ovary and testis

respectively; i, j, the ovary of the treated group; k, l, the testis of the treated group. OG, oogonia; OC, the oocyte; SG, spermatogonia; The green signals are TTX and the blue signals are Hoechst33258 (staining for nucleic acid), the white dotted-rectangle indicate the magnified area, black arrows showed the germ cells, the white triangle arrows are indicated the TTX signals, the scale bar is 100, 200 and 50 μm. C, TTX signals of gonads in juvenile Takifugu flavidus after TTX administration. Data were shown as mean±SEM. *, indicated that ρ<0.05; **, indicated that ρ<0.001; ***, indicated that ρ<0.0001.

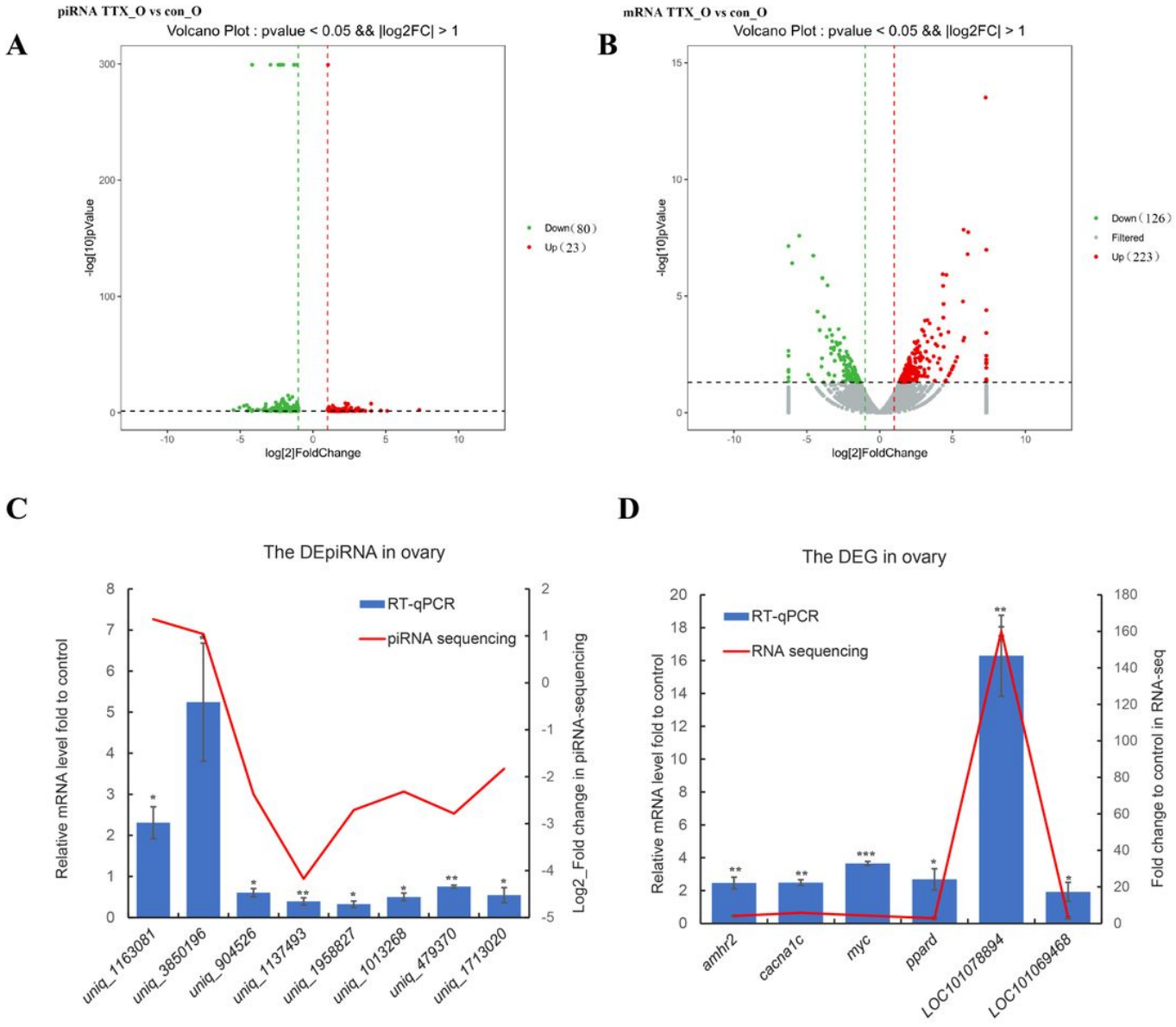

Figure 2

The Differential expression of piRNAs and mRNA in the TTX-treated ovary and control ovary. In piRNA/mRNA sequencing, the red dot represents up-regulated gene/piRNA, the green dot is down-regulated gene/piRNA, the gray dot is no significant difference gene/piRNA, DEG analysis threshold isρvalue < 0.05 && | log2 fold change | > 1; Expression levels of piRNA/mRNA measured with qRT-PCR

were depicted with column charts, and the red line represents the log2 fold changes/fold changes in sequencing. Error bars represent one standard deviation of three different biological replicates. the internal reference gene is U6 for piRNA and Gapdh for mRNA. set as **ρ < 0.01, ***ρ < 0.001 and ****ρ < 0.0001 vs controls. Statical significance is reported for each piRNA or RNA. DEpiRNA, differential expression piRNA; DEG, differential expression mRNA. A Volcano diagram of differential piRNA analysis in the ovary after TTX treatment; B, Differential expression of genes in the ovary of TTX treatment group and control group; C, Relationship between relative expression levels of selected four piRNA in the TTX-treated ovary and control ovary validated by qRT-PCR and log2fold changes derived using piRNA sequencing, control ovary as a calibrator; D, RT-qPCR and mRNA sequencing of selected three genes between ttx treated ovary and control, control ovary as a calibrator.

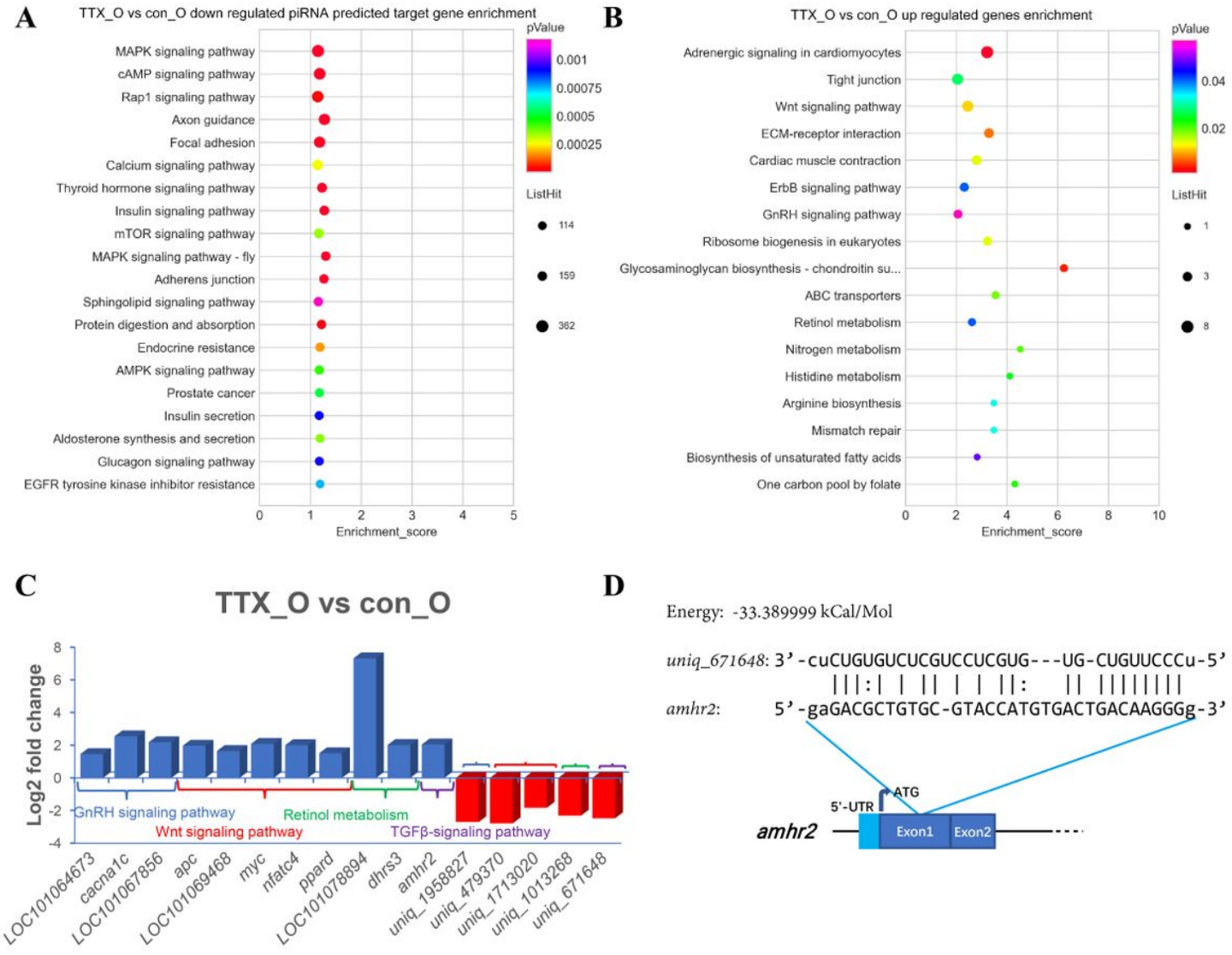

Figure 3

The impact of TTX on the piRNA and gene expression in the ovary of juvenile Takifugu flavidus. A, KEGG pathway of the target mRNA of TTX down-regulated piRNA in the ovary, including MAPK signaling

pathway, cAMP signaling pathway, thyroid hormone signaling pathway; B, KEGG pathway of TTX up-regulated genes in the ovary, including tight junction,wnt signaling pathway, GnRH signaling pathway and retinol metabolism, some of these pathway involved in the ovarian development; C, The relative expression of RNA and piRNA in the ovary based on piRNA and RNA sequencing, TTX up regµlated genes which are enriched in the GnRH signaling pathway, wnt signaling pathway, retinol metabolism and. TGFβ-signaling pathway was involved target genes of TTX down-regulated piRNA. open brace in the same color indicated the same signaling pathway; D, The uniq_671648-target RNA (amhr2) pairing prediction by MiRnada. The light blue box showed the 5'-UTR sequence and the navy blue box showed the exons of the target gene.

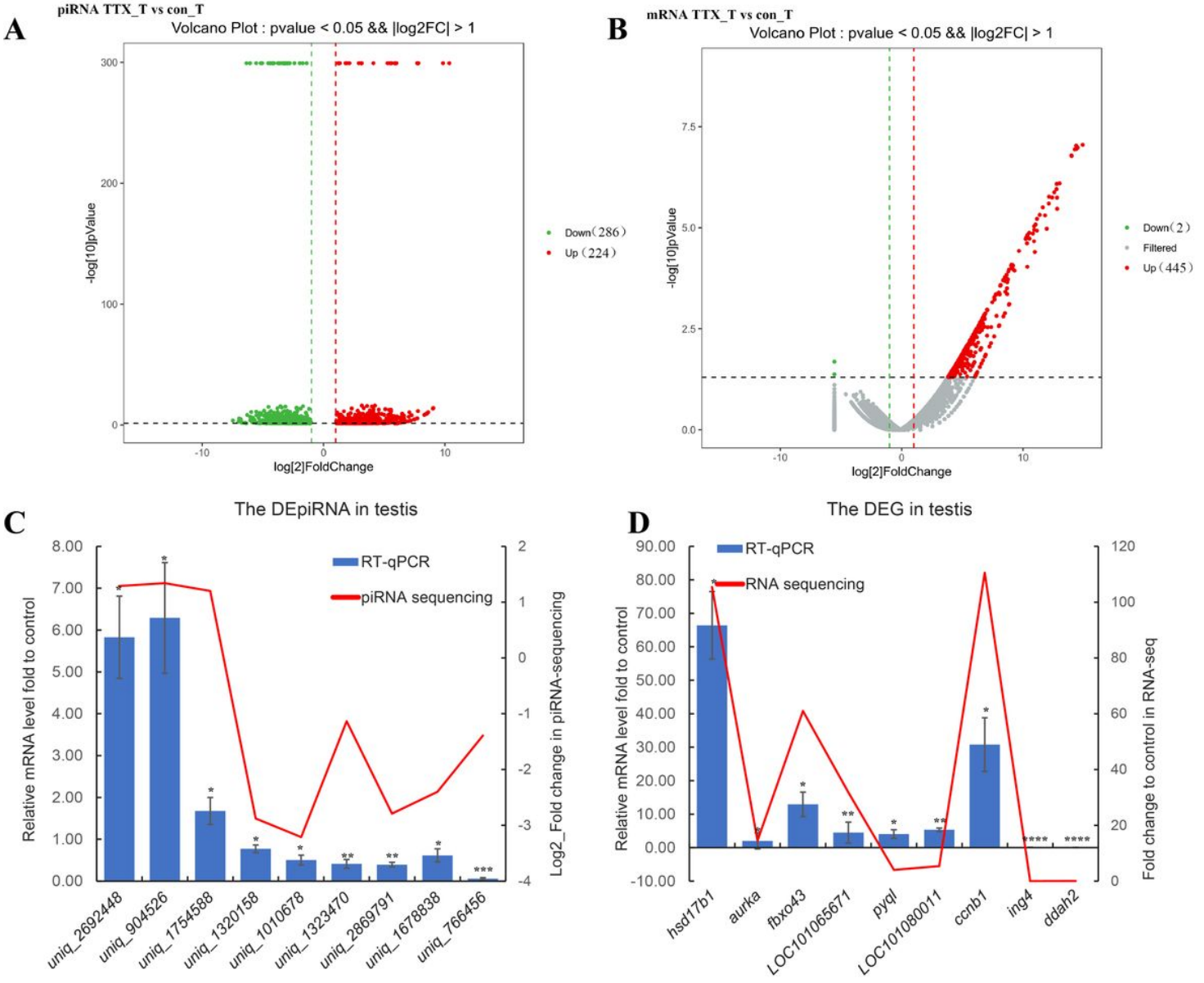

Figure 4

The Differential expression of piRNAs and mRNA in the TTX-treated and control testis. Expression levels of piRNA or RNA measured with qRT-PCR were depicted with column charts, and the dotted line

represents the log2 fold changes/fold changes in sequencing. Error bars represent one standard deviation of three different biological replicates. the internal reference gene is U6 for piRNA and gapdh for mRNA. set as **ρ < 0.01, ***ρ < 0.001 and ****ρ < 0.0001 vs controls. Statical significance is reported for each piRNA or RNA. DEpiRNA, differential expression piRNA; DEG, differential expression mRNA; the red dot represents up-regulated gene/piRNA, green dot is down-regulated gene/piRNA, gray dot is no significant difference gene/piRNA, DEG analysis threshold isρvalue < 0.05 & | log2 fold change | > 1. A Volcano diagram of differential piRNA analysis in testis after TTX treatment. B, Differential expression of genes in the testis of TTX treatment group and control group; C, Relationship between relative expression levels of selected piRNAs in the TTX-treated and control testis validated by qRT-PCR and log2fold changes derived using piRNA sequencing, control testis as a calibrator; D, RT-qPCR an RNA sequencing of selected genes between ttx treated testis and control testis, control testis as a calibrator.

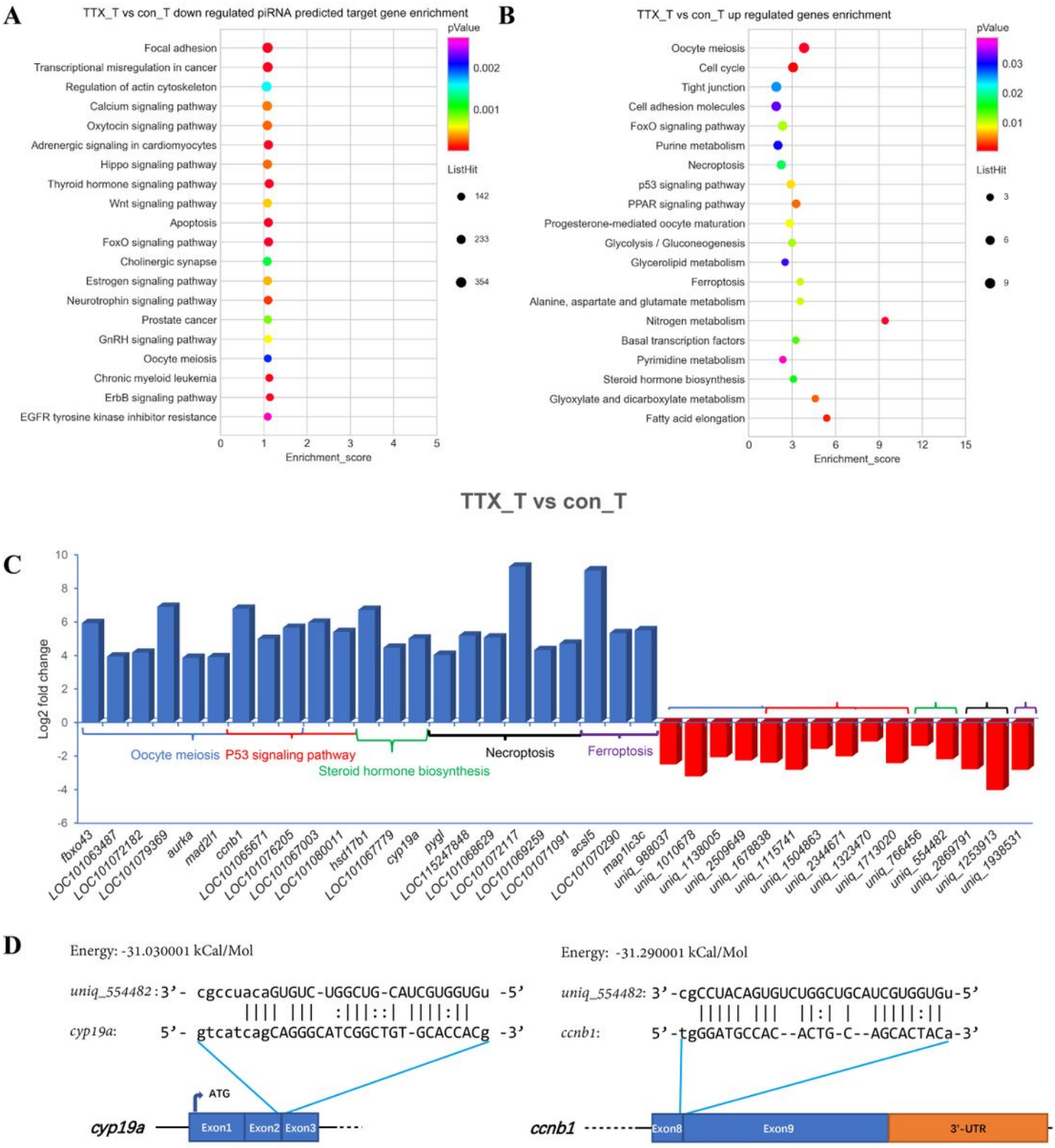

Figure 5

The impact of TTX on the piRNA and gene expression in the testis of juvenile Takifugu flavidus. A The KEGG pathway of down-regulated piRNAs target mRNA; B, TTX up-regulation RNAs in testis were enrichment; C, The relative expression of RNA and piRNA in the testis based on piRNA and RNA sequencing. TTX upregulated genes in the oocyte meiosis and steroid hormone biosynthesis way were predicted targets of TTX downregulated piRNAs in juvenile Takifugu flavidus testis. D, The uniq_554482-

target RNA (cyp19a and ccnb1) pairing prediction by MiRnada. The Yellow box showed the 3’-UTR sequence and the navy blue box showed the exons of the target gene.

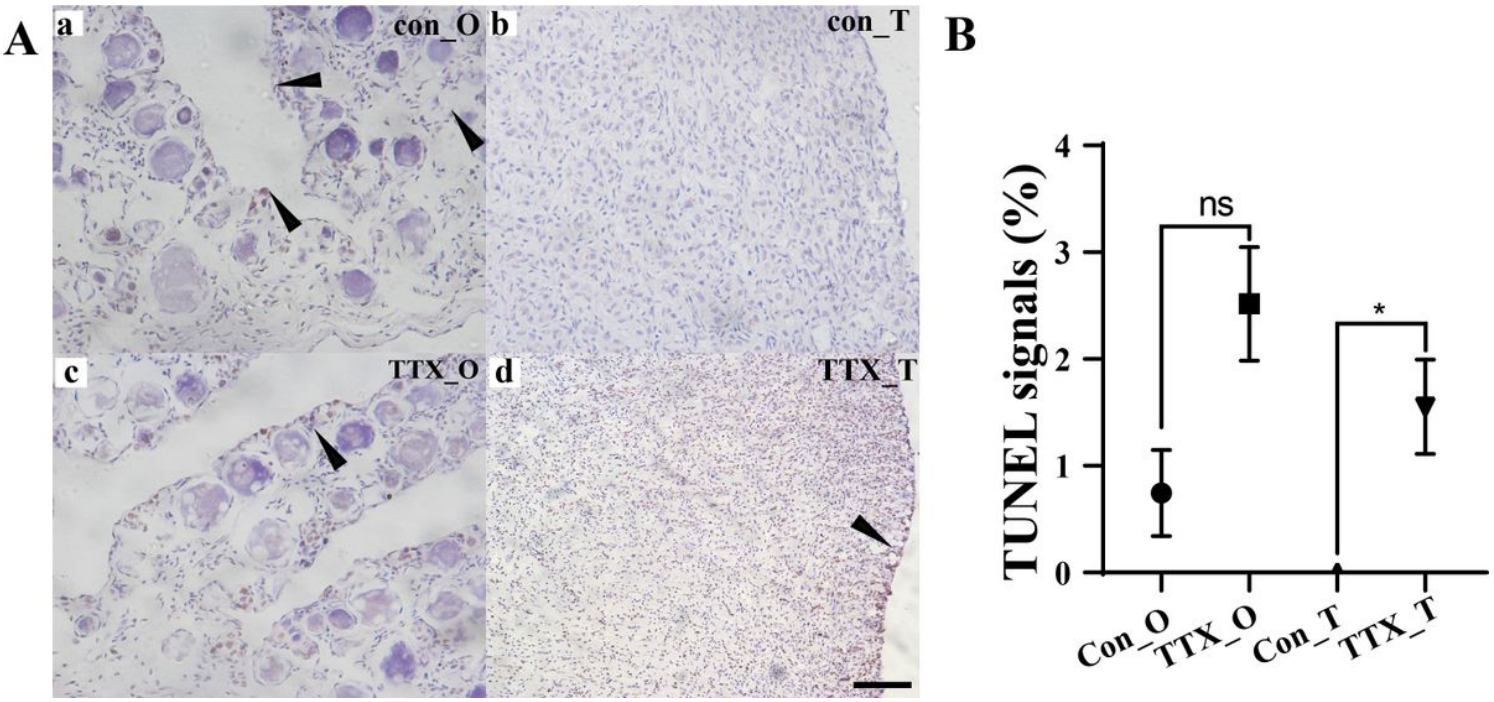

Figure 6

Apoptosis of Takifugu flavidus gonads after intramuscular injection of TTX. A TUNEL staining of Takifugu flavidus gonads after TTX administration. a, b, the testis the of control ovary and testis respectively; c, the TTX treated ovary; d, the TTX-treated testis. The dark brown signals are apoptosis signals by TUNEL staining and the black triangle arrows are indicated the positive signals, the scale bar is 50 μm. B, apoptosis ratio of gonads in juvenile Takifugu flavidus after TTX administration. Data were shown as mean±SEM, *, indicated that ρ<0.05

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1seqenceofuniqpiRNAs.xlsx
- Additionalfile2ListofDEpiRNAandDEGinovaries.xlsx
- Additionalfile3DEGpiRNAtargetmRNA.xlsx
- Additionalfile4KEGGanalysis.xlsx
- Additionalfile5ListofDEpiRNAandDEGintestes.xlsx
- Additionalfile6PrimersandforRTqPCR.xlsx
- Additionalfile7Figures1.docx
- Additionalfile8Figures2.docx