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Differential expression of two Piwil orthologs during embryonic and gonadal development in pufferfish, *Takifugu fasciatus*

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ABSTRACT

Piwil was an important regulator gene in germ cell division during gonadal development. Two Piwi-like genes, Piwil1 and Piwil2, were first cloned from *T. fasciatus*. The full-length cDNAs of Piwil1 and Piwil2 were of 2933 and 3394 bp, respectively. Piwil1 and Piwil2 possessed an open reading frame (ORF) of 2565 and 3138 bp, encoding 854 and 1045 amino acids, respectively. The tissue distribution analysis demonstrated that Piwil1 and Piwil2 were expressed at higher levels in gonad compared to other tissues (brain, liver, gill, etc.). The time-course dynamic expressions of Piwils during embryonic indicated that Piwil1 and Piwil2 were mainly enriched in the early embryonic development. In testis, the expression of Piwil1 and Piwil2 increased at first but then decreased at mRNA and protein levels. However, the expression of Piwil1 and Piwil2 in the ovary showed a downward trend from the beginning. In addition, the expression levels of Piwil1 and Piwil2 were weak in mature testes or ovaries. The immunohistochemistry analysis revealed that Piwil1 and Piwil2 were abundantly expressed in cytoplasm of spermatogonia, spermatocytes, oocyte I and oocytes II, which were mainly presented in the early stages of gonadal development. Our results suggested that Piwil was related to the differentiation of germ cells, and might play an important role in embryonic development. Therefore, our findings provided valuable information of Piwils in the reproductive cycle of *T. fasciatus*.

1. Introduction

Gametogenesis is central to sexual reproduction (Wang and Croll, 2004; Lim et al., 2013), which including spermatogenesis and oogenesis (Kowalczykiewicz et al., 2012). In invertebrates, gametogenesis is a process regulated by internal and external factors, but mainly influenced by internal factors (Zhang et al., 2014; Mueller et al., 2015), such as azoospermia-like (DAZL) (Gill et al., 2011), heat shock proteins (Hsp) (Wolgemuth and Gruppi, 1991), vasa (Feng et al., 2011; Hartung et al., 2014) and Piwi-like (Klein et al., 2016). Recently, it has been demonstrated that Piwi-like (Piwil) plays a crucial role in regulation during gametogenesis (Sugio et al., 2008), and contains the highly homologous PAZ and Piwi domain (Cox et al., 1999; Unajak et al., 2006), while Piwil is involved in a novel Piwi-interacting RNAs (piRNAs) pathway for gametogenesis and therefore less understood.

To date, a series of studies have demonstrated that Piwils (Piwil1 and Piwil2) are essential in gene silencing and transposon regulation during germ cell differentiation and gonadal development in animals (Klattenhoff and Theurkauf, 2008; Grentzinger et al., 2012; Kawaoka et al., 2012). Piwil is first identified and isolated in *Drosophila*, which functions as a crucial factor in maintaining self-renewal and division of germline stem cell (Cox et al., 1999). Mutations of Piwil proteins, as shown in mammals are limited to the male germline (Girard et al., 2006). In humans (*Homo sapiens*), four homologs of Piwils have been identified, including *Hiwi*, *Hiwi2*, *Hiwi3* and *Hili* (Qiao et al., 2002), which are specifically expressed in spermatocytes and round spermatids. The *Mus musculus* Piwil (*Miwi1*, *Miwi2*, and *Mili*) (Kuramochi-Miyagawa et al., 2001; Carmell et al., 2007) encode proteins that are specifically expressed in the cytoplasm of spermatocytes and spermatids. Research reported that spermatogenesis procedure stopped at the initial stage of spermatids after knockout of the *Miwi* in mouse (Carmell et al., 2007). Expression of the three Piwil (Piwil1, Piwil2 and Piwil4) mRNAs in porcine was proved to be tissue specific and restricted exclusively to the gonads (Kowalczykiewicz et al., 2012). Generally, Piwil has been studied extensively in mammals, but we are acquainting scarcely with its function in teleost. The research on the function of

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Piwil mainly concentrated in model organism of teleost (Bak et al., 2011), such as zebrafish (Tan et al., 2002) and medaka (Zhao et al., 2012). Zebrafish Piwil has two members; *Ziwi* (Piwil1) and *Zili* (Piwil2), both of them are expressed in female and male gonads. In addition, *Ziwi* mutation can lead to apoptosis of zebrafish germ cells (Houwing, 2009). For another, *Zili* is an important factor in the differentiation and meiosis of zebrafish reproductive cells (Ings and Gj, 2006). Moreover, lack of *Opiwi* can significantly reduce the number of PGCs in vivo and in vitro, and affect the distribution of PGCs in developing embryos of medaka (Li et al., 2012). Furthermore, sex steroid can regulate the expression of Piwil during embryonic development of *Xenopus* (Zhang et al., 2010). These researches indicating that Piwil are not only related to the differentiation of germ cells, but also plays an important role in embryonic development. However, the mechanism of Piwil in embryonic and gonadal development is not well deciphered.

As a widely distributed species in the South China Sea, the East China Sea, inland waters in China and Korean Peninsula (Akira et al., 2005), *Takifugu fasciatus* (*T. fasciatus*) is an important farmed fish with high commercial value (Liu et al., 2013). *T. fasciatus* was found to spawn once a year between Aprils and May (Hua and Chen, 1996). However, the culture efficiency of *T. fasciatus* has been plagued by developmental asynochronization, low gamete viability and slow maturation procedure, which seriously restricts the development of *T. fasciatus* industrialization. Therefore, to solve these problems, it is very necessary to carry out further study on the Piwil1 and Piwil2 in the breeding process of *T. fasciatus*.

In the present study, Piwil1 and Piwil2 of *T. fasciatus* were identified. Moreover, we aimed (1) to characterize the identified Piwil1 and Piwil2 at molecular level; (2) to elucidate their expressions at transcript level in both embryonic development and mature individuals; (3) to analyze their temporal expression profiles at mRNA and protein levels during different stages of gonadal development; and (4) to locate the expression distribution of Piwil1 and Piwil2. Overall, studies on the mechanism of Piwils in embryonic and gonadal development can provide a theoretical basis for gametogenesis and improving breeding efficiency of *T. fasciatus*.

2. Materials and methods

2.1. Experimental animals and fertilized eggs

All the experimental fish and fertilized eggs, supplied from Zhongyang Group Co., Ltd. of *T. fasciatus* (Jiangsu Province, China). Classification of gonad maturation state in this study refers to the previous research (Hua et al., 1999). According to the difference of gonad tissue slice (Fig. S3) and gonadosomatic index (GSI, Fig. 2) of experimental fish, gonad development is divided the process into five phases (I, II, III, IV and V). Fertilized eggs were derived from gametes produced by mature individuals and then cultured in hatchery barrels after artificial insemination.

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in China. This study was also approved by the Ethics Committee of Experimental Animals at Nanjing Normal University (grant No. SYXK 2015-0028, Jiangsu).

2.2. Tissue collection, total RNA extraction and cDNA synthesis

Briefly, experimental *T. fasciatus* (n = 6 for mature individuals) in the late preparatory phase were anaesthetized with an MS-222 solution (0.05%, Sigma, USA), and tissues (brain, gill, heart, intestine, muscle, spleen, liver, kidney, ovary and testis) were collected under the normal physiological conditions. In order to evaluate the role of Piwils in *T. fasciatus*, tissues of ovary and testis at the different stages of gonadal development were used for western blot and immunohistochemistry analysis. Samples of ten periods during embryogenesis were identified by microscopic examination (Fig. 4) and collected immediately for quantitative analysis. All of the samples were stored at −80 °C prior to further analysis.

Total RNA was extracted using High Purity RNA Fast Extract Reagent (BioTeke, Beijing, China) according to the manufacturer's instructions. Purified RNA was reversely transcribed into cDNA by HiScript™ QRT SuperMix (Vazyme, New Jersey, USA) and then immediately stored at −20 °C for subsequent quantitative real-time PCR (qRT-PCR) analyses.

2.3. Histological sections of the gonads

According to the method of reference (He et al., 2001), the gonad tissue was fixed in 4% paraformaldehyde for at least 24 h. Subsequently, dehydration was carried out at different concentrations of alcohol (75%, 85%, 90%, 95% and 99.5%). The dehydrated tissues were embedded in paraffin and then stored at −20 °C for chill-down. The thickness of the slice was 6–7 μm, and these samples were then used for HE (hematoxylin-eosin) staining and immunohistochemical analysis, respectively.

2.4. Piwil cloning, and phylogenetic analysis

To obtain the full-length cDNAs of Piwil1 and Piwil2 in *T. fasciatus*, total RNA from testis was used as template. Briefly, 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE were performed using the 5′ RACE System (Version 2.0, Invitrogen, US) and SMARTer™ RACE cDNA Amplification Kit (Clontech, US), respectively. Primers (Table S1) were designed based on NCBI databases derived from conserved regions of approximate species. The anchor primers were supplied along with the kit, and touchdown PCR was programmed according to the recommended conditions using 5′ and 3′ gene specific primers. The resulting PCR products were cloned into the pMD18-T simple vector and then subjected to sequence analysis. All sequences were assembled to obtain the full-length cDNAs of Piwils.

The software of Simple Modular Architecture Research Tool (SMART) was used to predict protein domain structure. Phylogenetic tree was constructed by the neighbor-joining (NJ) method of Molecular Evolution Genetic Analysis (MEGA 5.0) software. The number at each node indicated the percentage of bootstrapping after 1000 repetitions. The Ensembl and GenBank databases were used to identify related genes in other vertebrates to initially derive the syntenic relationships between species.

2.5. Real-time RT-PCR

The tissue distribution and temporal expression profiles of Piwil1 and Piwil2 in the different tissues of adult individual, different stages of gonadal and embryonic development were assessed by qRT-PCR. Table S1 listed all the primers for Piwil1, Piwil2 and β-actin of *T. fasciatus*. The experiments were carried out in triplicate with a total volume of 20 μL in ABI stepone™ plus (Applied Biosystems, USA), consisting of 4 μL of diluted cDNA template, 10 μL of Faststart Universal SYBR Green Master (Roche, Basel, Switzerland), 1 μL of each primer (6 mmol/μL) and 4 μL ddH_2O. Briefly, after a denaturation step at 95 °C for 10 min, the reaction was carried out with 40 cycles at a melting temperature of 94 °C for 10 s, and an annealing temperature of 55 °C for 30 s. β-actin of *T. fasciatus* was employed as the housekeeping gene, and the relative expression level of Piwils in *T. fasciatus* was determined by the $2^{-\Delta\Delta Ct}$ method. In the tissue-specific expression analysis, the calculated relative expression level of Piwils in each tissue of *T. fasciatus* was compared with its respective level in liver. In the different stages of gonadal development, the fold-change of I, II, III and IV were determined by comparing the expression of V phases. Similarly, the I phase of embryonic development was used as a reference to measure differences in other periods of expression.