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Short Communication

Human chorionic gonadotropin suppresses expression of Piwis in common carp (*Cyprinus carpio*) ovaries

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ABSTRACT

Piwi proteins are required for germline maintenance and gonad development. In this study, the cDNAs encoding Piwil1 and Piwil2 were cloned and sequenced from the common carp. The full-length cDNA of Piwil1 and Piwil2 were 3114 and 3421 bp, encoding 858 and 1034 amino acids including PAZ domain and PIWI domain, respectively. In addition, the Piwil1 and Piwil2 proteins shared high homology with other teleosts. Reverse transcriptase PCR revealed that the Piwi mRNAs were exclusively expressed in adult testes and ovaries. Using real-time PCR, expression study of different developmental profiles showed that Piwil1 and Piwil2 were down-regulated during pre-ovulation. Further, human chorionic gonadotropin treatment in ovaries (*in vivo*) and in cultured ovaries cells (*in vitro*) resulted in down-regulation of Piwi RNAs. These results suggest that the decreased expression which was regulated by hormone plays a crucial role during ovarian differentiation and development.

1. Introduction

Piwi belongs to Argonaute superfamily which is characterized by highly homologous PAZ and PIWI domains. Based on phylogenesis, the superfamily is divided into two subclasses – Ago subclade and Piwi subclade [3]. Ago is well studied for its function in pathway of microRNAs (miRNAs) and small interfering RNA (siRNAs) [5]. Until Piwi-interacting RNA (piRNA) has been elucidated recently, enormous progress has been made to unveil function and mechanism of Piwi proteins in piRNA pathway [1,13,23].

Piwi was initially isolated in *Drosophila* as a crucial factor in germline stem cells maintenance [3]. In *Drosophila*, Piwi mutations caused germline stem cell division failure. In mouse (*Mus musculus*), three Piwi-like proteins have been identified which dominated meiotic progression in testis [2,11]. Human (*Homo sapiens*) Piwi subfamily contains four homologous genes – Hiwi, Hiwi2, Hiwi3 and Hili. These proteins are specifically expressed in spermatocytes and round spermatids in human [19]. Ziwi (Piwi-like 1, Piwil1) and Zili (Piwi-like 2, Piwil2) were elucidated in zebrafish (*Danio rerio*) for the first time in fish [7,25]. Expression studies revealed that Ziwi and Zili were exclusively expressed in germline. Ziwi mutation triggered germ cell apoptosis and Zili mutation conduced to failure of germ cells differentiation [6,7]. Up to date, series of studies has reported that Piwi were identified in pig (*Sus scrofa*), *Xenopus tropicalis*, *Schmidtea mediterranea* and *Caenorhabditis elegans* [3,20,27,32]. However, mechanism of Piwi and its regulation in gonad development and fertilization is not well deciphering.

It is reasonable to believe that Piwi–piRNA complexes play a crucial role in reproductive cycles. Unlike well studied miRNA, piRNAs which interact with Piwi proteins were discovered until 2006 [1,13]. The piRNAs were located on different loci in genome, especially in transposable element. Thus, piRNAs function was determined mainly in repressing of transposable element transcripts [2,7]. Meanwhile, some piRNAs are derived from intronic or exonic regions. Recent research validated that a broad sets of piRNAs generated from 3′UTRs which suggested that 3′UTR piRNA may regulate transcripts via partially complementary sequences as miRNA in Ago-miRNA complexes [21]. Other studies showed that Piwi–piRNA complexes are essential in silencing, control of transcripts and mRNA regulation during differentiation and development of animal gonad [2,9,16,27,31].

In teleosts, gonad development and reproductive activities are controlled by the hypothalamic–pituitary–gonad (HPG) axis. Hypothalamic secretes Gonadotropin-releasing hormone (GnRH) regulates gonad development and final maturation through gonadotropins (GTH), including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [12,24]. However, almost all female fish exhibit reproductive dysfunction due to failure of LH released into bloodstream by pituitary in breeding condition [33]. Thus, exogenous hormones are employed to manipulate for spawn, including GTH, GnRH, luteinizing hormone-releasing hormone (LHRH), Human chorionic gonadotropin (hCG) and their analogs [4,18].

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Meanwhile, there has been a dearth of information about non-coding RNAs and their binding proteins during these processes that would aid in understanding germline development and maturation.

As a well-known food fish species, common carp (*Cyprinus carpio*) is also a species in studies of fish endocrinology and chemical exposure. Former studies have shown that HPG axis regulates gonad differentiation and development [14,26,29]. To address the role of Piwi proteins in common carp ovary, we cloned two Piwi homologous genes, and investigated their expression profiles in adult tissues. Piwil1 and Piwil2 expression patterns were identified during different stages of ovarian development. In addition, *in vivo* and *in vitro* studies were applied to examine the action of hCG on Piwil1 and Piwil2 expression in carp ovary.

2. Materials and methods

2.1. Experiment fish

Common carp were provided by the Engineering Center of Polyploidy Fish Breeding of the National Education Ministry located at Hunan Normal University. Fish were anesthetized with 2-phenoxyethanol. All tissues were excised from the fish, frozen in liquid nitrogen, and stored at −80 °C until further use.

2.2. Gene cloning of Piwi in common carp

Total RNA from adult common carp testis was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA) and then digested with DNase I (Fermentas, Vilnius, Lithuania) to eliminate residual DNA. Frist-strand cDNA was synthesized using AMV reverse transcriptase (Fermentas, Vilnius, Lithuania) with oligo $(dT)_{12-18}$ primer following the manufacturer's instruction.

The degenerated primers were designed based on conserved sequences of other teleosts (Table 1). Polymerase chain reaction (PCR) was carried out by using the primers listed in Table 1. PCR were performed 94 °C for 30 s, 53 °C for 30 s, 72 °C for 3 min with 30 cycles. The PCR product was cloned and sequenced to obtain core partial cDNA of Piwil1 and Piwil2, respectively.

Subsequently, RACE (Rapid Amplification of cDNA Ends) were performed using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) to amplify full length cDNAs. Specific nested PCR primers were designed base on the core partial sequences (Table 1). For 3′ RACE, the two amplification rounds conditions were both performed 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min with 30 cycles. For 5′ RACE, the two amplification rounds conditions were: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min with 30 cycles.

2.3. Sequence alignments and phylogenetic analysis

Phylogenetic analysis was conducted to compare with other vertebrates. Amino acids sequences were aligned by ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was performed using Neighbor-Joining method with 1000 bootstrap in Mega version 4.1 [10].

2.4. Tissue distribution

Total RNA was extracted from various tissues of adult common carp. Specific primers for Piwil1 and Piwil2 were designed according to the cloned sequences with Primer Express 3.0 (Applied Biosystems). β-Actin was used as internal control. The cDNAs of different tissues synthesis was described above. The PCR condition was as follow: 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and finally, a single elongation step at 72 °C for 10 min.

2.5. Real-time RT-PCR

To measure different transcripts expression, Real-time RT-PCR was used. β-Actin was used as endogenous control. Each test was repeated three times to improve the accuracy of the results in a Prism 7500 Sequence Detection System (Applied Biosystems). The procedure was: 50 °C for 5 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. The analysis of relative mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method [15]. Finally, the dissociation curves were used to examine whether the PCR products were indeed specific.

2.6. Developmental change of Piwi

Comparative expressions of Piwil1 and Piwil2 mRNAs in ovaries were examined by Real-time RT-PCR. Common carp in differential developmental periods were obtained and divided into five groups: 0^+ year group [January, Body weight (BW) = 58 ± 1.95 g, gonadosomatic index (GSI) = 0.064 ± 0.011, $n = 4$] and four 1^+ year groups (November, BW = 423 ± 21.1 g, GSI = 0.137 ± 0.010, $n = 4$; January, BW = 471 ± 17.6 g, GSI = 0.201 ± 0.012, $n = 4$; February, BW = 613 ±

Table 1
Primers sequences used in the polymerase chain reactions.

Gene	Primer name	Sequence (5′–3′)	Application
Piwil1	Piwil1-F	TMWGTGGGYTTTGTATCAGT	Partial cDNA cloning
	Piwil1-R	CCMACWCCATCTCGRTAM	Partial cDNA cloning
	Piwil1–3′ out	ACAATCAGCAAACCTCAAGCACT	3′ RACE
	Piwil1–3′ inner	GCGGTTCTCCAAGTGCGTCT	3′ RACE
	Piwil1–5′ out	TGTGCTTTGCCCAGAGTCTCCTC	5′ RACE
	Piwil1–5′ inner	CGGGACTCCATAGGTGGCTTGA	5′ RACE
	Piwil1 RT-F	AGCACAGGCTGACGATTTGG	RT-PCR and realtime RT-PCR
	Piwil1 RT-R	CAGAATGATCAGGCCCACAA	RT-PCR and realtime RT-PCR
Piwil2	Piwil2-F	ATGGAKCCAARGCGRCC	Partial cDNA cloning
	Piwil2-R	TYACAGAAARAAYAGTTTCTCTGAA	Partial cDNA cloning
	Piwil2–3′ out	GGTTCGAGACCCCTCCATCA	3′ RACE
	Piwil2–3′ inner	AACTGCTGGGCTGTTTTCTATCC	3′ RACE
	Piwil2–5′ out	TGATGGTCGGCTTAGACTGGA	5′ RACE
	Piwil2–5′ inner	CTCTGCCCCATGTCTTTGC	5′ RACE
	Piwil2 RT-F	AACAACTGCTGCACAACATCAAC	RT-PCR and realtime RT-PCR
	Piwil2 RT-R	ACAGTGCTGATGGAGGGGTCT	RT-PCR and realtime RT-PCR
β-Actin	β-Actin-F	GCCCTGCCCCATGCCATCCT	RT-PCR and realtime RT-PCR
	β-Actin-R	AGTGCCCATCTCCTGCTCGA	RT-PCR and realtime RT-PCR