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Research Paper

# DNA methylation level of *cyp19a1a* and *Foxl2* gene related to their expression patterns and reproduction traits during ovary development stages of Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

*Foxl2* and *cyp19a1a* genes are crucial for the ovarian development, and *Foxl2* could play a direct regulatory role on *cyp19a1a* transcription. In this study, we aimed to study DNA methylation status and mRNA expression patterns of *Foxl2* and *cyp19a1a* genes during ovarian development of female Japanese flounder. The relative expression level of *cyp19a1a* and *Foxl2* gene during the gonadal development stages was measured by quantitative PCR. Moreover, DNA methylation status in the promoter and coding regions of the two genes was detected by bisulfite sequencing. The estradiol-17β (E2) was measured by radioimmunoassay. The results showed low expression levels of *cyp19a1a* and *Foxl2* genes in stages II and V, while the highest expression levels were detected in stage IV. The variation trend of the methylation level of all CpG sites in promoter and exon 1 of *cyp19a1a* gene and three CpG rich regions in coding region of *Foxl2* gene was negatively associated with their expression levels during the ovarian development. In addition, two CpG sites in promoter and seven CpG sites in exon 1 of *cyp19a1a* were on the putative transcription factors binding sequence. Further studies showed that the forkhead domain, which is important for *Foxl2* binding to *cyp19a1a* was located in the F1 and F2 region. These results provide a powerful theoretical basis for the regulatory mechanism on *Foxl2* regulating *cyp19a1a* and promoting gonadal differentiation towards the female pathway, and further reveal that *Foxl2* and *cyp19a1a* play a vital role in the female Japanese flounder gonad development.



## 1. Introduction

Aromatase cytochrome P450 (P450arom), the product of *Cyp19* gene, is a sex steroid hormone and a key component of the enzymatic aromatase complex converting androgens to estrogens (Conley and Hinshelwood, 2001; Séralini and Moslemi 2001). It has been proved that two types of P450arom were found in brain and gonad in many teleosts. The *cyp19a1a* encodes the gonad-specific aromatase, while *cyp19a1b* encodes the brain-specific aromatase (Gelinas et al. 1998; Sudhakumari et al. 2005; Tang et al. 2010; Trant et al. 2001; Valle et al. 2002). It was reported that *cyp19a1a* expression level was higher in ovary than in testis during sex differentiation of non-mammalian vertebrates (Jeyasuria and Place 1997; Kitano et al. 1999; Smith et al. 1997; Yoshida et al. 1996). In fish, it has been suggested that estrogen plays a vital role in sex differentiation and gonad development. P450arom plays a key role in estrogen production and ovary development (Chang et al. 2005). In Nile tilapia, the expression of *cyp19a1a* at all ovarian stages suggests an important role for estrogen in female sex determination and maintenance of phenotypic sex (Tao et al. 2013). The *cyp19a1a* promoter contains a cAMP-responsive element (CRE-like sequence) and two Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF1) binding sites in teleosts such as the medaka (*Oryzias latipes*), zebra fish (*Danio rerio*), and gilthead bream (*Sparus aurata*). Moreover, luciferase reporter assay suggested that cAMP activated *cyp19a1a* gene transcription via the CRE-like sequence in vitro (Yamaguchi et al. 2007). The analysis revealed that gonadotropins (GTHs) induced the *cyp19a1a* mRNA expression in ovary (Kagawa et al. 2003; Wong et al. 2006). Therefore, *cyp19a1a* gene transcription may be regulated by GTHs through cAMP pathway in ovary development of many vertebrates. Wen et al. (2014) reported that adult *Paralichthys olivaceus cyp19a1a* expression was much higher in the ovary than that in the testis ($P < 0.01$), the *cyp19a1a* promoter CpGs, were methylated more in the testis than that in the ovary. This demonstrated that the methylation level of *cyp19a1a* could repress its transcripts expression. Sexual dimorphic expression indicated that *cyp19a1a* was a key sex-related gene.

The forkhead transcription factor gene 2 (*Foxl2*), containing a 110 amino acid long conserved DNA binding region (FH domain), is the first known sexual dimorphic marker (Caburet et al. 2012).

*Abbreviations:* cyp19a1a, Cytochrome P450 19a1a; Foxl2, Forkhead box L2; E2, estradiol-17β; CRE, cAMP-responsive element; Ad4BP, Ad4 binding protein.

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Recent reports have described the cloning of *FoxL2* from the medaka (*O. latipes*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), half smooth tongue sole (*Cynoglossus semilaevis*), brown spotted reef cod (*Epinephelus merra*), and Japanese flounder (*Paralichthys olivaceus*), and have shown expression in ovary (Alam et al. 2008; Baron et al. 2004; Dong et al. 2011; Nakamoto et al. 2006; Wang et al. 2004; Yamaguchi et al. 2007). Research has suggested that *Foxl2* was involved in transcriptional regulation of ovarian aromatase gene along with some additional partners such as Sf1 or cAMP (Galay-Burgos et al. 2006; Guiguen et al. 2010; Wang et al. 2007; Yamaguchi et al. 2007). Wang et al. (2007) studied on *O. niloticus* (Nile tilapia) and reported that Foxl2 protein directly binds to *cyp19a1a* promoter through the interaction of its forkhead domain with Ad4 binding protein, and endogenous factors, such as Ad4BP/Sf1, were needed to enhance this effect. Thus, Foxl2 was involved in early ovarian development and differentiation through interactions with *cyp19a1a* and thereby, influencing estrogen production specifically in the female gonad (Wang et al. 2007).

DNA methylation, an important epigenetic factor, could modulate gene expression. One CpG dinucleotide is found in both cAMP-responsive element (CRE) and Ad4 binding protein (Ad4BP) recognition sequence of *cyp19a1a* promoter and these two CpG sites were located in CpG rich regions. The forkhead domain (FHD) of *Foxl2* gene was located in two CpG rich regions (namely F1 and F2) of its coding region. DNA methylation could influence chromatin structure, DNA conformation, DNA stability, and the interaction between DNA and protein, thus regulate gene expression (Tornaletti and Pfeifer, 1995). We hypothesize these CpG sites mentioned above may be involved in the control of *cyp19a1a* gene transcription. In the European sea bass, the DNA methylation levels in juvenile males are twice as much as that of females in the promoter of *cyp19a1a* (Navarro-Martín et al. 2011).

Japanese flounder (*P. olivaceus*) is one of the most important economic teleosts in China. It has XX (female)/XY (male) sex determination system and the female grows faster than the male. At the present, gynogenesis Japanese flounder are popular in productive practice. The transcriptome of flounder gonads was analyzed by Fan et al. (2014), which provided further insights into sex determination and gonadal development. Sex-related genes, including *Foxl2* and *cyp19a1a*, have been cloned in Japanese flounder to study their functions in gonadal differentiation (Kitano et al. 1999; Yamaguchi et al. 2007). Yamaguchi et al. (2007) found that *Foxl2* could regulate the expression level of *cyp19a1a*, but the molecular mechanism and epigenetic mechanism of the sex-related genes regulation in different ovary development stages is still unclear. The present study focused on adult female *P. olivaceus* of different gonad development stages and investigated the gene relative expression level and DNA methylation patterns of CpG rich regions in *Foxl2* gene and promoter and exon1 of *cyp19a1a* gene within ovary. The study is aimed to clarify the molecular mechanism of *Foxl2* regulating *cyp19a1a* and their functions in ovary development from epigenetic perspective.

## 2. Materials and methods

### 2.1. Experimental fish and data collection

Healthy adult female Japanese flounder (20 months) were obtained from a local aquatic farm every 20 days. At least six fishes were taken randomly every time and sixty were sampled finally. All the fishes were divided into 4 different stages according to its ovarian development. In our experiment and data analysis, 3–5 fishes were used in each stage. They came from a full-sib family and were reared under the same controlled conditions. All fish were anesthetized using MS-222, and then blood was collected from the caudal vein using a heparinized 1 ml syringe. Tissue samples were removed and stored at −80 °C for genomic DNA and total RNA isolation. Gonadosomatic index (GSI) was calculated (GSI = [gonad weight/(body weight-viscera weight)] × 100). Ovary development of the same batch fish has been studied by Ding et al. (2013) in our lab.

### 2.2. RNA isolation and RT-PCR

RNAiso reagent (TaKaRa, Japan) was used to extract total RNA. The concentration of extracted total RNA was spectrophotometrically measured. Reverse transcription was carried out using a two-step method with PrimeScript™RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). The synthesized cDNA was stored at −20 °C for later use.

### 2.3. qPCR

The relative expression levels of *cyp19a1a* and *Foxl2* mRNA were determined using total RNA extracted from ovary of Japanese flounder. Quantitative PCR was performed via Roche LightCycler480 (German) and SYBR Premix Ex Taq™ (TliRNaseH Plus) Kit (Takara, Japan, Code no. RR420A), according to the manufacturer's protocols. Cyp19-F, Cyp19-R, Foxl2-F, Foxl2-R and 18S-F, 18S-R were primers used for qPCR analysis respectively (Table 1). The amplification efficiency of the three primer pairs were over 99%. The 20 μl mixture of PCR consisted of 10 μl SYBR®Premix Ex Taq (TliRNaseH Plus), 0.4 μl PCR Forward Primer, 0.4 μl PCR Reverse Primer, 2 μl cDNA template, add RNase-free water to 20 μl. The PACAP qPCR conditions were as follows: denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing at Tm for 30 s, and extension at 72 °C for 30 s. 18S ribosomal RNA, as reference gene, was amplified under the same conditions. Each sample was run in triplicates. Negative control was also run by using all the qPCR reagents without the cDNA template, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). A probability level of $P < 0.05$ was considered statistically significant.

### 2.4. Analysis of genetic structure and amino acid sequence of cyp19a1a and Foxl2

Online software ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to find *cyp19a1a* and *Foxl2* gene open reading frame (ORF). Transcription factor was predicted using TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html). The conserved gene sequence of *Foxl2* was identified with the other species including *Silurus meridionalis* (GenBank accession no. EF0l5396), *O. niloticus* (GenBank accession no. AY554172), *D. rerio* (GenBank accession no. XM_693823), *O. mykiss* (GenBank accession no. AY507927) and *O. latipes* (GenBank accession no. AB252055) by multiple sequence alignment. The transcription factor binding site of *cyp19a1a* promoter was analyzed according to Yamaguchi et al. (2007).

### 2.5. Genomic DNA isolation

Genomic DNA was extracted from ovary samples at ovarian development stages II to V using Marine Animal DNA Kit (CWBIO). The DNA purity and concentration were measured using UV spectrophotometer (Amersham, American), and their integrity was evaluated by agarose gel electrophoresis (Ding et al. 2013). The DNA was stored at 20 °C until needed.

### 2.6. DNA bisulfite modification and analysis

In each stage 3–5 fishes were used to perform the bisulfite modification. DNA samples (200 ng) were sodium bisulfite-modified using the Methylamp™ DNA Modification Kit (QIAGEN) following the manufacturer's instructions. The CpG rich regions of *cyp19a1a* (GenBank accession no. AB304921) promoter and exons and *Foxl2* (GenBank accession no. AB303854) coding region were identified by online MethPrimer design software (http://www.urogene.org/methprimer/). Primers were

**Table 1**
Nucleotide sequences of primers used for Real Time PCR in the experiment.

| Primer name | Sequence (5′ to 3′) | Product size (bp) | Tm (°C) | Accession no. |
|---|---|---|---|---|
| Cyp19a1a | F:TTGGGAGCAAGCAGGGACT<br>R:CAGCGCAGCAAACTGAGGA | 220 | 65 | AB017182.1 |
| Foxl2 | F: CTTCACTCATCGAGGTTGC<br>R:GGCTTCTCCGACACTTTCT | 209 | 61.7 | AB303854.1 |
| 18S | F:ATTGACGGAAGGGCACCAC<br>R:ATGCACCACCACCCACAGA | 134 | 65 | EF126037.1 |

designed according to the known sequences using Oligo 6.0 (Table 3). The PCR products were cloned into a pEASY-T1 vector, for each fish typically 8 clones (range 7–10) were sequenced to determine the methylation level. To evaluate the efficiency of bisulfite modification, we calculated the percentage of the number of converted cytosines on the total number of cytosines (excluding cytosines of CpG dinucleotides). The formula is as follows:

The conversion percentage = [the number of converted cytosines (excluding cytosines of CpG dinucleotides)]/[the total number of cytosines (excluding cytosines of CpG dinucleotides)] × 100%.

### 2.7. $E_2$ radioimmunoassay (RIA)

Blood was centrifuged for 15 min at 4 °C at 10,000 rpm. Plasma was removed and stored at −20 °C. Plasma $E_2$ levels were determined by $^{125}$I radioimmunoassay, using diagnostic kits from Diagnostic Products Corporation (TianjinNine Tripods Medical and Bioengineering Co., Ltd., Sino–US joint venture enterprise). The sensitivity of E2 is 5–46 pg/ml. The assay error is shown in Table 2.

### 2.8. Statistical analysis

Data reported were expressed as means ± standard error. All RQ expression data were log-transformed to ensure normality. Data was analyzed by one-way ANOVA followed by Duncan's multiple range tests to determine significant differences between samples using SPSS 10.0. The correlations between gene expression, methylation extent, and hormone changes were initially examined by spearman tests using SPSS 10.0 (SPSS Co. Ltd., Chicago). The correlation coefficient was calculated by Excel. Statistical significance was determined at $P$-value <0.05.

## 3. Results

### 3.1. Expression of Foxl2 and Cyp19a1a mRNA in female Japanese flounder

Fig. 1 showed the expression level of *Foxl2* and *Cyp19a1a* in the ovary of Japanese flounder during the ovarian development stages. From agarose gel electrophoresis (Fig. 1A), we can see that *cyp19a1a* and *foxl2* was highly expressed at stages II, III, IV, V. In Fig. 1B, *Cyp19a1a* expression level increased from stage II to stage IV, with the highest expression quantity in stage IV ($P < 0.05$). There was a minor decline at stage V ($P < 0.05$). Correlation analysis demonstrated that *Foxl2* mRNA expression had a positive correlation with *cyp19a1a* mRNA expression (r = 0.53).

**Table 2**
The definition of human estradiol of the plasma in RIA.

| Index | Intra-assay error (n = 20) | | | Inter-assay error (n = 20) | | |
|---|---|---|---|---|---|---|
| | I | II | III | I | II | III |
| Mean value(pg/ml) | 36.2 | 121 | 630 | 40.4 | 117 | 618 |
| S.D | 2.9 | 4.9 | 23 | 3.1 | 5.8 | 29 |
| C.V% | 8.0 | 4.0 | 3.7 | 7.7 | 5.0 | 4.74 |

### 3.2. Structure analysis of cyp19a1a and Foxl2 gene

The predicted CpG rich region in *cyp19a1a* promoter (GenBank Accession no. AB303853) was 169 bp in length, including nine CpG sites, one cAMP- responsive element (CRE) and one Ad4 binding protein (Ad4BP) (Fig. 2A). This study found that the CpG site in position -223 bp of *cyp19a1a* promoter was located at a recognition sequence for cAMP-responsive element (CRE) and that the CpG site in position -141 bp was located at a recognition sequence for Ad4 binding protein (Ad4BP). Fig. 2B showed that exon1 of *cyp19a1a* coding region had eleven CpG sites, and seven of them were located at or near putative binding sites for a series of transcription factors such as CDPCR, GCN-4, AP-1, AML-1a, GATA-1, RREB-1 and GATA-2.

It was predicted that *Foxl2* gene had three CpG rich regions, F1 (including 23 CpG sites located at -77 bp to +234 bp), F2 (including 21 CpG sites located at +279 bp to +664 bp) and F3 (including 25 CpG sites located at +684 bp to +886 bp) delete. The conserved sequence, FH domain, of *Foxl2* gene was located at +139 bp to +468 bp, encoding 111 amino acids (Fig. 3).

### 3.3. Relationship between DNA methylation levels of cyp19a1a promoter and exon1 and cyp19a1a expression level during ovarian development stages of Japanese flounder

The PCR products by bisulfite modification were evaluated by agarose gel electrophoresis and the result showed that all products were in accordance with the anticipated objective strap size (Fig. 4A). Part of the sequencing diagram was shown in Fig. 4B. Evaluation of the efficiency of bisulfite treatment showed that C were all converted to T in all copies of three different samples of CpG dinucleotide sequence analyzed. This implies that the DNA modification procedure was very efficient.

The CpG dinucleotides methylation status of CpG rich regions in promoter and coding region of *cyp19a1a* was detected by bisulfite sequencing. The DNA methylation patterns were shown in Fig. 5. There are nine CpG sites in *cyp19a1a* promoter region. Differential methylation levels for each CpG position were determined. As is shown in Fig. 6A, the DNA methylation of every CpG site declined from stage II to stage III, and then increased from stage IV to stage V. In addition, the CpG sites at −223 bp and −141 bp were also in the cAMP-responsive element binding protein and the Ad4 protein binding sequence. The change trend of eleven CpG sites in *cyp19a1a* exon1 was shown in Fig. 6B. DNA methylation levels for each CpG position decreased at first, then raised, and the peak appeared at stage II, especially in specific seven CpG sites located at the sequence of transcription factor binding sites.

Results suggested that the average methylation levels of stage II and stage V were higher than stage III and stage IV as well as the methylation levels of promoter and exon1 (Fig. 7). The relationship between DNA methylation levels of *cyp19a1a*, its promoter and exon1 and gene expression were given in Fig. 7. Methylation level of *Cyp19a1a*, its promoter and exon1 showed strong negative correlation with gene expression; they were high in stage II and declined in stage III or IV, finally increased in stage V.

**Table 3**
Nucleotide sequences of primers used for BS-PCR in the experiment.

| Primer names | Sequence (5′-3′) | Product size (bp) | Tm (°C) | Accession no. |
|---|---|---|---|---|
| Cyp19a1a Promoter | F:TTTAATTAGATTTTTTTGATGTTTAG<br>R:CCTTAAAAAACTAAATTTATACCAA | 169 | 50 | AB304921.1 |
| Cyp19a1a Exon1 | F:TGTAGTTTTGTGTAGGTTGTTTTTA<br>R:ATTCTCCTATCTATATAACTCCAAA | 229 | 53.7 | |
| Foxl2 Exon1 | F:TAGTAGTAGTAGTATTTTTATTTAT<br>R:CTTACTAATAATATACTAATAAATAC | 339 | 49.2 | AB303854.1 |
| Foxl2 Exon2 | F:GAATAGTATTAGATATAATTTTAGTT<br>R:ATAAAACTAAACTACAAATACTTAAA | 261 | 49.4 | |
| Foxl2 Exon3 | F:TTTAGTTTTATGAATAATTTATGGT<br>R:AATAAAAACAATACATCATAAACAA | 346 | 48.9 | |

### 3.4. The change trends of methylation levels of CpG dinucleotides of three CpG rich regions and Foxl2 gene expression during ovarian development stages of Japanese flounder

There are three CpG sites in *Foxl2* gene of *P. olivaceus*. As shown in Fig. 8, the methylation level of each CpG site in *Foxl2* gene is relatively low as compared to that in *cyp19a1a* gene, but the total methylation levels of three CpG rich regions in *Foxl2* gene has the same change trend with those of *Cyp19a1a* gene. The methylation level of *Foxl2* gene is relatively high at stage II, then declined at stages III and IV, and returned to the level as similar as stage II at stage V (Fig. 9). Methylation level of *Foxl2* ORF had the same change trend with the total methylation levels of three CpG rich regions, while the methylation level of *Foxl2* 5'UTR is relatively high at stage II, then declined at stage III, and returned to the level as similar as stage II at stage IV, and has the highest methylation level at stage V. The relationship between DNA methylation level of three CpG rich regions and *Foxl2* gene expression was also given in Fig. 9. The *Foxl2* average methylation level was opposite to its gene expression during the ovarian development ($r = -0.91$). Methylation level of *Foxl2* ORF showed an opposite change trend with gene expression.

As shown in Fig. 10, the CpG sites of *Foxl2* 5'UTR have three transcription factor binding sites, located in the last four CpG sites respectively. The methylation level of the forkhead domain was analyzed. Forkhead domain has two parts, part of F1 (+139 bp to +234 bp) and part of F2 (+279 bp to +468 bp). In the histogram we can see that the methylation level of the forkhead domain, part of F1 and part of F2 has a significant difference at different stages, while more obvious changes of F1 part were observed from stage IV to stage V.

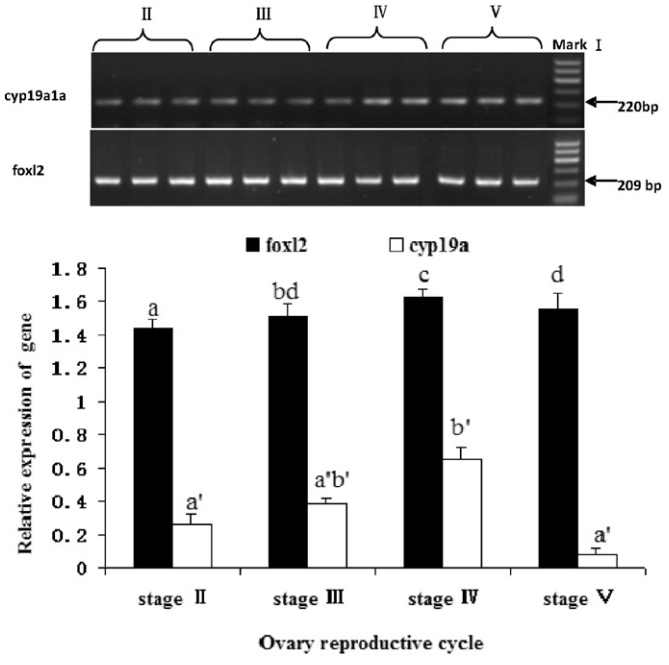


**Fig. 1.** The result of RCR production electrophoresis of *cyp19a1a* and *Foxl2* (A). Mark I was used to indicate the size of the fragment. Ovarian mRNA expression of *Foxl2* and *cyp19a1a* from ovarian development stages II to V in Japanese flounder (B). Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

### 3.5. The change trends of cyp19a1a and Foxl2 gene expression during ovarian development stages of Japanese flounder

Fig. 11 showed that the methylation level of *cyp19a1a* had strong negative correlation with its gene expression ($r = -0.80$); the change trend of *Foxl2* average methylation level was opposite to its gene expression during the ovarian development. The transcription level of *Foxl2* gene increased firstly and then descended with the tendency in accordance with the change trend of *cyp19a1a* gene.

### 3.6. The changing trend of serum $E_2$ level during ovarian development stages of Japanese flounder

Our previous study showed the change of serum estrogen level corresponding with ovarian development stage. In Fig. 12, it was shown that $E_2$ level was low in stage II and stage V, but its highest level was recorded in stage IV. The result showed that *cyp19a1a* expression level has the same change trend with $E_2$ level.

## 4. Discussion

In the present study, P450aromA (*cyp19a1a* gene) was highly expressed from stage II to stage V, it significantly increased from stage II to stage IV, and then slowly declined at stage V. Such results were similar to those previously reported in teleosts. In *Pelteobagrus fulvidraco*, transcript abundance of *cyp19a1a* was high in stage III to V during the ovarian cycle. The highest peak of *cyp19a1a* expression emerged at stage IV and the lowest expression level was recorded at stage VI (Liu et al. 2011). At stage I-III low level expression of *cyp19a1a* mRNA of *Epinephelus coioides* was recorded, and was enhanced significantly in stages IV and V, and then decreased considerably in stage VI (Hu et al., 2012). The serum $E_2$ content in *E. coioides* had a significant surge and was at its peak at stage IV along with oocyte development. In a study conducted on channel catfish (*Ictalurus punctatus*), *cyp19a1a* was found to be involved in endogenous E2 biosynthesis, thus participated in the whole process of ovary development. Our study found that *cyp19a1a* gene and $E_2$ content had the same change tendency. It has been reported that *cyp19a1a* expression plays a pivotal role in the processes of estrogen synthesis, which control ovary development,

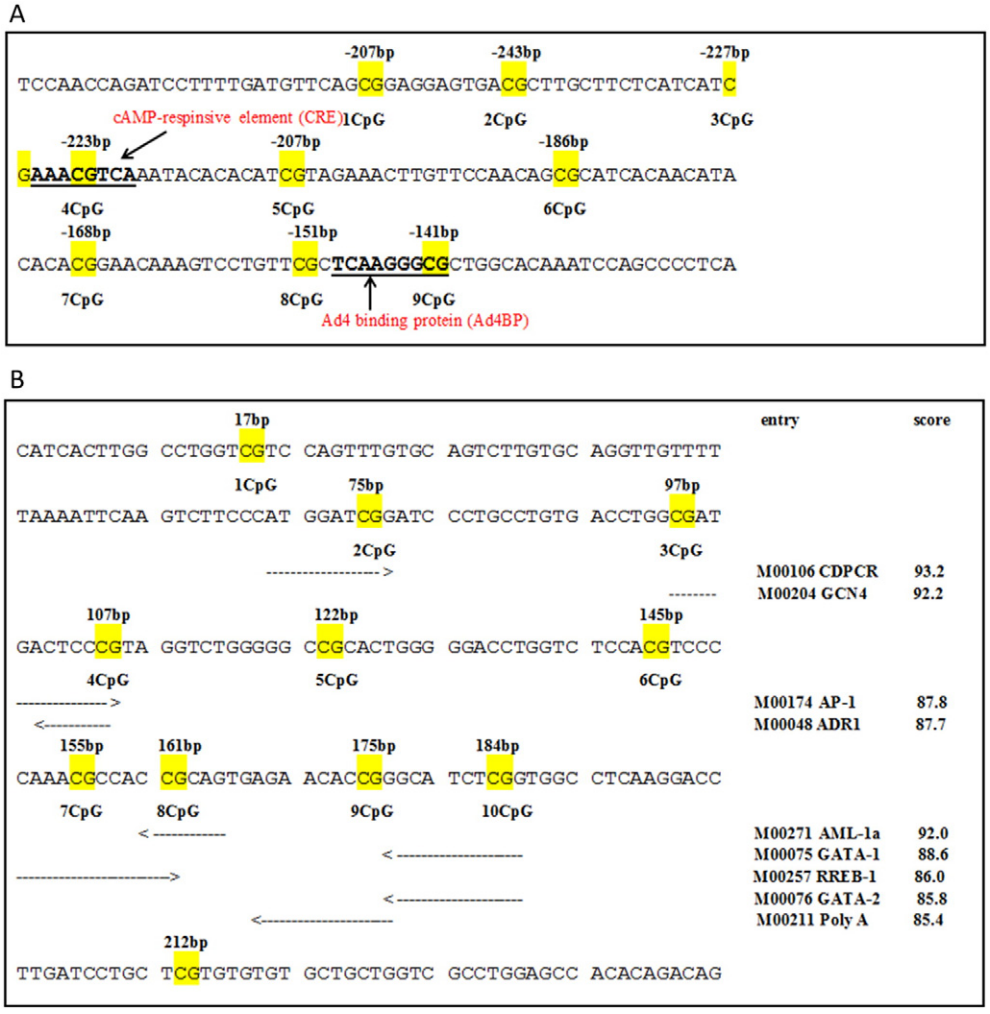


**Fig. 2.** The gene structure analysis of *cyp19a1a*. The yellow boxes indicate CpG dinucleotide sites on promoter of *cyp19a1a* gene (A). The binding sequences of the cAMP-responsive element (CRE) and Ad4 binding protein (Ad4BP) are marked with an underscore. Numbers with a minus sign indicate CpG positions with respect to the transcription starting site. The CpG sites in the exon 1 of *cyp19a1a* gene are marked with yellow boxes (B). The transcription factor is forecast as in the right of the gene sequence. The dotted line indicates the consensus binding sequence of the transcription.

maturation and female reproduction in teleosts (Guiguen et al. 2010). We could deduce that *cyp19a1a* played an important part in estrogen biosynthesis during the reproductive cycle of female Japanese flounder.

*Foxl2* gene was overexpressed in the reproductive period of female *P. olivaceus*, and the expression level of *Foxl2* was positively correlated with that of *cyp19a1a*. The studies on many mammal species such as rat (Pannetier et al. 2003), goat (Pannetier et al. 2005), human (Cocquet et al. 2002) and other vertebrates such as bird (Govoroun et al. 2004), turtle (Loffler et al. 2003), fish (Baron et al. 2004; Wang et al. 2004) and so on found that *Foxl2* mRNA was exclusively expressed in ovary during breeding season. Yamaguchi et al. (2007) documented that *P. olivaceus Foxl2* mRNA was expressed in small amounts in the gonads of genetic female juveniles, and was scarcely detected in the gonads of genetic male juveniles, while after the sex-reversal, *Foxl2* mRNA was highly expressed in phenotypic females but not in phenotypic males. This signified that *Foxl2* gene had a role to play in ovarian development. Transient

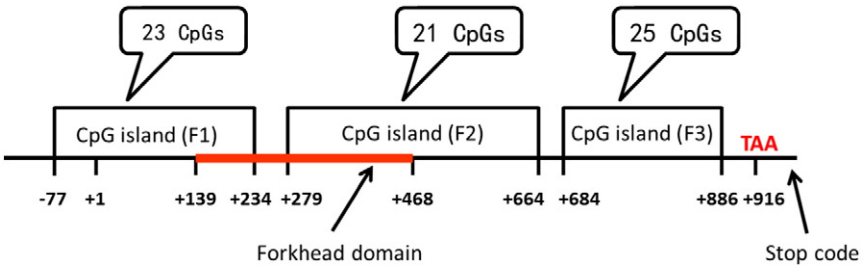


**Fig. 3.** The gene structure analysis of *Foxl2*. The long black line indicates the *foxl2* gene structure (C) and the three empty black boxes indicate the F1、F2 and F3 CpG rich region (i.e. CpG island). The FH domain (from +139 bp to +468 bp) is marked with a saffron yellow line, across F1 and F2 CpG rich region. The stop codon was located at +916 bp site.

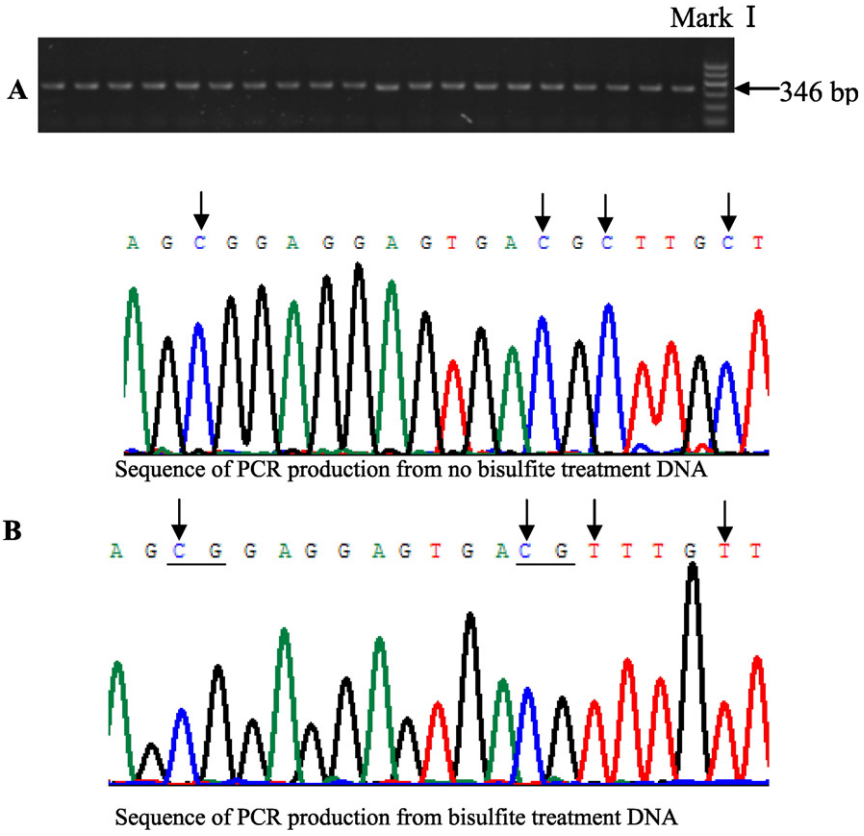


**Fig. 4.** The result of PCR production electrophoresis of bisulfite treatment DNA in CpG rich region (A) and its sequence (B).

transfection assay suggested that *Foxl2* could activate *cyp19a1a* mRNA expression directly (Yamaguchi et al. 2007), which is consistent with reports in goats (Pannetier et al. 2006) and tilapia (Wang et al. 2007). In addition, Wang et al. (2007) reported that two pathways are involved to mediate estrogen synthesis: 1) binding of Foxl2 to cyp19a1a promoter directly; 2) binding Foxl2 to Ad4BP/SF-1, transcriptional regulators, to

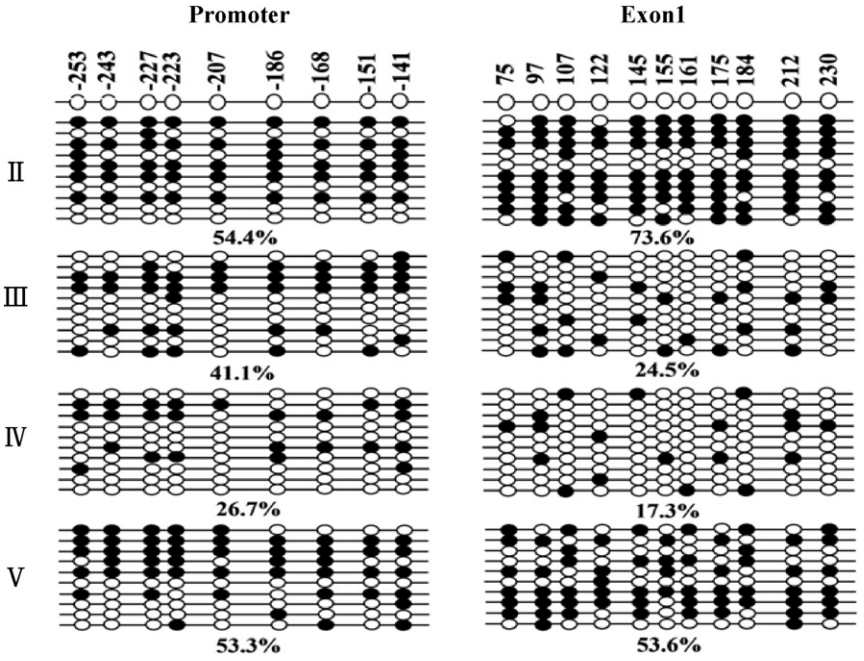


**Fig. 5.** DNA methylation patterns of promoter and exon 1 in *cyp19a1a*. Open and filled circles denote unmethylated and methylated positions, respectively. Each line represents one sequenced clone. Numbers with a plus or minus sign indicate CpG positions with respect to the transcription starting site. Average methylation was calculated for all CpG sites in each stage. 3–5 fish samples were used, and for each fish typically 8 clones (range 7–10) were used to determine DNA methylation levels.

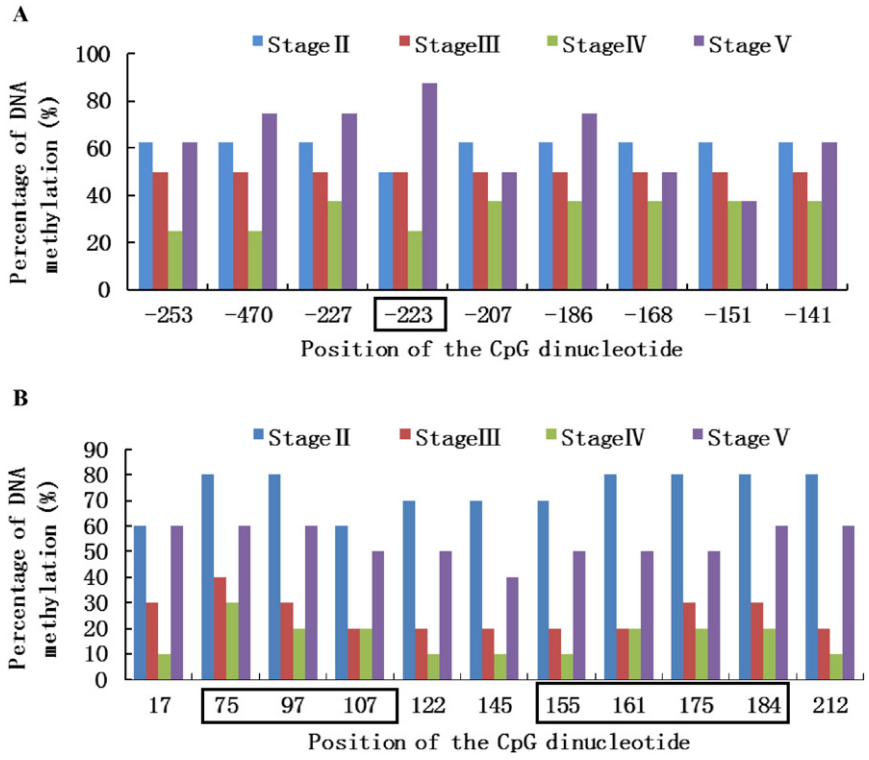


**Fig. 6.** The DNA methylation level of every CpG site of the promoter (A) and exon1 (B) of *cyp19a1a* at different ovarian development stage. The CpG sites in the red boxes were in the binding sequence of the transcription factors. The CpG site at -223 bp was in the cAMP-response element binding protein (CREB) binding sequence. The CpG site at -141 bp was in the Ad4 protein binding sequence. The seven CpG sites of *cyp19a1a* exon1 were located at the sequence of transcription factor binding sites.

enhance cyp19a1a transcriptional activity. DNA methylation levels of nine CpG sites in CpG rich region of *cyp19a1a* promoter were determined by DNA bisulfite modification method. The results showed that the change trend of total DNA methylation level of CpG island dropped first, and then rose, corresponding with ovarian development, and every CpG site basically had the same change trend. But it showed the opposite tendency for *cyp19a1a* transcription level, i.e., DNA methylation level was the lowest, but *cyp19a1a* mRNA expression was the highest at stage IV in female Japanese flounder. It was interesting that −223 bp CpG dinucleotide was located at the cAMP- responsive element binding protein recognition sequence. Earlier studies illustrated that the cAMP could activate *cyp19a1a* gene transcription activity by binding to the cAMP-like sequence of

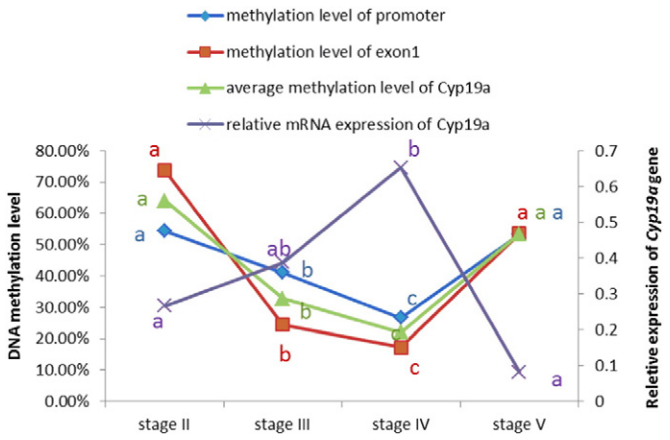


**Fig. 7.** The correlation between gene expression and CpG methylation level of *cyp19a1a* gene at different ovarian development stage. Values represent mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

*cyp19a1a* promoter in vitro (Yamaguchi et al. 2007). The −141 bp CpG site was located at Ad4BP recognition sequence. DNA methylation could make chromatin condensable and then repress transcription (Boerboom et al. 1999; Fitzpatrick and Richards 1991; Fürbass et al. 2001). It is speculated that in stages II and V, high CpG methylations at the position of −223 bp and −141 bp lead to the decrease of the combination rate of the cAMP and Ad4BP corresponding with their recognition sequences, and then lowered *cyp19a1a* transcription activity; while in stage IV, DNA methylation level decreased to the minimum, the binding rate of the cAMP and Ad4BP correspondence with their recognition sequences was improved and gene expressing activity also increased. But further studies are needed to verify the combination rate of the cAMP and Ad4 binding proteins.

It is evident that DNA methylation of the coding region could strongly repress gene expression (Irvine et al. 2002; Hsieh 1997). Site-specific methylation in the *Arabidopsis phyA*' gene coding region played a vital role in maintaining *phyA*' silencing. However, hypomethylation at a specific CG site could apparently activate phyA irreversibly (Rangani et al. 2012). In teleosts, the methylation levels of whole CpG sites of *cyp17-II* were inversely correlated with the expression of ovarian cyp17-II gene expression, which regulate the development of ovary (Ding et al. 2013). Our study identified the methylation level of eleven CpG sites in exon1 of the coding region of *cyp19a1a* gene. Results demonstrated that the methylation level of every CpG site in this region appeared to change regularly, and had negative correlation with the gene expression of *cyp19a1a*. We hypothesized that the DNA methylation pattern of exon1 could alter the efficiency of transcription. According to predicted putative transcription factors binding sequence, seven CpG sites in exon1 of the coding region of *cyp19a1a* gene were on the putative transcription factors binding sequence. In Nile tilapia (*O. niloticus*), the methylation of specific E-Box CpG site is negatively related to the mRNA expression of GH in pituitary gland (Zhong et al. 2014). This implied that the DNA methylation level of the seven CpG sites in coding

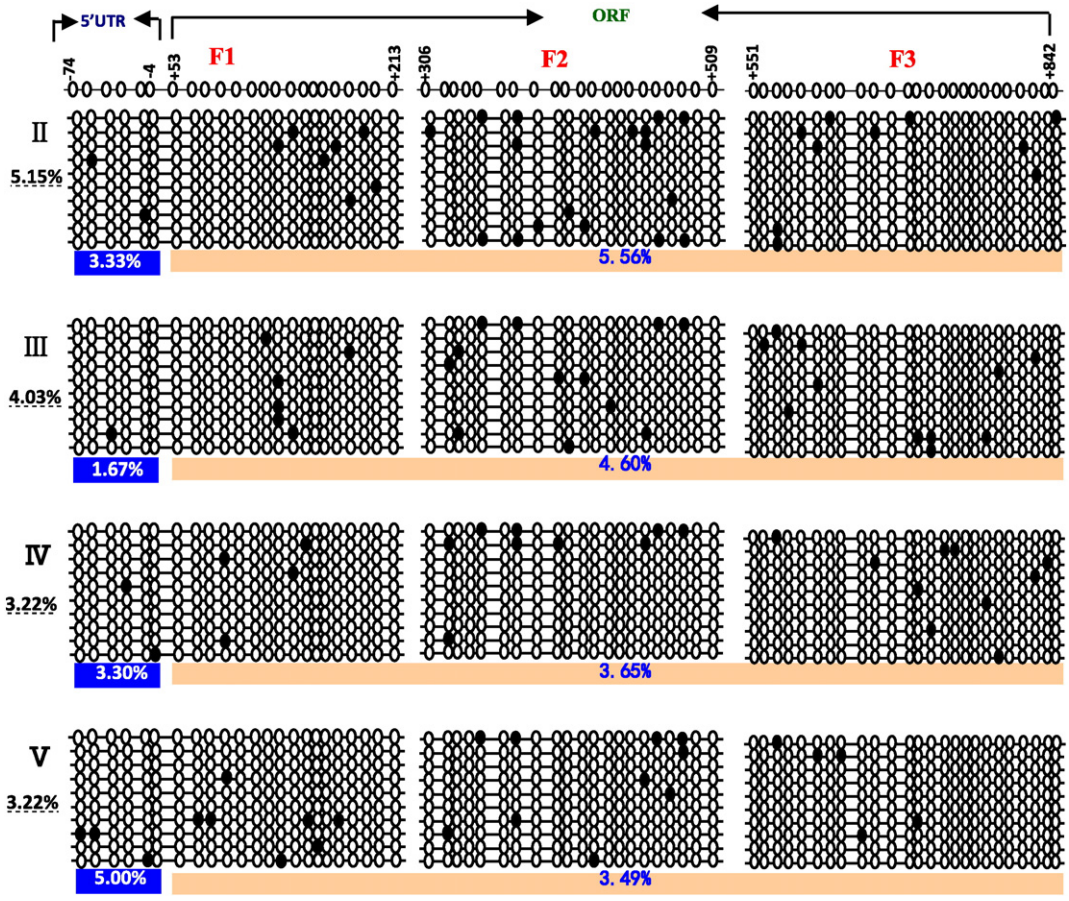


**Fig. 8.** DNA methylation patterns of three CpG islands in *Foxl2*. An open circle represents an unmethylated CpG, and a solid circle represents a methylated CpG. Each line represents one sequenced clone. The first line indicates the localizations of studied CpG sites related to the sequence of *Foxl2*. The percentage indicates the methylation level (under the line), calculated as the number of methylated CpG sites per total number of CpG sites in each stage.

region could alter the efficiency of transcription factor binding and affecting efficiency of gene expression finally.

*Foxl2* could regulate gene transcription either by binding directly to the promoter of *cyp19a1a* or interacting with Ad4BP/SF-1. We detected the DNA methylation patterns of three CpG rich regions in the coding region of *Foxl2* gene in the gonads of female Japanese flounder. The total DNA methylation level of the three CpG islands was low as shown in Fig. 9, but appeared regularly changing, and had negative correlation with the gene expression of *Foxl2* mRNA. The DNA methylation content was relatively high in the early stage of ovary development, and was minimum at stage IV and increased at stage V. This implied that the DNA methylation pattern of CpG sites in the coding region could influence *Foxl2* mRNA expression.

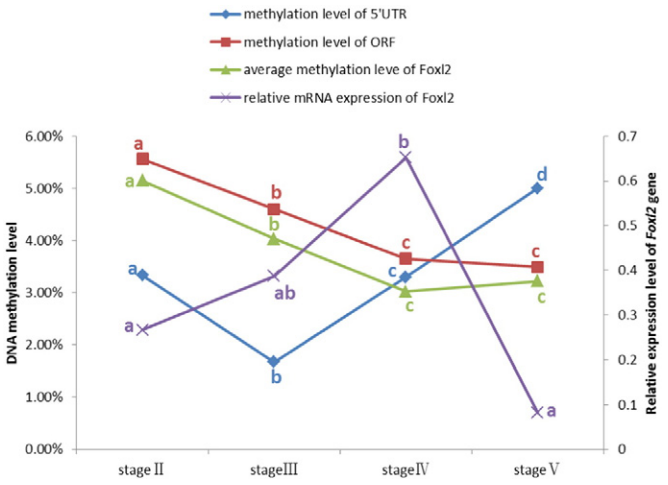


**Fig. 9.** The change trend of gene expression and CpG methylation level of *Foxl2* gene at different ovarian development stage. Values represent mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

## 5. Conclusion

This study investigated the expression patterns of *Foxl2* and *cyp19a1a* gene during the ovary development. The results revealed that the trend of *Foxl2* gene expression was consistent with that of *cyp19a1a* gene expression, entailing that *Foxl2* gene may be involved in the regulation of *cyp19a1a* gene expression. The DNA methylation level revealed that two CpG sites in promoter were located in cAMP-responsive element (CRE) and Ad4 binding protein (Ad4BP) recognition sequences, suggesting the influence on binding efficiency of CRE and Ad4BP, thus affecting gene expression. The whole methylation levels of CpG rich regions were inversely correlated with the *Foxl2* gene expression during ovary development, indicating that the DNA methylation pattern in the coding region could influence *Foxl2* mRNA expression. We speculated that epigenetic mechanism of *Foxl2* regulated *cyp19a1a* transcription: (1) *Foxl2* expression was regulated by the DNA methylation level of the CpG sites in its coding region; (2) the binding efficiency of the transcription factors in the promoter and coding region of *cyp19a1a* were influenced by DNA methylation. Our research may help to elucidate the molecular mechanism of fish development from the epigenetic point of view.

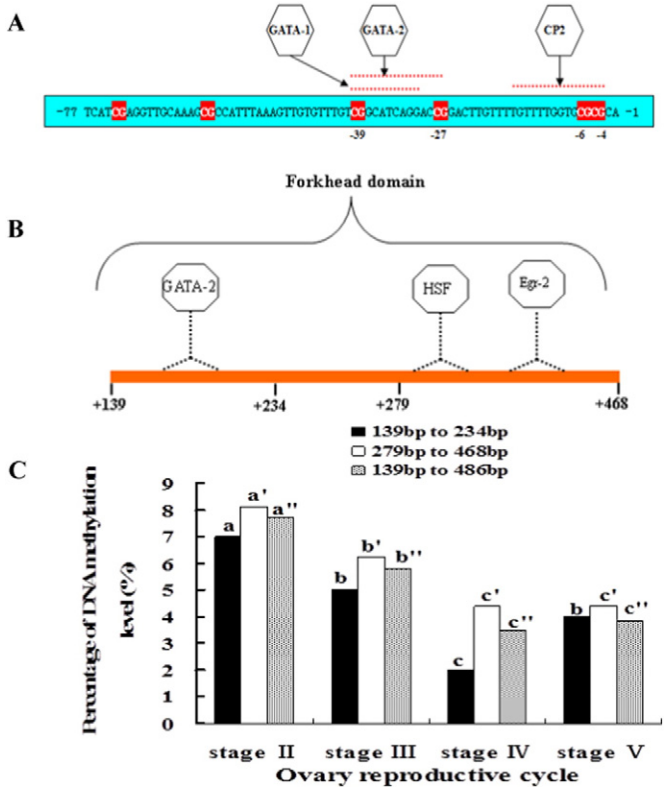


**Fig. 10.** The methylation level of the forkhead domain changed with the ovary development. The blue close box refers to part sequence of the *foxl2* gene (from −77 nt to −1 nt) with three transcription factor binding sites in the 5'UTR (A). Saffron yellow close box indicate the forkhead domain (from 139 nt to 468 nt) and the methylation level was divided into two parts, +139 bp to 234 bp and +279 bp to +468 bp respectively (B). The methylation level of every divided part at different ovarian development stage (C).

## Conflicts of Interest

The authors declare no conflict of interest.

## Acknowledgments

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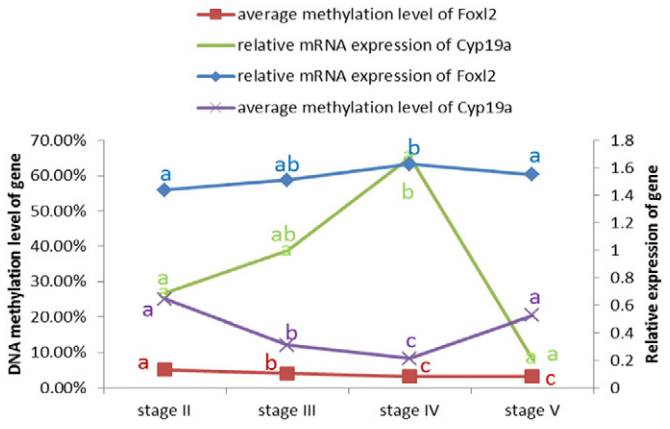


**Fig. 11.** The change trends of *cyp19a1a* and *Foxl2* at different ovarian development stage. Values as mean ± SEM. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

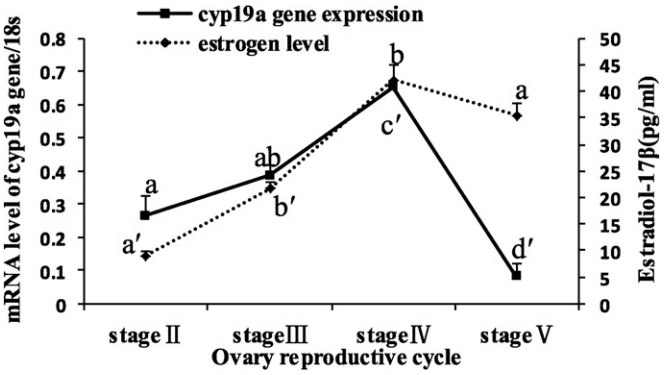


**Fig. 12.** The change trends of the *cyp19a1a* mRNA level and estradiol-17β concentration in ovary reproductive cycle.

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