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Study on intragenic methylation of the aromatase P450 gene from different rice field eel tissues

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The gonads of ricefield eels show differential expression of brain and ovarian aromatase P450 as compared other tissues. We determined whether these differential expression patterns were related to the level of genomic DNA methylation by detecting DNA methylation in different tissues with seven pairs of primers. As a result, we found that the two types of aromatases in eel testis and ovary were all methylated. By contrast, in peripheral blood, some regions of both aromatases were demethylated, and the CpG islands of ovarian aromatase in the kidney were demethylated. To determine if there were demethylation epialleles in the tissues expressing aromatase P450 gene methylation, we designed an artificial adaptor and a series of primers in a two-step amplification of the methylated tissue-specific aromatases. The results suggested that there was evidence of demethylation epialleles in most of the tissues where the aromatase P450 gene was methylated. Most of the demethylated regions in the blood were located at heterozygous methylation sites, whereas there were barely any demethylation epialleles in the methylated regions. This study demonstrated that the aromatase P450 gene was universally heterozygous in some regions of different tissues. In these tissues, the methylation state, demethylation state and extent of demethylation exerted some regulatory effects on the expression of aromatase. To determine whether the expression of brain and ovarian aromatase P450 was related to its differential methylation sites, the expression of the two types of aromatase was detected in different tissues using semi-quantitative PCR, and we found that both of aromatases was low expressed in most experiment tissues. The expression of ovarian aromatase was relatively high in blood and kidney and weakly detectable expression was found in other tissues. The brain aromatase was weakly expressed in all combinations. We suggested that gene expression of aromatase was weakly influenced by methylation of the internal sequence.

Keywords: Aromatase P450, methylation, ricefield eel, gene expression.

INTRODUCTION

Ricefield eels (*Monopterus albus*) belong to the *Monopterus* species, *Synbranchiformes* family, *Synbranchiformes* order, *Actinopterygii* subclass, and the *Osteichthyes* phylum. Widely distributed in countries and regions such as China, America and Oceania, ricefield eel is an important economic freshwater fish in China. It is an animal characterized by natural unidirectional sex reversal, which remains female since childhood to its first sexual maturation, but turns intersex and finally male after spawning. Therefore, ricefield eel species are important in fish breeding research to study sex determinative mechanisms.

Aromatase is an important member of the P450 family, which is a critical enzyme for estrogen synthesis and does so by catalyzing some androgens like testosterone and rostadienone into estrogens like estrone and estradiol. Thus, we chose the aromatase P450 gene as the target gene of eel related research.

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There are two types of the aromatase P450 gene found in research on fish, including ovarian aromatase P450 and brain aromatase P450 [1-4]. Yu et al. isolated ricefield eel ovarian aromatase P450 from fresh ovary, and identified this gene in the brain [5]. Li *et al*. [6] isolated ricefield eel brain aromatase P450, and found relatively high expression in the brain and testis, low expression in skin, and no detectable expression in the liver, heart, small intestine and muscle. We conclude from the above observations that both types of aromatase P450 expressed in ovary and testis are different genes, and are both expressed in the ricefield eel brain. Yann Guiguen *et al.* believed that ovarian aromatase P450 was the key to trigger and sustain ovary and testis differentiation, since blocking the expression of ovarian aromatase 450 could induce virilization [7]. Diotel *et al*. believed that brain aromatase P450 was only expressed in radial glial cells and distributed in neural tissues like the hypothalamus, spine and preoptic area [7]. This group also believed that brain aromatase P450 was controlled by estrogen and aromatized androgen, and involved in the reproduction of fish in addition to regulating brain sexual differentiation and sexual

activities. These observations were discordant with the conclusions of others who also thought that brain aromatase P450 was also expressed in the testis [3]. There has not been a reasonable explanation for the differences.

DNA methylation is an important modification of nucleic acids that shuts off gene activity, while demethylation induces gene reactivation and expression. Therefore, DNA methylation is a critically important area of epigenetics research, with relevance to understanding diseases like cancer, senescence and dementia. The major product of DNA methylation is 5-methyl cytosine (5-mC), which is ubiquitous in the genome of eukaryotes. The latest research findings in the field of human genomic methylation, suggests that most cells show stable methylation patterns, and 70-80% of the CpGs are methylated. It is believed that although every CpG can potentially change their methylation status, only a fraction of them are regulated and ready to change their methylation status [9]. Other groups are convinced that CpG methylation and demethylation are associated with gene expression although the degree of DNA methylation is not well correlated with gene expression [10].

To determine whether the differential expression of aromatase P450 in the gonad organs and other tissues is related to its differential internal methylation status, our current research study used methylation sensitive *Hpa*II to treat genomic DNA of the ovary, testis, degenerated ovary and other tissues. Further, we amplified them by segments and investigated the methylation status of both types of aromatase by comparing the amplification products before and after digestion. Additionally, we annealed the digested genomic DNA with an artificial adaptor. Appropriate primers were designed according to the sequences of the adaptors to amplify these fragments so that we could verify whether these methylation sites were in the apparent heterozygous state, which would imply the coexistence of both methylation and demethylation.

MATERIALS AND METHODS

The search for a CpG island

There were two searching results relevant to the genomic DNA sequence of aromatase P450 found on the NCBI website. One of them was EU840259, with a full-length sequence of 4869 bp, which was of the ovarian type, and uploaded on July 29, 2008. The other was EF640940, which was of the brain type, with a full-length sequence of 3754bp, and uploaded on May 19, 2008. No CpG island sequences were found following an analysis with the genomic DNA CpG island analyzing software provided by the European EBI website (http://www.ebi.ac.uk/emboss/cpgplot/).There were a total of 11 CCGGs found in the sequence in EU840259 and 10 CCGGs short sequences in EF640940. We designed several pairs of primers (Table.1) for each sequence that essentially covered all of the potential methylation regions. The orarian aromatase P450 is divided into 3 amplified regions and the brain aromatase P450 is divided into 4 amplified regions.

Genomic DNA extraction in different ricefield eel tissues

The ricefield eel samples were collected at September 2014. Ten samples (five male and five female)were obtained from Central Fish Nursery Farm of Fresh Water Fishery Research Institute of Chinese academy of Fishery Sciences.

In this procedure, 450 μl STE buffer (150 mmol/L NaCl, 50 mmol/L Tris and 1 mmol/L EDTA), 12.5 μl 10% SDS and 10 μl proteinase K (20 mg/ml) were added to 0.1 g tissue (muscle, liver, kidney, blood, testis, ovary and degenerative ovary), mixed and digested at 55°C overnight. The mixture was extracted with phenol/chloroform, precipitated with equal volumes of isopropanol, and then centrifuged at 12000 rpm for 20 min. The pellet was washed twice with 70% ethanol, dissolved in 60 μl double distilled water, and resolved on a 0.8% agarose gel to measure the mass and concentration of DNA. Since eels are bred in captivity as edible fish in China, and samples were taken after sacrificing eels by cervical dislocation, there were no ethical issues with regard the experimental animals involved in this research.

Genomic DNA digestion in different ricefield eel tissues

The total volume of the digestion system was 20 μl, including 2 μl sample genomic DNA, 2 μl (10 u/ul) restriction endonuclease *Hpa*II, 2 μl 10x digestion buffer, and 14 μl double distilled water. One blank control without restriction endonuclease and the other control with restriction endonuclease *Msp*I were included in each group. The mixture was digested for 4 hrs at 37°C.

Sequence amplification and methylation statistics of aromatase P450

The total volume of the amplification system was 25 μl, including 1.0 μl template, 2.5 μl 10×PCR buffer, 5 pmol forward primer, 5 pmol reverse primer, 1.5 μl dNTPs (2.5 mmol), 2U Taq DNA polymerase, and deionized water to 25 μl. The

amplification procedure was as follows: an initial denaturation at 94°C for 2 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 58°C for 45s, elongation at 72°C for 1min), and a final elongation at 72°C for 10 min. The PCR products were detected on 1.0% agarose gels.

Table 1. Primers for amplification of different regions in brain and gonadal aromatase P450.

Primer for ovarian aromatase P450		Primer for brain aromatase P450	
F11	tgtttaccacaacgagggaggg	F21	Gtgagcttttgaagctactgtaatg
R11	tttaaatgtccttgggcaaac	R21	tatgagcctgtgcccttgact
F12	ggttggagtgtattgggatgga	F22	cagtggtcactaatcaacttgg
R12	gatgaaactctggaccgtctgt	R22	cagcaaactgaggacatccac
F13	gacaggtgcccagtcctttctt	F23	gttaaccctgtgtgcagagaa
R13	tcccaattcctcgtgtcacgct	R23	cctgtagcaactgcagctcc
		F24	gaccgatgatatcatagatggc
		R24	tcaagcccttatgagggcaaact

To measure aromatase P450 methylation, the common PCR products of the blank control group without restriction endonuclease, and the group digested with restriction endonuclease were designated as methylation regions, while the regions in the PCR product of the blank control group, but not in the product of the digested group were demethylation regions.

Epiallele amplification of aromatase P450

The 20 μl the digestion product was precipitated with 50 μl of 100% ethanol, centrifuged at 12 000 rpm for 10 min to obtain the pellet, and dried at room temperature to remove ethanol. Next, 14 μl of distilled water, 2 μl of 10x T4 ligase buffer, 2 μl of T4 ligase and 2 μl of the adaptor sequence (50 mm adaptor) were added, mixed and incubated at 16°C for 4 hrs. The adaptor sequence was 5-cgagtcggctggctcctcaattgtatc-3, and 5-tcagccgaccgaggagttaacatag-3.

The total volume of the first step amplification was 25 μl, including 1.0 μl of the template, 2.5 μl of 10×PCR buffer, 10 pmol of the first step primer (caattgaggagccagccgact and the forward primer, 5 pmol each), 1.5 μl of dNTPs (2.5 mmol), 2U taq DNA polymerase, and deionized water to a final volume of 25 μl. The amplification procedure was as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 58°C for 45s, elongation at 72°C for 1 min), and a final elongation at 72°C for 5 min.

The total volume of the second step amplification was 25 μl, including 1.0 μl of the PCR product from the first step, 2.5 μl of 10×PCR buffer, 10 pmol of the second step primer which was one of the four primers of: ttgaggagccagccgactcggt, ttgaggagccagccgactcgga, ttgaggagccagccgactcggc, ttgaggagccagccgactcggg and the forward primer at 5 pmol each, 1.5 μl of dNTPs (2.5 mmol), 2U of Taq DNA polymerase and deionized water to a final volume of 25 μl. The amplification procedure was an initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification as follows: denaturation at 94°C for 30 s, annealing at 66°C for 45s, elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min. The PCR products were detected with a 1.5% agarose gel. To confirm whether the PCR product was the target digestion product, the PCR product was annealed to a T vector and subsequently sequenced.

Semi-quantitative PCR of aromatase P450

Samples of different tissues of ricefield eel was added to 500μL RNAiso Plus reagent. and was incubated for 5minutes at room temperature. The mixture was added 100μL of chloroform and shaked vigorously and then was centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase of the sample was removed into a new tube. Equivalent volume of 100% isopropanol was added and the tube was shaked vigorously. The sample was centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was collected and washed by 75% ethanol (500μL). The sample was centrifuged at 7500g for 5 minutes at 4°C and the wash was discarded and the residue was dried. The RNA pellet was then dissolved in DEPC water and was detected by electrophoresis analysis in 1.0% Argrose gel.

cDNA synthesis was according to M-MLV First strand cDNA Synthesis Kit(Sangon Biotech, SK2435).

Semi-quantitative PCR primers: actin, cttcgacatcacggctgatgg/caggacctgtatgcttcagg; brain aromatase P450, gttaaccctgtgtgcagagaa/ cctgtagcaactgcagctcc; ovarian aromatase P450, gacaggtgcccagtcctttctt/tcccaattcctcgtgtcacgct.

Adjust the concentration of the different cDNA

and the primers to make sure all amplification have the nearly equivalent result, then detect the brain and ovarian aromatase P450. 0.5μL Primers(10mmol/L), 0.5μL dNTPs(10mmol/L), 0.5μL *Taq* enzyme(5U/L), total volume 25.0μL. 94.0ºC for 5minutes, (94.0 ºC for 30s, 58.0 ºC for 30s, 72.0 ºC for 45s) 29 cycles, 72.0 ºC for 5minutes. Electrophoresis analysis in 1.5% Argrose gel.

RESULTS

Methylation detection of different regions in aromatase P450 sequence

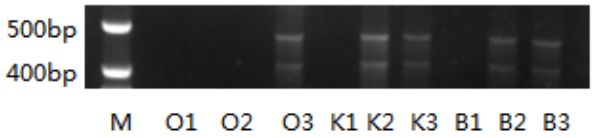

Fig. 1. Methylation amplification of aromatase from different tissues; B:blood; O: Ovary, K: Kidney: 1:digested with MspI, 2: digested with Hpa I 3: contrast.

Figure 1 represents the amplification result of region 3 in the ovarian aromatase P450. There was no band in the lanes digested with MspI of three samples, while there were bands seen in the control group. But there were bands seen in the lanes digested with Hpa II of kidney and blood. This suggested that there was demethylation of region 3 in the blood and kidney. There were no bands in ovary suggesting methylation status .

Methylation analysis of aromatase P450 in different tissues

Table 2 described the statistics of methylation in different tissues. It could be seen that most CCGG regions in both types of aromatase P450 were methylated. All of the CCGG regions of both aromatase P450 genes found in the testis, ovary, degenerative ovary, muscle and liver were methylated. Ampilied region 2 and 3of ovarian aromatase P450 of blood were demethylated while region 1 was methyalted. Ampilied region 1 and 2 of ovarian aromatase P450 of kidney were methylated and region 3 was demethyalted. Ampilied region 2 and 4 of brain aromatase P450 of blood were demethylated while region 1 and 3 were methyalted. All amplified regions of brain aromatase P450 of kidney were methylated. The result of ten samples suggested methylation patterns of two genes in different tissues may be universal

Table 2. Methylation analysis of two types of aromatase P450.

	Ovarian Aromatase P450			Brain Aromatase P450			
Amplified region	1	2	3	1	2	3	4
Blood	+	—	—	+	—	+	—
Liver	+	+	+	+	+	+	+
Kidney	+	+	—	+	+	+	+
Muscle	+	+	+	+	+	+	+
Ovary	+	+	+	+	+	+	+
Testis	+	+	+	+	+	+	+
Degenerated ovary	+	+	+	+	+	+	+

Table 3. Methylation analysis of the two types of aromatase.

	Ovarian Aromatase P450			Brain Aromatase P450			
Amplified region	1	2	3	1	2	3	4
Female	+	—	—	+	—	+	—
Male	+	—	—	+	—	+	—

Table 3 show intragenic methylation patterns of two aromatase P450 gene of blood from five male individials and five female individual. No differences were found between them.

Amplification of the aromatase P450 epiallele

If there is indeed coexistence of methylation and demethylation epialleles of a diploid nature, methylation fragments should be theoretically detected after digestion and amplification. It transpired, that we did not know if there were also demethylation epialleles in the above mentioned methylation positive samples. To further study these samples and to determine if there were demethylation epialleles, we designed an artificial adaptor that could be annealed with the genomic fragments that were digested by *Hpa*II. Moreover, we designed a first step amplification primer according to the adaptor sequence. In case there were four possible bases downstream of CCGG, we conducted a two-step amplification of the samples that were connected with adaptors, and sequenced the amplification product. If there was an obvious amplification band of the second step primer (with anyone as its selective base) in the amplification

result, we concluded coexistence of methylation and demethylation epialleles.

Table 4. Amplification of the epiallele of two types of aromatase.

	Ovarian Aromatase P450			Brain Aromatase P450			
Amplified region	1	2	3	1	2	3	4
Blood	—			—		—	
Liver	—	—	+	—	—	—	+
Kidney	—	+		—	+	—	+
Muscle	—	+	+	—	+	—	+
Ovary	—	+	+	—	+	—	+
Testis	—	+	+	—	+	—	+
degenerated ovary	—	—	+	—	—	+	+

In Table 4, (+) means that there was an amplification product, (–) means that there was no amplification product, and blank represents regions demonstrated to be de-methylated in the previous experiment. It could be seen that most regions that were de-methylated in the blood had demethylation epialleles in other tissues, while most methylated regions in the blood did not have demethylation epialleles. Thus, we believe that there might be two types of CCGG region in aromatase P450; one of the regions is essentially permanently methylated, while the other is dynamically methylated. Furthermore, they are relatively stable. However, there is also a very low rate of demethylation in the permanently methylated regions, and complete methylation in dynamically methylated regions of some tissues.

Semi-quantitative PCR of two aromatase P450

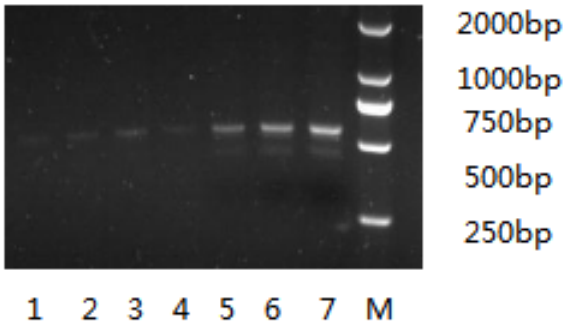

Fig. 2. Semi-quantitative PCR of ovarian aromatase P450 in seven tissues. 1: degenerated ovary, 2: muscle, 3: liver, 4: testis, 5:ovary, 6: kidney, 7: blood, . Ovarian aromatase P450 expressed more in peripheral blood, less in kidney and ovary, little in other tissue.

According to our previous experience, using different pairs of primers to detect the expression level of a same gene by quantitative PCR will lead to different results. To avoid this, we use the more easy and intuitive semi-quantitative PCR to detect the expression differences of aromatase P450 in different tissues. We use *actin* of ricefield eel as inner control and adjust the amplification factors to get the consistent results in different tissues, and then detect the expression of two types of aromatase P450. The result suggested that brain aromatase P450 expressed rarely in all experiment tissues. The highest expression level of ovarian aromatase P450 shown in Fig.2 was found in peripheral blood, followed by kidney and rarely in the rest. A slight difference in expression of ovarian aromatase in blood and kidney was found among ten samples, but higher than other tissues. Difference in other tissues can't be detected because of low expression. We also can't find difference of brain aromatase among ten samples with the same reason.

DISCUSSION

The result that most of CCGG regions of two aromatase P450 genes were methylated, was consistent with the conclusions from a previously published human genomic study (Michael, *et .al*, 2013)[9]. The result suggests that most CCGG regions in the ricefield eel genome were most likely methylated. In the methylation study of the human genome, the methylation of CpG islands is normally given greater emphasis. However, since there are no CpG islands in the both aromatase P450 sequences, we believe that part of the CCGG sequence outside of the CpG islands are involved in the regulation of methylation.

Our result of methylation research contradicts observations made by Yu *et. al.*, [5] and Li *et al.*, [6]. The difference is that we can't find intragenic demethylation of ovarian aromatase P450 of which the expression can be detected in ovary. But the result of semi-quantitative PCR shown expression of ovarian aromatase P450 can be detected in ovary, which was consistant with that of Yu *et al*. [5]. So we think there was no direct correlation between the extent of methylation of the internal sequence and the expression of both of these genes in these ricefield eel tissues（testis, ovary, degenerative ovary, muscle and liver). From Figure 2, we found the expression of ovarian aromatase in kidney was slightly lower than that in blood. Because amplified region 2 was methylated in kidney while

demethylated in blood, we think intragenic methylation can effect the expression of some genes to some extent in special tissues.

Nearly all tissues can detect the weak expression of brain aromatase P450. High intragenic demethylation of brain aromatase P450 in blood don't improve the expression of the gene. Nicolas Diotel *et al.* held the opinion that brain aromatase is directly related to adult sex hormone levels, and is only expressed in the adult brain tissue [7]. However, this research was not consistent with his conclusion. We tend to think that the high expression of brain aromatase P450 in the brain tissue should require the addition of regulation mechanisms and intragenic methylation may have weak regulatory effect on the expression.

Both research reports of Yu *et. al.* [5] and Li *et al.* [6] don't involve the expression of both aromatases in blood. The result shows that the tissue in which expression of ovarian aromatase P450 is the highest is blood. It is consistent with the intragenic demethylation of the gene. We guest that the activity of aromatase P450 in the blood should be relatively high, playing an important role in lowering blood androgens. To confirm whether there was a difference in the methylation status between either type of aromatase in male and female eel blood, we collected and compared the blood of male and female eels individually, but failed to find a difference. Thus, the expression of blood cell aromatase P450 had no direct effect on the sexual reversal of the eel, and the existence of other effects remains to be further elucidated. Guiguen *et al*. [8] believed that ovarian aromatase P450 played a role in testicular differentiation, since the decline in aromatase P450 was essential for testicular development. We don't think the aromatase in blood plays an important role in sex reversal of rice field eel. Maybe their main role is to maintain stable blood concentration of hormone [8].

Zhang Y *et al.* [18] considered that the methylation of the CpG island of long-distance cAMP element and the TATA box of the short-distance SF1/Adrenergic Binding Protein 4, acetylation and trimethylation of histone 3 in regulation region I and II and phosphorylation of cAMP element binding protein could affect the expression of aromatase . Our result suggested that inner methylation and demethylation of CpG island of aromatase P450 may also have slightly regulation. The biological significance of this kind of regulation needs further digging [18].

The study shows both aromatase P450 genes in some cells is apparently heterozygous, although we cannot be certain that aromatase P450 is at an apparent heterozygous state in every cell of the tissues with the allele.

Most of the reports regarding epialleles were focused on paramutations [11-16]. Arteaga-Vazquez *et al.* [16]. reported a pair of epialleles "b1" (low expressed B' and high expressed B-I) specifically expressed in maize. Methylation of a seven-repeat sequence located 100 Kb upstream of the b1 coding region played an important role in establishing and maintaining B' gene silence. Arteaga-Vazquez *et al.* [16] and Wolfgang & Joachim [17] reported a pair of epialleles P1-rr and P1-pr in the Myb-like transcription factor, which mediates the coloring of flowers in maize. The difference between them was the methylation state in the regulatory regions [17].These results were collectively focused on the methylation study of some special sequences, and were mostly based on previous studies with no direct reference value for the methylation state and function of most genomic CCGG regions. Our results showed that there were corresponding demethylation sequences for partially methylated genomic CCGG regions of aromatase P450. Consequently, we believe that epialleles could exist in many areas outside the regulatory region, which might serve to regulate gene expression.

There are many reports studying aromatase in fish, and most results are paradoxical, reflecting a very complicated mode of regulation that has evolved. This research showed that aromatase is a crucial enzyme that is not only regulated by its upstream and downstream regulative sequences, but also maybe by the methylation and demethylation of its internal CCGG regions. The apparent heterozygous state of partially methylated regions might be vital to the regulation of gene expression.

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