MOLECULAR CHARACTERIZATION AND EXPRESSION ANALYSIS OF *OREOCHROMIS NILOTICUS* SERPIND1 (HEPARIN COFACTOR II) CDNA

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ABSTRACT

Heparin cofactor (HCII) is a member of serine protease inhibitors (SPIs) family of genes which play a fundamental physiological role in different biological processes including hemostasis. Therefore, this study aimed to cloning a partial nucleotide sequence of Nile Tilapia HCII cDNA and examine its expression level in the liver tissue following the intracoelomic injection of benzo-a-pyrene BaP. The cloned nucleotide sequence contained 972 bp representing a 666 bp of the Open Reading Frame (ORF) encodes for 221 amino acids and a stop codon and a 306 bp of the 3′ noncoding region. The amino acids sequence comparison and phylogenetic tree analysis showed a close relationship between tilapia and *Pundamilia nyererei* serpind1 sequences. Realtime-PCR results showed high expression level (6.2) of Serpind1 mRNA in the liver of the BaP-treated fish compared with the control group suggesting the physiological role of HCII in tilapia. The study proposed the use of tilapia fish as a mammalian hemostasis model depending on the hemostatic genes homology analysis.

Key words: HCII, Nile Tilapia, BaP, Cloning, Realtime-PCR.

INTRODUCTION

Serine Proteinase INhibitors (Serpins) are a huge family of protease inhibitors having around 350–400 amino acids length. Most of the serpin family members involved in angiogenesis, blood coagulation, inflammation, and tumor suppression through inhibiting the activity of serine proteases (SPs) (Heit *et al.,* 2013). Some serpins playing an immense role in migration, phagocytosis, and elimination of cancerous cells (Mangan *et al.,* 2008).

'SERPIN' was designated as the gene symbol for humans and other species. Based on phylogenetic relationships, the serpin superfamily is divided into 16 clades named A–P, with clades A–I representing human serpins (Silverman *et al.,* 2001). There are a variety of members being in each clade, for example, in the symbol SERPINA1, the 'A' referencing the clade and the '1' is the gene number within the clade (Wright, 1993). Up to date, around 3000 serpin genes were cloned from many species, including higher animals, plants, and viruses (Gagaoua *et al.,* 2015).

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The Heparin Cofactor II (HCII) gene (known as SERPIND1 in humans) is a serpin that belongs to clade D or group 2. It has been characterized in many vertebrate species, such as humans, rabbit, cow, African clawed frog, European flounder, and zebrafish (http://www.ncbi.nlm.nih.gov/entrez/). The main function of HCII is inhibiting thrombin (Parker and Tollefsen, 1985). Thrombin is involved in many physiological processes including coagulation, and thus HCII controls all the biochemical processes in which thrombin is involved (He *et al.,* 2002).

Nile Tilapia (*Oreochromis niloticus*) is an African freshwater cichlid which affected by toxicants that exist as complex mixtures closely associated with the sediment such as heavy metals and polycyclic aromatic hydrocarbons (PAHs). Benzo-a-pyrene (BaP) is an omnipresent Polycyclic Aromatic Hydrocarbon (PAH) which generated as a byproduct of organic combustion and has gotten the foremost consideration in fish species, with a major focus on hepatic metabolism (Varanasi *et al.,* 1989). Previous studies showed the Carcinogenic effect of BaP to fish following oral (Hendricks *et al.,* 1985) and waterborne exposures (Hawkins *et al.,* 1988).

BaP is a ligand of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor found in the inactive form as a cytosolic complex with two 90 kDa heat shock proteins (HSP90) and c-SRC protein kinase (Meyer and Perdew, 1999). Upon ligand binding, AHR dissociates from the heteromeric

complex and translocates into the nucleus to dimerize with the AHR nuclear translocator (ARNT) to form the ligand-bound AHR/ARNT complex. The ligand/AHR/ARNT complex recognizes and binds to specific xenobiotic responsive elements leading to the transcriptional activation of several genes including members of the cytochrome P450 family of enzymes (e.g. CYP1A1, CYP1B1) which in turn catalyze the conversion of BaP into reactive metabolic intermediates.

The use of molecular biomarkers such as gene expression changes in the field of marine environmental monitoring playing an important role in recognition of exposure and impact of different pollutants (Malins and Ostrander, 1994). Although Serpins are a group of extensively studied protease inhibitors (Harish and Uppuluri, 2017), there is very little information about their role in *Oreochromis niloticus*. Therefore, the present study aimed at cloning of partial serpind1 cDNA sequence from the liver of *Oreochromis niloticus* fish following the intraperitoneal injection of benzo (a) pyrene (BaP) and investigate BaP-induced serpind1 gene expression in liver tissue of the same fish. This will facilitate future research on the structure of serpind1 gene family in tilapia fish and the molecular mechanisms by which BaP affecting the homeostasis process in tilapia.

MATERIALS AND METHODS

1. Experimental fish
Thirty male nile tilapia (500 ± 10 g) were provided by a local fish farm in Japan. Fish were acclimated for one week at 28 °C with normal feeding. After the acclimatization period, fish were divided into control and treated groups with 15 fish in each group. The treated group were injected intracoelomically with BaP (100mg/ kg body weight) while the fish in the control group were injected with the corn oil as a vehicle. One day after the injection, fish in both groups were sacrificed; liver samples were taken, stored in liquid nitrogen at -80°C till analysis.

2. Molecular Characterization of a partial tilapia Serpin1D1 cDNA sequence.

2.1. RNA extraction and Reverse transcription
Isogen reagent (Nippon Gene Co., LTD.) was used for total RNA extraction from the liver tissue of tilapia according to the manufacture's protocol. Spectrophotometer was used to assess the RNA samples purity and concentration as described by Sambrook and Russel (2001), A260/A280 ratio ranging from 1.7 and 1.9. cDNA was synthesized using Superscript II reverse transcriptase (GibcoBRL, USA) to generate 5'-RACE-Ready and 3'-RACE-Ready first strand cDNA using a SMART[TM] RACE cDNA amplification kit (Clontech, USA) according to the manufacture's protocol.

2.2. Oligonucleotide primers and PCR amplification of Tilapia Serpin1D1 cDNA
Two primer sequences, sense (F) and antisense (R), were designed using highly conserved regions of different fishes serpind1 sequences retrieved from GenBank/NCBI data bank. The primers sequences are; Forward primer (5'- AAGAGTGTGGATCC AAATATGG -3'), reverse primer (5'-AGAAATGACTGTTTCACTTCAGCC-3') .PCR reaction was carried out in a final volume of 25 μl containing the DNA sample (1μl), forward and reverse primers (1μl each), PCR master mix (12.5 μl) and distilled water (9.5 μl). The cycle conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, then 72° C for 5 minutes for further extension. The PCR product was electrophoresed on 1% agarose gel and the resulting fragments were visualized by UV transillumination. The DNA band of the 972 bp length was excised, purified using GFX PCR DNA and a gel band purification kit (GE Health Care, UK), cloned into PT7BlueT- vector (Novagen, USA). Direct sequencing for the Purified plasmids was done by dye terminator cycle sequencing using an ABI PRISM dye terminator cycle3130 xl DNA sequencer.

2.3. Phylogenetic analysis of tilapia Serpin1D1
The following serpind1 mRNA sequences were retrieved from the Genbank and used to assess the phylogenetic tree of serpin1D1 gene family; XM_026186216 (*Astatotilapia calliptera* serpind1), XM_012865588 (*Fundulus heteroclitus* serpind1), XM_005924414 (*Haplochromis burtoni* serpind1),XM_017412364 (*Kryptolebias marmoratus* serpind1),XM_004547002 (*Maylandia zebra* srpind1), XM_011478884(*Oryzias latipes* serpind1), XM_024275125(*Oryzias melastigma* serpind1), XM_028577377 (*Perca flavescens* serpind1), XM_005738200 (*Pundamilia nyererei* serpind1)

3. Serpind1 gene expression in tilapia liver after intracoelomic injection of BaP.
RT-qPCR was used to detect the transcript expression pattern of liver- serpind1. The reaction was done using Fast Start Essential DNA Green Mater (Roche, 06402712001) and LightCycler Nano system (Roche Applied Science). β-actin was utilized as an internal reference gene for expression analysis. Primer sequences for both tilapia serpind1 and β-actin genes were listed in Table 1. Cycle conditions were as follows; one cycle of 95℃ for 10 min, 35 cycles of 95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec, one cycle of 95℃ for 30 sec; and one cycle of 60℃ for 20 sec, 95 ℃ for 20 sec. The geometric mean of the internal control genes was used in the quantification analysis of gene expression according to Hellemans equation (Hellemans *et al.,* 2007).

RESULTS

1. Tilapia serpind1 cDNA partial sequence analysis.

Partial cDNA sequence of serpind1 was characterized in tilapia (*Oreochromis niloticus*). The sequence contained 972 bp representing a 666 bp of the Open Reading Frame (ORF) which encoding for 221 amino acids and a stop codon and a 306 bp of the 3′ noncoding region (Figure 1). Sequence alignment of this sequence with the previously mentioned sequences was done by Clustal W (Thompson *et al.,* 1994) using Lasergene Megalign program, version 15, 2018 (DNASTAR Inc) and has been registered in the GenBank/NCBI data bank with an accession number MN117958.

2. Amino acids sequence comparison

The comparison of amino acid sequences of tilapia serpind1 protein with that of the other species mentioned earlier presented in Table 2. The results recorded that the tilapia serpind1 sequence is closest to that of the *Pundamilia nyererei* fish (97.6) while the lowest identity was found with *Oryzias latipes* fish (69.2).

3. Phylogenetic analysis of tilapia serpind1 protein.

A phylogenetic tree was constructed between the amino acid sequences of serpins through Clustal W (Thompson *et al.,* 1994) using Lasergene Megalign program, version 15, 2018 (DNASTAR Inc). Figure 2 clearly shows a close relationship between tilapia serpind1 and *Pundamilia nyererei* serpind1.

4. Realtime-PCR results

The results show high expression level (6.2) of Serpind1 mRNA in the liver of the BaP-treated fish compared (Figure 3) with the control group with almost no expression detected in other tissues (gills, heart, muscle, intestine, data not shown here).

Table 1: Real-time PCR primers of tilapia serpind1 and β-actin genes.

Gene	Primer description	Sequence (5'-3')	Product size
Serpind1	F	5'-CTCCCTTACATAACTCAAAGCACA-3'	117 bp
	R	5'-AGAAGCGTGCTCAGAAAACAG-3'	
β-actin	F	5'-GGGTCAGAAAGACAGCTACGTT-3'	143 bp
	R	5'-CTCAGCTCGTTGTAGAAGGTGT-3	

Table 2: Percent identities of deduced amino acid sequences of serpind1 gene subfamilies.

	P. nyererei	A. calliptera	F. heteroclitus	H. burtoni	K. narmoratus	M. zebra	O. latipes	O. melastigma	P. flavescens
O. niloticus	97.6	96.9	76.2	96.6	77.9	96.7	69.2	70.9	83.2
P. nyererei		99.7	82.0	99.3	84.6	99.7	81.4	80.4	86.2
A.calliptera			78.1	99.2	80.7	100.0	76.4	75.9	83.7
F. heteroclitus				75.7	83.2	76.2	72.7	73.2	80.5
H. burtoni					78.8	99.1	73.5	73.5	83.5
K.marmoratus						78.9	72.9	73.6	82.5
M.zebra							73.4	73.4	83.7
O.latipes								86.5	79.7
O.melastigma									79.5

Figure legend.

Figure 1: Partial Nucleotide sequence (972 bp) of tilapia serpind1representing a 666 bp of the Open Reading Frame (ORF) which encoding for 221 amino acids and a stop codon and a 306 bp of the 3′noncoding region.
Figure 2: Phylogenetic tree of serpind1 cDNAs using the amino acid sequences.
Figure 3: Serpind1 mRNA levels in liver tissue of control and of BaP treated tilapia fish
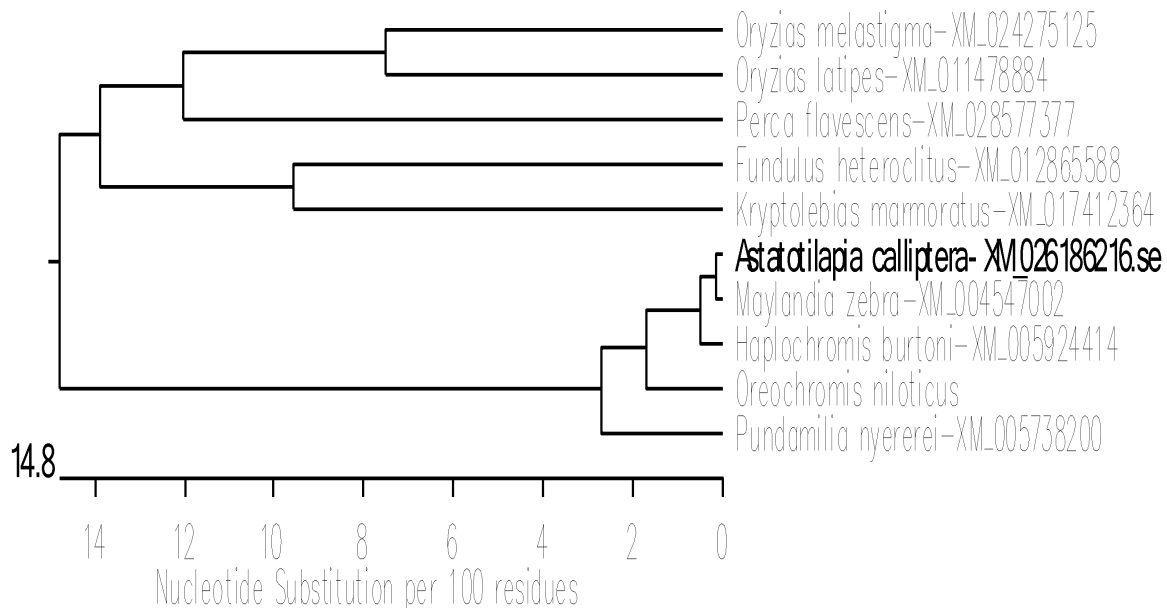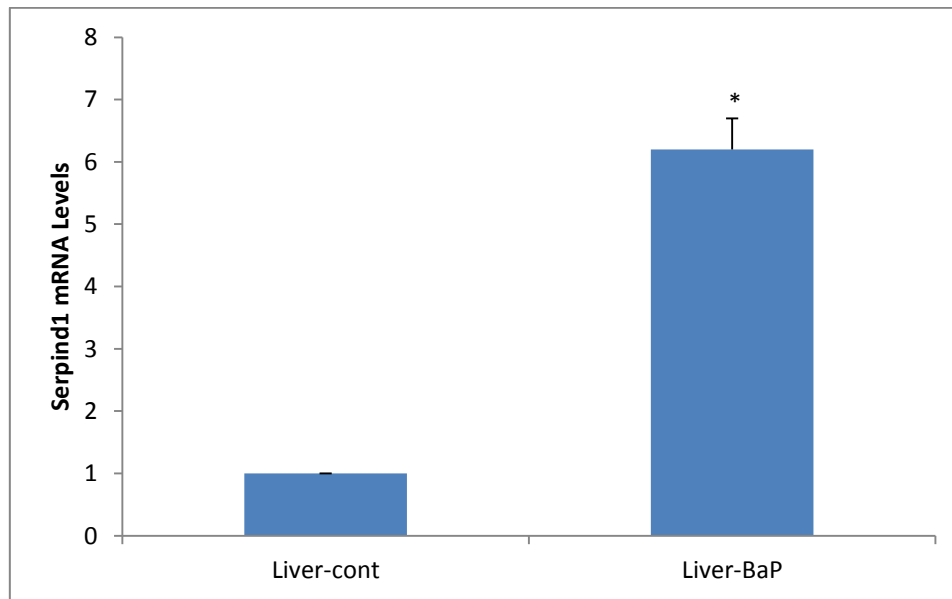

Figure 1:

Figure 2:

Figure 3:

DISCUSSION

The Serine proteinase inhibitors (Serpins) superfamily playing an important role in many fundamental biological processes including blood coagulation, cell, cell migration, inflammation, angiogenesis, and tumor suppression (Kumar, 2009).

Hemostasis (the process which causes the bleeding stop) is a feature by which the vertebrates respond to vascular injury and pathophysiology (Umasuthan *et al.,* 2001). Serpins including HCII are involved in different biochemical pathways of hemostasis (Umasuthan *et al.,* 2001). The biochemical properties HCII have been well studied in mammals. However, in lower-order vertebrates such as the teleost, there is rare information about both molecular structure and physiological role of this gene. Realtime-PCR results indicated higher expression of HCII mRNA in the liver samples of fish treated with BaP compared with the control samples.

In this study, a partial sequence of heparin cofactor II cDNA was cloned and characterized in nile tilapia fish and its transcription response in the liver was investigated after the intracoelomic injection of BaP. The phylogenetic tree revealed a close relationship between tilapia HC II and *Pundamilia nyererei* HCII. There is an agreement between the order displayed in the tree and the evolutionary order of the species involved, the sequence homology and the phylogenetics confirm that tilapia HCII belongs to the teleost serpin, clade D.

Several studies stated that a major amount of HCII mRNA is transcribed in the human liver (Kamp *et al.,* 2001). Similar results were obtained in our study indicate that the liver contributes to a large extent of circulatory HCII.

Liver is the main organ for BaP metabolism in fish causing the formation of carcinogenic metabolites and cytotoxicity (Andersson and Koivusaari 1985; Gill and Walsh 1990). After intraperitoneal injection, the maximum level of the BaP was reached in the fish liver after 6 h, then decrease gradually until 24 h, followed by new increased bioaccumulation of liver BaP with the maximum metabolization level in the liver after only 24 h exposure. (Banni *et al.,* 2009).

Thrombin is a serine protease enzyme which catalysis the conversion of fibrinogen into fibrin and so aids in the formation of a blood clot. It transduces signals through protease-activated receptors (PARs) to activate the neutrophils and platelets aiming at enhancing the release of pro-inflammatory factors such as chemotactic agents, cytokines and growth factors (the mediators affect the inflammation). This explains how coagulation proteases and inhibitors including HCII associated with the inflammation by controls thrombin activity (Shrivastava *et al.,* 2007).

In conclusion, this is aimed at identification and characterization of a partial sequence of novel heparin cofactor II (HCII) from nile tilapia (*O. niloticus*). Phylogenetic analysis showed high similarity between tilapia HCII and the clade D, HCII serpin members. Constitutive HCII mRNA expression was recorded in liver tissue following intraperitoneal injection of BaP, suggesting its physiological importance. Our results proposed the use of tilapia fish as a mammalian

hemostasis model depending on the hemostatic genes homology analysis.

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استنساخ وتحديد التتابع النيوكلوتيدى ودراسة التعبير الجينى لشريط الحمض النووى المكمل للفصيلة D من العائلة ١ لفوق فصيلة Serpin فى أسماك البلطى النيلى

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HCII هو واحد من جينات عائلة جين ال Serpin المسئولة عن معظم العمليات الحيوية داخل الجسم فيما فيها عملية وقف النزف. لذلك أجريت هذه الدراسة بمعمل التكنولوجيا الحيوية البحرية – كلية مصايد الأسماك- جامعة كاجوشيما – باليابان- بهدف عزل وتصنيف شريط الحمض النووى المكمل (cDNA) لجين (HCII) من سمكة البلطي النيلي بعد حقن مادة البنزو بيرين وكذلك دراسة التعبير الجينى لنفس الجين فى نسيج الكبد باستخدام تقنية التفاعل المتسلسل بأنزيم البلمرة الآني Realtime -PCR. أوضحت النتائج أن الطول الجزئى لشريط الحمض النووى المكمل ل HCII هو٩٧٢ زوج من القواعد منها ٦٦٦ زوج من القواعد تمثل جزء من منطقة ال Open Reading Frame التى تترجم الى ٢٢١ حمض أمينى بالأضافة الى كود النهاية '٣ المكونة من ٣٠٦ زوج من القواعد. وكانت هذه هى المرة الأولى التى يتم فيها عزل وتصنيف شريط الحمض النووى المكمل لجين HCII من اسماك البلطى النيلى وقد تم ايداع الجين فى بنك الجينات تحت رقم ايداع MN117958 . أوضحت نتائج التعبير الجينى لجين ال HCII فى أنسجة الكبد باستخدام تقنية التفاعل المتسلسل بأنزيم البلمرة الآني وجين البيتا اكتين كمقياس داخلي انه تم الزيادة في التعبير الجينى فى نسيج الكبد فى المجموعة المحقونة بمادة البنزوبيرين مقارنة بالمجموعة الضابطة. أوصت الدراسة باستخدام اسماك البلطى النيلى كنموذج لدراسة الية عملية وقف النزف فى الثدييات نظرا للتشابه الكبير فى تركيب جين فى كلا من اسماك البلطى والثدييات.