

cDNA cloning and expression of vitamin-K Epoxide Reductase (VKOR) gene from orange spotted grouper *Epinephelus coioides*

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The cDNA sequence encoding Vitamin-K Epoxide Reductase (VKOR) was constructed from hypothalamus and pituitary cDNA libraries of Orange spotted Grouper *Epinephelus coioides*. VKOR gene contains 610 nucleotides including the open reading frame (ORF) of length 399 bp. ORF was started with ATG codon at the position of 199 and terminated with a stop codon TAA of 585. ORF sequence had 128 amino acids and the predicted protein molecular weight is approximately 14.08 kDa (GenBank: FJ939281.1). Expression of VKOR gene in different tissues was further examined using one-step RT-PCR. RT PCR result revealed that, VKOR mRNA expression was detected only in liver, kidney, hypothalamus and pituitary. VKOR gene sequence of *E. coioides* aligned with related sequences by multiple sequence alignment by clustal X and phylogenetic tree was generated by Neighbor Joining method using Genious v 5.4. *E. coioides* VKOR had the highest identity and homology with the puffer fish, *Tetraodon nigroviridis*, *Takifugu rubripes* and Zebra fish *Danio rerio*. Based on the VKOR expression in the kidney, liver hypothalamus and pituitary of the seven year's old (sex reversal stage) *E. coioides*, it may be involved in sex reverse.

[**Keywords:** *Epinephelus coioides*, Vitamin-K Epoxide Reductase, cDNA cloning]

Introduction

Vitamin K Epoxide Reductase (VKOR) mediates recycling of vitamin K 2,3 epoxide to vitamin K hydroquinone, an essential co-substrate for modification of multiple glutamic acid residues to γ -carboxyglutamate in vitamin K-dependent proteins such as the coagulation factors II, VII, IX, and X, protein C, S, and Z, Matrix Gla protein (MGP), and osteocalcin¹. VKOR complex gene located on chromosome 16 at band p11.2 encodes the VKOR enzyme, a small transmembrane protein of the endoplasmic reticulum. Transcription of VKORC1 occurs primarily in the liver; however, smaller amounts of VKORC1 are present in the heart and pancreas². They target the warfarin anticoagulants, is an 18.2-kDa protein^{3,4} with 3 transmembrane domains⁵. VKOR expression in arteries is prominently in the tissues under angiogenesis-related physiologic conditions such as in fetal heart and in pathologic conditions such

as ventricular aneurysm caused by myocardial infarction and tumor tissues⁶.

The VKOR gene encodes several isoforms, and it will be important to characterize the activity and expression pattern of each isoform. Millions of people worldwide use warfarin to inhibit coagulation; therefore, it is important to characterize further VKOR as it may lead to more accurate dosing or to the design of safer, more effective anticoagulants than are currently available³. Pseudogenes of VKORC1 and VKORC1L1 were cloned in human, mouse and rat genomes. VKORC1 seems to be conserved in vertebrates, as it is present in human, rodents and fish. Notably, a close homologue is present in *Anopheles gambiae* but not in the *Drosophila* genome, although a gene encoding γ -carboxylase has been described in the fruit fly⁴. Researchers were already cloned and sequenced the VKOR protein families in several fish species such as *Takifugu rubripes* (Japanese puffer fish), *Tetraodon nigroviridis* (puffer fish), *Danio rerio* (zebrafish), *Ictalurus punctatus* (channel catfish) and *Salmo salar* (Atlantic salmon).

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The orange-spotted grouper, *Epinephelus coioides*, is a marine fish widely cultured in Southern China. It is a protogynous hermaphroditic fish. Females reach sexual maturity at about 5 years of age when they grow to be approximately 55–75 cm in body length and the transition of sex from female to male begins at about 7 years of age⁷. Present study, SMART cDNAs was synthesized for encoding the VKOR gene from the mRNA of four year old *E. coioides*. Further VKOR gene was amplified from different tissues and determined the sequencing, homology with different species and expression.

Materials and Methods

Pituitary and hypothalamus of the 7-year-old male orange-spotted grouper were excised and immediately dipped to RNA LaterTM for RNA (QIAGEN) at room temperature for 1 h for RNA extraction. Total RNA was extracted using SV total RNA isolation system (Promega, USA). The quality of RNA was measured at A 260 nm and the purity from the ratio A260:A280 nm by Photo Biometer (Eppendorf).

SMART cDNA were synthesized from 50 ng of total RNAs according to the previous work⁷⁻⁹ using the Switching Mechanism At 5' end of RNA Transcript (SMART) cDNA Library Construction Kit (Clontech). The cDNAs were ligated to pGEM-T vector (Promega) and the plasmids were used to transform *Escherichia coli* DH5 α super competent cells. A clone was screened by VKOR specific primers using LD- PCR programme.

DNA sequencing of the positive clones were performed in United Gene Company, Shanghai, China using M13+ and M13- primers. Nucleotide sequence identity was analysed using BLAST tools in European Bioinformatics Institute website and Clustal X for multiple sequences alignment. Phylogenetic analysis performed with Geneious Pro¹⁰. Phylogenetic tree was generated based on the 24 Nucleotide sequence of Vitamin K epoxide reductase complex gene families, VOKR genes and Hypothetical proteins. Neighbor joining method has used to create distance matrix.

Total SV RNAs of different tissues such as hypothalamus, pituitary, liver, kidney, spleen, fat, heart, muscle, cerebellum, midbrain, medulla oblongata, ovary and testis were extracted and constructed the SMART cDNA. One pair of primers (F: 5'- GAATTCATGTGCGACGTGAGCAG -3'; R:

5'- CTCGAGTTAGTCCTGCTTGCCTGAAGCTG - 3') to amplified the ORF region and a common forward primer for (5'- AGCAGTGGTATCAACGCAGAGT GGCCATTACGGCCGGG-3') RACE amplification were synthesized (Sangon, Shanghai). Using the different primers, amplification reaction were performed in volume of 25 μ L containing 1 μ L c DNA as template, 0.2 μ M each primer, 0.5 U Taq DNA polymerase (MBI Fermentas), 0.1 μ M of d NTP, 1 X buffer for Taq DNA polymerase. Each PCR cycle included denaturation at 94 °C for 40 sec, annealing at 60° C for 30 sec and extension at 72 °C for 1 min. Primers have been tested with c DNA obtained from pituitary and hypothalamus of the *E. coioides* after 35 cycles the PCR products were run in the 1% agrose gels containing ethidium bromide.

Results

VKOR was successfully amplified using cDNA constructed from pituitary and hypothalamus of the orange spotted grouper *E. coioides* as template for obtained the full sequence by RACE PCR. The 5' RACE PCR amplification generated a single c DNA fragment of 610 bp, including the open reading frame (ORF) of length 399 bp. The open reading frame was started with the ATG codon at position of 199 and terminated with a stop codon TAA of the position 585 (Fig. 1). The ORF sequence had 128 amino acids and the predicted protein molecular weight is approximately 14.08 kDa and the ORF sequence was deposited in NCBI gene bank (GenBank accession no: FJ939281.1). PCR amplification of the first strand cDNA of the 5' RACE (610 bp) and ORF (399 bp) were cloned successfully to the p GEM-T easy vector and sequenced (Fig. 2a & b).

The comparison of amino acid alignments, their identities and homology of VKOR were given in the Figures 3 & 4 respectively. The *E. coioides* VKOR had maximum identity with the puffer fish *Tetraodon nigroviridis*, Japanese puffer fish *Takifugu rubripes* followed by Zebra fish *Danio rerio* respectively. The phylogenetic analysis revealed that, the VKOR family were classified in to four monophytic groups based on the similarity. The group (G1) consist Ephinephaleus, Taikafu, Tetraodon VKOR family; G2 consisted Ailuropoda, Human, Xenopus VKOR family; G3 consist Anoplopoma, Dicentrarchus, Ictulus, Ixodes, Daphnia, Trichoplax VKOR family and finally G4 had Branchiostoma, Arvicola, Rattus etc.

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1                               GGCTCACAGGCGAACTCG 18
19 CTGCGTGTGTATGCGTGTCTCCAGCTAGGCCAGAGAGTGATGGCGGCGCCCGTCCTGAGA 78
79 GTGTCCACCCCTCGGTGGGAAAAGAATAGCCAGGCTTCTCGTCTGCCTGCTGGGCATACTG 138
139 CTATCTCTATATGCCCTTCCACGTCGAGAGGGAAAAGGCCAGGACCCAGTTACAAGGCT 198
199 ATGTGCGACGTGAGCAGCTCCATCAGCTGTTCAAAAGTGTTCAAGGTGGGGTCGA 258
1  M C D V S S S I S C S K V F S S R W G R 20
259 GGATTTGGACTCCTGGGCTCAATTTTTGGAAATGACAGCGCACTGAACCAACCCAACAGT 318
21  G F G L L G S I F G N D S A L N Q P N S 40
319 GTCTACGGAATTGTCTTTTATGCCTTTCAGCTCCTTCTAGGAATGACTGTCAAGTGAATG 378
41  V Y G I V F Y A F Q L L L G M T V S A M 60
379 GCCGCCCTTATTCTCATGACGACATCCATCTTGTCGGTGGTGGGTTGCTCTACCTGGGC 438
61  A A L I L M T T S I L S V V G S L Y L G 80
439 TACATCCTCTACTTTGTCCTAAAGGACTTCTGCATCATCTGCATCACCACATATGCGCTG 498
81  Y I L Y F V L K D F C I I C I T T Y A L 100
499 AACTTCATTCTCTTTATTCTCAACTACAAGCGACTGGTTTACTTGAACGAGGCCTGGAAG 558
101 N F I L F I L N Y K R L V Y L N E A W K 120
559 CAGCAGCTTCAGGCCAAGCAGGACTAAGCTGTCTCGAGAACGGCAAGAAAAACAAGATAAAC 618
121 Q Q L Q A K Q D STOP 128
619 AAACAAAAGAGAAAAAAAAGAAATGTGACCTCTGAACATTTTGACTTGCCACCAGGAGAC 678
679 CTTGCTTCCTAACAATGTTTATTCTGCTGTGTGTCGAAAAAAGAAAAAAGAAAAA 738
739 AAAAAA 745

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Fig. 1—The complete c DNA sequence and deduced amino acid sequence of the VKOR like gene.

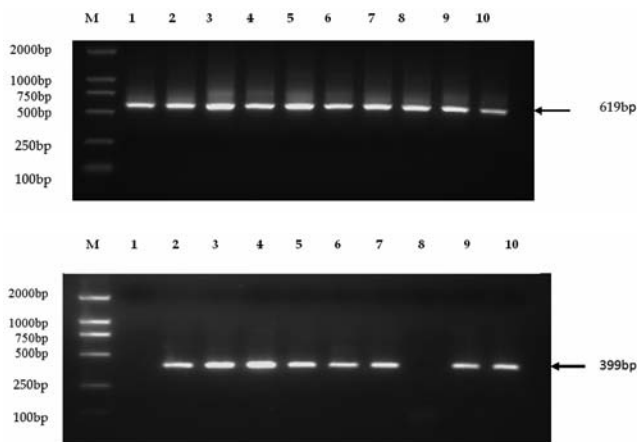


Fig. 2 a & b—PCR screening of VKOR like gene from clones (pGEM-T/BL21 DE3) using 5' RACE primer (a) and ORF primer (b)

Tissue specific gene expression of VKOR in different tissues such as liver (L), kidney (K), spleen (S), fat (F), heart (H), muscle (Mu), pituitary (P), hypothalamus (Hy), cerebellum (C), midbrain (MB), medulla oblongata (MO), ovary (O) and testis (T) was

studied by RT PCR using the primer 5' RACE and gene specific ORF forward and reverse primers. The VKOR like gene is abundantly expressed in liver, kidney, pituitary and hypothalamus. There is no expression observed in spleen, fat, heart, muscle, cerebellum, midbrain, medulla oblongata, ovary and testis (Fig. 5a & b).

Discussion

VKORC1 is a member of a large family of homologues that are represented among plants, bacteria and archaea, as well as vertebrates and arthropods¹⁰. Even though the function of vitamin K epoxide reductase (VKOR) has been reported since 1974¹¹, the VKOR gene has only been cloned after 2000^{3,4}. Wang *et al.*⁶ cloned the VKOR ORF gene to pGEMT easy vector and transfect into tumor cell line A549 and H7402. The tumour cells did not promote the cell proliferation and conclude that, VKOR may also be involved in angiogenesis. VKORC1 seems to be conserved in vertebrates, as it is present in human, rodents and fish. In the present study, the different

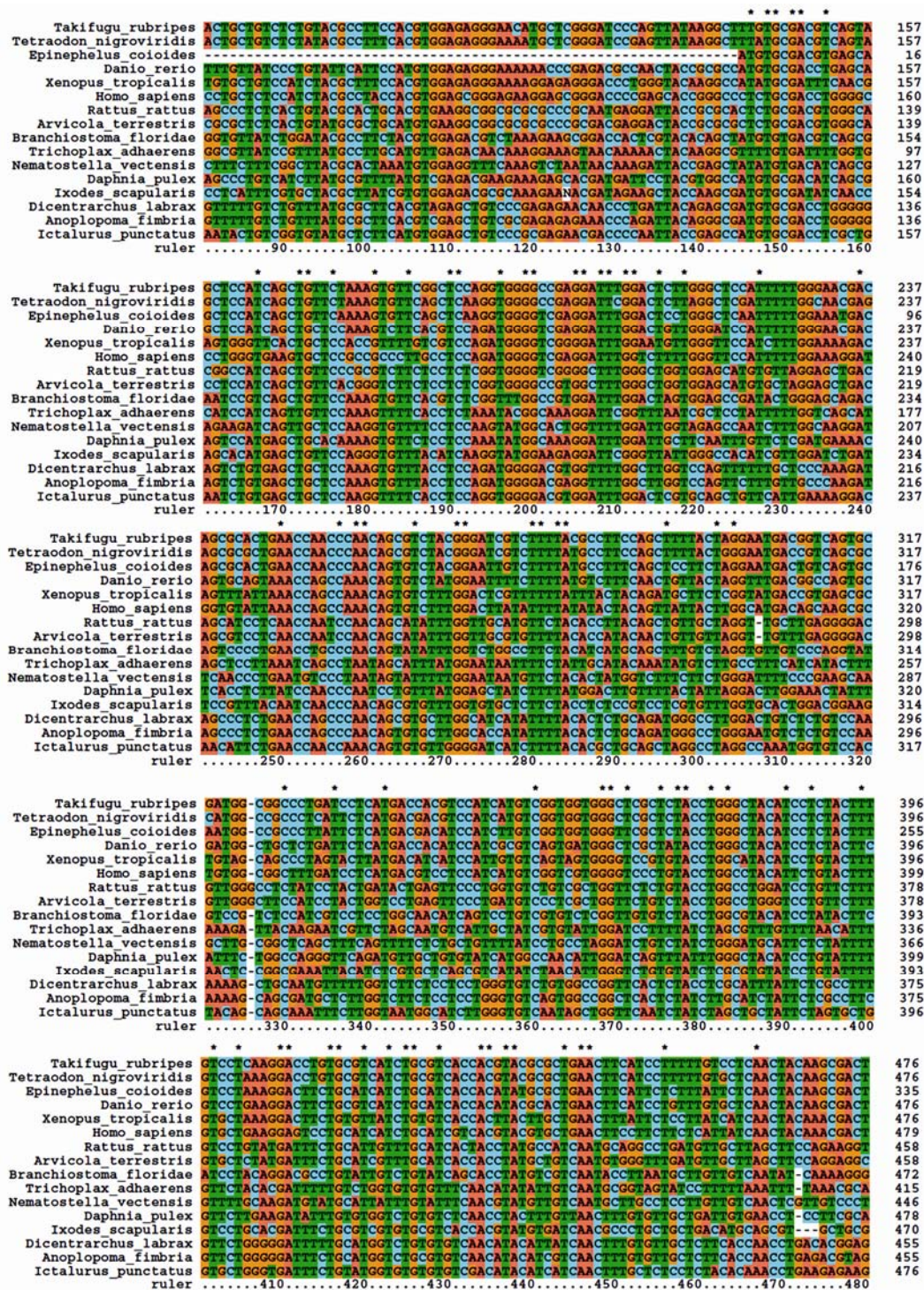


Fig. 3—Amino acid alignment identities of VKOR like gene of orange-spotted grouper *E. coioides* with its homologues

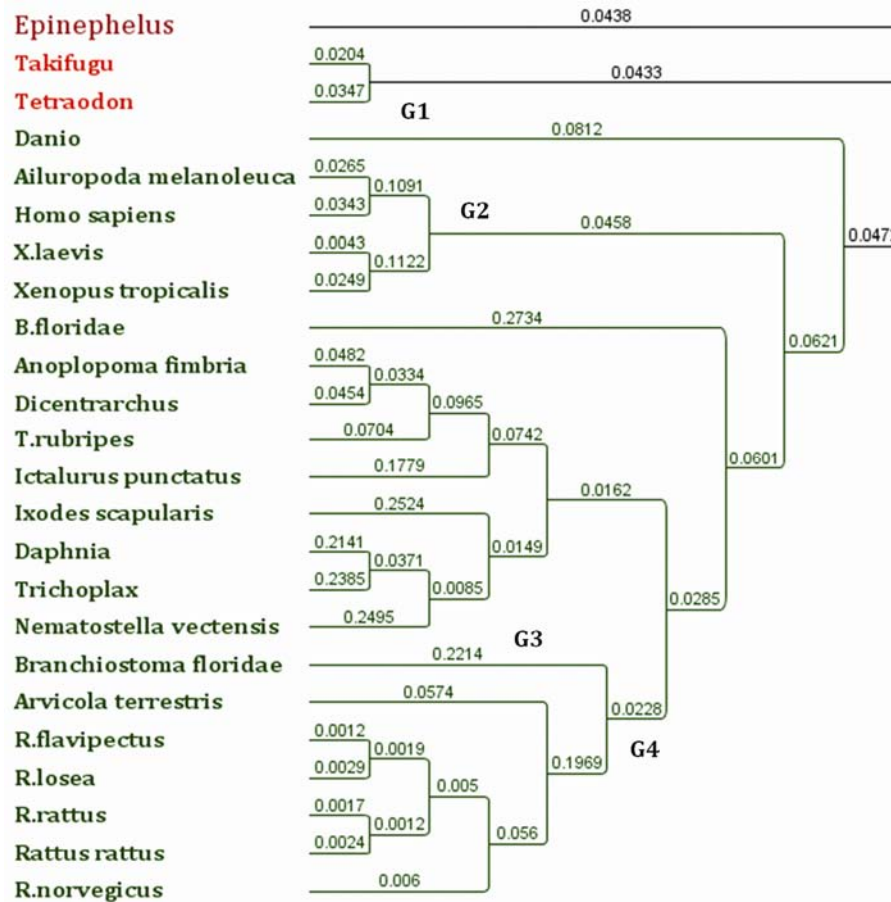


Fig. 4—Phylogenetic analysis of the nucleotide sequence of VKOR like gene of grouper *E. coioides* with that reported in other animals by Neighbor Joining method using Geneious Pro analysis.

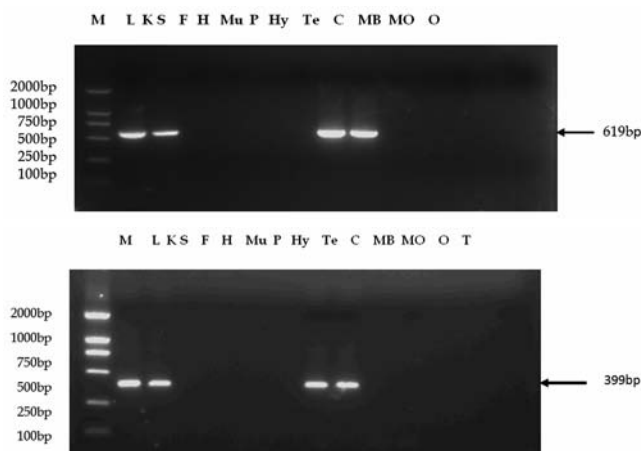


Fig. 5 a & b—Tissue distribution of VKOR like gene from different organs such as liver (L), kidney (K), spleen (S), fat (F), heart (H), muscle (Mu), pituitary (P), hypothalamus (Hy), cerebellum (C), midbrain (MB), medulla oblongata (MO), ovary (O) and testis (T) by RT-PCR analysis using 5' RACE primer (a) and ORF primer (b)

primers for full gene and specific ORF amplifications successfully amplified the genes at 610 bp and 399 bp respectively. Unfortunately the protein was not expressed in the highly expression vectors such as BL21DE3 and pGEX-KG and its predicted protein molecular weight is approximately 14.08 kDa. Tblastn searches with VKORC1 detected paralogous human (BC027734) and mouse (AK009497) genes, each with 50% protein identity. Human, mouse and rat VKORC1L1 proteins were highly conserved, sharing roughly 97% identity⁴. Also the VKOR subunit protein was successfully cloned in *Thermoproteus uzoniensis*, *Bifidobacterium longum* and *Vulcanisaeta moutnovskia*^{13,14}.

An orthologue of the VKORC1 gene was present in mice and the orthologues in rat and in *T. rubripes* were established by homology searches and polymerase chain reaction with reverse transcription (RT-PCR). The corresponding proteins share 79–84% identity with the human protein⁴. Among the

vertebrates, the available human, rat and mouse orthologs of VKORC1 were obtained, along with the chicken, *Xenopus*, *Takifugu* and Zebra fish orthologs from genomic and cDNA sequences. These are all relatively straight forward except for the VKORC1 ortholog in the chicken. This protein is rather divergent and the gene is not present in the draft genome assembly¹⁵. Rost *et al.*⁴ also noted that there is a paralog of VKORC1 called VKORC1-like 1 (VKORC1L1) in these vertebrate genomes and that this gene/protein is actually more conserved in sequence across the vertebrates than this VKORC1. The present study, *T. nigroviridis* hypothetical protein and *T. rubripes* Vkorc1-like protein 1 fell into the one group (G1), which is descended from *E. coioides* VKOR gene, and the length of the branch leading to the that node is 0.043, Gene sequence in Group 1 (G3) and Group 2 (G4) forms separate monophyletic groups, which is interconnected with *B. floridae*, and the length of the branch leading to the that node is 0.028. G3 and G4 were descended from the G2 Monophyletic group with 0.062 distances, all the G1, G2 and G3 were diverged from *Danio rerio* vitamin K epoxide reductase complex. We are concluding that *T. nigroviridis* gene may express as like VKOR protein gene as the closely descend from from *E. coioides* VKOR gene. We found two clusters in G4 cluster with one Branch Branchiostoma floridae hypothetical protein and second cluster has five nodes with 6 vitamin K epoxide reductase complex subunit 1 of various species. Hence *B. floridae* gene may function as like vitamin K epoxide reductase complex subunit 1 as it is closely diverged from G4 with 0.19 distances. G3 has two clusters with 8 branches, as hypothetical clustered with vitamin K epoxide reductase complex genes we can assume the function of Hypothetical proteins in G3 would similar like vitamin K epoxide reductase complex. G3 and G4 diverged from *B. floridae* hypothetical protein with close distance it would express like vitamin K epoxide reductase complex genes. Thus Hypothetical proteins in G2clusterd with vitamin K epoxide reductase gene families we can assume the same function as like hypothetical proteins cluster in G3 and G4.

VKOR was highly expressed in human fetal heart and extremely up-regulated in tissues from human ventricular aneurysm caused by myocardial infarction, indicating that VKOR may mediate angiogenesis in late fetal and early postnatal periods

of development⁶. Romero *et al.*¹⁶ also found the highest expression in fetal and adult liver, followed by fetal heart, kidney and lung, adult heart and pancreas and concluded that, its expression seems to be broader. Multiple clones representing the putative VKOR cDNA have been reported from almost every tissue, with the greatest number from lung, pancreas, nerve and liver³. Hazeleit and Preusch¹⁷ also studied the distribution of VKOR and its sensitivity to warfarin in the whole microsomes from warfarin-resistant strain rats and the result revealed that, activity on a per gram tissue basis was highest in kidney, adrenal, spleen, lung, testes, and epididymis at a level about 1/20th of that present in liver microsomes. The present study, the grouper *E. coioides* VKOR also highly expressed in the liver, kidney, pituitary and hypothalamus like mouse. The reason for VKOR expression in liver is: the carboxylation undergo in liver in the presence of Vitamin K as co-factor. The *E. coioides* VKOR not only expressed in liver at higher levels, it also expressed in kidney, pituitary and hypothalamus. The present results also confirmed that, it may be secreted in pituitary and its active forms passed through the VKOR pathway to hypothalamus, liver and finally kidney. Most well-known vitamin K-dependent proteins (VKDPs) are the coagulant factors II, VII, IX, and X, produced by the liver, they are converted into their biologically active forms by the carboxylation of glutamic acid residues, a process requiring vitamin K as a cofactor¹⁸. Substrates of carboxylase are the so-called vitamin K-dependent proteins, which are involved in diverse physiological processes such as blood coagulation, bone and soft tissue mineralization, and cellular proliferation¹⁹.

Grouper *E. coioides* VKOR cDNA contains 610 nucleotides including the open reading frame (ORF) of length 399 bp was successfully cloned and expressed from the pituitary and hypothalamus. The *E. coioides* VKOR also expressed in kidney and liver and these findings revealed that, the VKOR protein may secreted from the neuro-secretory cells of the pituitary and hypothalamus region and carboxylation occurs in the liver in the presence of Vitamin-K. Function of *E. coioides* VKOR was not understood in the present study and it may be involved in bone and soft tissue mineralization, cellular proliferation and act as antioxidants during sex reversal. Further studies need to find out the function of the VKOR in grouper *E. coioides* at

different stages and the expression related to the influence of warfarin etc. Also Zebra fish/Medaka embryonic developmental studies need to find out more functions.

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