Molecular Cloning and Characterization of a Novel Gene, *EJO*3, Putatively Involved in the Ovarian Development of Chinese Mitten Crab (*Eriocheir Japonica Sinensis*)

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Abstract In the present study, we performed suppression subtractive hybridization (SSH) combining cDNA macroarray to identify genes putatively involved in the ovarian development of Chinese mitten crab. A novel gene, *EJO*3 (GenBank accession number: AY185919) whose expression is higher at stage III than that at stage II was cloned by 5′ and 3′ RACE. The ORF (open reading frame, ORF) of the cDNA of *EJO*3 consisted of 1 368 bp encoding 455 amino acids. The pI/Mw deduced from the amino acids sequence was 5.79/50 000. Protein coded by *EJO*3 gene was probably a secretion protein with a VWD domain. The differential expression of *EJO*3 was further confirmed by Northern blot analysis. Expression profile analysis showed that *EJO*3 is highly expressed in ovary and there is little or no expression of *EJO*3 in intestine, heart, muscle and hepatopancreas. Results of this study have made an important foundation to further elucidate the molecular mechanism of the ovarian development of Chinese mitten crab.

Key words: suppression subtractive hybridization (SSH), cDNA macroarray, *Eriocheir japonica sinensis*, ovary, *EJO*3
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中华绒螯蟹卵巢发育相关的新基因 *EJO*3的克隆和特征分析

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[摘要] 运用了抑制性差减杂交和 cDNA芯片技术鉴定中华绒螯蟹卵巢发育相关基因, 并用 5′和 3′RACE的方法克隆了一个与 II期相比在卵巢发育的 III期高表达的新基因 *EJO*3 (GenBank登录号: AY185919). *EJO*3基因的开放阅读框(ORF)长 1 368 bp, 编码 455个氨基酸. 从氨基酸序列推算的 *EJO*3的等电点和相对分子质量分别为 5.79和 50 000. 生物信息学分析表明, *EJO*3可能是一个含有 VWD结构域的分泌蛋白. *EJO*3在 II期和 III期卵巢的差异表达用 Northern blot进行了进一步验证. 组织表达谱分析表明 *EJO*3在卵巢高表达, 在肠、心脏、肌肉和肝胰腺中弱表达或不表达. 本研究结果为进一步深入研究中华绒螯蟹卵巢发育的分子机制奠定了重要的前期工作基础.

[关键词] 抑制性差减杂交, cDNA芯片, 中华绒螯蟹, 卵巢, *EJO*3

Chinese mitten crab *Eriocheir japonica sinensis* is a commercially important aquatic animal in China and other Asia countries. In 2005, the total production of market-sized crab in China reached over 400 000 tonnes[1].

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In recent years, sexual precocity has been a very popular phenomenon during the breeding process of the mitten crab and resulted in heavy yield losses in many areas of China[2]. Previous researches concerning Chinese mitten crab were mainly concentrated on larval culture, diseases, diets, nutrition[3-6] and population studies[7-9]. The molecular basis of the ovarian development is not clear so far.

The primary purpose of this study is to clone genes related the ovarian development of Chinese mitten crab. According to the morphology, color, and growth of the oocyte, the development of the ovary is divided into 6 stages[10]. Suppression subtractive hybridization (SSH) combining cDNA macroarray assay was performed using stage II and stage III ovaries. The full length cDNA sequence was cloned by 5′ and 3′ RACE (rapid amplification of cDNA end, RACE) and its differentially expression was further verified by Northern blot analysis. Tissue expression profile was investigated by RT-PCR analysis. In addition, the characteristics of protein encoded by the cloned gene were preliminarily analyzed by bioinformatics. Results of this study have made an important foundation to further elucidate the molecular mechanism of the ovarian development of Chinese mitten crab.

1 Materials and Methods

1.1 Experimental animals

The mitten crabs with ovarian development at stage II and stage III were obtained from Anhui Fisheries New Technology Research Institute of China.

1.2 Total RNA and mRNA isolation

Total RNA was extracted from stage II and stage III ovaries of the mitten crab using Trizol reagent (Gibco). mRNA was purified from the total RNA using Oligotex™ mRNA mini kit (Qiagen) following the protocol supplied along with the kit. Double-stranded cDNAs were prepared by reverse transcription of mRNA following the steps outlined in the Super SMART PCR cDNA synthesis kit (Clontech, Palo alto, USA).

1.3 Construction of subtracted cDNA library

SSH was performed with mRNA purified from stage II and stage III ovaries using Clontech PCR-Select™ cDNA Subtraction Kit (Clontech). The subtracted library was constructed by ligating the subtracted PCR products into PinPoint plasmid vectors (Promega), and transforming the ligation mixtures into *E. Coli* JM109.

1.4 Screening the subtracted library using macroarray

Differentially expressed genes in the subtracted library were identified primarily using macroarray. Briefly, inserts in the PinPoint plasmid vectors were amplified using primers designed according to sequences flanking the cloning site (SP6: 5′-ATTTAGGTGACACTATAGAA-3′; PinPoint 5′-CGTGACGCGGTGGGCG-3′). The amplified fragments were robotically printed onto nylon membranes. The probes were prepared by incorporation of ^{33}P-labelled dATP in a reverse transcription reaction using purified mRNAs from stage II and III ovaries.

1.5 Sequencing and analysis of the cloned ESTs

The plasmids containing the differentially expressed genes identified by the cDNA macroarray were isolated using mini-preps (QIAprep Spin Miniprep Kit, Qiagen), and the inserts were sequenced using an ABI 310 automatic sequencing machine. Sequences obtained were searched against GenBank and SwissProt databases to search homologies. The novel expression sequence tags (ESTs) were deposited in the EST database.

1.6 Cloning the full length cDNA sequence of *EJO3*

To obtain the full-length cDNA of the differentially expressed genes, double-stranded cDNAs of the crab ovaries at stage III were synthesized with SMART™ cDNA Library Construction Kit (Clontech) according to the manufacturer's instructions. Primers according to EST003 (dbEST accession number: CA591895) were designed. The sequences of the designed primers were as follows: P33-3: 5′-GTGGCGGCGGTGAAGTCATCG-3′; P33-5: 5′-GCGAACTCTCCGGCGATGTGG-3′. The PCR conditions consisted of 30 cycles of denaturing at 95℃ for 1 min, annealing at 58℃ for 30 s and extension at 72℃ for 2 min. At the end of the last cycle, the PCR mix was incubated at 72℃ for another 10 min for the completion of DNA synthesis. Prior to the 30 cycles, the tem-

plates were denatured at 95℃ for 4 min. The PCR products were subcloned to T-easy vectors (Promega) and sequenced.

Sequences obtained from 5′ and 3′ RACE, as well as the sequence of EST003, were spliced. To further verify the splicing sequence, primers named L2 (L2: 5′-TCAGAGGTTGGGGTGCTTCGTG-3′) and R2 (R2: 5′-TAACGAGTGTACAGATGCTTTGAG-3′) were synthesized according to the spliced sequence. The PCR conditions were as follows: denaturation at 95℃ for 30 s, annealing at 58℃ for 30 s, and extension at 72℃ for 2 min. The first cycle had a denaturation period of 4 min. The last cycle had an extension period of 20 min. 30 cycles of PCR were performed, and the PCR products were purified and subcloned in T-easy vectors and sequenced.

1.7 Bioinformatics analysis

The ORF of the *EJO*3 cDNA sequence was found using DNA club software. The SwissProt database was searched for the homology analysis of the translated amino acid sequence. The signal peptide was analyzed using SignalP V1.1 software. pI/Mw was computed using Compute pI/Mw tool. Subcellular localization of *EJO*3 was analyzed using PSORT II software. Motif analysis was performed using ProScan software.

1.8 Northern blot assay

20 μg of total RNA was electrophoresed and transferred to a nylon membrane. ^{33}P-labelled probes for *EJO*3 and β-actin were hybridized sequentially using standard protocols. The hybridization signals were detected and quantitated with PhosphoImager and ImageQuaNT software programs (Molecular Dynamics).

1.9 Expression profile of *EJO*3

The tissue expression profile of *EJO*3 was determined using RT-PCR screening. Multiple tissue cDNAs including hepatopancreas, heart, intestine, muscle and ovary were prepared. β-actin was used as an internal control. PCR conditions were as follows: denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s and extension at 72℃ for 1 min. The first cycle had a denaturation period of 5 min. The last cycle had an extension period of 7 min. 30 cycles of PCR were performed and the PCR products were analyzed after eletrophoresis.

2 Results and Discussion

2.1 Subtracted library construction and differential screening

A subtracted cDNA library including 863 clones was constructed using the SSH method. After screening with cDNA macroarray, 104 differentially expressed clones were obtained from the subtracted cDNA library. Seven independent sequences were obtained after sequencing 20 clones randomly. Homology analysis of the seven sequences showed that no significant matching sequence was found, so they were deduced to be novel ESTs and deposited in the EST database. Among these ESTs, EST003 (accession number: CA591894), whose expression at stage III was higher than that at stage II, was selected to be studied further.

2.2 Cloning the full-length cDNA of *EJO*3

To obtain the full length cDNA of EST003, RACE PCR was performed using double-stranded cDNAs synthesized form stage III ovaries. As shown in Fig. 1A, products of 5′ and 3′ RACE PCR were about 900 and 750 bp, resceptively. After being ligated to the T-easy vector, fragments of RACE PCR were sequenced. The obtained sequences were spliced with the sequence of EST003. A spliced cDNA of 1 514 bp in length with the overlap sequence of 109 bp and 196 bp in 5′ and 3′ end, respectively, was obtained (Fig. 1B). The full-length cDNA of EST003 was amplified successfully with primers designed according to the spliced 1 514 bp sequence (Fig. 1C). The nucleotide sequence and the deduced amino acid sequence of *EJO*3 are shown in Fig. 2.

2.3 Bioinformatics analysis

The cDNA sequence of *EJO*3 was blasted in SwissProt, and no significant matching sequence was found. So it was deduced as a novel gene. The sequence of *EJO*3 was deposited in GenBank and the accession number is AY185919. The ORF of *EJO*3 was between 39-1 406 bp with the length of 1 368 bp encoding 455 amino acids.

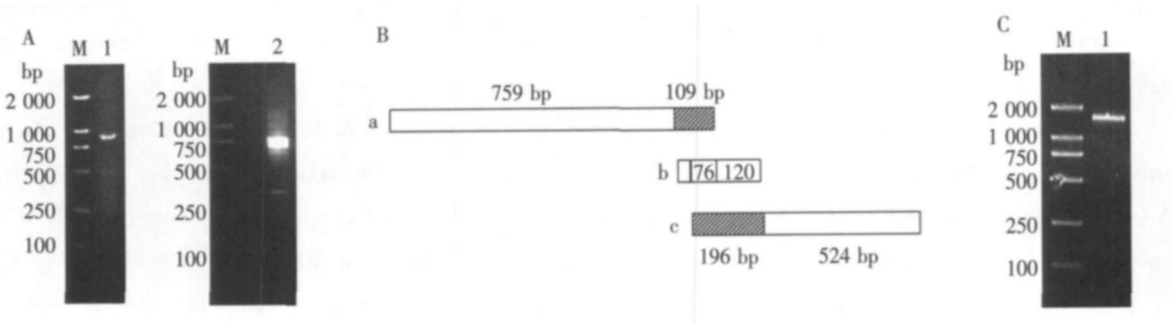

Fig 1　Cloning the full length cDNA of *EJO* 3 gene

图 1　*EJO*3基因全长 CDNA的克隆

A. 5′ and 3′RACE products of *EJO* 3 gene from Chinese mitten crab ovaries M: DNA Marker; 1: 5′RACE; 2 3′RACE; B. Strategy for he splicing of *EJO* 3 gene a 5′RACE; b EST003; c 3′RACE; C. Amplification of the full length cDNA of *EJO*3 M: DNA Marker; 1: *EJO*3.

(Fig 2). Amino acid sequence analysis using Compute pI/Mw tool indicated that pI/Mw of EJO3 protein was 5.79 and 50 000, respectively. Result of SignalP VI. 1 analysis showed that EJO3 is probably a secretion protein with a signal peptide of 20 amino acids. Cellular localization analysis using PSORT II also showed that EJO3 is an extracellular protein, which is consistent with the result of Signal peptide analysis. ProScan software analysis showed that EJO3 protein has nine glycosylation sites, one cAMP- and cGMP-dependent protein kinase phosphorylation site, seven Protein kinase C phosphorylation sites, seven Casein kinase II phosphorylation sites, and one VWD domain between amino acid 103 and 278.

VWD domain of proteins is responsible for normal multimerisation and optimal secretion[11, 12]. Since *EJO*3 has a VWD domain, it is possible that this domain has a role in the secretion of this protein and *EJO* 3 may execute its function as a multimer. Secreted, extracellular proteins usually are needed for coordination of complex biological processes such as differentiation and wound healing. As a secretion protein, EJO3 may have a role in cell adhesion, migration, differentiation or signal transduction and play an important function during the ovarian development of the mitten crab. This needs to be verified by further studies.

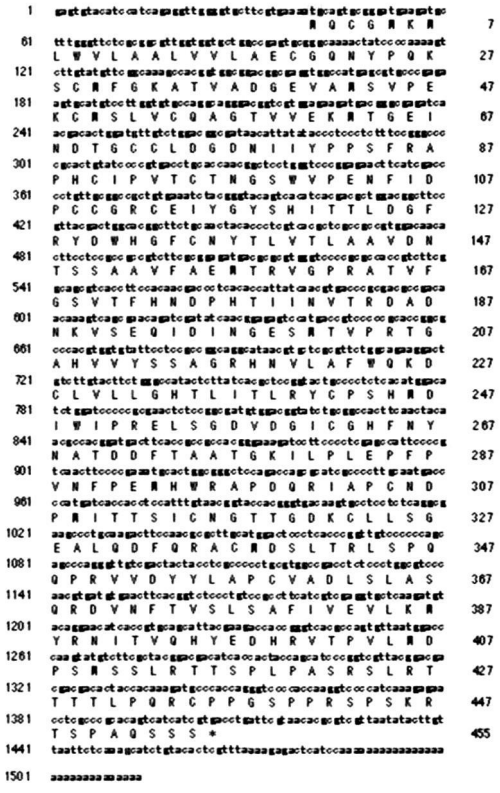

Fig 2　Nucleic acid and deduced amino acid sequences of *EJO* 3 gene

图 2　*EJO*3基因的核酸序列及推断的氨基酸序列

Gray letters indicate start codon; asterisk indicates stop codon

2.4　Northern blot assay

To further verify the differential expression of the *EJO* 3 gene during the development of crab ovary, Northern blot was performed. Results of the Northern blot analysis showed that the expression of *EJO* 3 was higher at stage III than that at stage II (Fig 3), indicating the differential expression of *EJO*3 gene.

2.5　Expression profile of *EJO*3

*EJO*3 is expressed in ovary and intestine tissues, while its expression in intestine was very faint. There was no expression of *EJO*3 in heart, muscle and hepatopancreas tissues (Fig 4). This implied that *EJO*3 is a specifically expressed gene and it might play a pivotal role in ovarian development of Chinese mitten crab.

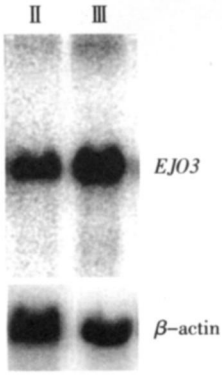

Fig.3 Northern blot analysis of *EJO3* gene in ovary at stage II and stage III

图 3 *EJO3* 基因在 II 期和 III 期卵巢的 Northern blot 分析

Total RNA of the crab ovaries at stage II and stage III was isolated, electrophoresed, and transferred to a nylon membrane. ^{33}P-labelled probes for *EJO3* were hybridized using standard protocols; β-actin was used as internal control. The hybridization signals were detected and quantitated with Phosphoimager and ImageQuaNT software programs.

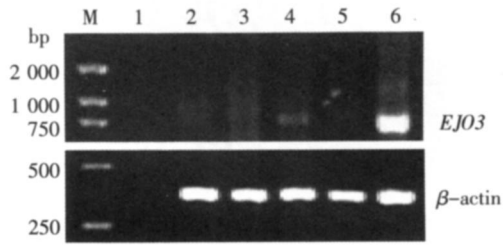

Fig.4 Expression profile of *EJO3* gene

图 4 *EJO3* 基因的表达谱

RNA of heart, muscle, intestine, hepatopancreas, and ovary of the mitten crab was isolated, and cDNA was synthesized with the isolated RNA. PCR was performed using the synthesized cDNA, and the PCR products were analyzed by eletrophoresis. β-actin was used as internal control. M: DNA Marker; 1: negative control; 2: heart; 3: muscle;4: intestine; 5: hepatopancreas; 6: ovary.

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Effects of Osthole on the Proliferation, Cell Cycle and Apoptosis in Human Breast Cancer Cells

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Abstract: Osthole, a natural coumarin derivative, is extracted from the fruit of *Cnidium monnieri* (L.) Cusson, a therapeutic agent in traditional Chinese medicine. Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death in women, in developed and increasingly in developing countries. The objective of this study is to investigate the effects of Osthole on the proliferation, cell cycle and apoptosis in human breast cancer cells. Antiproliferative activity of Osthole was measured with the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was performed to observe cell cycle distribution and apoptotic body appearance. Our results show that Osthole is effective in inhibiting cell proliferation, promoting G1 phase arrest and inducing apoptosis in breast cancer cells. These findings indicate a need for further evaluating Osthole as an antitumor agent to treat breast cancer patients.

Key words: Osthole; breast cancer; proliferation; cell cycle; apoptosis
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蛇床子素对人乳腺癌细胞增殖、细胞周期及凋亡的影响

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[摘要] 蛇床子素是从传统的中药蛇床子的果实中提取的香豆素类衍生物. 在发达国家以及发展中国家, 乳腺癌是发病率和致死率较高的女性肿瘤之一. 本研究的目的是调查蛇床子素对人乳腺癌细胞的增殖、细胞周期以及凋亡的影响. 蛇床子素的抗细胞增殖活性用 MTT 法测定. 细胞周期的分布以及细胞凋亡用流式细胞术测定. 本研究结果表明, 蛇床子素对抑制乳腺癌细胞的增殖、促进 G1期阻滞以及诱导细胞凋亡有明显作用. 该结果提示有必要进一步研究和评估蛇床子素在乳腺癌治疗中的作用.

[关键词] 蛇床子素, 乳腺癌, 增殖, 细胞周期, 凋亡

Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death in women in developed countries and increasing in developing countries[1]. With 1 million new cases diagnosed in the world annually, it accounts for 18% of all female malignancies[2]. Despite its pervasive impact, the etiology of breast

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