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博士学位论文

拟穴青蟹细胞膜脂筏结构相关基因SpFLT-1的克隆、表达特性及其与病原入胞机制的相关性研究

Cloning, expression and characterization of membrane lipid rafts related gene SpFLT-1 and its functional study during pathogens endocytosis in Scylla paramamosain

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摘 要

拟穴青蟹(Scylla paramamosain)是我国最重要的海水养殖蟹类之一，具有重要的营养价值和经济价值。集约化的养殖模式导致了近年来拟穴青蟹的疾病不断爆发，其中以弧菌等革兰氏阴性菌为主的细菌性病原是导致青蟹发病的重要诱因之一。然而由于对病原微生物感染拟穴青蟹的致病机理知之甚少，目前对其尚无有效的免疫防治措施。因此，阐明病原微生物的致病途径对于控制拟穴青蟹疾病爆发具有重要的意义。

病原微生物在长期的进化过程中发展出一整套逃避、干扰、抑制和对抗宿主免疫系统的机制，其中利用宿主的细胞膜结构和受体蛋白进入胞内是病原微生物感染宿主的重要途径之一。为了研究病原在入侵拟穴青蟹过程中与宿主作用的关键蛋白，进而为有效阻断病原微生物入侵途径提供理论依据，本论文比较系统地研究了细胞膜脂筏结构相关基因SpFLT-1的结构特点、组织器官分布特性以及溶藻弧菌诱导后的体内表达模式，并通过体外培养原代青蟹血淋巴细胞，初步探讨了SpFLT-1在弧菌内吞过程中可能发挥的功能，该项研究为初步阐明病原微生物感染拟穴青蟹的致病机制奠定了理论基础。取得的结果如下：

1 成功构建了LPS刺激拟穴青蟹血淋巴细胞SSH cDNA文库。本试验以雌性拟穴青蟹为对象，利用抑制差减杂交技术(Suppression subtractive hybridization, SSH)构建了脂多糖(Lipopolysaccharide, LPS)刺激下血淋巴细胞cDNA文库，文库总容量为1.2×105 cfu。随机选取了721个克隆子进行测序，共获得271个上调表达基因。利用AmiGO基因释义工具，将所有271个基因按照参与的生物学过程进行分类，其中179个基因分别参与6个不同的生物学过程，92个基因属于完全未知功能的基因。值得一提的是，上述271个基因中只有18个基因(6.6%)在甲壳类动物中报道过，其余253个基因均是首次在拟穴青蟹中发现。从该文库中筛选获得细胞膜脂筏结构相关基因flotillin-1(SpFLT-1)。

2 克隆获得拟穴青蟹膜脂筏结构基因flotillins家族成员SpFLT-1的全基因序列和另一成员SpFLT-2的全长cDNA序列。SpFLT-1的全长cDNA序列包括1278 bp的开放阅读框(Open reading frame, ORF)、42 bp的5'非翻译区(Untranslated region,

UTR)和103 bp的3'UTR三部分(GenBank登录号: FJ774690)。预测该基因编码426个氨基酸，编码的蛋白分子量为47 kDa。SpFLT-1基因包含9个外显子和8个内含子，其DNA侧翼片段长度为1310 bp(GenBank登录号: JX228176)。SpFLT-2的全长cDNA序列包括1317 bp的ORF、339 bp的5'UTR和171 bp的3'UTR三部分(GenBank登录号: KC865733)，预测其编码439个氨基酸，编码的蛋白分子量为48 kDa。

3 以纯化的拟穴青蟹SpFLT-1原核表达产物制备了特异性强且效价高的多克隆抗体。根据SpFLT-1基因的全长cDNA序列，构建了pET-28a(+)/SpFLT-1融合表达载体，并成功在大肠杆菌中诱导表达出重组蛋白。纯化的蛋白经质谱鉴定为目的蛋白。利用纯化好的目的蛋白制备了特异性强、效价高的多克隆抗体，可以用于后续蛋白功能的相关研究。

4 分析了拟穴青蟹SpFLT-1和SpFLT-2基因的组织表达分布特性以及在青蟹不同发育阶段的表达模式。结果表明SpFLT-1基因在各组织器官中表现出组成型表达特点，血淋巴细胞中表达量最高；SpFLT-2基因在各组织器官中表达水平较一致。SpFLT-1 mRNA在青蟹胚胎发育早期表达量较高，伴随着生长发育进程其表达量逐步降低；SpFLT-2基因在溞状幼体I期表达量最高。SpFLT-1和SpFLT-2两个基因的转录水平在卵巢发育早期表达量最高，随着拟穴青蟹逐渐性成熟过程，两个基因转录水平有降低的趋势。

5 研究了SpFLT-1蛋白在拟穴青蟹部分组织器官中的表达及分布特征。选取了SpFLT-1 mRNA表达水平较高的5个组织器官: 卵巢、鳃、胸神经团、心脏、中肠，利用Western-blot技术研究了SpFLT-1蛋白表达模式。SpFLT-1蛋白在中肠中表达量最高。免疫组织化学(Immunohistochemistry, IHC)试验结果显示SpFLT-1蛋白广泛分布于青蟹卵泡上皮细胞、鳃丝上皮细胞层、胸神经团神经细胞、心肌外膜以及中肠的上皮细胞层中。

6 分析了溶藻弧菌感染拟穴青蟹后SpFLT-1 基因在血淋巴细胞和鳃中转录和翻译水平变化。结果显示，青蟹感染溶藻弧菌3 h后，SpFLT-1 mRNA在血淋巴细胞和鳃中显著上调表达。梯度密度离心和Western-blot结果发现SpFLT-1蛋白不仅分布于青蟹血淋巴细胞和鳃上皮细胞的膜脂筏结构中，还可能在胞内表达并发挥特定的功能；SpFLT-1蛋白与弧菌蛋白之间可能相互作用，二者作用时间短暂。结果提示

SpFLT-1在青蟹血淋巴细胞和鳃细胞中可能是溶藻弧菌入侵宿主细胞的关键蛋白。7 初步探讨了SpFLT-1基因在拟穴青蟹血淋巴细胞内吞溶藻弧菌过程中的作用。在体外成功培养拟穴青蟹原代血淋巴细胞的基础上，利用RNA干扰技术(RNA interference，RNAi)特异性地抑制SpFLT-1 mRNA表达。RNAi试验后，SpFLT-1 dsRNA处理组与对照组相比细胞的内吞效率降低，该结果进一步从体外验证了SpFLT-1可能是溶藻弧菌入侵宿主细胞关键蛋白的推测。

关键词：拟穴青蟹；血淋巴细胞；溶藻弧菌；SpFLT-1；内吞

Abstract

The mud crab, Scylla paramamosain is one of the most important marine breeding crabs in China, with vital nutritional and economic value. The animals are usually raised in ponds at high densities which has led to disease epidemics in recent years. Bacteria especially Gram-negative bacteria (e.g. Vibrios), are one of the leading pathogens infecting these crabs. However, the pathogenesis is poorly understood and there are no effective immune control measures yet. Therefore, clarifying the pathogenic pathway of pathogens will play a significant role in the control of disease outbreak in these crabs.

Pathogenic microorganisms can enter the host cell by using the membrane structure and receptors during the process of confrontation with the host immune system. This is an important way by which pathogens infect the host. The purpose of the present study is to investigate the key proteins involved in the pathogenic invasion process which could provide us a theoretical foundation on effectively blocking the way for pathogens infection. In this study, a complete cDNA sequence of membrane lipid rafts related gene SpFLT-1 was cloned, its gene organization, tissue-specific distribution as well as its expression patterns during the Vibrio alginolyticus infection process were also determined. Furthermore, preliminary study of SpFLT-1 gene function during the process of hemocytes endoctosis V. alginolyticus in S. paramamosain was performed in vitro by culturing the primary hemoctyes. This study provided a theoretical foundation to clarify the pathogenesis for pathogens infecting S. paramamosain. The results were as follows:

1 A forward suppression subtractive hybridization (SSH) cDNA library was successfully constructed from the hemocytes of S. paramamosain in response to bacterial lipopolysaccharide (LPS) with content of 1.2×105 cfu. A total of 721 clones on the middle scale in the SSH library were sequenced. Among these

genes, 271 potentially functional genes were recognized based on the BLAST searches in NCBI and were categorized into seven groups in association with different biological processes using AmiGO against the Gene Ontology database. Among 271 genes, 179 (66.1%) were annotated to be involved in different biological processes, while 92 genes (33.9%) were classiﬁed as an unknown-function gene group. It was noted that only 18 of the 271 genes (6.6%) had previously been reported in other crustaceans and most of the screened genes showed less similarity to known sequences based on BLASTn results, suggesting that 253 genes were found for the ﬁrst time in S. paramamosain. From the library, a membrane lipid rafts related gene SpFLT-1 was identified.

2 The full-length DNA of SpFLT-1 and cDNA of SpFLT-2 were obtained. The cDNA sequence of SpFLT-1 comprises the open reading frame (ORF) of 1278 bp, 5' untranslated region (UTR) 42 bp and 3' UTR 103 bp (GenBank accession number: FJ774690). The gene encodes 426 amino acids and the molecular weight of the protein is 47 kDa. SpFLT-1 gene contains nine exons and eight introns and the DNA flanking fragment length is 1310 bp (GenBank accession number: JX228176). The full-length cDNA sequence of SpFLT-2 includes an ORF 1317 bp, 5' UTR 339 bp and 3' UTR 171 bp (GenBank accession number: KC865733). It encodes 439 amino acids and the putative molecular weight of the protein is 48 kDa.

3 Prokaryotic expression and purification of SpFLT-1 recombinant protein and the preparation of the specificity and high titer polyclonal antibodies. According to the full-length cDNA sequence of SpFLT-1, the fusion expression vector pET-28a(+)/SpFLT-1 was constructed and the recombinant protein was successfully expressed. The protein was purified in gel slices and it was confirmed by mass spectrometry. A specificity and high titer polyclonal antibody were prepared which can be used for subsequent protein function study.

4 The tissues distribution characteristics and different developmental stages expression patterns of SpFLT-1 and SpFLT-2 genes from S. paramamosain was investigated. SpFLT-1 gene showed constitutive expression feature and the highest expression level is in hemocytes. SpFLT-2 gene also distributed in various tissues and organs, and the expression levels were more consistent. SpFLT-1 mRNA showed high expression levels in early embryonic development process and the expression gradually reduced during the late stages. The two genes transcription level were relatively higher in ovaries of young crabs than in mature ones.

5 The SpFLT-1 protein expression pattern in partial tissues. Five organs were selected, all of which showed high SpFLT-1 mRNA expression levels: ovaries, gills, thoracic ganglion mass, heart and midgut gland for studying SpFLT-1 protein expression by Western-blot. The highest expression level of SpFLT-1 protein was in the midgut gland. The immunohistochemistry (IHC) results showed SpFLT-1 protein was widely distributed within the follicle epithelial cells, the gill epithelial cells, thoracic ganglion nerve cells, myocardial adventitia and intestinal epithelial cells.

6 Analysis of SpFLT-1 gene transcription and translation level in hemocytes and gills of S. paramamosain after challenged with V. alginolyticus. The results showed that SpFLT-1 mRNA in hemocytes and gills was significantly upregulated after 3 hours of infection V. alginolyticus. SpFLT-1 protein was distributed not only in the lipid rafts domain of hemocytes and gill epithelial cell membrane. Further, this protein may also play specific function in intracellular components. Density gradient centrifugation results suggested possible interactions between SpFLT-1 protein and Vibrio proteins,and the interation time was temporary. These results suggested that SpFLT-1 may play a key role during V. alginolyticus entry into host cells of S. paramamosain.

7 The preliminary study of SpFLT-1 gene function during the process of

hemocytes endoctosis V. alginolyticus in S. paramamosain. The primary hemocytes of S. paramamosain were successfully cultured in vitro, and SpFLT-1 mRNA expression was specifically inhibited by RNA interference (RNAi) in the hemocyts. It was found that SpFLT-1 dsRNA group showed a lower endocytosis rate than the control group. The results further validated the results from the in vitro study suggested that SpFLT-1 may act as a key protein during V. alginolyticus invasion of the host cell.

Keywords: Scylla paramamosain; Hemocytes; Vibrio alginolyticus; SpFLT-1; Endocytosis

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