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Short communication

Identification of the *MmeHairy* gene and expression analysis affected by two SNPs in the 3′-untranslated region in the clam *Meretrix meretrix*

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ABSTRACT

As a bHLH transcriptional repressor, Hairy-related proteins can bind to DNA sites in target gene promoters and negatively regulate gene transcription. In the present study, the full-length cDNA of *Hairy* was obtained from the clam *Meretrix meretrix* (*MmeHairy*), and two SNPs in the 3′-untranslated region (UTR) of this gene, SNP1066 and 1067, were identified and characterized. Multiple sequence alignment and phylogenetic analysis revealed that MmeHairy belongs to the Hairy protein subfamily. Analysis of tissue expression patterns showed that the mRNA of *MmeHairy* had the highest expression level in the hepatopancreas. The expression levels of *MmeHairy* were up-regulated in the hepatopancreas after *Vibrio* challenge. Genotyping and quantitative analysis showed that the mRNA levels of *MmeHairy* were significantly different among individual clams with different genotypes at SNP1066 and 1067 ($P < 0.05$), which indicated that these two SNP loci may affect the expression of *MmeHairy* and could be used as candidate markers for future selection in *M. meretrix* breeding programs.

1. Introduction

As one type of bHLH transcriptional repressor, Hairy-related proteins contain the bHLH domain, the Orange domain and the carboxyl-terminal tetrapeptide motif WRPW [1–3]. The bHLH domain is approximately 60 amino acids, which comprises a DNA-binding region and dimerization region [4–8]. The Orange domain is approximately 30 amino acids and serves as an additional interface for protein interactions [9]. The WRPW motif recruits active Groucho/transducin-like Enhancer of split (Gro/TLE) family proteins, which are transcriptional corepressors, to target gene promoters for Hairy-related proteins [3]. Hairy-related proteins include the *Drosophila* Hairy, Deadpan and Enhancer of split [E(spl)] proteins and mammalian HES proteins [3,8]. Based on the comparison of full-length protein sequences, Davis and Turner [9] divided the Hairy-related proteins into two distinct subfamilies, Hairy and E(spl) protein subfamilies. As important targets of the Notch signaling pathway, Hairy-related proteins are involved in cell proliferation, differentiation and apoptosis in various tissues where the Notch signaling pathway plays fundamental roles [10–14]. In addition, Notch-Hairy pathways have links with the NF-κB signaling pathway [15,16], the TGF beta signaling pathway [17] and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway [18].

Gene expression is regulated by multiple factors, among which single nucleotide polymorphisms (SNPs) are important. Being co-dominant, highly abundant and easily genotyped by high-throughput analysis, SNPs have been widely applied to molecular genetic analysis [19]. Given that the SNPs can affect traits of interest, marker-trait association analyses have been conducted in fish and shellfish recently [19–23]. Based on location, SNPs could be divided into coding SNPs and non-coding SNPs [24,25], and the coding SNPs attract more attention for causing alteration of protein structure and stability [26–28]. However, many studies had suggested that the SNPs in non-coding regions, such as the promoter, 5′-untranslated region (5′-UTR) and 3′-UTR, would also affect gene expression [29–31], and the mechanism involved may be that they can affect the affinity of some transcriptional activators and the splicing of mRNA and enzyme activity. Therefore, SNPs have the

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potential to be functional polymorphic variants, and studying SNPs involved in traits of interest is feasible and meaningful.

Meretrix meretrix is a commercially and ecologically important marine bivalve along the coastal and estuarine areas of south and southeast Asia [32]. *Vibrio* has been reported to be a leading agent causing mortalities of *M. meretrix*, so one of the most important issues in *M. meretrix* breeding programs is to breed highly *Vibrio*-resistant strains [33,34]. With regard to this matter, marker-trait association analyses have been conducted in *M. meretrix*. In a previous study, two adjacent SNPs located on contig16077 were identified to be associated with *Vibrio* resistance [35]. In addition, the protein predicted by contig16077 was homologous with transcription factor HES-4 in *Xenopus (Silurana) tropicalis*, which is a Hairy-related protein that acts as a DNA-binding transcriptional repressor [8]. The function of this contig and the *Vibrio*-resistant influence of these two SNPs attracted our attention. With the aims to identify the *MmeHairy* and analyze how these two SNPs inside affect *Vibrio* resistance, the full-length cDNA of *MmeHairy* was cloned from *M. meretrix*, and its expression levels with different genotypes were analyzed. The present study provides basic information for functional analysis of *MmeHairy* SNPs in *M. meretrix,* and implied that the *MmeHair* SNPs could be used as potential markers for selective breeding of this clam.

2. Materials and methods

2.1. Sample preparation and amplification of the cDNA sequence

A total of 127 *M. meretrix* clams with an average shell length of 45.2 ± 0.42 mm were bought from the aquatic market in Qingdao, China. The foot from 122 clams was dissected respectively and applied to DNA extraction, which were used for SNP genotyping. Additionally, the hepatopancreas was dissected respectively and stored at −80 °C for detection of *MmeHairy* mRNA expression in clams with different genotypes. To determine the tissue distribution of *MmeHairy* transcripts, samples of the foot, mantle, gill, adductor and hepatopancreas were collected from the other five clams and applied to RNA isolation and cDNA synthesis. Total genomic DNA was extracted with a special DNA extraction kit for marine animals (Tiangen, China) [33]. Total RNA was extracted by TransZol Up Total RNA Isolation Reagent (TransGen, China), following the manufacturer's protocol. The cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA) with oligo (dT) primer AOLP and BDA oligo listed in Table 1. Primers used in this study are all shown in Table 1.

One pair of primers, HairyF and HairyR, designed according to the sequence of contig16077 (TSA accession: JI273590), was used to amplify and verify the sequence of *MmeHairy* using Taq DNA polymerase (TaKaRa, Japan). The PCR was carried out on a PCR Thermal Cycler Dice TP-650 (TakaRa) with the following temperature parameters: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s, extension at 72 °C for 60 s, and a final extension step at 72 °C for 10 min. The PCR products were detected by electrophoresis on 1.5% agarose gels, and the expected products were purified and cloned into the PMD19-T simple vector (TaKaRa) and then sequenced at BGI Tech (China).

The SMART™ RACE cDNA Amplification Kit (Clontech) was applied to clone the full-length *MmeHairy* cDNA. A nested PCR approach was used for 5′- and 3′-RACE amplifications according to the manufacturer's recommended protocol with four reverse gene primers, Hairy5F1, Hairy5F2, Hairy3F1 and Hairy3F2. The PCR products of the expected size were cloned into the pMD19-T simple vector, sequenced and overlapped with the above sequence.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)
Aolp	GGCCACGCGTCGACTAGTAC(T)$_{16}$(A/C/G)
BDAoligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG
Hairy3F1	TACCTTGGGTCGGTCCAGGGCGT
Hairy3F2	GTGCCTAACACAAACTCTCCATCGG
Hairy5F1	CGGCATAAGCAGGCAGCCATTTT
Hairy5F2	CAACTGTCGGGTCACAAGTCATAGCCTG
HairyF	TTCAAACAGTTCAGAGGGTG
HairyR	TGCCAGAGGTCGGTAATG
HairyRTF	GCAGGTCAATCAGGCTAT
HairyRTR	GGTTGTTGGCACTGTTCT
actin-F	TTGTCTGGTGGTTCAACTATG
actin-R	GACTGATTTCTTACGGATG
Primer1F	TCGGTAATGAATATGTCTTGG
Primer1R	AGCAGCAAATCGTTCACACCT
Extension1	TTTTTTTTTTTTTTTTAATGAATATGTCTTGGCAATTT
Extension2	TTTTTTTTTTTTATTGAACTGTTTACAATGTAT

2.2. Sequence and phylogenetic analysis

The similarity of the resulting sequence was analyzed using the BLASTX and BLASTP programs (http://www.ncbi.nlm.nih.gov/blast/) and ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) program. The nucleotide sequence was translated into the amino acid sequence with the DANMAN 7.0 software package, and the functional domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). The isoelectric point and molecular weight of the deduced protein were determined using the "Compute PI/MW" tool on the ExPASY Server (http://www.expasy.org/tools) [36]. Based on amino acid alignments, multiple sequence alignments and a neighbor-joining (NJ) phylogenetic tree were constructed using ClustalX2 [37] and MEGA 5.0 [38], respectively. The robustness of each topology was checked by 1000 bootstrap replications. All the analyzed sequences used were retrieved from the GenBank database.

2.3. Quantitative detection of MmeHairy mRNA expression by RT-PCR

Real-time PCR was applied to quantitative detecting the *MmeHairy* mRNA expression using the QuantiFast SYBR Green PCR Kit (Qiagen, Germany). The sequence-specific primers HairyRTF and HairyRTR were used, and the real-time quantitative PCRs were conducted in 20 μl reactions containing 10 μl 2 × QuantiFast SYBR Green PCR Master Mix, 4 μl RNase-free water, 2 μl template cDNA and 2 μl each primer. The amplifications were carried out on a Mastercycler ep realplex 4S (Eppendorf, Germany) machine, with the following parameters: initial heat inactivation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s and combined annealing/extension at 60 °C for 30 s. The melting curve analyses were performed to verify the specificity of the products with continuous fluorescence reading from 65 to 95 °C at a ramping rate of 0.3 °C/s. The *M. meretrix β-actin* was used as an internal control gene for sample loading and normalization, and the real-time quantitative PCRs were conducted as described above with primers actin-F and actin-R. Four clams were used, and the measurements were performed with 4 replicates, respectively.

2.4. Vibrio challenge and MmeHairy expression analysis

To investigate the mRNA expression levels of *MmeHairy* after *Vibrio* challenge, another 60 adult clams (46.51 ± 0.32 mm in shell length) were bought and cultivated in laboratory conditions at 26.5 °C under continuous aeration for one week with condensed