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Short communication

Ubiquitin-conjugating enzyme E2-like gene associated to pathogen response in *Concholepas concholepas*: SNP identification and transcription expression

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ABSTRACT

Ubiquitin-conjugated E2 enzyme (UBE2) is one of the main components of the proteasome degradation cascade. Previous studies have shown an increase of expression levels in individuals challenged to some pathogen organism such as virus and bacteria. The study was to characterize the immune response of *UBE2* gene in the gastropod *Concholepas concholepas* through expression analysis and single nucleotide polymorphisms (SNP) discovery. Hence, *UBE2* was identified from a cDNA library by 454 pyrosequencing, while SNP identification and validation were performed using *De novo* assembly and high resolution melting analysis. Challenge trials with *Vibrio anguillarum* was carried out to evaluate the relative transcript abundance of *UBE2* gene from two to thirty-three hours post-treatment. The results showed a partial *UBE2* sequence of 889 base pair (bp) with a partial coding region of 291 bp. SNP variation (A/C) was observed at the 546th position. Individuals challenged by *V. anguillarum* showed an overexpression of the UBE2 gene, the expression being significantly higher in homozygous individuals (AA) than (CC) or heterozygous individuals (A/C). This study contributes useful information relating to the UBE2 gene and its association with innate immune response in marine invertebrates.

1. Introduction

In conjunction with phosphorylation, ubiquitination process is one of the most studied post-translational modifications of proteins [1]. Ubiquitin is a protein of 76 amino acids which acts in sequence with three activating enzymes, known generically as UBE1, UBE2, UBE3 [2]. UBE1 enzyme forms an activated thiolester bond with ubiquitin, in an ATP-dependent reaction. For its part, UBE2 carries the activated ubiquitin from the UBE1-Ub complex to an UBE3 ubiquitin-ligase. The latter enzyme transfers the ubiquitin from the UBE2-Ub complex to a target protein or another ubiquitin. In general, UBE2 has the peculiarity of determining the type of ubiquitin chain assembled [3]. Therefore, UBE2 has a great relevance to determine the function of the ubiquitin chain, which is directly related with its structure, given by the different types of linkages that have been discovered [4]. Seven of the ubiquitin chain linkages, allowed by the connection between the UBE2 and UBE3 enzymes, involve internal lysines (K) that are linked to the carboxy-terminal diglycine of the consequent ubiquitin. Nevertheless, an eighth type of linkage was discovered, which is formed by an internal methionine of an ubiquitin with the carboxy-terminal carboxy group of the next one [5].

Previous studies have identified an association between the ubiquitination and the stimulation of genes cascades involved in the immune response. In this context, the up-regulation of genes involved in ubiquitination, such as *UBE2*, have related to increments in the activity of some key genes of the immune response like the cytokine TNF-α [6–9]. Recently, the progress of the study of this process has revealed novel conformations of linear ubiquitin chains, which are two main components known as LUBAC (linear ubiquitin assembly chains) and SHAIRPIN (SHANK-associated RH domain interacting protein). Those conformations are unique because of the role of the UBE3 in determining the type of formation of the ubiquitin chain. Reports suggest the expression of genes involved in the innate immune response increase when ubiquitination follows this structure [4,10]. These gene pathways are also activated in the absence of SHARPIN, even though gene transcription are significantly lower than those in the pathway with LUBAC activity [8]. In both cases, the role of UBE2 enzyme is crucial for the conformation of the structure that finally activates the transcription of immune-related genes.

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The immune response of various species has been studied under different approaches. One approach is to identify punctual polymorphism (SNPs) in candidate genes involved in immune response. It is previously reported that SNPs in these genes could alter the conformation of some genes, and therefore influence in the resistance/susceptibility of organisms to diseases [11]. Thousands of SNPs markers related to immune response genes have been discovered in different aquatic species, such as rainbow trout, oyster, turbot and common carps [12–15]. Additionally, an SNP in the serine protease inhibitor gene of the eastern oyster was associated with resistance/susceptibility to bacterial disease [16]. Numerous studies have evaluated the transcription patterns of candidate genes involved in innate immune response of marine species and have associated these patterns to the response to pathogens [17–24]. However, until now there have been no reports that associate SNP markers and its influence on the gene transcription pattern of candidate genes.

Concholepas concholepas, our model species for this study, is a marine gastropod found along the Chilean coast that has become commercially important [25–27]. Until now, the main focus of immunology related to *C. concholepas* is the production of a novel hemocyanin that has been useful for the study of cancer, due to its antitumor properties [28–30]. However, there are no studies aimed to characterize the genetic component of the immune response of *C. concholepas*, despite the availability of the EST database generated by 454 pyrosequencing [31]. Recently, we have performed a new 454 pyrosequencing with major genome coverage (unpublished data) and found candidate genes involved in innate immune response. Herein, Ubiquitin-conjugating enzyme E2 (*UBE2*) gene was partially identified in *C. concholepas* with a putative SNP variation. The aim of this study was to characterize the immune response of *UBE2* gene in the gastropod *C. concholepas* and to evaluate the association between the putative SNP-*UBE2* and changes in gene expression of *UBE2* in individuals exposed to *V. anguillarum*.

2. Materials and methods

2.1. Ubiquitin-conjugating enzyme E2 identification

UBE2 gene was identified from a *De novo* assembly using the ESTs database generated by Cárdenas et al. [31] for *C. concholepas*, and a new cDNA library pyrosequenced by our research team (unpublished data). Contigs were then annotated with CLC Genomic Workbench (CLC Bio, Aarhus, Denmark) with an *e*-value threshold of 1E-05. The "Ubiquitin-conjugating enzyme E2-like" gene sequence was submitted to NCBI Genbank database (Accession number JQ954530).

2.2. SNP discovery and validation

SNP mining was carried out from *De novo* assembly with CLC Genomic Workbench software (CLCBio, Aarhus, Denmark). The bioinformatic analysis showed a putative SNP variation within the UBE2 sequences. To validate the SNP, a pair of primers was designed to flank a 79 bp region using Geneious v5.1.7 software (Biomatters, Auckland, New Zealand). Genomic DNA was then extracted from 36 individuals according to the protocol described by Aljanabi & Martinez [32], and used for PCR amplification and high-resolution melting (HRM) analysis using the Eco Real-Time qPCR System (Illumina, San Diego, California, USA), and the Kapa HRM Fast PCR Kit (Kapa Biosystems, Boston, Massachusetts, USA) following the manufacturer's instructions. SNP genotyping was carried out with Eco-qPCR software using homozygous and heterozygous controls, identified by re-sequencing.

2.3. Gene transcription analysis

Two trial groups ($N = 18$ each one) were acclimatized for ten days before the experiments. All the individuals were adults and their sizes ranged between 7.2 and 8.7 cm in shell length. One group was challenged to *Vibrio anguillarum* by injection of 200 μL ($OD_{600} = 0.2$ bacteria) into the adductor muscle at exposure times of 2, 4, 6, 10, 24 and 33 h (1 $OD_{600} = 8 \times 10^8$ cells/mL). The remaining individuals were maintained as a control group and injected with filtered sterile seawater for each exposure time. Gill tissue was dissected and total RNA was extracted using the reagent Trizol (Invitrogen®, Life Technologies, USA). Then, RNA was retrotranscribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA). Finally, qPCR assays were performed by the ΔCt method on the StepOnePlus® Instrument (Applied Biosystems, Life Technologies, USA) using the Maxima® SYBR Green qPCR Master Mix (Thermo Scientific, USA) according to the manufacturer's instructions. The *elongation factor 1* gene (*EF1*) was selected as the housekeeping gene (HKG) due to its stable value inferred through the NormFinder algorithm [33]. The other HKGs assayed were *GAPDH*, *α-Tubulin* and *EF2*. Statistical analyses were performed using the qBasePlus software (Biogazelle, Zwijnaarde, Belgium).

3. Results and discussions

3.1. Partial characterization and validation of the SNP marker in the E2 gene

Four contigs from the *C. concholepas* EST database were annotated as "*ubiquitin-conjugating enzyme E2*" with an *e*-value score of 2,97E-29. The consensus sequence showed a partial *UBE2* sequence of 889 base pair (bp) with a partial coding region of 291 pb. Despite the pivotal role of ubiquitination related to the post-translational modifications of proteins in marine invertebrates, partial *UBE2* gene sequence has only been reported for the cephalopod *Sepia officinalis* as a housekeeping gene [34]. Furthermore, SNP variation (A/C) was observed and validated at the 546th position. Among the individuals genotyped by HRM analysis, 35 showed successful PCR amplification, 13 individuals being heterozygous, 15 homozygous AA and the remaining 7 homozygous CC (Fig 1). Up to now, SNP within the *UBE2H* gene has only been characterized and associated with a particular sclerosis disease in humans [35]. This is the first

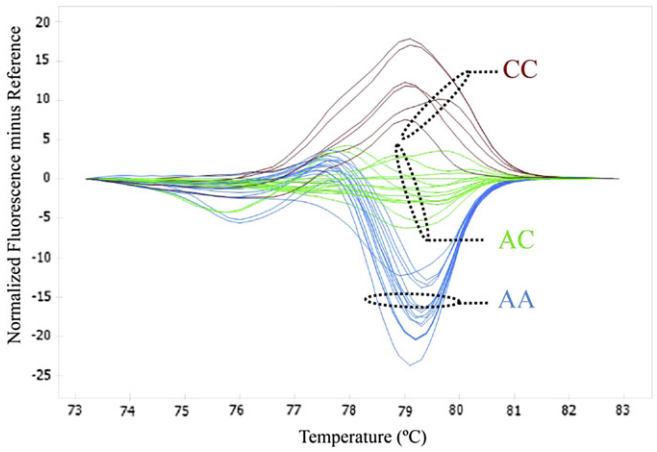

Fig. 1. Difference plots from high resolution melting analysis of *UBE2* gene in *C. concholepas* showing clear separation among homozygous and heterozygous genotypes.

time that a SNP variation is reported and validated for the *UBE2* gene for invertebrate marine species.

3.2. E2 gene transcription analysis

After 33 h post-treatment there were no mortalities among the individuals challenged by *V. anguillarum*. However, gene transcription analysis showed greater transcript abundance in challenged individuals than in control group. Significant differences ($p < 0.05$) between challenged and control groups were observed for the exposure times evaluated, reaching up to a 4-fold difference in transcript abundance (Fig. 2). In spite of this, there was no significant difference among the relative expression values in individuals exposed to pathogens at different time points ($p > 0.05$). Additionally, a comparative analysis showed that the relative abundance of the *UBE2* gene remained constant in both control and challenged individuals through exposition times. Before this study, there were no reports of gene transcription analysis of *UBE2* at different exposition times to a pathogen, therefore we could expect an increase trend of gene transcription in relation with those, nonetheless, we have not found that tendency. Previous reports have found similar results in other genes like in antioxidant genes in mussels exposed to a *Vibrio* challenge [36], and *Lysozyme* genes in Pacific whit shrimp exposed to the same pathogen [37]. Studies of *UBE2* gene expression in plants have associated this gene with differential expression between native and transgenic lines [38], while in salmonids, the relative abundance of the *UBE2* gene was compared in individuals with different nutritional states [39]. With respect to its expression levels in response to pathogens, Boname and Lehner reviewed the effects of some viruses and infections on the ubiquitin pathways [40]. In their work, they have summarized evidences of viral genes that are involved in the response to this kind of pathogens through the regulation of different components of the ubiquitination process pathway, including isoforms of the *UBE2* gene. Our study evidences the immune response of the *UBE2* gene of *C. concholepas* to *V. anguillarum* infection.

We observed the association between the SNP genotype and the transcriptional activity of UBE2. Herein, homozygous individuals (AA) had highest levels of relative abundance of this gene rather than the homozygous (CC) and heterozygous (A/C) individuals ($p < 0.05$) (Fig. 3). This SNP could be related with a major immune response of *UBE2* gene against marine pathogens in *C. concholepas*. A similar approach was reported by Kawaida et al. [41], but with respect to the *UBE3 ligase* gene. Nonetheless, this is the first time that an SNP marker within an immune-related gene was associated with its mRNA abundance levels in response to a marine pathogen. In conclusion, this study contributes relevant knowledge about *UBE2* and its association with the immune response in *C. concholepas*, and also gives novel insights with respect to changes in transcription expression of immune-related genes linked to specific SNP variations.

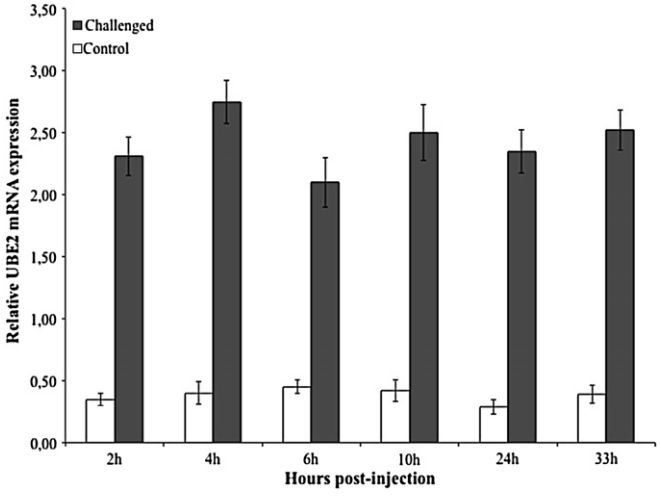

Fig. 2. *UBE2* mRNA expression in *C. concholepas* following *V. anguillarum* challenge. *Elongation factor 1* (*EF1*) gene was used as housekeeping gene and time 0 h was used as references samples. Vertical bars represent the mean ± SD. White indicates control groups and gray injected with bacteria. At each time point 3 individuals were sampled per group.

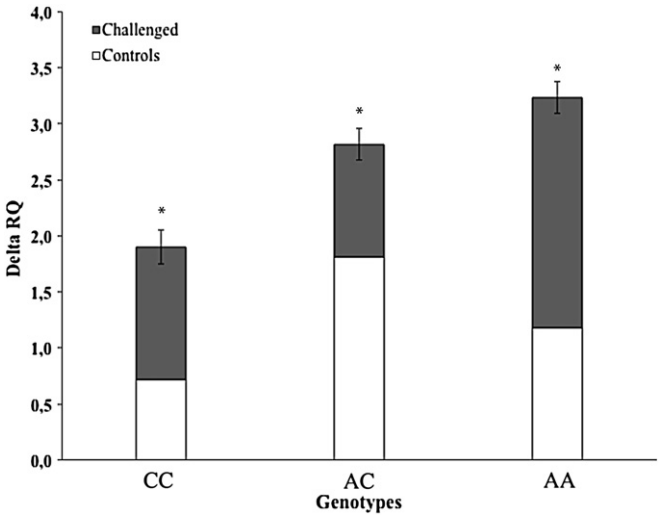

Fig. 3. Delta RQ among *UBE2* mRNA expression related to SNP's genotypes in *C. concholepas*. Vertical bars represent the differences of RQ values among control (white) and challenged (gray) individuals for each genotype. Among each group 6 individuals were sampled of each genotype. * Indicates significant differences according their genotypes.

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