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Molecular characterization and expression analysis of the first Porifera tumor necrosis factor superfamily member and of its putative receptor in the marine sponge *Chondrosia reniformis*

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ABSTRACT

Here we report the molecular cloning and characterization of the first Tumor Necrosis Factor homologous and of its putative receptor in the marine sponge *Chondrosia reniformis*: chTNF and chTNFR, respectively. The deduced chTNF amino acid sequence is a type II transmembrane protein containing the typical TNFSF domain. Phylogenetic analysis reveals that chTNF is more related to Chordata TNFs rather than to other invertebrates. chTNF and chTNFR are constitutively expressed both in the ectosome and in the choanosome of the sponge, with higher levels in the ectosome. chTNF and chTNFR mRNAs were monitored in sponge fragmorphs treated with $Gram^+$ or $Gram^-$ bacteria. chTNF was significantly upregulated in $Gram^+$-treated fragmorphs as compared to controls, while chTNFR was upregulated by both treatments. Finally, the possible chTNF fibrogenic role in sponge fragmorphs was studied by TNF inhibitor treatment measuring fibrillar and non fibrillar collagen gene expression; results indicate that the cytokine is involved in sponge collagen deposition and homeostasis.

1. Introduction

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine involved in a number of cell signalling pathways such as immune function, inflammation, apoptosis, cell differentiation and proliferation (Tracey et al. 2008). It belongs to a large family of structurally related proteins known as "TNF ligand superfamily" (TNFSF) (Wiens and Glenney, 2011). TNFSF members are type II transmembrane proteins with a conserved extracellular C-terminal TNF homology domain (THD). The THD signature is composed of 10 β-strands, which fold to originate a compact "jellyroll" topology. As many TNFSF members, TNF possesses a proteolytic cleavage site and, by the activity of specific metalloprotease/disintegrin/cysteine-rich protease (Blobel, 1997), it can act in two states: membrane bound, carrying out a local/autocrine activity, or extracellularly soluble, acting as a paracrine effector. The soluble form then can exist as a trimer bound to its receptor.

In mammals, the structure and function of more than nineteen TNFSF members have been described so far (Tansey and Szymkowski, 2009). Genes coding for TNFSF members have also been cloned and expression analysis data from various fish species including *Oncorhynchus mykiss* rainbow trout (Laing et al. 2001), *Salmo salar* Atlantic salmon (Morrison et al. 2007) and *Plecoglossus altivelis* Ayu fish (Uenobe et al. 2007) are already available. TNF molecules are recognized from a system of receptors grouped into the TNF receptor superfamily (TNFRSF), TNFRs are type I transmembrane proteins with one or more extracellular ligand-binding domains that contain a common fundamental structure with a set of cysteine-rich domains (CRDs) and a cytoplasmic region as cell function effector (Hehlgans and Pfeffer, 2005). In invertebrates the first evidences of the existence of cytokine-related factors were obtained by the evaluation of the sensitivity of invertebrate immunocytes to vertebrate cytokines or vice versa, demonstrating

Abbreviations: chTNF, *Chondrosia reniformis* Tumor Necrosis Factor; chTNFR, *Chondrosia reniformis* Tumor Necrosis Factor receptor; FNSW, filtered natural sea water.

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that invertebrate extracts could induce responses in vertebrate immune cells (Ottaviani et al. 1995). Search for homologies at the genomic level also suggests the existence of TNFSF members in various invertebrate species. The first molecular characterization of an invertebrate TNFSF ligand was reported in *Drosophila* fruit flies (Moreno et al. 2002) where the Eiger gene was identified. It encodes for a type II transmembrane protein with a C-terminal THD domain mainly expressed in the nervous system of these animals. Eiger (ligand) is recognized by Wengen (receptor) a TNFRSF member characterized by a unique extracellular cysteine rich domain (Kauppila et al. 2003; Igaki and Miura, 2014). More recently, TNFSF members have been isolated from marine arthropods as *Marsupenaeus japonicus* kuruma shrimp (Mekata et al. 2010) and *Litopenaeus vannamei* Penaeid shrimp, in the latter also a first TNF-TNFR system was described (Wang et al. 2012). The lowest taxonomic metazoan group in which a TNF-like gene was functionally described is the Mollusca, where a TNF homologous responsive to pathogenic infection *in Haliotis discus* has been cloned (De Zoysa et al., 2009).

Porifera is the most ancient phylum of the metazoans that diverged from the other metazoan phyla over 600 million years ago. Sponges are animals characterized by extreme structural simplicity and formed by relatively few cell types not yet well organized into conventional tissues (Simpson, 1984). In these first animals, the transition to multicellularity was accompanied by the development of defense mechanisms against invading pathogens (Wiens et al. 2005). In addition, most of the sponge species are characterized by abundance of prokaryotic endosymbionts (Sarà et al. 1998; Hentschel et al. 2010). For this reason these animals are provided with appropriate molecular tools allowing the recognition of sponge pathogenic bacteria and fungi as the lipopolysaccharide (LPS)-interacting protein identified on the sponge cell surface that acts as a receptor for Gram negative bacteria (Wiens et al. 2005). In the past, sponge immuno-reactivity towards TNF antibodies was reported on tissues of the *Geodia* genre (Pfeifer et al. 1992) suggesting the existence of TNF-like molecules in Porifera but, to date, no data regarding the molecular cloning of sponge cytokines and a clear evidence of their existence are reported. Since sponges are animals eliciting a growing biotechnological interest, especially in sponge mariculture (Bergman et al. 2011; Sipkema et al. 2005) aimed at the purification of a number of bioactive compounds for biomedical and industrial uses (Müller et al. 1986; Nicklas et al. 2009), it results extremely relevant to widen our knowledge on sponge defense mechanisms as well as on factors affecting their growth and development.

In the present study we report the molecular cloning of a first Porifera full-length cDNA (chTNF) coding for a TNFSF member in the marine sponge *Chondrosia reniformis*. The sponge tissue distribution of the cytokine transcript was analysed by quantitative PCR (qPCR). In order to establish the immunological role of the TNF-like protein and to evaluate the presence in sponges of a first ancient TNFR towards which the TNF-like protein may be directed to, the mRNA expression profile of the chTNF and of an identified putative sponge TNFR-like transcript were investigated after sponge fragmorph treatment with Gram positive (*Enterococcus faecalis*) or Gram negative (*Vibrio alginolyticus* and *Vibrio fluvialis*) bacteria. Finally, to investigate the possible involvement of sponge chTNF in collagen biosynthesis, the expression levels of a fibrillar and of a non fibrillar collagen gene were evaluated after fragmorph treatment with specific TNF inhibitors and after Gram positive bacterial infection.

2. Material and methods

2.1. Experimental animals

Specimens of *C. reniformis* were collected in the area of Portofino Promontory (Liguria, Italy) at depths of 10–20 m. During sampling and transport the temperature was maintained at 14–15 °C. Laboratory storage conditions are described in Pozzolini et al. (2014).

2.1.1. C. reniformis de novo transcriptome sequencing

C. reniformis chTNF and chTNFR transcripts were identified by *de novo* transcriptome sequencing approach.

Total RNA from *C. reniformis* was extracted using Isol-RNA Lysis (5 Prime GmbH, Hilden, Germany) using a whole freshly collected sponge, subsequently the poly-A fraction was isolated using Fast track MAG mRNA isolation kit (Life Technologies, Milan, Italy) according to the manufacturer's instructions. The obtained poly-A fraction was subjected to a second round of mRNA messenger purification in order to improve the purity level of the sample. Total RNA and further purified mRNA were assessed for quality on a Bioanalyzer 2100 (Agilent Inc., Santa Clara, CA, USA). 7 μg of purified *C. reniformis* mRNA were used for cDNA synthesis and normalization from Evrogen JSC Company (Moscow, Russian Federation).

5 μg of normalized cDNA were finally used for pyrosequencing reaction using a FLX Titanium 454 sequencer (Roche SpA, Milan, Italy) by BMR Genomics Company (Padova, Italy). Short nucleotide reads obtained were assembled by the MIRA software to produce error-free, unique contiguous sequences (contigs). The 19,678 transcripts obtained (isotig) were first aligned by BlastX to protein database Ref Seq prot (e-value < 0.00001, March 2013 release).

2.1.2. chTNF and chTNFR full-length cDNA identification and cloning

TransDecoder, which comes included in the Trinity software distribution (trinityrnaseq r20130225, Grabherr et al. 2011), was used to extract likely coding regions from transcripts and to identify CDS with homology to known protein domains via Pfam search. Pfam annotations were searched for the term "TNF", and four sequences (isotig 06410, isotig 14442, isotig 09381 and HZIY80A01AGWX1) were identified as containing the Pfam domains PF00229 or PF00020, the TNF (Tumor Necrosis Factor) family and the TNFR/NGFR cysteine-rich region (Tumor Necrosis Factor Receptor) respectively.

Further searches using Hidden Markov Model approach with HMMER v3.1b2 (http://hmmer.org/, last accessed May 20th, 2015) with profiles built with core sequences characterizing the PF00229 (37 sequences) and the PF00020 (52 sequences) domains, were performed to narrow the Pfam prediction, with 1e-5 as reporting threshold.

The sequence isotig 06410 identifying a putative complete cDNA sequence was identified as candidate TNF-like transcript (chTNF), whereas the sequence isotig 09381, with a complete putative cDNA as well, as candidate TNFR-like transcript (chTNFR).

Since the assembled sequences were products of *de novo* assembly, they were considered prone to errors, to confirm that the sequence represented a true gene product, experimental validation was performed by full-length chTNF and full-length chTNFR high-fidelity PCR amplification with Platinum Taq Polymerase High Fidelity (Life Technologies) using the couples of primers described in Table 1. The obtained PCR products were then cloned into pJET1.2/blunt vector for sequencing.