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Identification and functional characterization of a sTRAIL gene in mussel *Hyriopsis cumingii*

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) plays an important role in many biological processes including immune response and cell apoptosis. Here, we identified a soluble TRAIL homolog in a mussel species, *Hyriopsis cumingii* (designated Hc-sTRAIL), which shows significant structural and functional similarities to mammalian sTRAIL. Real-time PCR analysis shows that mussel TRAIL is ubiquitously expressed in various tissues and involved in the immune response of mussel. Study on its apoptotic effect indicates that Hc-sTRAIL can induce significant apoptosis in NCI-H446 cells and involved the caspase 3 pathway. This study provides new insight into the physiological function of Hc-sTRAIL.

1. Introduction

The apoptotic effects of tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL/Apo2L) or its extracellular domain—soluble TRAIL (sTRAIL) have been well studied in human (Pitti et al., 1996; Tang et al., 2011; Wiley et al., 1995; Yildiz et al., 2010). It can induce apoptosis in many kinds of cancer cells by binding to the death receptors (DR4 and DR5) (Yildiz et al., 2010). This interaction results in recruitment of adapter protein Fas-associated death domain (FADD) and procaspase 8, and leads to the formation of a protein complex called DISC (Death-Inducing Signaling Pathways). In this complex, procaspase 8 is proteolytically activated to caspase 8 (Choi et al., 2011; Shirley et al., 2011) and activated caspase 8 can induce cellular apoptosis through activation of the death receptor pathway and/or the mitochondrial pathway (Siegmund et al., 2011; Wang and El-Deiry, 2003). Both pathways eventually unite in the activation of caspase 3, the final executor of apoptosis, and result in cell death in various human cancer cell lines, while show little or no cytotoxicity to normal cells (Yildiz et al., 2010). Because of the selective cytotoxicity, TRAIL is regarded as a potential therapeutic molecule against cancer malignancies.

In addition, both TRAIL and sTRAIL play an important role in immune responses, and they can be induced by stimuli such as lipopolysaccharides (LPS), Rickettsia-like organism (RLO) and viruses (Collison et al., 2009; Halaas et al., 2000; Simons et al., 2007; Yang and Wu, 2010).

Despite the pro-apoptotic effects of TRAIL on human cancer cells and its roles in immune responses that have been well studied in various vertebrate species from fish to human (Abdalla et al., 2004; Gao et al., 2008; Wiley et al., 1995), little is known about its role in invertebrate mollusks (Yang and Wu, 2010). Particularly, there is no report about the pro-apoptotic effects of mollusk TRAIL on human cancer cells. On the contrary, several kinds of TNF members including Eiger, Wengen, TNF-α, FasL and LPS-induced TNF-a factor (LITAF) were identified in invertebrate species (De Zoysa et al., 2009; Kauppila et al., 2003; Park et al., 2008; Yang et al., 2012; Yu et al., 2007).

Hyriopsis cumingii (Lea) is an economically important freshwater mussel in China (Zhang et al., 2007). To understand the biological function of TRAIL in invertebrate species, a soluble form of TRAIL is cloned from *H. cumingii* (designated Hc-sTRAIL). Studies on the physiological functions of Hc-sTRAIL indicate that it plays a role in mussel immune system. Moreover, Hc-sTRAIL can induce apoptosis in human small cell lung cancer cell line NCI-H446 through caspase-3 apoptotic pathway.

2. Material and methods

2.1. Animals, tissue sampling, and immune challenge

Healthy mussels (*H. cumingii*) of body weight ranges from 150 to 180 g, were collected from a pearl mussel farm in Zhuji city (Zhejiang,

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China), and were cultured and fed with diatoms in a circular freshwater system for 7 days at 20 ± 2 °C. Ten mussels were used in each experimental condition. Mussels were challenged, by injecting into the adductor muscle, 100 μl of *Aeromonas hydrophila* (10^9 bacteria/ml, Institute of Hydrobiology, Chinese Academy of Sciences; diluted in sterile saline: 0.65% sodium chloride) with a syringe, or 100 μl of sterile saline

Fig. 2. Comparison on TNF domain from Hc-sTRAIL with other TRAIL proteins and their identity rates.

2.7. Roles of caspase 3 in Hc-sTRAIL-mediated apoptosis pathway

NCI-H446 cells were collected and divided into three groups: Hc-sTRAIL untreated, Hc-sTRAIL treated and caspase 3 inhibitor groups. Cells were treated with or without 100 ng/ml Hc-sTRAIL recombinant proteins for 24 h. In the caspase 3 inhibitor group, cells were pre-treated with 5 μmol/l Z-DEVD-FMK (R&D Systems, USA) for 1 h followed by Hc-sTRAIL treatment for 24 h. Cellular apoptosis was analyzed using Annexin-V/PI staining and flow cytometry analysis as stated above.

2.8. Statistical analysis

The relative expression levels of Hc-sTRAIL were detected using real-time PCR and were calculated according to the formula: $2^{(CT\ housekeeping\ gene - CT\ target\ gene)}$. Data were presented as the standard errors of the mean (S.E.M.). Differences were considered statistically significant when p values were less than 0.05.

3. Results and discussion

3.1. Cloning and sequence analysis of Hc-sTRAIL

Partial cDNA sequence of TRAIL gene (GenBank accession no. GU984232, NCBI) was cloned from the mussel species, *H. cumingii* (designated Hc-sTRAIL). TRAIL contains an ORF of 501 bp encoding for a putative protein of 167 amino acids, which displays a molecular mass of 18.4 kDa (Fig. 1). It has a TNF domain (from 9 aa to 166 aa) which is conserved among TNF superfamily members, and the cysteine residue (C117) is conserved in human sTRAIL, which is essential for its structure and pro-apoptotic activity (Bodmer et al., 2000). ClustalW pairwise comparison showed that the TNF domain of Hc-sTRAIL has over 98% similarity with either oyster CasTRAIL or human sTRAIL; and 62–72% similarity with sTRAIL from other vertebrates (Figs. 2, 3a). But it showed only 15–20% similarity with other types of TNF domains from invertebrate species (Fig. 3b). These results indicated that Hc-sTRAIL highly homologous to human sTRAIL, which is consistent with the study of CasTRAIL from oyster (Yang and Wu, 2010).

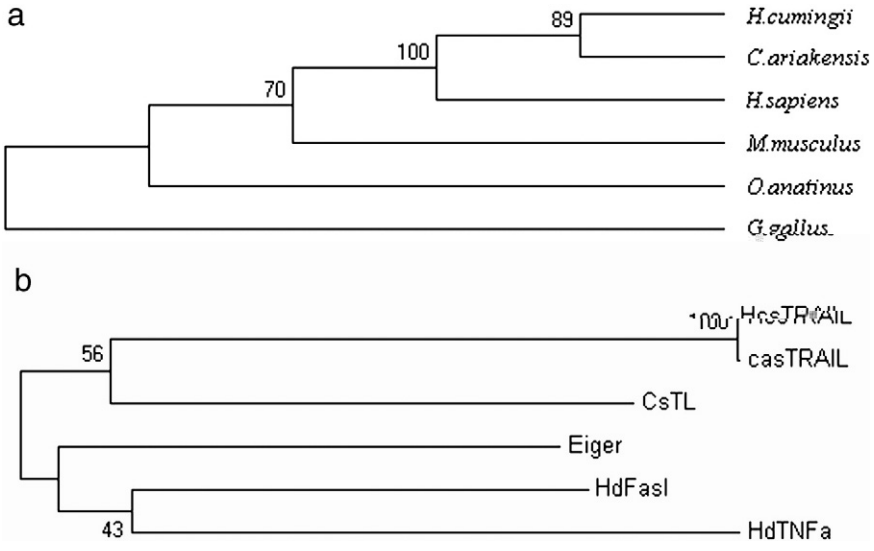

Fig. 3. Phylogenetic analysis of Hc-sTRAIL. (a) Phylogenetic tree for Hc-sTRAIL and other TRAIL proteins. The unrooted tree was built using the NJ method based on the alignment of TNF domain sequences. (b) Phylogenetic tree for Hc-sTRAIL and other TNF member proteins of invertebrates.

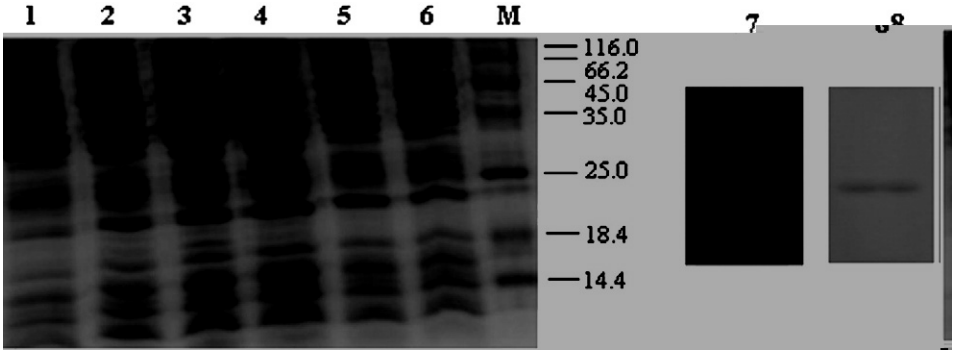

Fig. 4. SDS-PAGE and western blot analysis of Hc-sTRAIL. Bacterial proteins and recombinant fusion proteins were separated on 10% SDS-PAGE gels. A band with a molecular weight of about 20 kDa was detected using anti-His tag antibody. Lane 1, non-induced; Lanes 2–6, after induction by 0.01, 0.025, 0.05, 0.1 and 0.2 mM IPTG, respectively; M, molecular weight marker; Lane 7, purified recombinant proteins; Lane 8, western blotting of purified recombinant proteins using anti-His tag antibody.

3.2. Recombinant protein and western blotting

The Hc-sTRAIL recombinant protein was expressed in *Escherichia coli* BL21 (DE3) and purified with Ni-NTA affinity columns. Western blotting using anti-His tag antibody confirmed the presence of Hc-sTRAIL (~20 kDa) (Fig. 4).

3.3. Tissue distribution and expression analysis of Hc-sTRAIL

To determine the potential functions of Hc-sTRAIL, we examine the distribution of Hc-sTRAIL in various normal tissues (including hemocytes, gill, mantle, gonad and digestive gland) by quantitative real-time PCR. Results showed that Hc-sTRAIL was ubiquitously expressed in all examined tissues, and relatively higher expression levels were observed in hemocytes, gills and mantle (Fig. 5a), which is consistent with the fact that sTRAIL is involved in a broad range of important biological processes (Collison et al., 2009; Thorburn, 2007; Wiley et al., 1995; Yildiz et al., 2010). Interestingly, the expression level of Hc-sTRAIL mRNA in hemocytes increased sharply after *A. hydrophila* challenge and reached a peak level at 12 h (Fig. 5b). Meanwhile, no significant changes were detected in the control groups. In mussel and other invertebrates, hemocytes play a key role in the innate immunity system in which they act against microorganisms (Bachere et al., 2004; Canesi et al., 2002). A relatively high expression level of Hc-sTRAIL in normal hemocytes indicated that Hc-sTRAIL might be involved in the immune response of mussel. This hypothesis was supported by the observation of mussel challenged by *A. hydrophila* (Fig. 5b). Our previous study on CasTRAIL from oyster *Crassostrea ariakensis* indicated that oyster TRAIL might play key roles in transferring the stimuli of Rickettsia-like organism (RLO), an obligate intracellular Gram negative bacterium, to activate the p38 and then the transcription factor NF-κB, and NF-κB triggered the expression of subsequent cytokines and other molecules to against the RLO infection (Yang and Wu, 2010). But whether the similar mechanisms exist in the processes against *A. hydrophila* infection of Hc-sTRAIL, still needed to be clarified.

3.4. The role of Hc-sTRAIL in inducing cancer cell apoptosis

To investigate whether Hc-sTRAIL can induce apoptosis in mammalian cells, a cancer cell line, NCI-H446 (Fig. 6a), was treated with 100 ng/ml Hc-sTRAIL proteins. Cell viability assay showed that Hc-sTRAIL proteins caused a marked reduction in the cell number of NCI-H446 cells and reached to an average apoptotic rate of 23.7% at 24 h (Fig. 6c), which is similar to the results of human sTRAIL on NCI-H446 cells (Liu et al., 2007); Apoptosis of NCI-H446 was also verified by the presence of DNA fragmentation, the hallmark of apoptosis, within 24 h (Fig. 6b).

Previous studies showed that either TRAIL or sTRAIL can specifically induce apoptosis in various tumor cell lines through binding and activation of the death receptor pathway and/or mitochondrial pathway (Siegmund et al., 2011; Wang and El-Deiry, 2003). In both pathways, caspase 3 is ultimately activated and results in cell death (Qi et al., 2004; Yang et al., 2001). To further illuminate the molecular mechanisms underlying Hc-sTRAIL-induced apoptosis in NCI-H446 cells, Z-DEVD-FMK, a specific inhibitor of caspase 3 was used to examine the role of caspase 3 in Hc-sTRAIL-mediated apoptosis. As expected, cytotoxicity induced by Hc-sTRAIL on NCI-H446 cells could be blocked by Z-DEVD-FMK, in which the average apoptotic rate was significantly decreased to about 7.6% ($P < 0.05$) (Fig. 6c). Similar to human sTRAIL (Liu et al., 2007), Hc-sTRAIL could induce cell death in NCI-H446 cells, and caspase 3 plays a key role in Hc-sTRAIL-mediated apoptotic pathway.

In human, normal cells are believed to be resistant to TRAIL because of expressing higher levels of two TRAIL decoy receptors DcR1

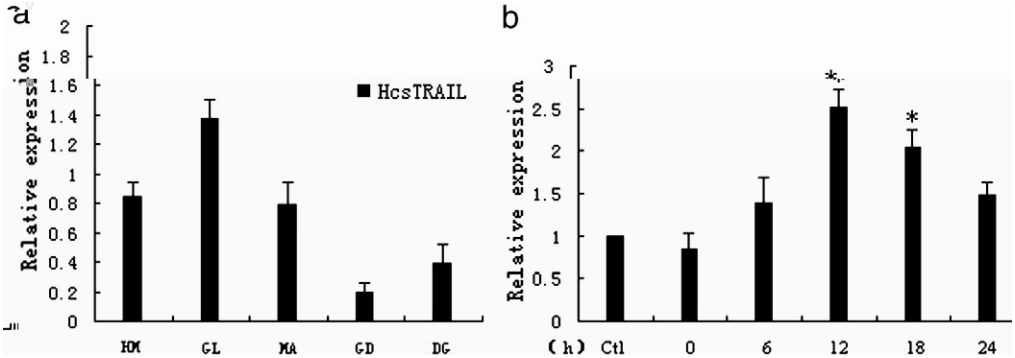

Fig. 5. Distribution and expression of Hc-sTRAIL. (a) Distribution of Hc-sTRAIL in various tissues. HM, hemocytes; GL, gill; MA, mantle; GD, gonad; DG, digestive gland. (b) Real-time RT-PCR analysis of the expression of Hc-sTRAIL in hemocytes with *Aeromonas hydrophila* challenge. Values presented as mean ± S.E. of independent experiments done in triplicates and analyzed by Student's *t*-test; *$P \leq 0.05$ when compared to control values.

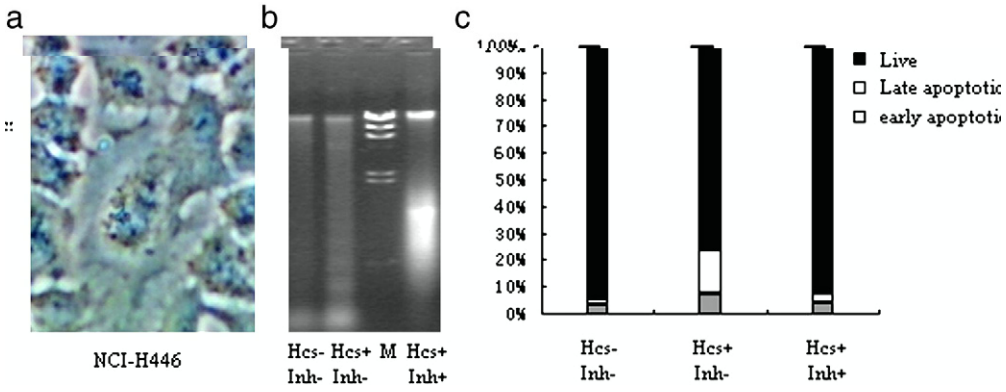

Fig. 6. Cytotoxicity of Hc-sTRAIL on tumor cells. (a) Culture of NCI-H446 cells. (b) DNA electrophoresis to detect the formation of DNA ladder at 24 h after Hc-sTRAIL addition with or without caspase 3 inhibitor group. (c) Flow cytometry analysis of apoptosis in Hc-sTRAIL untreated, Hc-sTRAIL treated and caspase 3 inhibitor groups. These experimental groups were represented with two parallels, and the average apoptotic rates in these groups were 4.5%, 23.3% and 7.6% respectively.

or DcR2 on their cell surface (Van Noesel et al., 2002). Our previous study showed that oyster sTRAIL (CasTRAIL) couldn't induce apoptosis in oyster hemocytes (Yang and Wu, 2010). This finding and the data in the present paper suggest that Hc-sTRAIL might also have no obvious cytotoxicity to normal mussel hemocytes.

In conclusion, a novel human sTRAIL homolog, Hc-sTRAIL, was identified from the mussel species, *H. cumingii*. Study on its physiological function indicated that Hc-sTRAIL was involved in the immune response of mussel and can induce cell death in cancer cells through the caspase 3 apoptotic pathway.

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References

Abdalla, S.A., Horiuchi, H., Furusawa, S., Matsuda, H., 2004. Molecular cloning and characterization of chicken tumor necrosis factor (TNF)-superfamily ligands, CD30L and TNF-related apoptosis inducing ligand (TRAIL). The Journal of Veterinary Medical Science 66 (6), 643–650.

Bachere, E., Gueguen, Y., Gonzaiez, M., Lorgeri, J., Garnier, J., Romestand, B., 2004. Insight into the anti-microbial defense of marine invertebrates: the penaeid shrimp and the oyster *Crassostrea gigas*. Immunological Reviews 198, 149–168.

Bodmer, J.L., Meier, P., Tschopp, J., Schneider, P., 2000. Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. The Journal of Biological Chemistry 275 (27), 20632–20637.

Canesi, L., Betti, M., Ciacci, C., Scarpato, A., Citterio, B., Pruzzo, C., Gallo, G., 2002. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAPK. Developmental and Comparative Immunology 26, 325–334.

Choi, H., Chang, J.W., Jung, Y.K., 2011. Peroxiredoxin 6 interferes with TRAIL-induced death-inducing signaling complex formation by binding to death effector domain caspase. Cell Death and Differentiation 18 (3), 405–414.

Collison, A., Foster, P.S., Mattes, J., 2009. The emerging role of TRAIL as key regulator of inflammatory responses. Clinical and Experimental Pharmacology & Physiology 36, 1049–1053.

De Zoysa, M., Jung, S., Lee, J., 2009. First molluscan TNF-alpha homologue of the TNF superfamily in disk abalone: molecular characterization and expression analysis. Fish & Shellfish Immunology 32, 625–631.

Gao, Y., Chang, M.X., Sun, B.J., Nie, P., 2008. TRAIL in the mandarin fish *Siniperca chuatsi*: gene and its apoptotic effect in HeLa cells. Fish & Shellfish Immunology 24, 55–66.

Halaas, O., Vik, R., Ashkenazi, A., Espevik, T., 2000. Lipopolysaccharide induces expression of APO2 ligand/TRAIL in human monocytes and macrophages. Scandinavian Journal of Immunology 51, 244–250.

Kauppila, S., Maaty, W.S., Chen, P., Tomar, R.S., Eby, M.T., Chapo, J., Chew, S., Rathore, N., Zachariah, S., Sinha, S.K., Abrams, J.M., Chaudhary, P.M., 2003. Eiger and its receptor, Wengen, comprise a TNF-like system in *Drosophila*. Oncogene 22, 4860–4867.

Liu, Y.J., Chen, F.H., Yu, Q., Han, J.B., Su, C.Q., Qian, Q.J., 2007. Antitumor activity of replication-defective adenovirus carrying human tumor necrosis factor-related apoptosis-inducing ligand gene on human small cell lung cancer cell line H446 in vitro. Chinese Pharmacological Bulletin 23 (6), 755–759.

Park, E.M., Kim, Y.O., Nam, B.H., Kong, H.J., Kim, W.J., Lee, S.J., Kong, I.S., Choi, T.J., 2008. Cloning, characterization and expression analysis of the gene for putative lipopolysaccharide-induced TNF-a factor of the Pacific oyster, *Crassostrea gigas*. Fish & Shellfish Immunology 24, 11–17.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A., Ashkenazi, A., 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. The Journal of Biological Chemistry 271, 12687–12690.

Qi, J.P., Wu, A.P., Wang, D.S., Wang, L.F., Li, S.X., Xu, F.L., 2004. Correlation between neuronal injury and caspase-3 after focal ischemia in human hippocampus. Chinese Medical Journal 117, 1507–1512.

Shirley, S., Morizot, A., Micheau, O., 2011. Regulating TRAIL receptor-induced cell death at the membrane: a deadly discussion. Recent Patents on Anti-Cancer Drug Discovery 6 (3), 311–323.

Siegmund, D., Mauri, D., Peters, N., Juo, P., Thome, M., Reichwein, M., Blenis, J., Scheurich, P., Tschopp, J., Wajant, H., 2011. Fas-associated death domain protein (FADD) and caspase-8 mediate up-regulation of c-Fos by Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) via a FLICE inhibitory protein (FLIP)-regulated pathway. The Journal of Biological Chemistry 276 (35), 32585–32590.

Simons, M.P., Nauseef, W.M., Griffith, T.S., 2007. Neutrophils and TRAIL: insights into BCG immunotherapy for bladder cancer. Immunologic Research 39, 79–93.

Tang, H.Y., Qin, Y., Li, J.Y., Gong, X.G., 2011. The scavenging of superoxide radicals promotes apoptosis induced by a novel cell-permeable fusion protein, sTRAIL:FeSOD, in tumor necrosis factor-related apoptosis-inducing ligand-resistant leukemia cells. BMC Biology 9, 18.

Thorburn, A., 2007. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway signaling. Journal of Thoracic Oncology 2461–2465.

Van Noesel, M.M., Van Bezouw, S., Salomons, G.S., Voûte, P.A., Pieters, R., Baylin, S.B., Herman, J.G., Versteeg, R., 2002. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. Cancer Research 62 (7), 2157–2161.

Wang, S., El-Deiry, W.S., 2003. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 22, 8628–8633.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A., Goodwin, R.G., 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3 (6), 673–682.

Yang, S.B., Wu, X.Z., 2010. Identification and functional characterization of a human sTRAIL homolog, CasTRAIL, in an invertebrate oyster *Crassostrea ariakensis*. Developmental and Comparative Immunology 34 (5), 538–545.

Yang, B., El Nahas, A.M., Thomas, G.L., Haylor, J.L., Watson, P.F., Wagner, B., Johnson, T.S., 2001. Caspase-3 and apoptosis in experimental chronic renal scarring. Kidney International 60 (5), 1765–1776.

Yang, S.B., Li, P.F., Mi, Z.X., 2012. LPS-induced TNFα factor (LITAF) in the snail *Cipangopaludina chinensis*: gene cloning and its apoptotic effect on NCI-H446 cells. Fish & Shellfish Immunology 32 (2), 268–272.

Yildiz, R., Benekli, M., Buyukberber, S., Kaya, A.O., Ozturk, B., Yaman, E., Berk, V., Coskun, U., Yamac, D., Sancak, B., Uner, A., 2010. The effect of bevacizumab on serum soluble FAS/FASL and TRAIL and its receptors (DR4 and DR5) in metastatic colorectal cancer. Journal of Cancer Research and Clinical Oncology 136 (10), 1471–1476.

Yu, Y., Qiu, L., Song, L., Zhao, J., Ni, D., Zhang, Y., Xu, W., 2007. Molecular cloning and characterization of a putative lipopolysaccharide-induced TNF-a factor (LITAF) gene homologue from Zhikong scallop *Chlamys farreri*. Fish & Shellfish Immunology 23, 419–429.

Zhang, Z.C., Wu, H., Di, L.Q., Chen, L., 2007. A review about freshwater mussel (Unionidae). Chinese Archives Traditional Chinese Medicine 25 (1), 121–123.