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Identification and functional characterization of interferon-γ-inducible lysosomal thiol reductase (GILT) gene in common Chinese cuttlefish *Sepiella japonica*

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ABSTRACT

Interferon-γ-inducible lysosomal thiol reductase (GILT) is a pivotal enzyme involved in the histocompatibility complex (MHC) class II-restricted antigen processing whereby it catalyzes the disulfide bond reduction in the endocytic pathway. Here, a novel GILT homologue termed as *SjGILT* firstly identified from common Chinese cuttlefish *Sepiella japonica*. SjGILT shared domain topology containing a signal peptide, a signature sequence $CQHGX_2ECX_2NX_4C$, an activate-site CXXC motif, two potential N-glycosylation sites and six conserved cysteins with its counterparts in other animals. *SjGILT* transcripts were constitutively expressed in all examined tissues in *S. japonica*, with the higher expression levels in immune-related tissues such as pancreas, intestines, liver and gills. Upon lipopolysaccharide (LPS) challenge, *SjGILT* transcripts were significantly induced in liver and gill tissues, and *SjGILT* protein transferred to late endosomes and lysosomes in HeLa cells. Further study showed that recombinant *SjGILT* had obvious thiol reductase activity demonstrated by reducing the interchain disulfide bonds of IgG under acidic conditions. Taken together, these results suggested that *SjGILT* may be involved in the immune response to bacteria challenge, and then might play an important role in the processing of MHC class II-restricted antigens in *S. japonica*.

1. Introduction

In mammals, exogenous antigens were firstly captured by endocytosed proteins internalized by antigen presenting cells (APCs), following delivered to the dosomal-lysosomal system where they are denatured, unfolded and degraded, generating major histocompatibility complex class II (MHC II)-binding epitopes [1,2]. Antigen processing and presentation by MHC class I and II molecules play important roles in immune responses [3]. In the progress of antigens processing and presentation, interferon-γ-inducible lysosomal thiol reductase (GILT) could facilitate the generation of MHC II-restricted epitopes by catalyzing disulfide bonds reduction via cellular proteases [4–6]. GILT lacked mice showed the deficient of MHC-II-restricted CD4 + T-cell responses to protein antigens that contain disulfide bonds [7].

GILT was first identified as an interferon γ-inducible protein in human monocytic cell line in 1988 [8]. It is constitutively expressed in APCs, such as monocytes, B cells, bone marrow-derived dentritic cells (DCs) [4,9,10], beyond that, it is also expressed in some immune cells for instance thymocytes and T cells [11]. In addition, it has been proved that GILT expression can be induced by interferon-γ in other cell types, such as fibroblasts, endothelial cells, tumour cells and melanoma cells, which activates Jak-STAT signal-ling pathway and in turn promotes GILT expression [12–14]. Human GILT is initially synthesized as a precursor in the endoplasmic reticulum, which contains a signal sequence and tagged with mannose-6-phosphate (M6P) residues. After that, the precursor is transferred by M6P receptor to the endosomal-lysosomal system [4,7], in which its N- and C-terminal propeptides are cleaved to yield the mature form of GILT and finally localized to the late endosomes and lysosomes [5]. Mature GILT contains an active-site CXXC motif, a signature sequence $CQHGX_2ECX_2NX_4C$, more than one N-glycosylation sites and several conserved cysteines [15,16]. In human, Cys-46 and Cys-49 make up the active-site CXXC motif, when thiol reductase activity initiates, Cys-46 initiates a nucleophilic attack on the disulfide bond in the antigens, the GILT substrate mixed disulfide intermediate is then generated. The intra-molecular attack from the Cys-49 thiol group then results in the release of the reduced substrate and oxidized GILT [4,5].

It is commonly considered that GILT plays function in MHC class II-restricted antigen processing, however, recent study revealed that it could also facilitate the cross-presentation (present antigen to $CD8^+$ T

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cells) of MHC I-restricted epitopes [17]. Besides the defined roles in antigen processing, GILT has also been found to regulate the cellular redox state by increasing the expression and activity of superoxide dismutase 2 (SOD2) and decreasing the content of reactive oxygen species (ROS), such as superoxide anion, and these changes are correlated with decreasing of cell proliferation [18]. In mammals, GILT is also found to be involved in the course of some malignant diseases, for instance, GILT significantly restricts the replication of murine leukaemia virus and human immunodeficiency virus type 1 [19,20], the low expression of GILT mRNA is associated with poor survival rates in patients with diffuse large B cell lymphoma and breast cancer [21,22].

GILT is evolutionarily highly conserved and GILT homologues have been widely found from higher chordates to lower plants. Despite all that, the indepth elucidations of its functional role are limited to vertebrates especially mammals only. In recent years, GILT homologues have been identified in lower vertebrates such as fish containing zebrafish [23], mefugu [24], mandarin fish [25], goldfish [26], largemouth Bass [27], silver carp [28], Chinese sturgeon [29] and clawed frog [30], as well as in some classes of invertebrates containing arthropods as tiger shrimp, fruit fly and mud crab [31–33], echinoderm as sea cucumber [34], molluscs as disk abalone and pearl oyster [35,36]. In these species, GILT has been shown to be involved in the innate immune defending against bacterial or viral pathogen infections. However, in cephalopods, the largest category of molluscs, there is not yet published literature ascribed to GILT to date.

Common Chines cuttlefish *Sepiella japonica* is an economically important cephalopod in the East China Sea and plays a key role in the marine ecosystem [37,38]. Due to the rapid growth, strong fertility and high feed conversion rate, cuttlefish breeding has been gradually rising in Zhejiang, Fujian and other coastal provinces in recent years. During the continuous expansion of *S. japonica* farming, disease caused by exogenous stimuli namely biotic and abiotic stresses occasionally burst in some cuttlefish farms. However, the information involved in immunity and immunoregulation mechanism of remains very unclear, which seriously hinders the sustainable development of cuttlefish culture. Here, a GILT homologue termed as *SjGILT* was identified in *S. japonica* for the first time. Following, its phylogenetic status, tissue distribution and temporal expression profiles, subcellular location and thiol reductase activity were comprehensively analysed. The present results will contribute to the elucidation of the functional role of GILT in *S. japonica*, and provide basis for the comprehensive interpretation of squid immune system.

2. Material and methods

2.1. Animals, challenge assays, and sampling

Adult *S. japonica* individuals, average body weight 117.1 g, were obtained from Shacheng breeding farm in Fuding, Fujian province, P. R. China. These cuttlefishes were acclimated to laboratory conditions (temperature, 24 ± 0.5 °C; salinity, 28 ± 1%, pH 8.0) for one week before treated. During the culture period, the cuttlefishes were fed daily with miscellaneous shrimps, and kept in aerated seawater that was changed daily.

When the challenge experiment initiated, 180 cuttlefishes were randomly divided into two groups, the lipopolysaccharide (LPS) challenge group and the control group. In challenge group, cuttlefish individuals were enterocoelia injected with 100 μL LPS (10 μg/mL in vehicle of PBS), while in control group, an equal volume of PBS was injected likewise. In order to alleviate the animal suffering and avoid spraying ink, all the cuttlefish individuals were hocussed using a non-lethal dose of MS-222 anesthetic (Sigma, USA). Both challenge and control groups consisted of three replicates, with 30 cuttlefishes in each replicate. After injection, these cuttlefishes were cultured with the same maintaining conditions as described above.

Three individuals of each replicate were sacrificed for liver and gill tissues collection at 0, 3, 6, 12, 24, and 36 h after challenge, following liver and gill tissues of one replicate were respectively pooled to reduce individual variation and to provide sufficient tissues for total RNA extraction.

Various tissues, including pancreas, intestines, brain, liver, muscle, gills, heart, optic lobes, and gonads were dissected from nine adult individuals to examine the tissue distribution of *SjGILT*. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using Total RNA Kit according to the manufacture's protocol (Tiangen). First-strand cDNA synthesis was carried out based on Promega M-MLV reverse transcriptase using oligo (dT)-adaptor as primer. The reaction was performed at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min.

2.3. Cloning of complete cDNA sequence of SjGILT

The complete sequence of *SjGILT* ORF was obtained by scanning the *S. japonica* transcriptional database. Following, the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) was employed to make a homology comparison, and specific primer pairs (Table 1) were designed to amplify the nucleotide sequence. Rapid-amplification of cDNA ends (RACE) was carried out to amplify the 5′ and 3′ untranslated regions (UTR) with specific and adaptor primers (Table 1). This experiment was conducted using the RACE cDNA Amplification Kit (Life Technologies, USA) with total RNA of gills as the template according to the manufacturer's protocol. The PCR products were sequenced using an ABI 3730 automated DNA sequencer, following the ORF sequence and the 5′ and 3′ -UTR sequences were assembled to obtain the *SjGILT* full length cDNA.

2.4. Molecular characterization and phylogenetic analysis

The physicochemical property, such as molecular mass and isoelectric point, was analysed using the Prot Param tool (http://www.expasy.org). The signal peptide was deduced on Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The conserved domains were predicted using the SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi) online tool. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and Clustal W software were used for the homology comparison. Phylogenetic trees were constructed using the neighbour-joining (NJ) method with the Mega 5.0 program [39]. SMART program was used for functional domains prediction.

Table 1
PCR primer pairs used in the present study.

Primer pairs	Sequence (5′ to 3′)	Usage
GILT	ATGCGCTACTGGATTTTACTTG TTATTTTTGGCATCTCTGCAGT	For ORF cloning
GILT-5′	GAACTCTCCCACAATA ATTGGCACATTGTTGCTCA CATGGCATAATTCTGAAG	For 5′RACE
GILT-3′	GTCAGAAATATGATCTTAACTATAATGA ACCAATATGTACCTTGGGTCACTCTAAA	For 3′ RACE
Real- GILT	TGGAACAAATGGACAGAGCGT GGTGCATTATTGGGCCAAGTT	For qPCR
β-actin	GCCAGTTGCTCGTTACAG GCCAACAATAGATGGGAAT	Internal reference
P1-GILT	CAGGATCCATGGCTGATGAACCAATTTC GACCTCGATTTTTGGCATCTCTGCAGT	For *pCMV-C-FLAG-SjGILT* construction
P2-GILT	CAGGATCCTCAGAATTATGCCATGTCCCTC GACCTCGATTTTTGGCATCTCTGCAGT	For *pET32a-SjGILT* construction

2.5. qPCR

The quantitative real time PCR (qPCR) was conducted on a 7500 Real Time PCR System (Applied Biosystems, USA), and in a final volume of 10 μl consisted of 0.4 μL each for F and R primers, 5 μL 2 × SYBR® Premix Ex Taq™ II, 0.4 μL cDNA sample (100 ng/μL), ROX II 0.2 μL and ddH_2O 3.6 μL. The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference [40]. In the tissue distribution analysis, the mRNA level of *SjGILT* was expressed as the ratio of their respective expression level in the heart, while in the challenge assay, expressed as fold changes by comparing the normalized gene expression level of *SjGILT* in LPS challenged cuttlefishes with that in the control cuttlefishes at the same time point. All samples were analysed in triplicate.

2.6. Plasmids construction

Specific primer pairs containing *Bam*H I and *Xho I* restriction enzyme cutting sites were designed to amplify the coding sequence with or without signal peptide (Table 1). After digested by restriction enzymes, the PCR products with signal peptide were cloned into pCMV-C-FLAG plasmid (Beyotime Biotech, China), while the PCR products without signal peptide were cloned into pET32a plasmid (Solarbio). The recombinant plasmids were designated *pCMV-C-FLAG-GILT* and *pET-32a-SjGILT* respectively.

2.7. Intracellular immunofiuorescence analysis

Immunofiuorescence staining was performed to detect the subcellular location of *SjGILT* in mammalian cell line HeLa cells according to Li et al. [26] and Cao et al. [28]. Briefly, HeLa cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO2. After 12 h seeding on slides (1×10^5/ml), HeLa cells were transfected with recombinant plasmid *pCMV-C-FLAG-SjGILT* using Lipofectamine 3000 kit (Invitrogen, USA) according to the manufacturer's instructions. After 12 h in culture, cells were stimulated with10 μg/ml LPS or PBS for 24 h. Afterwards, cells were fixed for 20 min at room temperature with 4% paraformaldehyde and then permeabilized in 0.2% Triton X-100 for 15 min. Subsequently, cells were incubated with mouse anti-FLAG antibody and rabbit anti-LAMP 1 anti-body (Proteintech, China). After incubation at 4 °C for 12 h, cells were stained with Alexa Fluor 488-conjugated goat anti-mouse IgG and AlexaFluor 549-conjugated goat anti-rabbit IgG (Proteintech, China). After incubation at room temperature for 1 h, the cells were counterstained with 4 μg/ml 4′ 6-diamidino-2-phenylindole (DAPI) at room temperature for 8 min. Confocal microscopy analysis was performed with Leica TCS SP5 II (Germany).

2.8. Prokaryotic expression and purification

The recombinant plasmid *pET-32a-SjGILT* was transformed into BL21 competent cells (Life Technologies) to express the fusion protein. The bacteria were inoculated in LB media with vigorous shaking at 37 °C. When the bacterial optical density reached absorbance 0.6 at 600 nm, the *SjGILT* expression was induced by isopropyl-beta-D-thiogalactopy ranoside to a final concentration of 1.0 mM. In the control group, the empty plasmid was used.

Eight hours after induction, the bacteria were harvested for expression analysis of recombinant proteins using sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE) assay. The *SjGILT* were purified by Ni-nitorilotriacetic acid (NI-NTA) affinity chromatography under denaturing conditions using the ProBond™ Purification System (Life Technologies). The proteins were refolded in 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM oxidized glutathione, and 0.5 mM reduced glutathione overnight at 4 °C [41]. The resulting proteins were filtered with a sterile 0.2 μm filter and stored at −80 °C. The protein concentration was measured using a NanoDrop 2000 spectrophotometer with an absorption at 280 nm.

2.9. Western blotting

A western blot assay was conducted to identify the fusion protein. Briefly, the purified sample was separated by SDS-PAGE using a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Life Technologies, USA) at 150 m A for 1.5 h in transfer buffer. The membrane was incubated overnight at 4 °C in the presence of anti-6 × His mouse antibody (Life Technologies, USA) after blocking with 5% (w/v) nonfat dry milk. The membrane was subsequently washed with Tris Buffered Saline Tween (TBST) buffer, incubated with AP-goat anti-Mouse IgG. Detection of the *rSjGILT* was conducted by X-ray film exposure with SuperSignal West Pico Substrate (Life Technologies, USA).

2.10. Analysis of thiol reductase activity

The thiol reductase activity of *rSjGILT* was determined based on the method described previously [33]. Briefly, purified *rSjGILT* was added to an acidic buffer (0.1% Triton X-100 in 100 mM NaCl, 50 mM acetate, pH 4.5) to a final volume of 100 ml and pre-activated with 25 mM dithiothreitol (DTT) at 37 °C for 10 min. Meanwhile, affinity-purified human IgG antibody (Solarbio, China) was denatured in 0.2% SDS by boiling for 5 min and diluted with 50 mM NaCl (containing 0.1% Triton). Then, 10 ml of denatured human IgG was co-incubated with 100 ml of pre-activated *rSjGILT* for 1 h at 37 °C. In this study, human IgG treated with 10 mM DTT was used as a positive control. Subsequently, the thiol reductase activity of *rSjGILT* was analyzed by non-reducing SDS-PAGE.

2.11. Statistical analysis

All the data were analyzed with Statistical Package for Social Sciences (SPSS) 17.0 (SPSS, Chicago, IL, USA) and represented as mean ± standard deviation (N = 3). The significant differences among groups were tested by one-way analysis of variance (ANOVA), and the results were deemed to be significant at $P < 0.05$.

3. Results

3.1. SjGILT cloning and molecular characterization

A GILT homologue termed *SjGILT* (accession number: MH513610) was firstly cloned from *S. japonica*, the gene owns a complete cDNA sequence of 1286 nucleotide residues, containing an ORF sequence coded of 771 nucleotide residues with a putative protein of 256 amino residues (Fig. 1). The calculated molecular mass of *SjGILT* is 29.5 kDa, and pI is 6.20. Homology comparisons showed that *SjGILT* had the highest sequence identity of 47% with its counterpart of *Lingula anatina*, followed by that of *Pinctada fucata* and *Acanthaster planci*, both with a sequence identity of 44%. There was also a 21-amino acid signal peptide at the N-terminus of *SjGILT* (Fig. 1), which was necessary for its transportation to lysosomal system. An activate-site CXXC motif, a signature sequence $CQHGX_2ECX_2NX_4C$ and two potential N-glycosylation sites were predicted in putative *SjGILT* amino sequence. Multiple alignments constructed using the mature peptides of GILTs obtained from NCBI database showed that the activate-site motifs and signature sequences were conserved in all examined species from lower arthropods to higher human (Fig. 2). However, the signal peptide was absent in Chinese alligator and fruit fly GILTs. In addition, six conserved cysteins were conserved in most examined GILTs although the second cysteine was omitted in two arthropods fruit fly and prawn (Fig. 2). Accession numbers of these used GILT homologues in this study

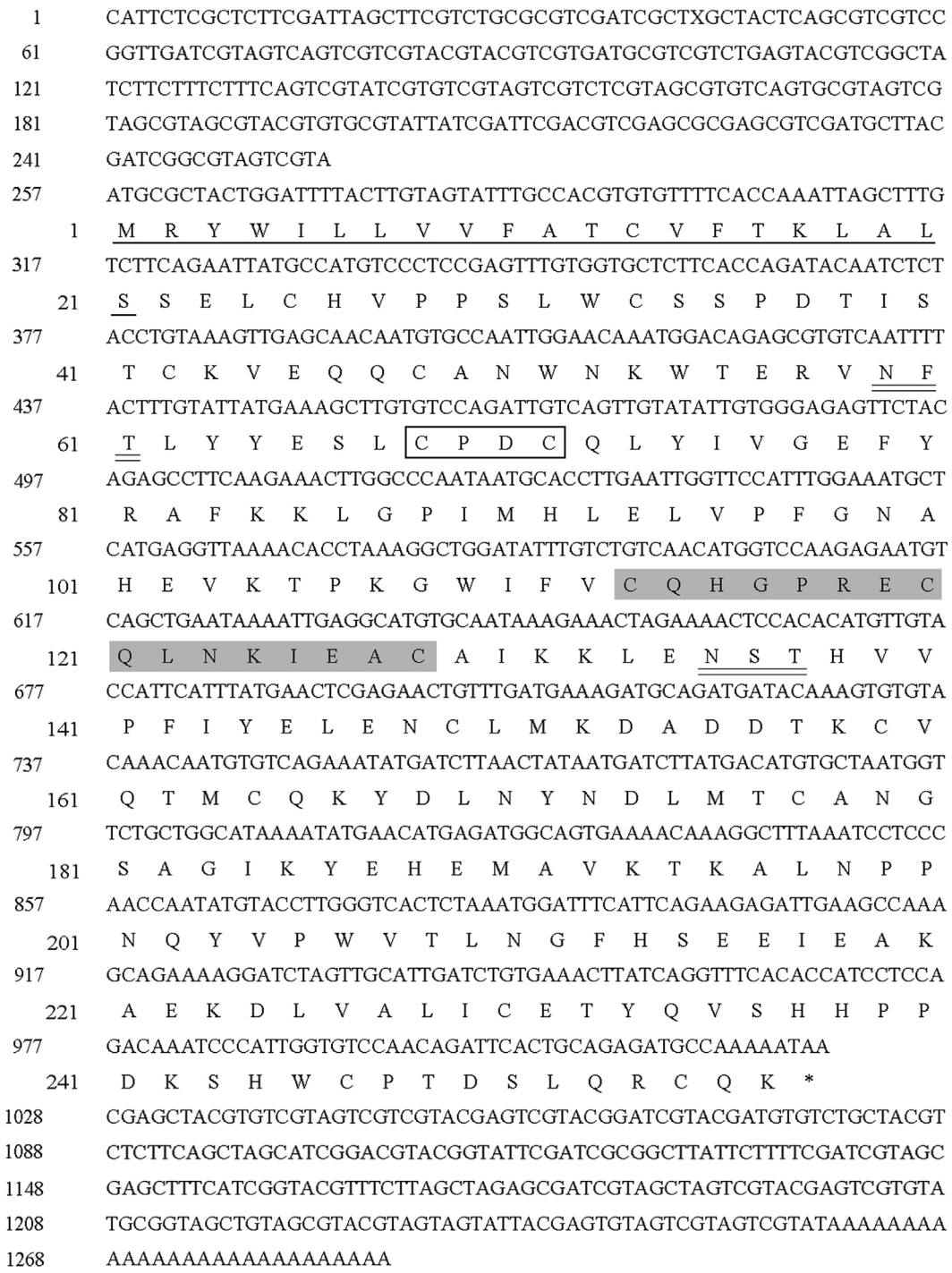

Fig. 1. The complete nucleotide and deduced amino acid sequences of gamma-interferon-inducible lysosomal thiol reductase (GILT) in *Sepiella japonica*. The nucleotide and deduced amino acid sequence of the complete cDNA were numbered on the left. The signal peptide was underlined. The GILT active-site CXXC motif was boxed. The GILT signature motif was shaded. The N-linked glycosylation sites were double underlined. The stop codon was tagged with asterisk.

are given in Suppl. Table 1.

3.2. Phylogenetic analysis

The phylogenetic status of *SjGILT* was analysed using Neighbor-joining method based on GILT sequences retrieved from NCBI database (Accession numbers used in this study are given in Suppl. Table 1). In the tree, *SjGILT* clustered into mollusc branch, suggesting the close relative of *SjGILT* with its counterparts from mollusc phylum. Despite of mollusc cluster, GILTs from chordates, cnidarians and arthropods respectively gathered together to construct specific clusters. Interestingly, chordates, cnidarians and molluscs clusters constructed one apparent branch in the tree while arthropods cluster constructed the other specific branch (Fig. 3).

3.3. Spatial and temporal expression of SjGILT transcripts

qPCR was employed to examine the transcriptional expression of *SjGILT* in nine adult tissues. As shown in Fig. 4, *SjGILT* were constitutively expressed in all examined tissues with the highest expression

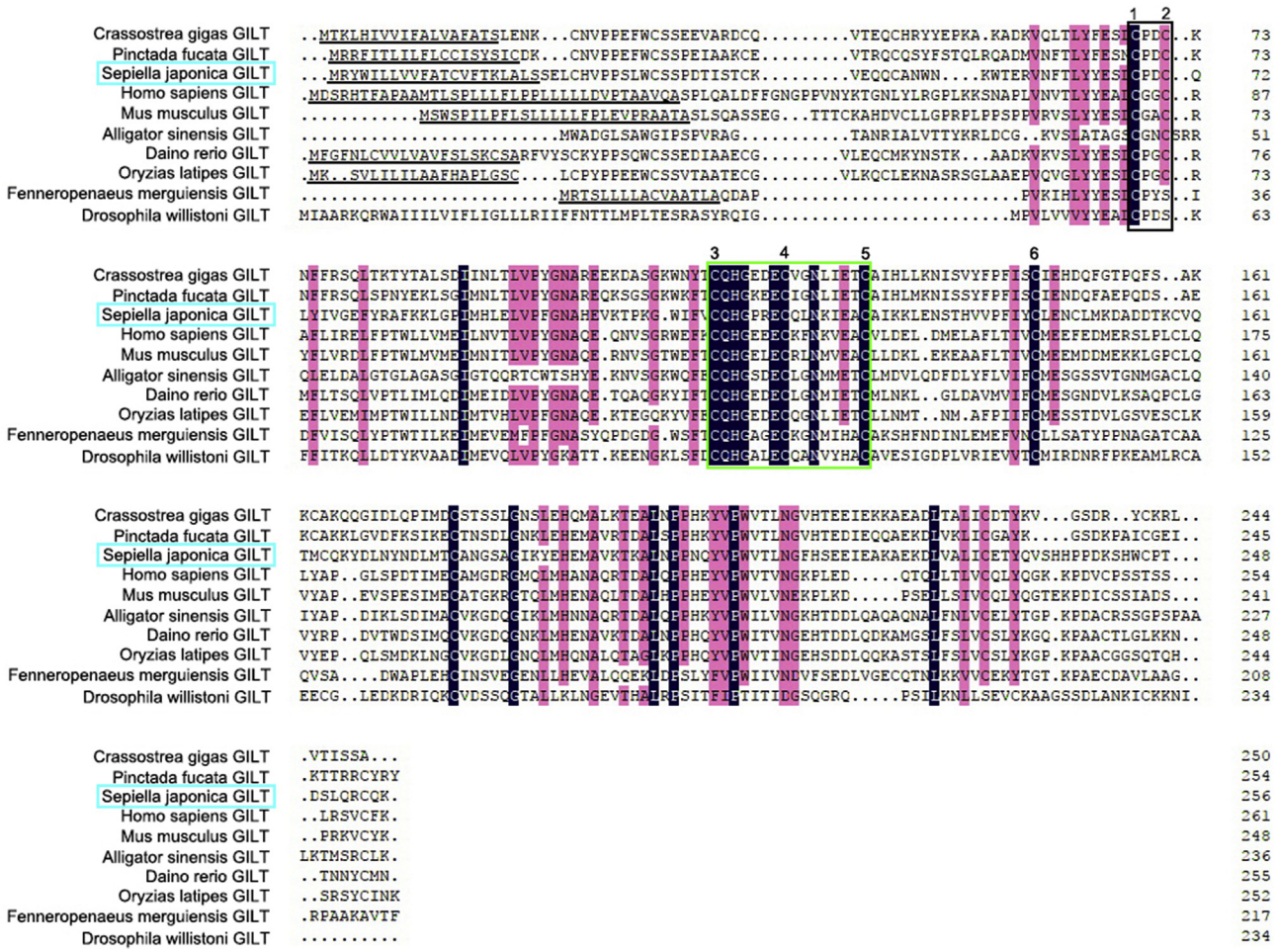

Fig. 2. Multiple alignments of *SjGILT* with other GILT homologues retrieved from GeneBank database. Completely conserved residues were marked with dark blue, surpass 75% conserved residues were marked with pink. The signal peptide was underlined. The conserved cysteines were digitaled. The GILT active-site and signature motif were boxed with black and green rectangle, respectively. *SjGILT* was boxed with blue rectangle. The gene destinations were listed on the left, and accession numbers were listed in Suppl. Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.).

level in pancreas, followed by intestines, liver, gills, gonads and optic lobes. Despite of these tissues, *SjGILT* also had a relatively high level of expression in both muscles and brains, and had the lowest expression level in heart.

Upon LPS challenge, in liver, the expression of *SjGILT* mRNA was significantly up-regulated at 8 h, with a 4.2-fold increase (Fig. 5). With the time elapsed, the level of *SjGILT* expression was gradually elevated and peaked at 24 h with a 20.9-fold increase. In gills, *SjGILT* transcription was sharply induced at 4 h, with a 11.2-fold increase, however, a weaker expression of *SjGILT* mRNA was detected at 8 h and 12 h, followed by a highest expression level of 15.8-fold increase at 24 h. After the time point of 24 h, the expression levels of *SjGILT* mRNA gradually decreased in both liver and gill tissues.

3.4. Subcellular localization of SjGILT

Immunofluorescence staining was performed to investigate the subcellular localization of *SjGILT* in HeLa cells. In normal condition, *SjGILT* distributed evenly in the cytoplasm of HeLa cells. After stimulation with LPS, the clustered fluorescent signals presented at the perinuclear region of HeLa cells, which showed the colocalization with LAMP-1, a marker for late endosomes and lysosomes, indicating that *SjGILT* translocated to late endosomes and lysosomes under the condition of LPS stimulation (Fig. 6).

3.5. Thiol reductase activity of SjGILT

In order to assess the thiol reductase activity of *SjGILT*, recombinant *SjGILT* proteins were efficiently expressed and successfully purified through SDS-PAGE and western blotting analysis (Fig. 7A). When the thiol reductase activity of *SjGILT* was examined, the denatured human IgG (160 kDa) was reduced into a heavy chain (H chain, 55 kDa) and a light chain (L chain, 25 kDa) with 10 mM DTT for 1 h incubation at 37 °C, pH 7.0, and this used as a positive control. After co-incubation with *rSjGILT* at pH 4.5 for 1 h, the denatured human IgG was reduced into a H chain and a L chain, indicating a thiol reductase activity of cuttlefish GILT (Fig. 7B).

4. Discussion

In human, the reductase activate site CXXC motif correspond to Cys-46 and Cys-49 in HsGILT [4]. Similar to reduction by thioredoxin, the N-terminal Cys-46 thiol group initiates a nucleophilic attack on a disulfide bond [5], resulted in the formation of a GILT-substrate mixed disulfide intermediate. Subsequently, the intermediate was attacked by the Cys-49, resulting in the release of the reduced substrate and oxidized GILT [5,42]. Functional domain prediction showed that putative SjGILT contains an activate-site CXXC motif, suggesting a likewise thiol reductase function correspondent to mammals. In addition, the

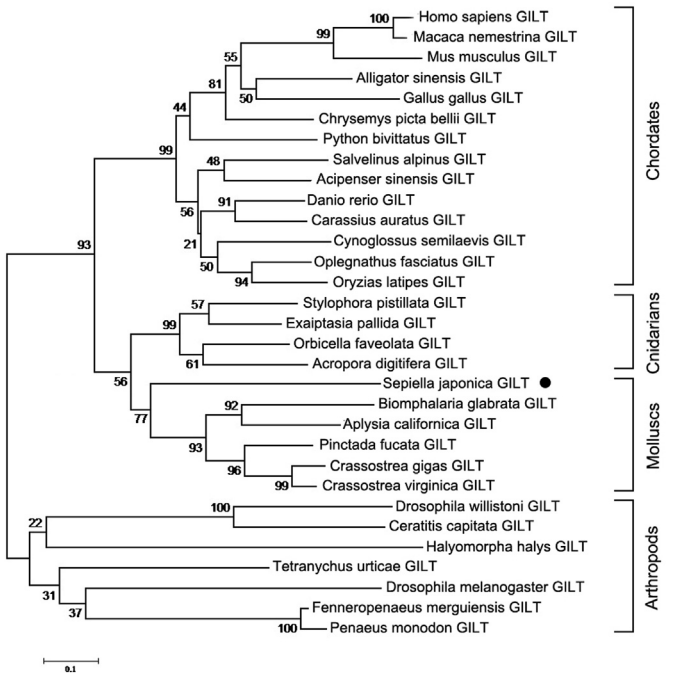

Fig. 3. Phylogenetic analysis of *SjGILT*. Phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap replications. The GenBank accession numbers used are listed in Suppl. Table 1.

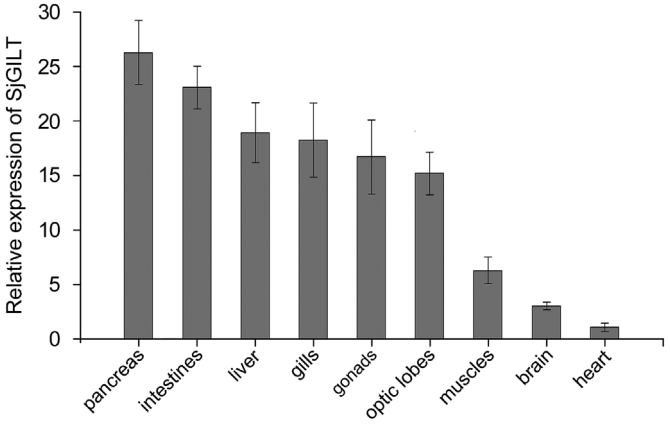

Fig. 4. Tissue distributions of *SjGILT* mRNA. β-actin gene expression was used as an internal control and the mRNA level was compared with expression level in heart to determine the relative fold change. Vertical bars represent the mean ± SD from nine sepias (n = 9).

presence of Asn-linked glycosylation sites suggest that they could theoretically be derivatized with mannose-6-phosphate (M6P), which has been considered necessary for transportation of GILT to lysosomal system [4]. In the phylogenetic tree, SjGILT was clustered with GILT homologues from mollusc species to construct a distinct clade. Multiple alignments showed that predicted motifs were overally conserved from lower arthropods to human. However, the C-terminal cysteine of the reductase active site is not conserved in two arthropods fruit fly and prawn, suggesting the expand function other than reduction. In addition, the function of signature sequence has not yet to be revealed to date. Through the scanning of GeneBank database, GILT homologues are found to be existence in primitive eukaryotes such as paramecium, and long before the development of adaptive immunity in jawed fish, suggesting that GILT has a fundamental role in cellular processes was adapted to facilitate antigen processing [42]. Taken together, these results suggested that SjGILT affiliated to GILT genefamily and could

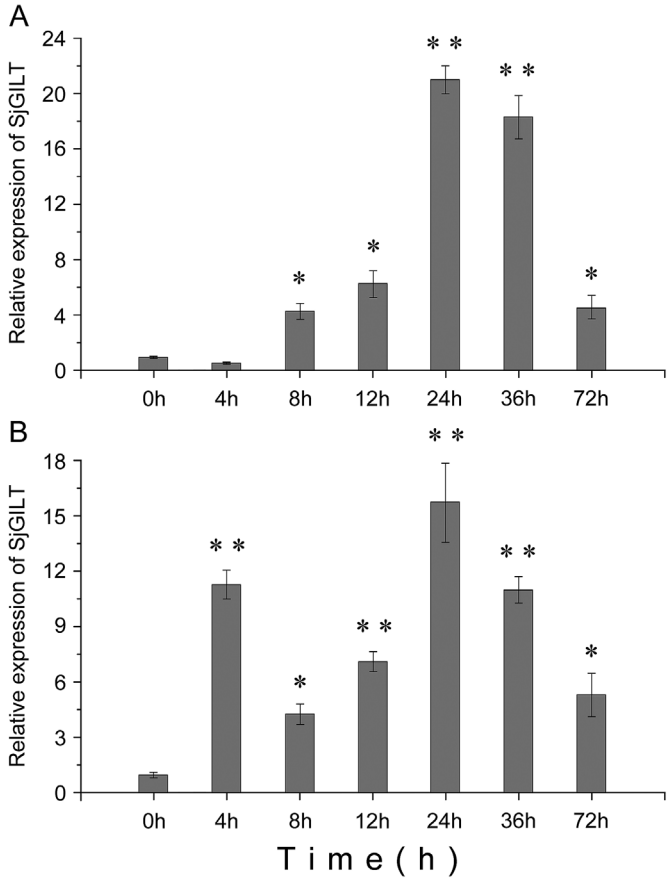

Fig. 5. Temporal expression of *SjGILT* mRNA in liver (A) and gills (B) after challenge with LPS. All data were normalized to the expression of β-actin gene. The expression levels were represented as fold changes by comparing the normalized gene expression level of LPS- challenged sepias with that of the control sepias at the same time point. The results were expressed as mean ± SD (n = 3). Significant difference relative to control was indicated with asterisk symbol (*P < 0.05, **P < 0.01).

play a parallel thiol reductase function with its counterparts in mammals.

SjGILT transcripts were constitutively expressed in all examined tissues in *Sepiella japonica*, similarly, ubiquitous expressions of GILT mRNA were also found in other animals such as in fish [28,29], clawed frog [30], sea cumumber [34], and these results further suggested that GILT may play a fundamental or diverse function role in cellular processes. Despite of the constitutive expression profile, *SjGILT* transcripts were high expressed in immune-related tissues such as pancreas, intestines, liver and gills. Further, the expression level of *SjGILT* mRNA was remarkably elevated by LPS challenge. The expressional change of GILT caused by LPS change was also observed in some fishes and invertebrates. In clawed frog, the GILT mRNA expression was significantly up-regulated in spleen and peripheral blood mononuclear cells (PBMCs) after LPS stimulation [30]. In zebrafish, GILT expression is obviously up-regulated in spleen and kidney after immunization with LPS, and no apparent change in heart, liver, muscle and intestines [23]. In goldfish, largemouth bass and silver carp, GILT mRNA were obviously up-regulated in spleen and head kidney cells after induction with LPS [26–28]. In addition, activated or inactivated bacteria could also induce the expression of GILT gene. In mud crab, three GILT-like genes are up-regulated in the hepatopancreas with *V. parahaemolyticus* infection [33], in sea cucumber, GILT mRNA was significantly up-regulated by inactivated *V. alginolyticus* and poly (I:C) in coelomocytes [34]. Collectively, these results proposed that GILT may be involved in the immune response to bacterial or virus challenge.

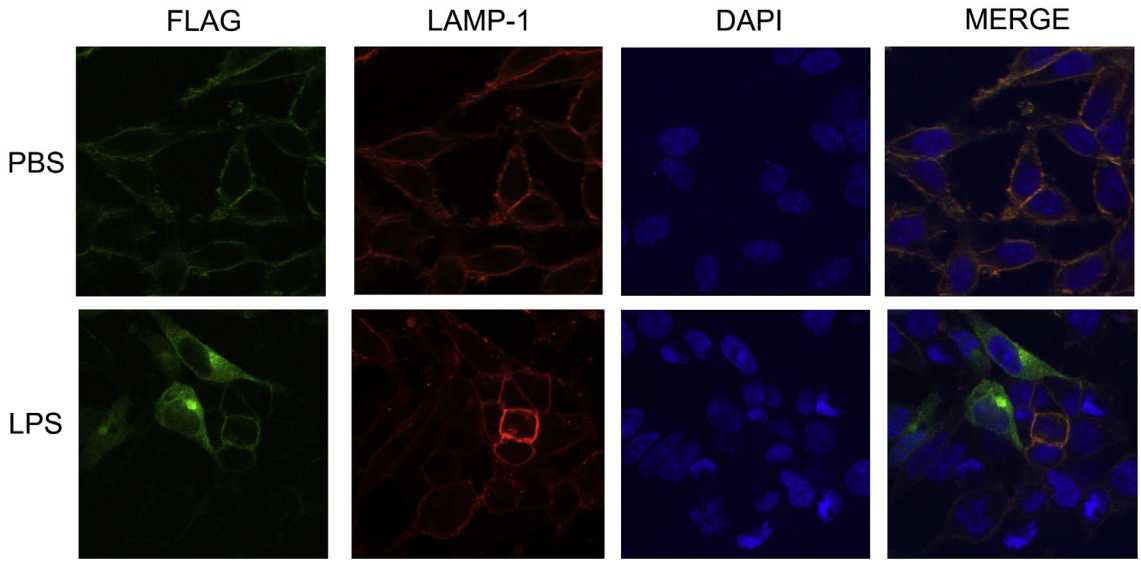

Fig. 6. The subcellular localization of *SjGILT* in HeLa cells stimulated with LPS. HeLa cells transfected with *pCMV-C-FLAG-GILT* were stimulated with LPS for 24 h and stained for FLAG-GILT and LAMP-1. The PBS treated HeLa cells were used as control. The merged images are displayed in the right panel.

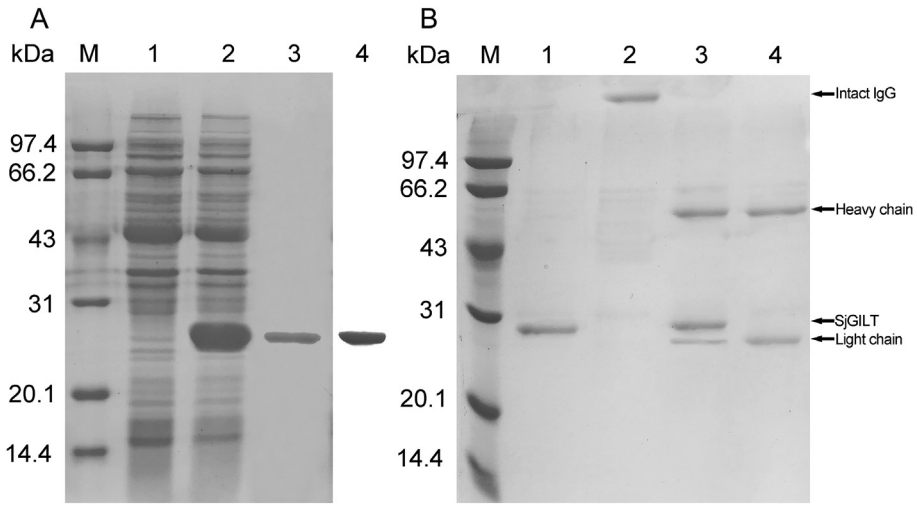

Fig. 7. (A) SDS-PAGE analysis of recombinant *SjGILT* protein expressed in E. coli BL21 (DE3). Lane 1 indicates cell lysates of bacteria transformed with empty pET28a under IPTG induction. Lane 2 represents cell lysates of bacteria transformed with *pET28a-SjGILT* under IPTG induction. Lane 3 shows the purified *SjGILT*. Lane 4 shows Western blot analysis of purified *SjGILT* using mAb against His6-tag. (B) *SjGILT* exhibits thiol reductase activity in vitro. M, protein marker; lane 1, purified *SjGILT*; lane 2, denatured purified human IgG; lane 3, human IgG incubated with *SjGILT* at pH 4.5; lane 4, human IgG treated with 10 mM DTT at pH 7.0 as positive control.

Localizations of the molecular components are important for our understanding of the protein function and molecular organization [43]. The subcellular localization of GILT has been checked in two fish species goldfish and silver carp. Upon LPS challenge, gGILT and ScGILT showed the colocalization with the late endosome/lysosome marker LAMP-1 at the perinuclear region of HeLa cells, which was consistent with the distribution of GILT in human APCs [7]. After infection with pathogens, exogenous antigens are internalized by APCs and are ultimately delivered to lysosomes via endocytic pathway. Here, a similar subcellular localization in late endosome/lysosome was also elucidated in *SjGILT*, hinting that *SjGILT* play an analogous thiol reductase function with its counterparts in fish and mammals in vivo.

Reduction is vital important for efficient antigen processing and presentation. GILT is a significant component in the generation of MHC class II restricted epitopes [1,2]. In contrast to thioredoxin, which is more efficient at neutral pH, GILT is optimally active between pH 4 and 5, consistent with its function being mediated in late endocytic compartments and lysosomes. Recently, the thiol reductase activity of GILT has been confirmed in some invertebrates such as in mud crab and sea cucumber [33,34]. In the present study, recombinant *SjGILT* reduced human IgG into H and L chains, and the reduction occurred at pH 4.5, suggesting an activate function of *rSjGILT*. This purified *rSjGILT* will allow for further investigation to reveal the role of this key enzyme in MHC class II-restricted antigen processing and presentation.

In conclusion, a GILT homologue termed *SjGILT* was identified and characterized from common Chinese cuttlefish *S. japonica*. *SjGILT* shared domain topology containing a signal peptide, a signature sequence $CQHGX_2ECX_2NX_4C$, an activate-site CXXC motif, two potential N-glycosylation sites and six conserved cysteins with its counterparts in other animals. *SjGILT* transcripts were constitutively expressed in all examined tissues in *S. japonica*, with the higher expression levels in immune-related tissues such as pancreas, intestines, liver and gills. Upon LPS challenge, *SjGILT* transcripts were significantly induced in liver and gill tissues, and *SjGILT* protein transferred to late endosomes and lysosomes in HeLa cells. Further study showed that recombinant *SjGILT* had obvious thiol reductase activity demonstrated by reducing the interchain disulfide bonds of IgG under acidic conditions. These results suggested that *SjGILT* may be involved in the immune response to bacteria challenge, and then might play an important role in the processing of MHC class II-restricted antigens in *S. japonica*.

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Appendix A. Supplementary data

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