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A novel liver-specific immunoglobulin heavy chain-like gene in a cartilaginous fish

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ABSTRACT

We identified a novel immunoglobulin (Ig) heavy chain-like gene (tsIgH) expressed in the liver of the banded houndshark *Triakis scyllium* by preliminary transcriptomic analysis. The tsIgH gene showed less than 30% of amino acid identities to Ig genes of the shark. The gene encodes one variable domain (VH) and three conserved domains (CH1-CH3) with a predicted signal peptide. Interestingly, this protein has only one cysteine residue in a linker region between VH and CH1 other than those required for the formation of the immunoglobulin domain. Genome sequencing revealed that each of the domains was encoded by a corresponding single exon, and the exon-intron structures of the homologues are conserved in the other cartilaginous fishes. By RT-qPCR analysis, the transcript of the tsIgH gene was observed only in the liver, while that of the IgM was mainly detected in the epigonal organ, liver, and spleen. The novel Ig-heavy chain-like gene in cartilaginous fish may provide new clues to the evolution of immunoglobulin genes.

Introduction

Cartilaginous fish possess three types of immunoglobulin heavy chain genes: IgM, IgW, and IgNAR [1]. The ancestors of cartilaginous fishes are thought to first acquire immunoglobulin genes after the appearance of jawed animals. Cartilaginous fish lack born marrow and a true lymphatic system. The fish spleen and thymus are lymphoid organs as in the cases in the other bony fishes, but they have unique organs such as the epigonal organ and Leydig organ [2]. In addition to those, the expression of Ig gene has been observed in some other organs such as the liver dependent on species and Ig isotypes [3]. In contrast, expression of IgM and IgNAR genes in the liver of adult animals has been reported in the banded houndshark, *Triakis scyllium* [4].

The vertebrate liver is a multifunctional organ. Among a variety of functions, it produces serum proteins to maintain homeostasis including biodefence. Certain serum proteins such as lysozyme are involved in biodefense against invasive pathogens. In the previous study, we identified the c-type lysozyme gene from the liver transcriptomic data of the banded houndshark [5]. By further in silico analysis, we identified a gene possessing 4 immunoglobulin (Ig) domains including one variable domain (VH) and three conserved domains (CH1-CH3).

In this study, we analyzed the deduced amino acid structure of the Ig heavy chain-like (tsIgH) gene. The genome sequence was determined and compared to a homologue identified in the deposited genome data of the other shark species. Furthermore, the tissue expression of the novel Ig-like gene was evaluated.

Materials and methods

Fish

All animal samplings were conducted according to the guideline issued by the Ministry of the Environment, Japan, and the Regulations on the Handling of Animal Experiments of Tokyo University of Marine Science and Technology. The organ pieces of the brain, epigonal organ, intestine, kidney, liver, muscle, pancreas, and spleen were collected from the anesthetized banded houndshark. Tissues were submersed in 500 μL of RNA Later (Takara, Japan) and stored at −80 °C.

cDNA and genomic dna sequencing

Total RNA was extracted using RNAisoPlus (Takara Bio, Japan) from the liver, and cDNA was synthesized using M-MLV RT (Invitrogen, USA) with oligo (dT) primer. All procedures were performed following the

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Table 1
Nucleotide sequences of the primers used in this study.

Name	Sequences
tsIgH_cloning_F	TGTACGGGGTAACATTGACTTATTT
tsIgH_cloning_R	TTATTTTCACATCAGGTGACTATCG
IgM_qRTPCR_F	CCCGAGAGATCTTCGTCAAG
IgM_qRTPCR_R	CTTCATGTCCCACCACACAG
tsIgH_qRTPCR_F	GAACCAACGGAAAGCAATGT
tsIgH_qRTPCR_R	TGCAAAGTATCGCATGAAGC
EF-1α_qRTPCR_F	AGATGGGCAAAGGCTCCTTC
EF-1α_qRTPCR_R	GCAACAATCAGCACAGCACA

The primer sets named "_cloning_" was used for cDNA and genome cloning, and those named "_qRTPCR_" were used for qPCR.

manufacturer's instructions.

The primer sets to amplify the ORF of the tsIgH gene were designed based on the NGS transcriptomic data for banded houndshark in the laboratory (Table 1). The cDNA fragments were amplified using ExTaq (Takara, Japan), and ligated into the pGEM-T easy vector (Promega, USA). The nucleotide sequences of the clones were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems).

The gene encoding the tsIgH was amplified by using genomic DNA as a template. Genomic DNA was purified from the muscle using NucleoSpin Tissue kit (Takara, Japan). The fragment was amplified using LA Taq (Takara, Japan), and then used to construct paired-end NGS libraries using the Nextera XT DNA Library Preparation Kit (Illumina, USA). The libraries were sequenced on Illumina MiSeq Reagent Kit v2 (300 cycles) (Illumina). Low-quality reads were removed using Fastp, version 0.23.2 [6] with -q 30 option. The trimmed reads were assembled using SPAdes v.3.13.1 [7]. The cotigs encoding the tsIgH were aligned manually.

qRT-PCR

Total RNA extraction and cDNA synthesis were performed from the organs as described above. Based on the sequences determined, we designed primer sets for the tissue expression analysis (Supplemental Table 1). For qPCR, THUNDERBIRD™ Next SYBR® qPCR Mix (TOYOBO, Japan) was used on a QuantStudio 1 real-time PCR system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. EF-1α (Accession number: LC704941) was used as an internal control. The relative expression levels of the target gene were calculated using the 2ΔΔCt method [8] and EF-1α was used as the reference gene for expression normalization. The expression level of each tissue was expressed as a percentage with the value of the tissue showing the highest level as 100.

Results and discussion

cDNA clone encoding tsIgH was sequenced (Fig. 1A; Accession no. LC754204). The gene showed 73% amino acid identity each to a hypothetical protein of the brownbanded bambooshark *Chiloscyllium punctatum* (Accession no. for the contig BEZZ01003013; protein ID, GCC18650) and immunoglobulin gamma-1 heavy chain-like of the whale shark, *Rhincodon typus* (Accession no. NC_063366). In addition, the protein was only 29.7% amino acid identity to the banded houndshark IgM. The protein possesses a predicted signal peptide, a VH, and 3 CH domains, while the shark IgM, IgNAR, and IgW possess 4, 5, and 6 CH domains, respectively [4]. Interestingly, the sequence possesses only one extra cysteine residue in the hinge region between VH and CH1 other than the pairs to form disulfide bonds in immunoglobulin domains. An extra cysteine residue between VH and CH1 was also observed in IgM and IgW of the banded houndshark but not in IgNAR [4]. On the other hand, an extra cysteine residue in CH1 is important to form covalent bonds between a heavy and light chain. It should be noted that an immunoglobulin gene, encoded by a non-somatically rearranged VDJ, possesses 2 additional cysteine residues in CH1, considered to form a tetramer of two heavy and two light chains [9].

The amplified genomic DNA fragments encoding the tsIgH gene were sequenced, while 4 regions potentially encoding repeats were not determined (Accession no. LC760724). The genome structure of tsIgH is composed of 6 exons (Fig. 1B). The Ig domains were encoded by a corresponding single exon. The exon-intron structure is conserved in the homologous genes of the brownbanded bambooshark (Accession no. BEZZ01003013 region from 12,279 to 21,923) and the whale shark (Accession no. NC_063366). It should be noted that the VH was encoded by a single exon and no segments showing VDJ-like structures were in the sequences. Additionally, each of the CH domains was encoded by an exon. The CH domains of nurse shark IgM also be encoded by single exons [10]. The tsIgH gene as well as the homologs in the sharks might have evolved from a common ancestral gene, which relates to the other immunoglobulin ancestral gene. The unique gene structure of tsIgH might pave the way for the history of the evolution of vertebrates' immunoglobulin genes.

In cartilaginous fishes, the sandbar sharks are known to have an Ig light chain gene with a fused variable and joining region in the variable domain (VL) [11]. The light chain genes of cartilaginous fishes are clustered, and the Ig light chain genes are also clustered. In each of the cluster cassettes, the VL of Ig light chain gene is encoded by a single exon, but the VL shows sequence diversity in each cassette. The Ig heavy chain-like gene found in the banded houndshark is also encoded by single exons for both VH and CHs, but it is not known whether the gene forms clusters. Additionally, the tsIgH gene forms an exon-intron structure, suggesting that the gene has evolved from the same ancestor with the other Ig genes, but not occurred by retro-transposition of mRNA which causes intronless genes [12].

By RT-qPCR analysis, the transcript of the tsIgH gene was observed only in the liver, while the mRNA levels of the IgM were the highest in the spleen followed by the epigonal organ and liver (Fig. 2). The liver is an organ that produces a large amount of serum protein. IgM is the most abundant in blood, which is account for more than 50% of serum proteins. The banded houndshark also possesses a high concentration of IgM in the serum [13]. It is speculated that the tsIgH might be secreted into the bloodstream, although the function is still unclear.

In conclusion, we identified a novel Ig-heavy chain-like gene in the banded houndshark. The protein possesses four Ig domains including one VH and three CHs. The VH domain is encoded by a single exon and does not show gene rearrangement. The gene is well conserved among different cartilaginous fishes, suggesting it has an important role across species.

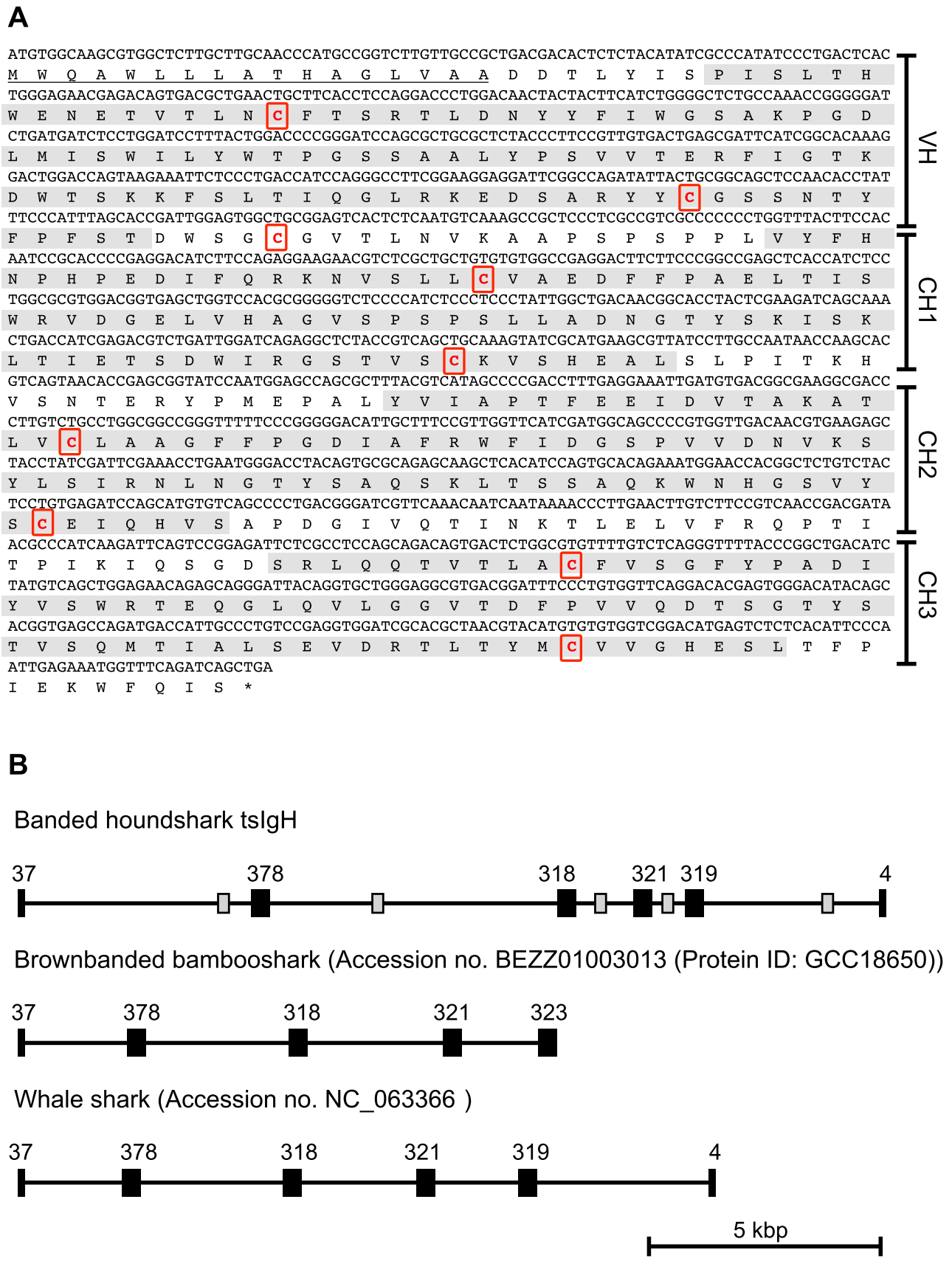

Fig. 1. Nucleotide and deduced amino acid sequences of the tsIgH gene (A) and the schematic exon-intron structures of the tsIgH and the homologous genes of the brownbanded bambooshark *Chiloscyllium punctatum* and the whale shark, *Rhincodon typus* (B). (A) A signal peptide was underlined. VH and CHs were indicated by gray boxes. Cysteine residues were highlighted by boxes. (B) Exons encoding the protein were indicated by black boxes, and the numbers on the boxes mean the size (bp). Gray boxes show the gaps.

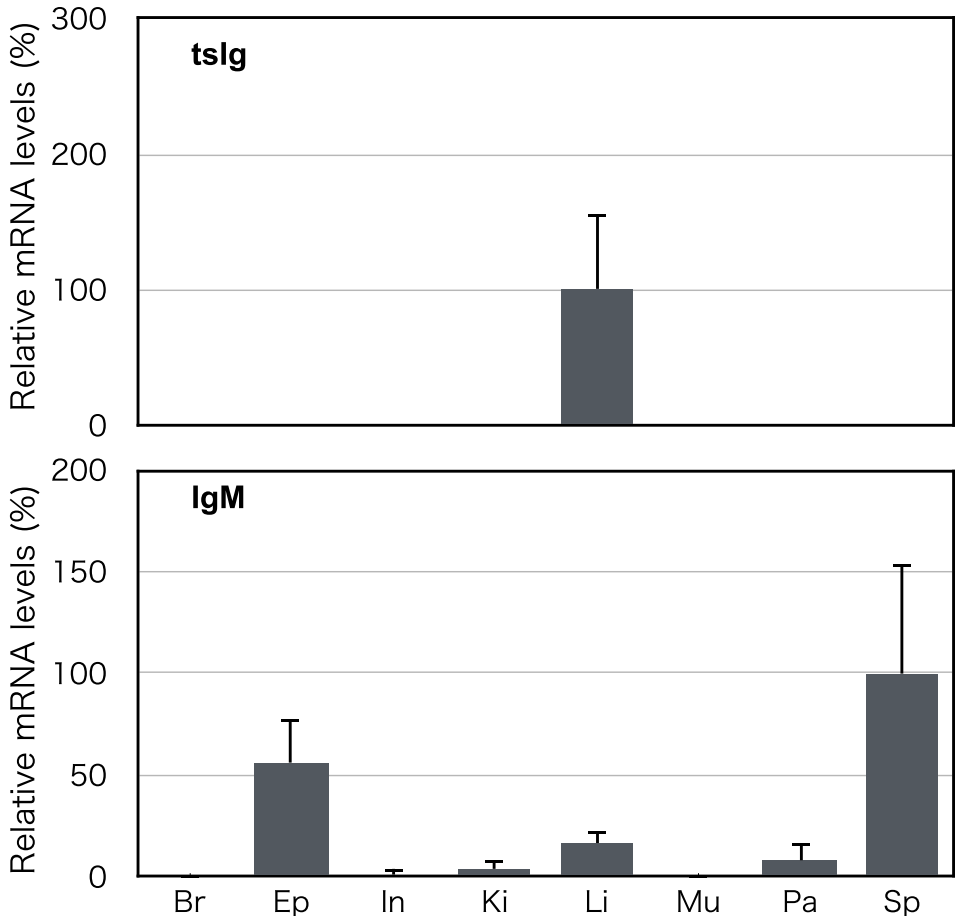

Fig. 2. mRNA levels of the tsIgH (A) and IgM (B) genes in various organs. The relative values are expressed as 100 for the highest value. Bars represent mean values for specimens ($n = 3$) and the error bar represents the standard deviation. Br: brain; Ep: epigonal organ; In: intestine; Ki, kidney; Li: liver; Mu: muscle; Pa: pancreas; Sp: spleen.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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