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Transcriptomic analysis of immunoglobulin novel antigen receptor (IgNAR) heavy chain constant domains of brownbanded bamboo shark (*Chiloscyllium punctatum*)

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ABSTRACT

Cartilaginous fish are the evolutionarily oldest group of animals which possess antibodies, T cell receptors and major histocompatibility complex (MHC). The immunoglobulin novel antigen receptor (IgNAR) found in cartilaginous fish is a heavy chain homodimer which lacks light chain. The presence of non-canonical cysteine molecules and lack of CDR2 region make it more significant. To synthesize active binding domains based on variable region of IgNAR (VNAR), knowledge on the constant region dynamics play a significant role. The IgNAR exhibit species variations in its primary sequence features; hence, this study was conducted to determine the IgNAR heavy chain constant domain of the brownbanded bamboo shark (*Chiloscyllium punctatum*). Peripheral blood leukocytes (PBL) isolated from adult bamboo sharks were used to synthesize a cDNA library. A total of four billion residues of two million sequences (average length 218.41 bp) were obtained. Assembled sequences were aligned with published cartilaginous fish IgNAR constant region sequences. Transcriptome analysis revealed two distinct types of IgNAR in the brownbanded bamboo shark. Also, constant-1 domain sequences displayed 13 unique sequences which may reflect the least number of IgNAR gene clusters. The phylogenetic analysis revealed the closest relationship with the nurse shark (*Ginglymostoma cirratum)* followed by the wobbegong shark (*Orectolobus maculatus*) which belong to the same order Orectolobiformes. Analysis of the constant domains of the brownbanded bamboo shark IgNAR revealed an evolutionarily conserved nature and this knowledge can be used to design primers for VNAR cloning. Furthermore, knowledge on the structural features in IgNAR constant domains that increase the stability could be useful in the process of stabilizing human immunoglobulins.

1. Introduction

The adaptive immune system plays a vital role in combating pathogens and evolved approximately 500 million years ago [1]. Cartilaginous fish (sharks, rays, and skates) are the first jawed fish species which possessed certain features of the adaptive immune system, such as immunoglobulins (Ig), the major histocompatibility complex (MHC), and T-cell receptors [2]. Furthermore, cartilaginous fish also exhibit recombinase-activating gene (RAG) function, somatic hypermutation, and antibody secreting B cells in epigonal, Leydig, and spleen tissues [3–6]. Three immunoglobulin types are identified in cartilaginous fish, namely IgM, IgW, and the immunoglobulin novel antigen receptor (IgNAR) [7]. Among them, IgM which is orthologous to a mammalian antibody was the first antibody identified since the 1960s and presents in both monomeric and pentameric forms [8]. The other isotype IgW has short and long forms and orthologous to IgD molecules in bony fishes and mammals [9,10]. The IgNAR is devoid of a light chain, like the camelid heavy chain antibody (VHH), and the variable region of the

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IgNAR (VNAR) in sharks is the smallest naturally occurring antibody-like molecule discovered to date [11].

The IgNAR structure consists of one variable (V) and five constant (C1–C5) domains in the secretory form [7,11]. The variable domain is attached to the constant (C) domain via a flexible hinge region which facilitates antigen binding [12]. The desirable properties, such as smaller size, higher solubility and stability, and ability to bind with enzyme active sites and antigen clefts, make the isolated VNAR domains as a potential candidate for immunotherapeutic agents and diagnostics [12–14].

Characterization of the IgNAR or VNAR genes in eight shark species, nurse sharks (*Ginglymostoma cirratum*), wobbegong sharks (*Orectolobus maculatus*), spotted catshark (*Scyliorhinus canicula*), smooth dogfish (*Mustelus canis*), banded houndsharks (*Triakis scyllium*), spiny dogfish (*Squalus acanthius*), horn sharks (*Heterodontus francisci*), and bamboo sharks (*Chiloscyllium plagiosum*) were recorded to date [11,15–21]. Most previous studies were concerned with the recombination and modification of the VNAR using molecular cloning and phage display methods. Although the constant region is less studied, it has a primary role in maintaining the stability of the antibody [22].

The structural model for the nurse shark IgNAR developed by Fiege et al. [22] indicated that dimerization was caused by the C1 and C3 domains to facilitate binding of the widely spaced variable domain with multiple epitopes; also, the disulfide bond between C3 and C4 enhanced the flexibility of the IgNAR stalk. They also suggested that the characteristics of the IgNAR constant domains should be further verified by investigating other shark species [22]. The C5 domain in nurse sharks is yet to be confirmed due to the lack of structural data [23]. Hence, the C domain sequence analysis of the brownbanded bamboo shark which belongs to the same taxonomic order Orectolobiformes as the nurse shark will be useful for further research.

In mammals, the immunoglobulin loci are arranged in a translocon organization, but in sharks, they are present as clusters. In each species, several clusters exist consisting of the variable (V), diversity (D), joining (J), and constant (C) exons which may be pre-joined in the germline [24]. Based on the earliest observation on nurse shark IgNAR variable domain sequence, two main types of IgNARs were reported. The type-I IgNAR consists of a non-canonical cysteine molecule in the framework (FW)-2 and FW-4 regions with two-to-four additional cysteine molecules in the complementarity determining region, (CDR)-3. In the type-II IgNAR, the non-canonical cysteine molecules are located in the CDR-1 and CDR-3 regions [11]. Despite this classification, several other shark species, such as small-spotted catsharks, banded hound sharks, and the wobbegong sharks, reportedly have a novel variant which lacks the non-canonical cysteine residue [15,17,19]. None of the species studied to date possess the type-I IgNAR except nurse sharks [15–20]. Therefore, classification of the IgNAR clusters based on the variable region alone might not be sufficient mainly due to the higher variability in the CDR regions. Hence, this study proposes the possibility of using the C1 domain sequence to identify IgNAR clusters due to its uniqueness compared to the other four C domains which share sequences similarity with IgW [4]. Furthermore, this study aimed to characterize the complete IgNAR heavy chain constant domain of brownbanded bamboo sharks (*Chiloscyllium punctatum*) and to determine its evolutionary relationship with other species.

The next-generation sequencing (NGS) method used in this study concomitantly revealed many sequences and saved time for cloning in expression vectors and sequencing. Based on the analysis of the C domains of brownbanded bamboo sharks, the presence of two distinct IgNAR types and 13 unique C1 sequences were identified. The findings of this study can be used for further studies on immunodiagnostic or immunotherapeutic agents using the brownbanded bamboo shark as a model organism.

2. Materials and methods

2.1. Sampling and cDNA library synthesis

Adult, male brownbanded bamboo shark (*Chiloscyllium punctatum*) reared at the Fisheries Laboratory, The University of Tokyo, Hamamatsu, Japan was used in this study. All the experimental procedures were conducted by the institutional animal ethics guidelines. Shark was anesthetized by spraying Ethyl 3-aminobenzoate methanesulfonate (MS 222) (Sigma-Aldrich/Merck, Darmstadt, Germany) at a concentration of 1 μg/ml, via the gill slits and oral routes [25]. The caudal vein was punctured by using an 18 G needle attached to a 10 ml sterile syringe containing 40 μl (40 units) of heparin. Approximately 8 mL of whole blood were collected and transferred to 15-ml falcon tubes to extract peripheral blood leukocytes (PBLs). The centrifugation method published by Smith et al. [26] was used to isolate the PBLs. Cells were stored in an RNAlater (ThermoFisher Scientific, MA, USA) solution at − 20 °C until total RNA extraction was achieved. The RNeasy mini kit (Qiagen, Hilden, Germany) was used to extract total RNA following the manufacturers' instructions. The extracted RNA was quantified by using the Qubit RNA Assay kit (Invitrogen, CA, USA) and its quality was tested by the Agilent 2200 TapeStation system (Agilent Technologies, CA, USA). To obtain the whole-transcriptome of the sharks, the TruSeq stranded mRNA, and the total RNA sample preparation kit (Illumina, CA, USA) was used, and the cDNA library was synthesized according to the Low Sample (LS) protocol under the manufacturers' guidelines.

In brief, the sample preparation workflow began with the preparation of a pooled sample consisting of 1.21 μg of total RNA from which to purify and fragment mRNA. The first-strand of cDNA was synthesized by adding the SuperScript II reverse transcriptase enzyme (Thermo Fisher Scientific, MA, USA), followed by the second-strand cDNA synthesis. Adenylation of the 3′ ends was performed to prevent ligation among the cDNA strands during adapter ligation. The adapter sequence GCCAAT was ligated and amplified by the polymerase chain reaction (PCR) using a thermal cycler (Veriti, Applied Biosystems, CA, USA). The final amplified product, over 500 bp in length, was gel extracted and purified for Illumina sequencing. The quality of the cDNA was verified using the Agilent 2200 TapeStation, and up to 4 nmol of cDNA were sequenced using the MiSeq Sequencing System (Illumina, CA, USA).

2.2. Sequence analysis

Raw Illumina sequences were analyzed by using several types of bioinformatic software as described below. The Illumina read quality was measured using the FastQC version 0.11.3 [27], and the adaptor sequences were trimmed by using the Trimmomatic software [28]. Sequence assembly was performed by the Paired-End reAd mergeR (PEAR) assembly [29] and Trinity software [30] using the default settings. The assembled reads were aligned using the local database composed of the National Center for Biotechnology Information (NCBI) GenBank published IgNAR constant domain gene sequences from five shark species and one ray species. Their species names and accession numbers are nurse sharks (*Ginglymostoma cirratum)* AAB42621.2, wobbong sharks *(Orectolobus maculatus)* ABB83616.1, small-spotted catsharks (*Scyliorhinus canicula*) AGG53259.1, banded houndsharks (*Triakis scyllium*) BAJ20185.1, spiny dogfish (*Squalus acanthias*) AES92985.1, and shovelnose guitarfish (*Rhinobatus productus*) - ray AAT02204.1.

The nurse shark IgNAR heavy chain constant region amino acid sequence was the reference for characterization of the brownbanded bamboo shark IgNAR constant domains. The transcriptome sequences from the PEAR and Trinity assembly software were compared, and nucleotide variations were determined. Consensus sequences were created by assembling the contigs using the CLC Genomics Workbench version 8.0.3 [31]. The ClustalW multiple alignment program was used

Table 1
Summary of the brownbanded bamboo shark transcriptome library indicating the sequence count and lengths before and after assembly by the Trinity or PEAR software.

	No of sequences	Total residue count	Minimum sequence length	Maximum sequence length	Average sequence length
Raw - Forward	20,599,176	4,499,047,447	35	301	218.41
Raw - Reverse	20,599,176	4,528,135,612	35	301	219.82
PEAR	19,890,817	4,855,711,172	50	592	244.12
Trinity	303,547	209,162,459	201	25,719	689.06

Table 2
Minor variation frequencies and respective amino acid changes in IgNAR constant heavy chain C1 domain.

Mutation[a]	Minor variation frequency %[b]	Polymorphism
c. 57 A > C (p. Glu19Asp)	32.2	Nonsynonymous
c. 73 A > G (p. Lys25Gly)	31.5	Nonsynonymous
c. 74 A > G (p. Lys25Gly)	31.5	Nonsynonymous
c. 180 G > T (p. Ser60Ser)	36.6	Synonymous
c. 181C > T (p. Pro61Ser)	41.5	Nonsynonymous
c. 238 A > C (p. Lys80Gln)	20.8	Nonsynonymous
c. 305 A > G (p. Lys102Arg)	42.9	Nonsynonymous

[a] Mutation nomenclature based on Ogino et al., [37].
[b] Minor variation frequency was calculated by the percentage value of the proportion of minor variations expressed per base site in C1 domain.

to align the constant domains of different species, and gene editing was performed by the BioEdit version 7.2.6.1 software [32]. The phylogenetic tree was created by the neighbor-joining method [33] with 1000 bootstrap replicates [34] using the MEGA v.7 software [35] and the evolutionary distances were calculated by the Poisson correction method [36].

3. Results

3.1. Transcriptome sequence assembly

The brownbanded bamboo shark cDNA library obtained using NGS (Illumina, CA, USA) consisted of 20 million reads of over 4 billion residues. The sequence assembly by Trinity formed 0.3 million contigs while the PEAR assembly merged up to 19 million Illumina paired-end reads. The average sequence length obtained by the Trinity software was much higher than using the PEAR assembler although the number of contigs was less in the Trinity assembly as displayed in Table 1.

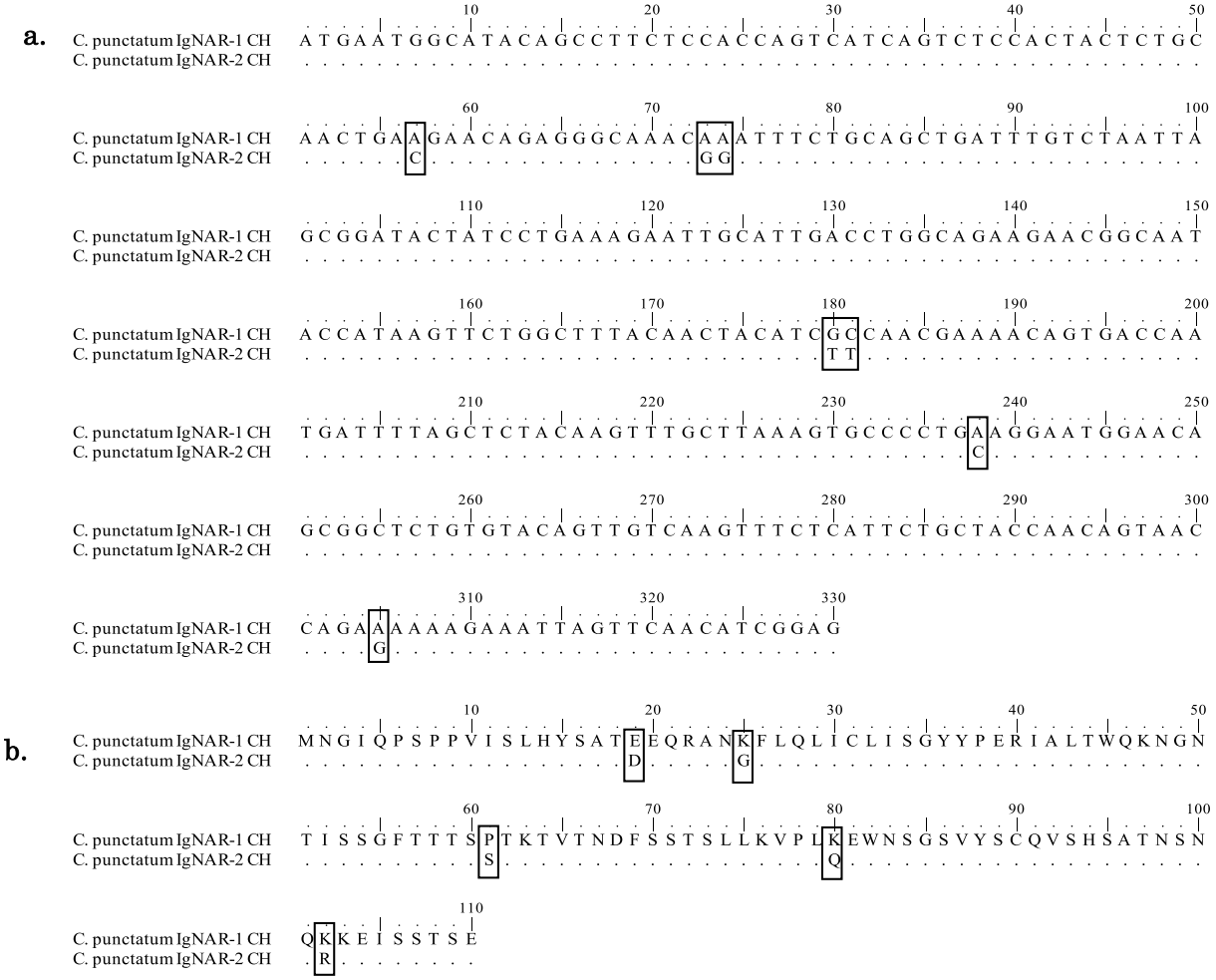

Fig. 1. Brownbanded bamboo shark IgNAR heavy chain C1 domain sequences indicating the presence of two IgNAR types: **a)** nucleotide sequence and **b)** amino acid sequence alignments indicating the polymorphic sites. Dots represent the identical residues, and rectangular boxes demarcate the polymorphic sites.

Table 3
Comparison of the bamboo shark IgNAR constant heavy chain protein sequence using the NCBI blastp with published cartilaginous species IgNAR sequences.

Species name	NCBI Accession number	Max. score	Total score	Query cover	E value	Identity
Nurse shark *(G. cirratum)*	AAB42621.2	938	996	99%	0.0	77%
Spotted wobbegong *(O. maculatus)*	ABB83616.1	869	1034	89%	0.0	79%
Spiny dogfish *(S. acanthias)*	AES92985.1	743	933	89%	0.0	67%
Banded houndshark *(T. scyllium)*	BAJ20188.1	705	1029	89%	0.0	65%
Small-spotted catshark *(S. canicular)*	AGG53259.1	655	952	89%	0.0	61%
Shovelnose guitarfish *(R. productus)*	AAT02204.1	612	668	89%	0.0	57%

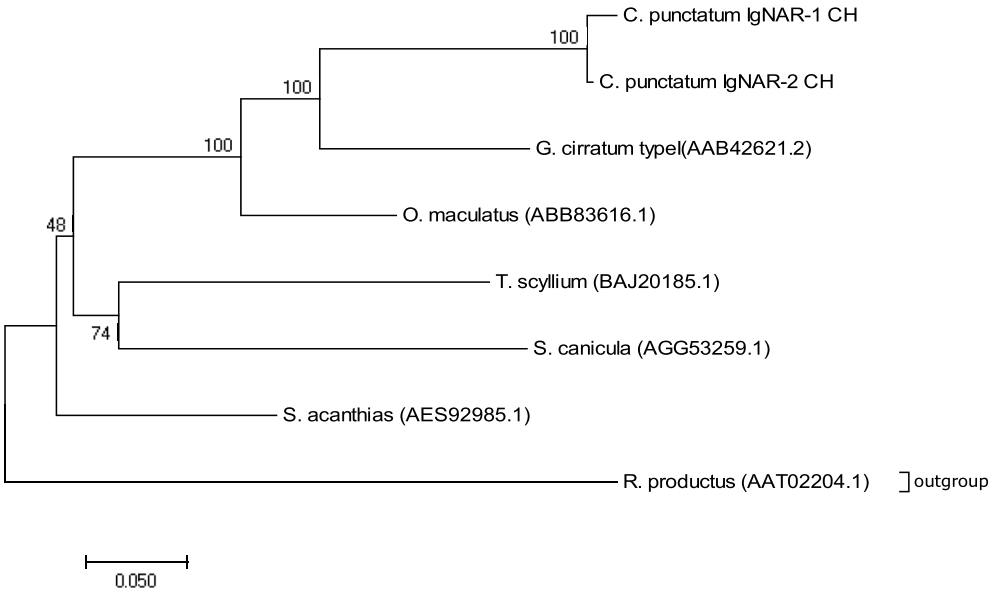

Fig. 2. Phylogenetic analysis of the IgNAR heavy chain constant region complete sequence of the brownbanded bamboo shark (IgNAR-1 CH and IgNAR-2 CH) compared to other cartilaginous fish species. *Rhinobatos productus* is a ray used as an outgroup. All species names and their accession numbers are indicated at their respective nodes. The genetic divergence is indicated by the branch length and scaled to 0.05. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree.

3.2. Characterization of the IgNAR constant domains

As per the alignment, 154 cDNA sequences of the PEAR-assembled library were aligned and mapped to complete IgNAR constant domains (secretory form) using the nurse shark (NCBI accession U18721) as the reference sequence. As a result, two representative secretory IgNAR constant heavy (CH) chain nucleotide sequences were obtained composed of 1758 base pairs coding 586 amino acids from the brownbanded bamboo shark (Figure S1 in Supplementary data). The Trinity-assembled IgNAR CH contigs were compared with the PEAR-merged sequences. A higher homology was revealed and confirmed by the ClustalW sequence identity matrix to be greater than 0.9. Based on the transcriptome sequence of C1, the brownbanded bamboo shark IgNAR was classified mainly into two types, i.e., the *C. punctatum* IgNAR-1 CH type and the IgNAR-2 CH type. A total of seven major polymorphic sites with minor variation frequency above 20% were distributed in the C1 domains of both IgNAR types with six nonsynonymous and one synonymous mutation (Table 2 and Fig. 1).

Based on the distribution of single nucleotide polymorphisms (SNPs), 13 unique IgNAR C1 gene sequences were identified (Figure S2 in Supplementary data). In the bamboo shark IgNAR C region (C1–C5), 199 SNPs were found that resemble 12.6% of the total IgNAR CH base positions. From the 154 PEAR-merged contigs analyzed in this study, 3.58% of polymorphisms were found in the C3 domain followed in decreasing order by C4 (2.73%), C2 (2.33%), C1 (1.65%), and C5 (0.8%) along the full length of the secretory IgNAR. Variable nucleotides are listed in the supplementary data table S1.

The average molecular weight of the entire IgNAR constant region of the brownbanded bamboo shark was 65.3 kDa. Based on the percentage of the total molecular weight, it is primarily composed of serine (11.58%), threonine (9.03%), and valine (8.01%). The least abundant amino acid in the brownbanded bamboo shark IgNAR constant domain is histidine (1.02%). The amino acid composition of the bamboo shark IgNAR was listed in the Supplementary data Table S2.

3.3. Phylogenetic analysis of the C. punctatum IgNAR constant region

The brownbanded bamboo shark IgNAR heavy chain constant region amino acid sequence compared with the NCBI blastp revealed a 77% identity to the nurse shark and was more than 57% comparable to other cartilaginous fish (Table 3). The phylogenetic analysis revealed the evolutionary relationship of the brownbanded bamboo shark with the nurse shark and the wobbegong shark which belong to the same taxonomic order (Orectolobiformes). Based on the phylogenetic tree, the IgNAR constant region of the brownbanded bamboo shark and the nurse shark shared a common ancestor with the wobbegong shark. The phylogenetic tree and the protein sequence alignment were obtained by using the MEGA 7 [35] and BioEdit [32] software as shown in Figs. 2 and 3, respectively.

3.4. Compatibility of the C. punctatum IgNAR constant region for immune modifications

Though IgNAR sequences are dissimilar to human immunoglobulin domains, the essential elements in the immunoglobulin fold are conserved. Like the analysis by Feige et al. [22], in this study, the brownbanded bamboo shark IgNAR constant domains (C1 to C5) were aligned with other cartilaginous fish CH domains and human IgG domains. (as illustrated in supplementary data Figure S3).

Similar to the previous study on nurse sharks, brownbanded bamboo shark IgNAR CH also possessed a tryptophan molecule which helps hydrophobic residues to form a tight core around the disulfide bridge [22]. Furthermore, molecules such as cysteine, tyrosine, phenylalanine and tryptophan along the C1-C5 domains in brownbanded

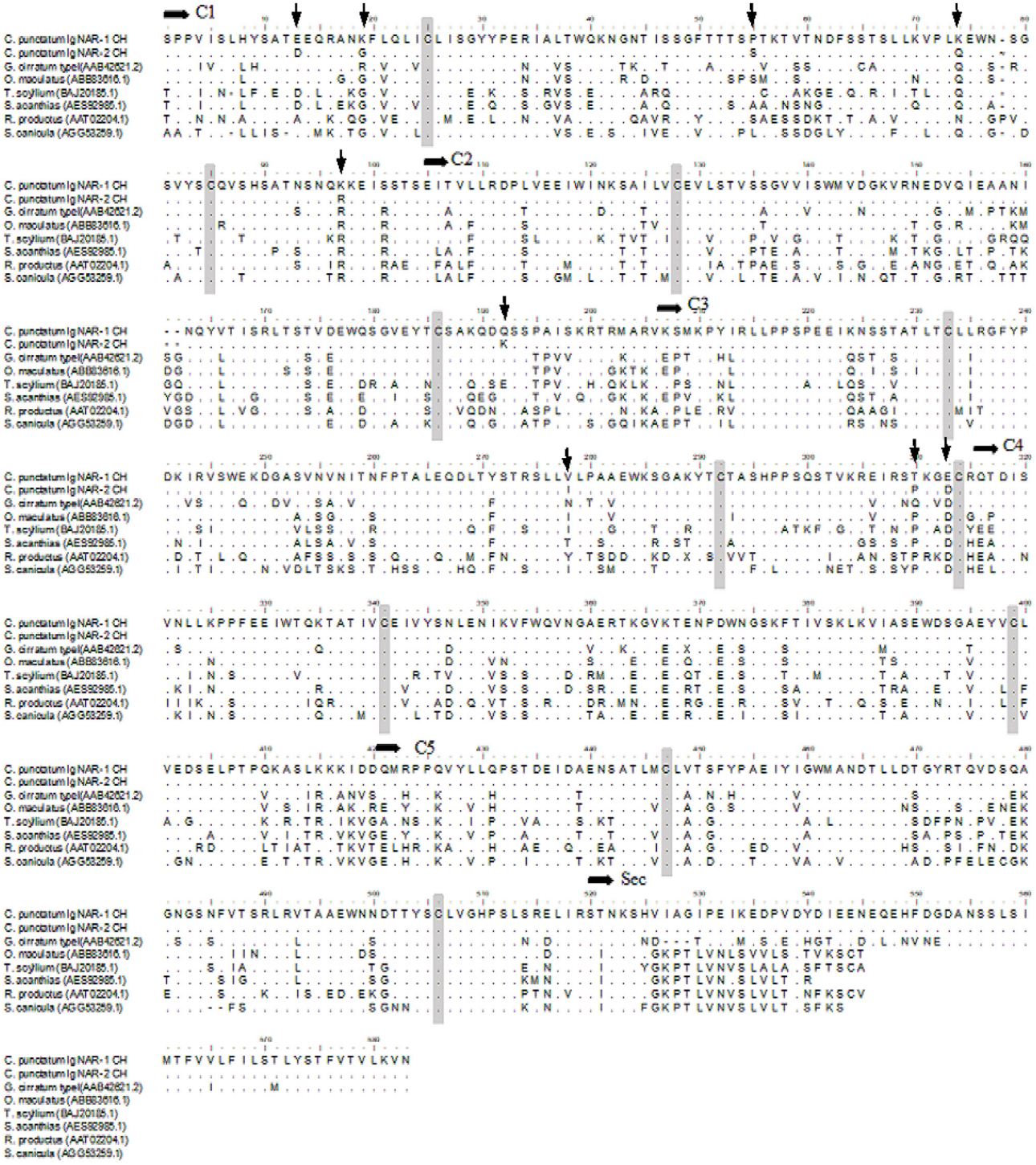

Fig. 3. The brownbanded bamboo shark IgNAR (*C. punctatum* IgNAR-1 CH and *C. punctatum* IgNAR-2 CH) constant region amino acid alignment compared to other cartilaginous fish species. NCBI accession numbers and species names are available at the left of the sequences. The black horizontal arrow marks the beginning of each constant and secretory domain (C1 to C5 and Sec). Gray areas indicate the conserved cysteine residues. Amino acids identical to the IgNAR-1 CH are marked with black dots and dashes represent gaps. Vertical black arrows indicate the nonsynonymous mutations in the IgNAR-2.

bamboo shark IgNAR marked the significance of evolutionary conservation and indicates the stability in nature which in turn can be used to modify human immunoglobulins. Among the differences with the human IgG domains, the IgNAR lacked proline residues which is essential to the mammalian immunoglobulin folding reaction [22,36]. However, in IgNAR constant domains, presence of hydrophobic core which surrounds the disulfide bridge and formation of helix interacting with aromatic residues lead to the stability and flexibility which lacks in human IgG.

4. Discussion

In this study, due to the complexity and the number of sequences, two software programs with different assembling systems were used to obtain a more accurate analysis. The Trinity assembly provided longer reads covering more than half of the entire IgNAR constant region while

the PEAR assembly merged the paired ends of the Illumina reads, giving rise to several short contigs with higher accuracy. Both analytical methods covered the entire IgNAR constant region transcriptome sequence and proved to be suitable to analyze NGS data.

Cartilaginous fish diverged from their jawed vertebrate ancestor approximately 450 million years ago and further divided into several lineages, particularly forming the superorder Galeomorphi (approximately 220 million years ago), and bringing the brownbanded bamboo shark (*C. punctatum*) into existence [36]. During the evolution of sharks, the Squalea and Glaleomorphii lineages experienced cluster expansion or contraction, resulting in a different number of clusters. In spiny dogfish, it appears that immunoglobulin clusters are arranged tandemly, indicating the possibility of duplication and expansion of the primordial cluster during meiosis due to unequal cross-over events [18]. Gene conversion may homogenize the immunoglobulin clusters to maintain high sequence identity, increasing the chance of more expansions or contractions. Similar observations have been made in the brownbanded bamboo shark IgNAR C1 domain transcriptome sequence, resulting in nucleotide variations in 13 sequences, which might indicate that the brownbanded bamboo shark may contain up to 13 independent IgNAR gene clusters. Interestingly, all five nonsynonymous mutations in the brownbanded bamboo shark IgNAR C1 domain were also found in other shark species which might have been retained during evolution. Because the IgNAR lacks the canonical hinge region, the C1 dimerization interface produces a wide angle, facilitating the variable domain to bind multiple epitopes [23], which is another reason for the increased complexity of the IgNAR C1 domain compared to the other constant domains. However, the overall number of nucleotide variations per domain is highest in the C3 domain in bamboo sharks possibly due to the formation of a narrow stalk by C3 dimerization which supports the flexibility of the IgNAR using a disulfide bridge between the C3 and C4 domains [23].

Molecular analysis and characterization of shark antibodies are essential not only due to its evolutionary history but also to the study of their existence in harsh conditions such as high urea concentrations in blood [7,36]. The brownbanded bamboo shark, similarly to other sharks, consisted of 11 conserved cysteine residues in its IgNAR constant domains. Furthermore, the alignment of the IgNAR constant domains with other cartilaginous fish and human IgG revealed that brownbanded bamboo sharks also possess conserved tryptophan and cysteine residues that are involved in the formation of disulfide bonds and structural folds [22].

The composition of amino acids in the IgNAR constant domains is significant for the stability of the antibody because it does not have support from the light chain domains [22]. The structural elements in the IgNAR constant domain could also influence the folding pathway of the antibody which increases the stability. This attribute makes it a suitable candidate as a therapeutic antibody, mainly due to the ability to transplant the stabilizing structural motif into other domains. Furthermore, the domains in the IgNAR are homologous with IgW, which is one of the oldest isotypes among immunoglobulins [7] and IgNAR is one of the immunoglobulin isotype which is responsible to antigen specific response upon immunization [13].

Understanding different classifications of the IgNAR is essential. Current categorization of the IgNAR is primarily based on the location of the non-canonical cysteine residue in the variable region [11]. Because the type I IgNAR is found only in nurse sharks, while all other species express the IgNAR type II and its variants [9], another means of categorization is necessary. Therefore, this research proposed the possibility of a novel classification of the IgNAR, based on the IgNAR C1 nucleotide variations. The polymorphisms in the C1 domain were considered due to the uniqueness in the IgNAR constant region compared to the other four domains which are derived from IgW [5,6]. The IgNAR C1 domain also plays a significant role in structural alterations that increase affinity against a specific antigen [22]. From the findings of this study, the brownbanded bamboo shark IgNAR-1 CH and IgNAR-2 CH sequences can be used to compare novel IgNAR types in future studies on cartilaginous fish.

This study was conducted using transcriptomic sequences obtained from next-generation sequencing (Illumina, MiSeq) and the results were compared by using two assembling methods. Both methods provided nearly similar outcomes, although the Trinity software provided longer sequence reads compared to the PEAR assembly procedure. This report is the first record of the detailed characterization of the brownbanded bamboo shark IgNAR constant domain to the best of our knowledge. Coding sequences of the *C. punctatum* IgNAR constant region were deposited at the NCBI GenBank. Accession numbers for IgNAR-1-CH and IgNAR-2-CH are MH813472 and MH813473 respectively.

5. Conclusions

The brownbanded bamboo shark (*Chiloscyllium punctatum*) IgNAR constant domain (secretory form) consisted of 586 amino acid residues (1758 bp) with a molecular weight around 65.3 kDa. Transcriptome analysis of the IgNAR CH domains revealed the presence of two primary IgNAR types and 13 unique sequences for C1 domain. The sequence information derived from this study could give insights of molecular mechanism of the IgNAR C domains against denaturation and that may be helpful for the designing of stable human immunoglobulins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.004.

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