論 文 の 内 容 の 要 旨

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論文題目 **Analysis of brownbanded bamboo shark (*Chiloscyllium punctatum*) immunoglobulin novel antigen receptor (IgNAR) as a potential immunotherapeutic**

（抗体医薬として期待されるイヌザメ（*Chiloscyllium punctatum*）免疫グロブリン新規抗原受容体 IgNAR の解析）

Immune system is important for animals to fight against invading pathogens and antigenic agents. It contains two parts mainly innate immunity and acquired immunity. During evolution, cartilaginous fish are the first jawed vertebrates having immunoglobulins (Igs) which is also called as antibodies. There are three types of Igs found in sharks namely, IgM, IgW and immunoglobulin novel antigen receptor (IgNAR). IgNAR was reported first by Greenburg et al., in 1995 as heavy chain homodimer. Since then many scientists focused on this antibody due to its advantageous characteristics in biomedical research.

There are only two naturally occurring heavy chain only antibodies (lacking light chain) were reported, one from camelids called VHH and the other one is IgNAR from cartilaginous fish. Both of these molecules have smaller variable regions of which variable region of IgNAR (vNAR) considered as the smallest antibody like molecule in nature (~ 12 – 15 kDa). The reasons for the smaller size are due to lack of light chain and devoid of second complementary determining region (CDR). IgNAR was found to be stable in harsh environments such as high temperature and pH variations. This might be an adaptation of the Ig which circulates in shark blood with high ammonia concentration. The higher stability was believed to be due to the presence of non-canonical cysteine molecules present in the variable domains of vNAR.

Use of monoclonal antibodies on diagnostics and treatments is becoming more popular recently. Due to larger molecular size of the conventional antibodies (eg: IgG 150 kDa), it is difficult to reach the epitopes particularly in cellular spaces and cancer cells. Hence scientists focused on engineering the antibodies to develop smaller, antigen specific and high affinity monoclonal antibodies. Variable region of shark IgNAR which comply with those features is being investigated to develop immunotherapeutics (anti-Ebola, anti-TNFα) and diagnostics (anti

malaria AMA1, anti-Cholera). Various techniques have been used to develop these nanobodies such as immunization of shark and development of semi-synthetic antibodies.

There are four types of IgNARs classified to date based on the presence and location of cysteine residue in the variable region. Currently several sharks and rays have been investigated for their IgNAR gene sequence. Although they all have non-canonical cysteine residues, type I IgNAR was reported only from nurse shark (*Ginglymostoma cirratum*). Most of the investigators also performed immunizations and it revealed that different shark species have been responding differently against antigens. Hence, the aim of this study was to develop a novel platform to isolate antigen specific vNAR from brownbanded bamboo shark (*Chiloscyllium punctatum*) by *in vivo* affinity maturation.

1. Characterization of brownbanded bamboo shark IgNAR constant region

IgNAR has two regions namely, the variable region and constant region. Constant region consists of five domains C1 to C5. These domains help the integrity of the IgNAR antibody and also maintains flexibility at the hinge region during antigen binding. Study on brownbanded bamboo shark focused on the evolutionary compliance with the conventional antibodies particularly human Igs. In this study, brownbanded bamboo shark (*Chiloscyllium punctatum*) was selected as the model species to characterize the IgNAR constant region sequence by next generation sequencing (NGS) method. Peripheral blood leukocytes (PBL) were used to isolate mRNA and synthesized cDNA for the library having 20 million raw reads (Illumina, MiSeq). Average sequence length after paired end assembly was 218.4 bp (35 - 301 bp) with total of 4.5 billion residues. Transcriptome analysis of complete constant region with five domains and conserved cysteine residues revealed the presence of two distinct types of IgNAR in brownbanded bamboo shark. The C1 domain alone displayed 13 unique sequences that might resemble the number of IgNAR CH gene clusters. Furthermore, phylogenetic analysis showed that the relationship with order Orectolobiformes, of which nurse shark (*Ginglymostoma cirratum)* and wobbegong shark (*Orectolobus maculatus*) as the highest similarity. The alignment of human IgG constant regions indicated the conserved nature of cysteine residues throughout the evolution of Igs. Through this study, understanding of the characteristics of IgNAR constant domains used to maintain its structural stability will become useful information for engineering monoclonal antibodies in the future.

2. Diverse response by vNAR of brownbanded bamboo shark against antigen

Shark vNAR is widely used in therapeutic and diagnostic research due to its smaller size which enables tissue penetration, simple architecture and higher stability of the molecule that helps antibody engineering easier and cost effective. Developing highly specific and higher affinity antibody is important to minimize adverse effects. Presence of cysteine in variable region is useful to maintain structural integrity and stabilize the antigen binding regions. Number of cysteine residues also play an important role in the formation of diverse antigen-binding surfaces. Therefore, studying the responses against antigen and screening cysteine expression in CDR domains is important prior to synthesis of the therapeutic or diagnostic nanobodies. This study aimed at understanding the diverse nature of vNAR from brownbanded bamboo shark and the individual response against antigen at various time of exposures. Adult sharks were immunized to determine antigenic responses of vNAR by injecting hen egg lysozyme (HEL) and collected the peripheral blood leukocytes (PBL) for cDNA library synthesis. Three-step tailed

PCR was designed and vNAR library was obtained using Illumina amplicon sequencing. The vNAR library with 72% productive sequences was consisted of 8.41 GB data (19.3 million sequences). More mutations were found in CDR-1 than CDR-3 which is typical for type II IgNAR when affinity maturation occurs due to somatic hypermutation. However, CDR1 and CDR3 variations were reduced as number of antigen exposures increased. Type II IgNAR sequences were abundant in only one shark and found different patterns expressed in different individuals. Interestingly, type I sequence found in one shark after first exposure to antigen at a very low expression level (1/100,000). In addition to nurse shark this is the first report of type I IgNAR from another species. There was an increase of IgNAR titer along with vNAR sequences obtained after HEL exposure. This study reveals the diversity of vNAR among individual sharks and also variability during the number of exposures. Hence it is suggestive of determining the highly antigen specific vNAR gene sequence prior to development of monoclonal antibodies from shark origin. This study shows the platform of synthesizing large cDNA library by amplicon sequencing and reduces the time, labour and cost of conventional cloning methods.

3. Transcriptome analysis revealed overall positive immune response against antigen

The transcriptome analysis and RNA-Seq data provides insights to gene expressions and reveals lot of information. Currently few shark species including nurse shark tissues such as heart, spleen, kidney were studied. Genomic study on Elephant shark (*Callorhynchus milii*) showed that adaptive immune system of cartilaginous fish has T helper cells that were highly restricted and unconditional antigen-binding ability. Furthermore, it lacks CD4 and related cytokine receptors as well. However very few studies have done on transcriptomic analysis of shark species and almost none of the studies investigated the immune response before and after antigen exposure in brownbanded bamboo shark. The aim of this study was to perform transcriptome analysis on the response of PBL from brownbanded bamboo shark against HEL. The RNA-seq library was prepared from PBL of pre-immunized (control) and immunized brownbanded bamboo sharks. Nearly 40 million clean sequence reads were obtained including 56 % immunized, 46 % control sequences. After antigen exposure, genes on negative regulation of Ig production was down regulated while regulation of complement activation, lectin pathway and regulation of cellular defense response were upregulated. Based on the transcriptomic analysis brownbanded bamboo shark shows great adaptive immune response against antigen which makes it a suitable candidate for immune research.

4. Strategy to synthesize brownbanded bamboo shark vNAR based monoclonal antibody

Development of monoclonal antibody is trending these days having around 20 – 30 products in the market and over 100 in clinical trials. It is predicted to be increasing and advancing with the use of smaller, recombinant antibody fragments and engineered antibody variants such as nanobodies. Shark vNAR having interesting features fit for immunotherapy is a potential candidate and this study performed to develop a platform to synthesize monoclonal antibody from brownbanded bamboo shark vNAR origin. Adult sharks were immunized with hen egg lysosome (HEL) and cDNA amplicon library prepared using mRNA from peripheral blood leukocytes. After paired end assembly unique sequences were listed based on the abundance and expression during prior and after immunization were compared. The sequences expressed after third immunization with higher abundance were selected and obtained anti-HEL vNAR model (nurse shark anti-HEL vNAR was used as template). Using Chimera (UCSF) software, strength of antigen binding by the number of contacts between antigen-antibody complex was

determined. In order to synthesize ant-HEL nanobody, the highest affinity vNAR gene sequence was inserted into mammalian expression vector pEHX1.1-N.S.-IgNAR-Fc (from Omasa lab, Osaka University). The protocol has been developed to express the vNAR antibody using chicken hamster ovary (CHO) cell line. This novel platform for isolation and expression of antigen specific vNAR may be useful for the development of monoclonal nanobodies from shark origin at less cost and time compared to the conventional methods such as phage display.

5. Future research potential

Shark IgNAR was first reported in 1995 by Flajnik and co-workers. Since then many studies have been carried out on characterization of IgNAR in different shark species and synthesis of antigen specific vNAR by immunization or molecular engineering. However, there are few drawbacks of using shark vNAR; firstly, due to the smaller size (~12 – 15 kDa) it is easily filter through kidney glomeruli causing less retention time (or half-life), secondly, adverse effects due to rejection of the antibody from host. Hence this study focused on developing IgNAR against albumin, which is a main plasma protein in mammals with higher molecular weight (66.5 kDa). A combination of HEL and bovine serum albumin (BSA) were injected to brownbanded bamboo sharks and synthesized a cDNA library with amplicons covering full-length vNAR gene sequences. Similar to the previous study with HEL, potential anti-BSA vNAR gene sequences were determined by using anti-HSA vNAR template from spiny dogfish. The target binding residues such as, Trp, Asp, Tyr and Pro were prominent in anti-BSA vNAR CDR3 sequence in brownbanded bamboo shark. According to ELISA, plasma IgNAR response against HEL became less compared to BSA. The sequences derived from this study will be useful for the expression of nanobodies using CHO cells in further research.

Overall, this research investigated brownbanded bamboo shark naiive IgNAR and immunized variations. The transcriptome analysis will provide insights to the antigenic response by PBLs. In addition, development of novel platform to synthesize full length vNAR amplicon library and screening highly specific gene sequences will be useful in developing nanobodies with minimal adverse effects in the future.