2014 2nd International Conference on Sustainable Environment and Agriculture
IPCBEE vol. 76 (2014) © (2014) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2014. V76. 22

Analysis of Expressed Sequence Tags (EST) Obtained from Common Carp, *Cyprinus carpio* L., Head Kidney Cells after Stimulation by CpG Oligodeoxynucleotides

Asmi Citra Malina AR Tassakka [1+] and Masahiro Sakai [2]

[1] Faculty of Marine Science and Fisheries, Hasanuddin University, Indonesia
[2] Faculty of Agriculture, University of Miyazaki, Japan

Abstract. We analyzed genes expressed from head kidney of common carp *Cyprinus carpio* L. treated with CpG oligodeoxynucleotides. The results of single-pass sequencing of expressed sequence tags (ESTs) from 88 clones (AU312478-AU312561) from kidney cDNA are presented. Out of 88 clones 84 (95.5 %) matched with nucleic acid and/or amino acid sequences, whereas the remaining 4 (4.5 %) clones did not show any significant homology to the sequences in the databases. Immune related cDNA clones identified from kidney were granulin2, CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, lysozyme C, interleukin-4 receptor alpha chain, cathepsin L preproprotein, CD9 protein and Granulin 1 were identified.

Keywords: CpG Oligodeoxynucleotides, Carp, Expressed Sequence Tags, Kidney.

1. Introduction

Expressed sequence tags (ESTs) analysis, which survey sequences contained in cDNA libraries, is a powerful approach for identifying new genes and profiling gene expression in tissue or cells. In recent years, EST sequence resources are rapidly growing in molecular databases. However, ESTs from teleosts account for less than 5% of all ESTs in the databases. Over 50% of the teleosts were generated from model fish, such as zebrafish and fugu, while EST resources are lacking for most fish species. The first fish EST survey included tissues of healthy winter flounder (*Pleuronectes americanus*), lumpfish (*Cyclopterus lumpus*) and halibut (*Hippoglossus hippoglossus*) [1]. Further EST analysis have been performed on immune-relevant tissues from species such as winter flounder [2], Atlantic salmon (*Salmo salar*) [3] and red sea bream [4]. In order to maximize the discovery of genes with roles in immunity, ESTs have also been obtained from leucocytes of carp treated with a mitogen [5], kidney of Japanese flounder treated with peptidoglycan [6], kidney of rainbow trout and Japanese flounder infected with viruses [7] and catfish (*Ictalurus punctatus*) infected with bacteria [8]. The lack of EST resources in cultured fish species prohibits use of modern functional genomic approaches for the study of growth, development, stress biology and molecular breeding.

Common carp is a widely cultured marine fish species in Asian countries and a cheap source of protein. However, diseases of the cultured fish have occurred frequently and losses due to infectious diseases limit profitability and development of aquaculture. The use of antibiotics has partially solved the problem but has raised concerns of antibiotic residues in fish and polluting environment and antibiotic resistance development. There is extensive interest in enhancing resistance of cultured fish to diseases. The screening for immune-relevant functional genes in fish is very important for identifying the molecular mechanism for disease resistance.

[+] Corresponding author. Tel.: +62411586025
E-mail address: citramalina@gmail.com

There are evidences stating that oligodeoxynucleotides (ODNs) containing cytosine-phosphodiester-guanine (CpG) motifs are very potent inducers of the innate immune system. There are no reports about EST study in the CpG-ODNs stimulated carp. This initiated us to construct a cDNA library from mRNA isolated from head kidney (HK) cells of carp that have been stimulated by CpG-ODNs and to identify the DNA sequences of 88 clones.

2. Materials and Methods

2.1. Fish and Immunostimulant

Common carp (mean weight=100g) was obtained from Sunaso Fisheries farm in Miyazaki, Japan. The fish were acclimated in aerated fresh water tank at 20 °C under natural photoperiod and fed for two weeks prior to the experiment.

The CpG-ODN **A** (GCTAGACGTTAACGTT**)** & **B (**ATCGACTCTCGAACGTTCC) were suspended in phosphate buffer saline [PBS (10 μg/100 μl)] and used for administration**.**

2.2. Construction of cDNA Library

Fish were injected with CpG-ODN A and B at a dose of 10 μg/fish intraperitoneally. Six fish of each group were sampled for the RNA isolation at 1, 5 and 7 days after injection. The head kidney phagocytic cells of the carps were isolated according to the modified method [9]. The stimulated head kidney cells were pooled together and stored in ISOGEN (Nippon Gene, Japan) at –80 °C for mRNA extraction. The mRNAs from the three different time periods were isolated using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). These were subsequently pooled to ensure complete coverage of expressed genes in the allotted time frames (1, 5 and 7 days) and used to construct a cDNA library. The cDNA library was prepared with a cDNA Synthesis Kit (Invitrogen, USA), with a *Not*I /*Sal*I adapter, and constructed in the pSPORT 1 vector, according to instructions of the manufacturers.

2.3. Plasmid Preparation and Nucleotide Sequencing

The recombinant plasmid DNA was isolated by the alkaline lysis method [10]. The cDNA clones were sequenced using ThermoSequenase with M13 forward or M13 reverse primers or both and an automated DNA sequencer LIC-4200L (Li-Cor, USA).

2.4. Data Analysis

The sequences were compared with sequences in the database using the BLASTX algorithm [11]. Assignment of putative identities for EST's required a minimum *P* value of 10^{-5}. EST's that putatively matched known genes were catalogued into five general categories (cell organism defense, cell structure/ motility, cell signaling/cell communication, metabolism and unclassified) based on the putative functions of known genes [12].

3. Results and Discussion

A total of 88 cDNA clones isolated from a cDNA library of kidney cells of common carp injected with CpG oligodeoxynucleotides (CpG-ODNs) were partially sequenced. The numbers of genes sequenced and the numbers in different functional groups are shown in Table 1. All the sequences were registered in GenBank under accession numbers AU312478-AU312561. Each EST was assigned to the functional group of its most commonly known function.

Out of 88 clones 84 (95.5 %) matched with nucleic acid and/or amino acid sequences, whereas the remaining 4 (4.5 %) clones did not show any significant homology to the sequences in the databases. The proportion of transcripts associated with each of the broad categories is as follows: Cell organ/defense (14.3 %), Cell structure/motility (5.9 %), Cell signaling/cell communication (50.0 %), metabolism (16.7 %), and unclassified (13.1 %).

Among all identified genes, 79 genes appeared only once and 5 genes were present more than once. The first three most frequently identified clones were α-globin (n=5), lysozyme C (n=4) and β−globin (n=3). The

cDNA libraries had a total of 9 clones related to immune function. The frequency of each identified clone involved in defense mechanism in the CpG oligodeoxynucleotides cDNA libraries is shown in Table 1.

Table 1: List of identified EST's from head kidney cDNA of common carp stimulated with CpG oligodeoxynucleotides

Clone number	Putative identification	Accession number	Closest species	Accession number of closest species	Identity	Frequency
	Cell/organism defense					
HK11	Granulin2	AU312488	Zebrafish	Q9ZC9	17/27 (62%)	1
HK16	CCAT/enhancer binding protein	AU312493	Zebrafish	208261B	76/234 (32%)	1
HK18	Immunoglobulin heavy chain variable region	AU312495	Rabbit	AF177003 1	15/30 (50%)	1
HK21	Lectin	AU312498	Tomato	302272A	19/52 (36%)	1
HK30	Lysozyme C	AU312507	Common carp	Q9IBG5	143/145 (98%)	4
HK53	Interleukin 4-receptor alpha-chain	AU312530	Horse	Q8MIR9	18/48 (37%)	1
HK60	Cathepsin L preproprotein	AU312537	Common carp	Q75SZ8	216/237 (91%)	1
HK61	CD9 protein	AU312538	Rainbow trout	2905394B	39/103 (37%)	1
HK81	Granulin1	AU312558	Zebrafish	Q90ZD4	30/92 (32%)	1
	Cell structure/motility					
HK9	Cytochrome C oxidase subunit I	AU312486	Carp	Q85E84	124/225 (55%)	1
HK25	Myosin IC heavy chain	AU312502	Amoeba	O61080	42/144 (29%)	1
HK29	Extension-like protein	AU312506	Tomato	272335A	21/48 (43%)	1
HK49	Cytochrome C oxidase polypeptide III	AU312526	Common carp	COX3 CYPCA	185/203 (91%)	1
HK72	Cytochrome C oxidase subunit II	AU312549	Common carp	Q85BZ2	164/228 (71%)	1
	Cell signaling/ Cell communication					
HK2	Ribosomal protein L23a	AU312479	Catfish	Q9YU4	101/108 (93%)	1
HK3	Ribosomal protein S11	AU312480	Chicken	Q98TH5	85/91 (93%)	1
HK6	Tax responsive element binding protein 107	AU312483	Chicken	2803253A	76/89 (85%)	1
HK7	Adenine nucleotide translocase	AU312484	African clawed frog	2711156A	65/97 (67%)	1
HK12	AAT-1 protein	AU312489	Human	2824403A	21/56 (37%)	1
HK13	Ribosomal protein S17	AU312490	House mouse	B02044 1	96/105 (91%)	1
HK14	Vimentin (growth-related gene)	AU312491	Zebrafish	2503277A	69/130 (53%)	1
HK15	60S ribosomal protein L39	AU312492	Catfish	RL39 ICTPU	46/51 (90%)	1
HK17	40S ribosomal protein S18	AU312494	Zebrafish	Q8JGS9	105/106 (99%)	1
HK20	Retinal homeobox protein	AU312497	Fruit fly	RX DROME	12/26 (46%)	1
HK23	40S ribosomal protein S3a	AU312500	Catfish	Q90YS1	35/81 (43%)	1
HK24	6S ribosomal protein L35	AU312501	Zebrafish	Q8JHJ1	74/86 (86%)	1
HK27	16 ribosomal protein	AU312504	House mouse	MUSRPS16 1	79/116 (68%)	1
HK28	Ribosomal protein	AU312505	Dog	Q9XSU3	69/77 (89%)	1
HK31	40S ribosomal protein S15	AU312508	Catfish	Q90YQ9	97/145 (66%)	1
HK32	Insulin receptor substrate-2	AU312509	Human	IRS2 HUMAN	20/48 (41%)	1
HK33	Nucleolar GTP-binding protein 1	AU312510	Human	Q9BZE4	86/160 (53%)	1
HK35	Ubiquitin and ribosomal protein S27a	AU312512	Zebrafish	Q7SXA3	79/136 (58%)	1
HK36	Proline-rich proteoglycan	AU312513	Norway rat	B48013	15/32 (46%)	1
HK37	60S ribosomal protein L23	AU312514	Human	RL23 HUMAN	131/140 (93%)	1
HK38	40S ribosomal protein S12	AU312515	African clawed frog	RS12 XENLA	49/105 (46%)	1
HK39	Similar to ribosomal protein L13a	AU312516	African clawed frog	Q7ZY48	52/136 (38%)	1
HK40	Ribosomal protein L27a	AU312517	Catfish	2905383K	71/187 (37%)	1
HK41	60S ribosomal protein L13a	AU312518	Salmon	R13A SALTR	133/146 (91%)	1
HK42	60S ribosomal protein L8	AU312519	Zebrafish	AAH65432	200/210 (95%)	1
HK43	FK506 binding-protein	AU312520	Tobacco hawkmoth	Q9U4Z3	65/82 (79%)	1
HK45	Zinc transporter-like3 protein	AU312522	House mouse	2816292A	20/57 (35%)	1
HK48	Keratin-associated protein	AU312525	Human	27134320	26/77 (33%)	1
HK50	Sperm histone P2 precursor	AU312527	House mouse	HSP2 MOUSE	26/104 (25%)	1
HK55	Ribosomal protein L4	AU312532	Catfish	2905383E	178/218 (81%)	1
HK56	Ribosomal protein L7a	AU312533	Catfish	2905383K	192/248 (77%)	1
HK57	Guanine nucleotide binding protein	AU312534	Pig	GBAK CAVPO	242/268 (90%)	1
HK63	Transcription factor ken	AU312540	Fruit fly	T00119	18/54 (33%)	1
HK64	ribosomal protein L23a	AU312541	Zebrafish	Q6Q416	24/34 (70%)	1
HK67	Sterol regulatory element binding protein	AU312544	Chicken	Q90ZMS	20/47 (42%)	1
HK69	FR01 and FR2-like protein	AU312546	Mouse	Q9FGS9	16/38 (42%)	1
HK71	40S ribosomal protein L2	AU312548	Catfish	RS2 ICTPU	48/93 (51%)	1
HK73	ribosomal protein L5b	AU312549	Catfish	2905383G	132/206 (64%)	1
HK74	Elongation factor 1-alpha	AU312551	Common carp	Q800W9	157/198 (79%)	1
HK76	Proline-rich protein MP4	AU312553	Mouse	S19560	16/33 (48%)	1
HK83	40S ribosomal protein S2	AU312560	Spoon worm	RS2 URECA	159/172 (92%)	1
HK84	Profilaggrin	AU312561	Human	CAD39823	45/173 (26%)	1
	Metabolism					
HK1	Alpha-globin	AU312478	Common carp	013136	80/93 (86%)	1
HK5	Beta-globin	AU312482	Common carp	Q98851	100/109 (91%)	2
HK22	Harmonin a1	AU312499	Rat	Q6PPF3	26/81 (32%)	1
HK34	Alpha-globin	AU312511	Common carp	013139	108/108 (100%)	3
HK46	Aspartate-semialdehyde dehydrogenase	AU312523	Pseudomonas	DHAS PSEAE	18/41 (43%)	1

HK47	ATP synthase 6	AU312524	Barbus	Q9B1G8	166/206 (81%)	1
HK54	Beta-globin	AU312531	Common carp	P70073	10/109 (91%)	1
HK59	ATP synthase 6	AU312536	Barbus	Q9B7L6	106/173 (61%)	1
HK62	NADPH Flavin reductase	AU312539	Human	2006199A	119/203 (58%)	1
HK66	Membrane alanyl aminopeptidase	AU312543	Norway rat	A32852	96/180 (53%)	1
HK79	Alpha-globin	AU312556	Common carp	013138	48/63 (76%)	1
	Unclassified					
HK10	Cys-rich protein	AU312487	Protozoa	1806371A	13/33 (39%)	1
HK19	Hypothetical protein	AU312496	Nematode	Q09636	16/46 (34%)	1
	C27D6.4 in Chromosome II					
HK26	Hypothetical protein	AU312503	Japonica cultivar group	Q6YUW2	14/28 (50%)	1
HK58	Male enhanced antigen 1	AU312535	Mouse	Q9DOQO	44/94 (46%)	1
HK65	Hypothetical protein	AU312542	Nematode	T34010	18/52 (34%)	1
HK68	Hypothetical protein	AU312545	Pyrococcus	C75154	25/70 (35%)	1
HK70	Sequence 1773 from patent EP 1270724	AU312547	Human	AX647581 1	23/88 (26%)	1
HK77	Hypothetical protein SC04250	AU312554	Streptomyces	Q9L0P1	41/132 (31%)	1
HK82	Novel protein	AU312560	Zebrafish	BX942844 4	134/229 (84%)	1
HK78	Hypothetical protein	AU312555	Zebrafish	AAH59454	129/216 (59%)	1
	Hypothetical alpha/beta hydrolases structure					
HK8	containing protein	AU312557	Mouse	Q8BHA3	20/23 (80%)	1

The 9 clones found related to cell/organ defense were granulin2 (AU312488), CCAAT/enhancer binding protein (AU312493), immunoglobulin heavy chain variable region (AU312495), lectin (AU312498), lysozyme C (AU312507), interleukin-4 receptor alpha chain (AU312530), cathepsin L preproprotein (AU312537), CD9 protein (AU312538) and Granulin 1 (AU312558).

EST analysis is an efficient and fast method for gene discovery. Like in mammals, the immune system of fish is also composed of non-specific and specific immune defense. The head kidney (HK) is an important organ for specific and non-specific immune defenses. Several immune-defense related peptides and proteins including specific and non-specific antimicrobial agents, activators and regulators of the immune system were detected in the head kidney leucocytes. In the present study, carp injected with CpG-ODNs were used for EST analysis with the expectation that the expression of biodefense related genes due to the immunostimulant treatment would be detected. A cDNA library of carp HK was constructed and the 88 ESTs were analyzed to identify immune-related genes. Of the 84 known genes, 9 immune-relevant genes were identified: CCAAT/enhancer binding protein, immunoglobulin heavy chain variable region, lectin, lysozyme C, interleukin-4 receptor alpha chain, cathepsin L preproprotein, CD9 protein and Granulin 1 were identified.

Fish treated with immunostimulants are likely to have activated macrophages and lymphocytes. In this study, most of identified immune-related genes might be related to the activation of macrophages or lymphocytes. The functions of some of the immune genes are described below:

a. CCAAT/enhancer binding proteins (C/EBP) are a family of leucine zipper transcription factors that are critically involved in the regulation of normal cellular differentiation and function in multiple tissues. Since the original isolation and cloning of the first C/EBP protein in 1998, there have been many reports on the functions of C/EBP in higher organism, reflecting their significance and importance in normal biological process. C/EBP β and ε in vertebrates have direct roles in the mediation of the immune system and regulate expression of acute phase (AP) proteins, particularly with the up regulation of cytokines and/or the terminal differentiation and function of macrophages (C/EBP β) and granulocytes (C/EBP ε Recently, Tucker et al. [13] have cloned C/EBP in Japanese flounder (*Paralichthys olivaceus*). They found that C/EBP β and C/EBP ε were expressed in Japanese flounder haemapoietic organs and leucocytes. They also suggested that C/EBP β has a more general role in inflammation as expression of C/EBP β was greatly induced in a number of tissues following inflammatory stimuli.

In this study, stimulation by CpG-ODNs induced the expression of C/EBP β suggesting its role in the mediation on the inflammatory response. The EST study of leucocytes from HRV infected fish [14] detected C/EBP ε and β indicating that these C/EBP genes may have a similar immune related function to those reported for mammalian species. C/EBP is also one of transcription factor that is activated in the signaling mechanism of CpG DNA by TLR 9, which directly up-regulate cytokine/chemokine gene expression.

b. Lectins are a range of diverse molecules, broadly classified as calnexin, C-, L-, P-, I-, R-, and S-type lectins, with C-type lectins classified into various sub-groups. Lectins are believed to mediate pathogen recognition, which can lead to neutralization of the invading organism during the early stages of an infection. As lower vertebrates and invertebrates do not posses a strong adaptive immunity, their survival depends on the strong non-self recognition molecules. Most recent works have emphasized the possible role of lectins as non-self recognition molecules in vertebrate and invertebrate immunity. Savan et al. [15] have cloned a new C-type lectin from common carp.

c. Lysozyme is important to inhibit the growth and invasion of infectious pathogens as well as the complement, interferon, C-reactive protein, transferring and lectin located in mucus, serum, and many organs fish. Lysozyme C was the most abundantly expressed immune-related genes during this analysis. In fish, lysozyme-C and its variants have been cloned in carp [5], and Zebrafish [16].

d. One particularly useful group of molecules that is not presented by many teleost equivalents is the cluster of differentiation (CD) markers. Antibodies to these cell surface molecules are extremely useful for identifying and isolating sub-populations of lymphocytes that cannot be differentiated by morphology alone. In addition, these molecules often play integral roles in cell adgesion, signaling and activation. CD9 has been cloned and characterized in Atlantic salmon and rainbow trout [17]. CD9 is a tetraspanin family member with a wide tissue distribution that seems to have a specific role in leucocytes, particularly granulocytes, macrophages, pre-B cells and T cells [17].

It is clear from the present study that CpG-ODNs could stimulate the immune-related genes in Common carp head kidney cells. In another report on the analysis of expressed genes in Japanese flounder injected with peptidoglycan [6], immune related genes isolated included CC chemokine, hepatic lectin, and immunoglobulin heavy and light chains. The difference in expression of immune-related genes compared to the present study probably relates to the kind of immunostimulants used and the time of sampling. This work should contribute to a better understanding of the network system of immune- and biodefense-expressed genes in fish.

4. Acknowledgements

This study was supported in part by Grant-in-Aid for Scientific Research (C) (14560156), a Grant in Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS).

5. References

[1] Z. Gong, Z. Hu, Z.Q. Gong, R. Kitching, C.L. Hew. Bulk isolation and identification of fish genes by cDNA clone tagging. *Mol. Mar. Biol. Biotechnol*. 1994, **3**: 243-251.

[2] S.E. Douglas, J.W. Gallant, C.E. Bullerwell, C.Wollf, J. Munholland, M.E. Reith. Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. *Mar. Biotechnol*. 1999, **1**: 458-464.

[3] G.C. Davey, N.C. Caplice, S.A.Martin, R. Powell. A surfey of genes in the Atlantic salmon (*Salmo salar*) as identified by expressed sequence tags. *Gene*. 2001, **263**: 121-130.

[4] S-L. Chen, M-Y. Xu, S-N. Hu, L. Li. Analysis of immune-relevant genes expressed in red sea bream (*Chrysophrys major*) spleen. *Aquaculture*. 2004, pp. 115-130.

[5] R. Savan, M. Sakai. Analysis of expressed sequence tags (EST) obtained from common carp, *Cyprinus carpio* L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concavalin-A. *Comp. Biochem. Physiol. B*. 2002, **131**: 71-82.

[6] T. Kono, M. Sakai. The analysis of expressed genes in the kidney of Japanese flounder, *Paralichthys olivaceus*, injected with the immunostimulant peptidoglycan. *Fish Shellfish Immunol*. 2001, **11**: 357-366.

[7] T. Kono, M. Sakai, S.E. Lapatra. Expressed sequence tag analysis of kidney and gill tissues from rainbow trout (*Oncorhynchus mykiss*) infected with infectious hematopoietic necrosis virus. *Fish Shellfish Immunol*. 2000, **2**: 493-498.

[8] M.M. Kocabas, P. Li, D. Cao, A.Karsi, C. He, A. Patterson, et al. Expression profile of the channel catfish spleen:

analysis of genes involved in immune functions. *Mar. Biotechnol*. 2002, **4**: 526-536.

[9] R. Braun-Nesje, G. Kaplan, G. Sejelid. Rainbow trout macrophages in vitro: morphology and phagocytic activity. *Dev. Comp. Immunol*. 1982, **6**: 281-291.

[10] J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York, 1982.

[11] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman. Basic local alignment search tool. *J. Mol. Biol*. 1990, **215**: 403-410.

[12] D. M. Hwang, A.A. Dempsey, R.X. Wang, M. Rezvani, J.D. Barrans, K.S. Dai, H.Y. Wang, H. Ma, et al. A genome-based resource for molecular cardiovascular medicine: Towards a compendium of cardiovascular genes. *Circulation*. 1997, **96**: 4146-4203.

[13] C. S. Tucker, I. Hirono, T. Aoki. Molecular cloning and expression of CCAAT/enhancer binding protein in Japanese flounder *Paralichthys olivaceus*. *Dev. Comp. Immunol*. 2002, **26**: 271-282.

[14] T.Aoki, B. Nam, I. Hirono, E. Yamamoto. Sequence of 596 cDNA clones (565.977 bp) of Japanese flounder (*Paralichthys olivaceus*) leucocytes infected with Hirame Rahbdovirus. *Mar. Biotechnol*. 1999, **1**: 477-488.

[15] R. Savan, M. Endo, M. Sakai. Characterization of a new C-type lectin from common carp *Cyprinus carpio*. *Mol. Immunol*. 2004, **41**: 891-899.

[16] F. Liu, Z. Wen. Cloning and Expression Pattern of the Lysozyme C Gene in Zebrafish. *Mech. Dev*. 2002, **113**: 69-72

[17] K. Fujiki, J. Gauley, N. Bols, B. Dixon. Cloning and characterization of cDNA clones encoding CD9 from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics*. 2002, **54**: 604-609.