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Short communication

Molecular characterization and expression analysis of galectins in Japanese pufferfish (*Takifugu rubripes*) in response to *Vibrio harveyi* infection

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ABSTRACT

Galectins are a family of proteins with conserved carbohydrate recognition domains (CRDs) that bind to specific glycans, including the glycans on the surface of pathogens, and therefore play a role in cytokine secretion, cell activation, migration, adhesion and apoptosis. Currently, galectins have been extensively studied in mammalian species but rarely studied in teleost fish species. In this study, a total of 12 galectin genes were characterized to understand the molecular mechanisms of galectin function in Japanese pufferfish (*Takifugu rubripes*). Phylogenetic analyses and syntenic analyses confirmed their correct annotation and suggested the strongest relationships to tetraodon. Furthermore, expression analyses were conducted in healthy tissues of Japanese pufferfish and after infection with *Vibrio harveyi* in the intestine, liver and spleen. The results showed that galectin genes were widely expressed in all examined tissues; however, most of the galectin genes were highly expressed in mucosal tissues (skin, gill and intestine). Moreover, majority of the galectin genes were significantly regulated after *V. harveyi* infection in the intestine, liver and spleen, suggesting that galectins were involved in the immune response to *V. harveyi* infection in Japanese pufferfish. This study established the foundation for future studies of galectin gene functions.

1. Introduction

Lectins usually refer to a range of carbohydrate-binding proteins that are associated with extensive biological functions. The interaction of protein carbohydrates mediated by lectin is recognized as the key component of innate immunity and is used not only for pathogen recognition but also in various biological processes [1,2]. Galectins are soluble lectins characterized by a conserved carbohydrate recognition domain (CRD) that specifically recognizes β-galactosides (a carbohydrate structure) and are nonclassical secretory proteins [5]. Galectins are categorized into three structural types: (1) prototype galectins that contain a single CRD, (2) tandem repeat-type galectins that contain two distinct CRDs in a polypeptide, and (3) chimera-type galectins that contain a proline- and glycine-rich domain at the N-terminal region and a CRD at the C-terminal region. These galectins are involved in a variety of phenomena such as signal transduction, cell differentiation, immunity, and tumorigenesis [3–5]. In mammals, wide involvement in the immune response and tumorigenesis has been discovered for galectins [6]. Research showed that Gal3 played an important proinflammatory role in promoting the activation of T lymphocytes in Con-A-induced hepatitis [7]. Moreover, based on an experiment with Gal3- "knockout" mice, Gal3 was also involved in the damage of immune-mediated B-cells [8]. In teleost fish, the first galectin was identified in electric eel (*Electrophorus electricus*) [9]. Currently, the number of fish identified with galectins continues to increase, which play an important role in the innate immune defense [10]. In Nile tilapia (*Oreochromis niloticus*), experimentally infected with *Streptococcus agalactiae*, Gal8 was significantly upregulated in the spleen after 5 days, indicating its involvement in the immune response to bacterial infection [11]. In rock bream (*Oplegnathus fasciatus*), a recombinant Gal2 protein had hemagglutinating potential and possessed affinity toward lactose and galactose. Additionally, the recombinant protein also agglutinated and bound potential pathogenic bacteria and a ciliate [12].

Japanese pufferfish (*Takifugu rubripes*) is a commercially valuable species in the aquaculture industry of China, Korea and Japan [13]. However, during the breeding process, outbreaks of diseases were big problems which could lead to huge economic losses, particularly vibriosis caused by vibrios [14]. Several studies related to immune genes

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have been conducted in Japanese pufferfish, including neuromedin U (Nmu) [15], interleukin [16], and T-cell receptor (TCR) [17]. However, no study regarding the functions and effects of galectins in Japanese pufferfish has been conducted. Currently, the Japanese pufferfish genome has been completed sequenced [18], however, many of the genes were annotated by automated computational analysis, which makes confusion with naming of some genes, especially in galectins. For example, three gal3 genes were all annotated with galectin-3-like, making it hard for further functional analyses. In this study, we systematically characterized the galectin family genes in Japanese pufferfish for the first time. Additionally, expression profiles were conducted on galectin members in Japanese pufferfish healthy tissues and the expression patterns of the galectin members in the intestines, liver and spleen after infection with *Vibrio harveyi*. Our results will provide the basis and inspiration for further functional research of galectins in Japanese pufferfish.

2. Materials and methods

2.1. Sequence search and analysis

The galectin genes in Japanese pufferfish were identified by searching NCBI and our previous transcriptome data. Briefly, TBLASTN similarity searches were conducted against RNA-seq database to identify all galectin gene-related sequences using all available sequences of galectins genes from human (*Homo sapiens*), mouse (*Mus musculus*), tropical clawed frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), channel catfish (*Ictalurus punctatus*) and tetraodon (*Tetraodon nigroviridis*) retrieved from the NCBI and Ensembl as queries with a cutoff E-value of e-5. The retrieved sequences together with all uncharacterized Japanese pufferfish galectin mRNA sequences downloaded from the NCBI were further analyzed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) for the generation of coding sequences. The predicted amino acid sequences from ORF predication were further verified by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI nonredundant protein sequence database. The conserved domains and signal peptides were identified using the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/). ExPASy server was used to detect the N-glycosylation sites.

2.2. Phylogenetic analysis

Full length sequences of galectin genes from Japanese pufferfish and other species including human, mouse, zebrafish, channel catfish and tetraodon were used to conduct the phylogenetic analysis. Multiple alignments of protein sequences were performed using ClustalW with default parameters. Phylogenetic and molecular evolutionary analysis was conducted with the maximum likelihood method using Molecular Evolutionary Genetics Analysis software (MEGA 7). Jones-Taylor-Thornton (JTT) and gamma-distributed rate with invariant sites (G + I) models was chosen based on the alignment results [19]. Gaps were removed by “Use all sites” treatment. Bootstrap tests with 1000 replicates were performed to evaluate the phylogenetic trees.

2.3. Syntenic analysis

Syntenic analysis was executed by location evolution relationship among neighboring genes, it was conducted to provide additional evidence for orthologies of galectin genes in Japanese pufferfish. Shared syntenies were assessed based on comparisons of the genes, the order on the genomic neighborhood that harbors galectin members with multiple gene copies were scanned for accurate annotation and additional phylogenetic evidence. The neighbor genes of the Japanese pufferfish galectin genes were retrieved from the NCBI genomic database. The conserved syntenic blocks of zebrafish and tetraodon were captured from the Ensembl Genome Browser. Based on the collected genomic organization of galectin genes in these species, syntenic analyses map was drawn according to the proportional relationship of neighbor genes position.

2.4. Sample collection of healthy Japanese pufferfish

In order to characterize the expression pattern of galectin genes in different tissues of Japanese pufferfish, nine tissues of healthy Japanese pufferfish were collected. Japanese pufferfish fingerlings (average body weight 15.4 g and average body length 8.6 cm) were purchased from an aquatic farm in Dalian, China. Before sample collection, fish were reared in the laboratory and acclimatized for two weeks at a temperature of 20 °C. 30 fish were anesthetized with tricaine methanesulfonate (MS-222) in seawater, then brain, kidney, heart, skin, gill, muscle, spleen, intestine, and liver were collected, and flash-frozen in liquid nitrogen, and then stored at −80 °C refrigerator.

2.5. Bacterial infection and sample collection

To characterize the innate immune response of the galectin genes against bacterial infection in the host, *V. harveyi* challenge was conducted. Before the experiment, fish were reared in the laboratory and acclimatized for two weeks at a temperature of 20 °C. *V. harveyi* was reisolated from a symptomatic Japanese pufferfish with lesions on the skin and was identified before culture. The challenge experiment was conducted by intraperitoneal injection, a total of 72 fish were injected, the treated group and control group were each injected with 36 fish. The experimental fish was injected with 0.1 ml of *V. harveyi* at a concentration of 1×10^7 CFU/ml. The control group was injected with an equal amount of physiological saline. Intestine, liver and spleen tissues were collected in both control and treated groups at 24 h, 48 h and 96 h after the challenge. At each sampling time-point, tissue samples were collected from each fish within three replicate tanks. Samples were flash-frozen in liquid nitrogen and then stored at −80 °C until extraction of RNA.

2.6. Total RNA extraction and reverse transcription

Prior to RNA extraction, three biological replicate samples were homogenized using mortar and pestle under liquid nitrogen. Total RNA was extracted using an RNA prep pure Tissue Kit (TIANGEN, Beijing) according to the instructions. Agarose gel electrophoresis was used to detect the integrity of total RNA. The purity and concentration of RNA were measured on an NV3000 spectrophotometer (VASTECH, USA). All extracted RNA samples had an A260/280 ratio between 1.8 and 2.1, and the concentration was greater than 150 μg/ml. First-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit (TaKaRa, Dalian) according to the instructions (1 μg of RNA per 20 μl reaction).

2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Dalian) on a 7500 Real-Time PCR System (Applied Biosystems, USA). Gene-specific primers were designed by Primer5 software based on the galectin mRNA sequences of the Japanese pufferfish. Japanese pufferfish β-actin gene was used as the housekeeping gene. All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Three biological replicate RNA samples from healthy and infected tissues were analyzed for gene expression. The results were analyzed with Relative Expression Software Tool (REST) based on the cycle threshold (Ct) values generated by quantitative real-time PCR [20]. For the analysis of gene expression in healthy tissues, Ct values of each galectin gene in brain was used as control group and β-actin was used as reference for normalization of the relative expression. For the analysis of gene expression following bacterial infection, treatment group of different time-points were compared with control group for statistical

Table 1
Characteristics of Japanese pufferfish galectin genes.

Gene name	mRNA size (bp)	5'UTR (bp)	3'UTR (bp)	Amino acids	Accession no.	N-glycosylation sites
Gal2a	492	74	7	136	XM_003964530.2	1(NPSN)
Gal2b	615	32	187	131	XM_003972267.1	1(NFSI)
Gal3a	1926	210	594	373	XM_011614877.1	2(NPTA,NPSA)
Gal3ba	977	91	163	240	XM_011619910.1	3(NPTW,NPSG,NRSD)
Gal3bb	1531	109	348	357	XM_011611921.1	1(NPTI)
Gal4	1556	113	84	452	XM_011608661.1	1(NMTR)
Gal8a	1009	20	35	317	XM_003977467.2	4(NPTV,NSTS,NSSD,NGTH)
Gal8b	843	158	355	109	XM_011616179.1	1(NGSH)
Gal9	1360	141	178	346	XM_011616914.1	4(NITI,NYTS,NGSF,NGTQ)
GRP-a	929	70	349	169	XM_011612658.1	0
GRP-b	1184	164	483	178	XM_011603086.1	0
GRP-c	942	52	452	145	XM_003976220.2	0

significance using a pair-wise fixed reallocation randomization test in the REST software [20]. Expression differences between groups were considered significant when p-value < 0.05.

3. Results

3.1. Sequence analysis of Japanese pufferfish galectin genes

In this study, a total of 12 galectin genes including galectin2a (Gal2a), galectin2b (Gal2b), galectin3a (Gal3a), galectin3ba(Gal3ba), galectin3bb(Gal3bb), galectin4 (Gal4), galectin8a (Gal8a), galectin8b (Gal8b), galectin9 (Gal9), galectin-related protein a (GRP-a), galectin-related protein b (GRP-b), and galectin-related protein c (GRP-c) were analyzed in Japanese pufferfish, and their characteristics were summarized in Table 1, which contained mRNA length, open reading frame, 5′UTR and 3′UTR, the length of the amino acid sequence of each protein, accession number, and the number of N-glycosylation sites of each gene. Gla2 and Gal8 each has two copies, whereas Gal3 and GRP each has three copies. No signal peptide has been found in all 12 galectin genes, which was consistent with their non-classical secretory pathway. Different numbers of N-glycosylation sites were found in Japanese pufferfish galectin genes, Gal8a and Gal9 contained four N-glycosylation sites, Gal3ba contained three N-glycosylation sites, Gal3a owned two N-glycosylation sites, Gal2a, Gal2b, Gal3bb, Gal4 and Gal8b each owned only one N-glycosylation sites, while no N-glycosylation site has been found in all three GRP genes.

3.2. Phylogenetic analysis of Japanese pufferfish galectin genes

Phylogenetic analysis was conducted to assist with the annotation of galectin genes as well as understand the phylogenetic relationships of Japanese pufferfish galectin genes with other species. Overall, phylogenetic analysis placed all galectin genes into the expected phylogenetic cluster with other organisms (Fig. 1). Two main clades were observed: one clade included Gal2 and Gal3, and the other clade included Gal4, Gal8, Gal9 and GRP. Collectively, tetraodon had the closest phylogenetic relationship with Japanese pufferfish, and all branch nodes were supported by high bootstrap values. In addition, Gal4 and Gal9 were confirmed with their correct annotation based on phylogenetic tree.

3.3. Syntenic analysis of Japanese pufferfish galectin genes

In order to provide further support for annotation of Japanese pufferfish galectins genes with multiple copies, syntenic analyses were conducted in Gal2, Gal3, Gal8 and GRP. Since the strongest phylogenetic relationships were shared with tetraodon and zebrafish, syntenic analysis with tetraodon and zebrafish were executed to provide additional evidence for orthologies of galectin genes. As shown in Fig. 2, conserved syntenic blocks were identified for all galectin genes from zebrafish, tetraodon and Japanese pufferfish, confirming the results from the phylogenetic analysis. For instance, Gal3a in Japanese pufferfish, zebrafish and tetraodon shared the same neighboring genes of synj2bp (synaptojanin 2 binding protein) and gmfb (glia maturation factor, beta). Japanese pufferfish Gal3ba was located between timm9 (translocase of inner mitochondrial membrane 9) and jmjd7 (jumonji domain containing 7), which was highly conserved with that of tetraodon and zebrafish. In addition, Japanese pufferfish Gal3bb shared the same neighboring gene with zebrafish Gal3b of plcb4 (phospholipase C beta 4) and mdga2 (MAM domain containing glycosylphosphatidylinositol anchor 2a). Taken together, the syntenic analyses provided evidence for supporting the annotation and nomenclature of galectin genes in Japanese pufferfish.

3.4. Expression analysis of galectin genes in healthy Japanese pufferfish

The expression of 12 galectin genes was determined by quantitative real-time PCR in nine healthy tissues, including brain, kidney, heart, skin, gill, muscle, spleen, intestine, and liver. As shown in Fig. 3, Japanese pufferfish galectin genes were expressed in all examined tissues and exhibited distinct tissue expression patterns. The brain was used as the control tissue to calculate the relative fold changes among different tissues. Specifically, most of the Japanese pufferfish galectin genes were expressed poorly in classical immune tissues (kidney, spleen and liver); however, Gal9 was highly expressed in these tissues, with increases of 25 fold in the kidney, 34 fold in the spleen and 54 fold in the liver. The lowest expression of Gal3bb, Gal8b, GRP-a, and GRP-c was observed in the spleen. Clearly, most of the galectin genes were highly expressed in mucosal tissues (skin, gill and intestine). For example, the highest expression level of Gal3ba was in the gill (91 fold increase), followed by skin (80 fold) and intestine (63 fold). For Gal2a, expression increased 54 fold in the skin and 17 fold in the intestine; for Gal4, the increase was 66 fold in the skin and 31 fold in the intestine; for Gal8a, the increase was 50 fold in the skin and 38 fold in the intestine; and for GRP-c, the increase was 19 fold in the skin and 19 fold in the gill. The highest expression level of Gal2b was observed in the gill (Fig. 3). To summarize, the high expression levels of galectin genes in mucosal tissues suggested that they played important roles in Japanese pufferfish mucosal immunity.

3.5. Expression analysis of galectin genes after V. harveyi infection

The expression profiles of Japanese pufferfish galectin genes were determined in the intestine, liver and spleen after *V. harveyi* infection. The gene expression levels were measured for 12 galectin genes at 24, 48 and 96 h postinfection (Fig. 4), and expression differences were assessed between treatment and control groups since the control did not alter significantly (Fig. S1). In intestine, significant upregulation was observed in four genes (Gal2a, Gal2b, Gal3ba and Gal4) at 24 h after *V. harveyi* infection. The extent of expression differences in these genes was between 3.7 and 8.7 fold changes. At 48 h after *V. harveyi* infection,

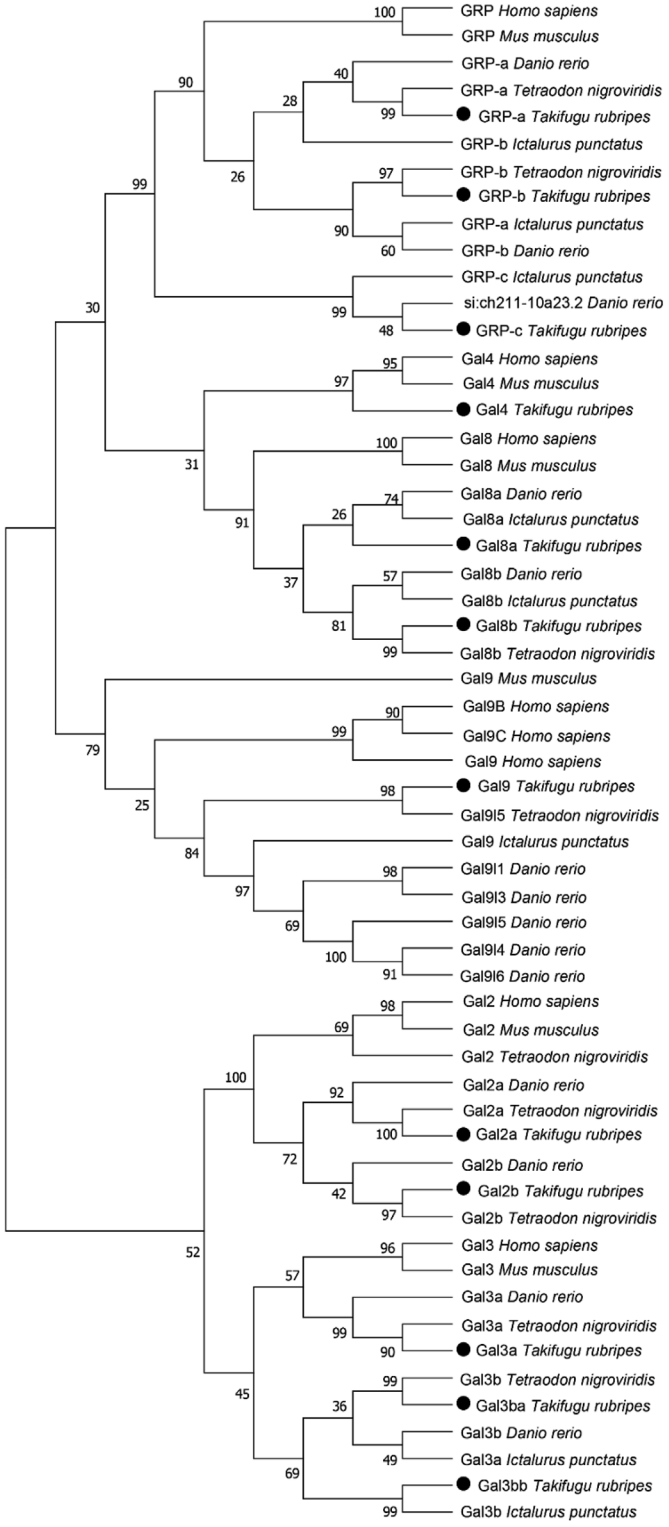

Fig. 1. Phylogenetic analysis of Japanese pufferfish galectin genes. The phylogenetic tree was constructed by MEGA 7 software using the maximum likelihood method. Bootstrap tests with 1000 replicates were performed to evaluate the phylogenetic trees. The accession numbers of each sequence are Gal2 *Homo sapiens*, NP_006489.1; Gal3 *Homo sapiens,* NP_002297.2; Gal4 *Homo sapiens,* NP_006140.1; Gal8 *Homo sapiens*, NP_006490.3; Gal9 *Homo sapiens*, NP_033665.1; Gal9B *Homo sapiens*, NP_001036150.1; Gal9C *Homo sapiens*, NP_001035167.2; GRP *Homo sapiens*, NP_054900.2; Gal2 *Mus musculus*, NP_079898.2; Gal3 *Mus musculus*, NP_001139425.1; Gal4 *Mus musculus*, NP_034836.1; Gal8 *Mus musculus*, NP_001277984.1; Gal9 *Mus musculus*, NP_034838.2; GRP *Mus musculus*, NP_776113.1; Gal2a *Danio rerio*, NP_998059.2; Gal2b *Danio rerio*, NP_956808.1; Gal3a *Danio rerio*, XP_704272.2; Gal3b *Danio rerio*, NP_999858.2; Gal8a *Danio rerio*, XP_003200715.1; Gal8b *Danio rerio*, XP_002665300.3; Gal9l1 *Danio rerio*, NP_956366.1; Gal9l3 *Danio rerio*, NP_001001817.1; Gal9l4 *Danio rerio*, NP_001034900.1; Gal9l5 *Danio rerio*, NP_001096100.1; Gal9l6 *Danio rerio*, XP_001921992.1; GRP-a *Danio rerio*, NP_001038765.1; GRP-b *Danio rerio*, NP_001007175.1; si:ch211-10a23.2 *Danio rerio*, NP_001139237.1; Gal3a *Ictalurus punctatus*, XP_017330798.1; Gal3b *Ictalurus punctatus*, XP_017311937.1; Gal8a *Ictalurus punctatus*, XP_017311810.1; Gal8b *Ictalurus punctatus*, XP_017330908.1; Gal9 *Ictalurus punctatus*, NP_001187343.1; GRP-a *Ictalurus punctatus*, XP_017319935.1; GRP-b *Ictalurus punctatus*, XP_017312606.1; GRP-c *Ictalurus punctatus*, XP_017319506.1; Gal2 *Tetraodon nigroviridis*, ENSTNIP00000022743; Gal2a *Tetraodon nigroviridis*, ENSTNIP00000015603; Gal2b *Tetraodon nigroviridis*, ENSTNIP00000021998; Gal3a *Tetraodon nigroviridis*, ENSTNIP00000022545; Gal3b *Tetraodon nigroviridis*, ENSTNIP00000005147; Gal8b *Tetraodon nigroviridis*, ENSTNIP00000020623; Gal9l5 *Tetraodon nigroviridis*, ENSTNIP00000007648; GRP-a *Tetraodon nigroviridis*, ENSTNIP00000004794; GRP-b *Tetraodon nigroviridis*, ENSTNIP00000012721; Gal2a *Takifugu rubripes*, XP_003964579.1; Gal2b *Takifugu rubripes*, XP_003972316.1; Gal3a *Takifugu rubripes*, XP_011613179.1; Gal3ba *Takifugu rubripes*, XP_011618212.1; Gal3bb *Takifugu rubripes*, XP_011610223.1; Gal4 *Takifugu rubripes*, XP_011606963.1; Gal8a *Takifugu rubripes*, XP_003977516.1; Gal8b *Takifugu rubripes*, XP_011614481.1; Gal9 *Takifugu rubripes*, XP_011615216.1; GRP-a *Takifugu rubripes*, XP_011610959.1; GRP-b *Takifugu rubripes*, XP_011601388.1; GRP-c *Takifugu rubripes*, XP_003976269.1.

Gal2a and Gal3ba remained significantly upregulated, and the upregulation increased to 8.8 fold and 12.7 fold, respectively. Significant downregulation was observed in three genes (Gal8a, Gal8b and Gal9) of 3.8 fold, 5.0 fold and 2.3 fold, respectively. At 96 h after *V. harveyi* infection, Gal3ba still remained significantly upregulated, as well as Gal8b and Gal9 remained significantly downregulated, with fold change of 6.1 fold, −3.8 fold and −2.2 fold, respectively. Significant downregulation was also observed in Gal3a (2.0 fold) and GRP-b (2.3 fold) (Fig. 4).

In liver, the only significantly downregulated gene was Gal3bb (−2.4 fold) at 24 h, while Gal4, Gal8a and Gal9 were significantly upregulated with 4.7 fold, 5.8 fold, and 7.2 fold. At 48 h, Gal4 (3.3 fold), Gal8a (7.4 fold) and Gal9 (10.3 fold) were still significantly upregulated, in addition, significant upregulation was also observed in Gal2a (2.2 fold). Gal2a, Gal4, Gal8a and Gal9 were further significantly upregulated at 96 h, with expression differences of 2.7 fold, 10.3 fold, 11 fold and 9.6 fold, while Gal8b (3.2 fold) and GRP-a (4 fold) were significantly downregulated at 96 h (Fig. 4).

In spleen, significant upregulation was observed in Gal3ba (3.1 fold), Gal3bb (5.8 fold) and Gal9 (3.6 fold) at 24 h after *V. harveyi* infection, while significant downregulation was observed in Gal8a (3.7 fold) and GRP-b (4.5 fold). At 48 h, Gal2b, Gal3ba, Gal4 and Gal9 were significantly upregulated with 12.2 fold, 1.8 fold, 2.2 fold and 2.2 fold, and the only significantly downregulated gene was Gal8b (2.2 fold). At 96 h, significant upregulation was observed in Gal3bb (2.5 fold), and Gal9 (2.3 fold), while Gal8b (2.4 fold) remained significantly downregulated (Fig. 4).

4. Discussion

Galectins are ubiquitous in living organisms [3], which associated with many functions, and recognize and integrate the carbohydrates on the surface of viruses, bacteria and protozoan parasites [21]. Other functions include involvement in apoptosis of T cells [22], phagocytosis [23], inflammatory response [4,24] and virus immunity [25]. Although galectins have been extensively studied in mammals, they remain poorly studied in teleosts. In the present study, we characterized 12 galectin genes in Japanese pufferfish according to sequence analysis, phylogenetic analysis and syntenic analysis. Expression profiling was

Fig. 2. Syntenic analyses of galectin genes from Japanese pufferfish, tetraodon and zebrafish. (A) galectin2, (B) galectin3, (C) galectin8, (D) GRP. The abbreviations of gene names are ankrd40, ankyrin repeat domain 40; wfikkn2a, info WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2a; gal2a, galectin2a; cacna1i, calcium channel, voltage-dependent, T type, alpha 1I subunit; sgsm3, small G protein signaling modulator 3; wfikkn2, WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2; cyth4b, cytohesin 4b; fam83fb, family with sequence similarity 83, member Fb; cacna1l, calcium voltage-gated channel subunit alpha1 I; gal2b, galectin2b; elfn2a, extracellular leucine-rich repeat and fibronectin type III domain containing 2a; cacna1ab, calcium channel, voltage-dependent, P/Q type, alpha 1A subunit, b; cacna1a, calcium voltage-gated channel subunit alpha1 A; elfn2, extracellular leucine-rich repeat and fibronectin type III domain containing 2; fam83fl, protein FAM83F-like; cyth4, cytohesin 4; arid4a, AT-rich interaction domain 4A; timm9, translocase of inner mitochondrial membrane 9; gal3b, galectin3b; fbxo34, F-box protein 34; jmjd7, jumonji domain containing 7; atg14, autophagy related 14; plcb4, phospholipase C beta 4; mdga2a, MAM domain containing glycosylphosphatidylinositol anchor 2a; mdga2, MAM domain containing glycosylphosphatidylinositol anchor 2; pacs2, phosphofurin acidic cluster sorting protein 2; srsf5a, serine/arginine-rich splicing factor 5a; synj2bp, synaptojanin 2 binding protein; gal3a, galectin3a; gmfb, glia maturation factor, beta; cnih1, cornichon family AMPA receptor auxiliary protein 1; cdkn3, cyclin dependent kinase inhibitor 3; srsf5, serine and arginine-rich splicing factor 5; rrm2, ribonucleotide reductase regulatory subunit M2; gal8a, galectin8a; psmc6, proteasome 26S subunit, ATPase 6; ube2j1, ubiquitin conjugating enzyme E2 J1; cldn20, claudin 20; tiam2b, T-cell lymphoma invasion and metastasis 2b; zdhhc14, zinc finger, DHHC-type containing 14; gal8b, galectin8b; scaf8, SR-related CTD-associated factor 8; tiam2a, T-cell lymphoma invasion and metastasis 2a; kbtbd11, kelch repeat and BTB domain containing 11; rbbp4, retinoblastoma binding protein 4; zbtb8os, zinc finger and BTB domain containing 8 opposite strand; tiam2, T-cell lymphoma invasion and metastasis 2; grp-a, galectin-related protein a; vps54, VPS54, GARP complex subunit; pus10, pseudouridylate synthase 10; cct4, chaperonin containing TCP1 subunit 4; c1d, C1D nuclear receptor corepressor; actr2, ARP2 actin related protein 2 homolog; grp-b, galectin-related protein b; ugp2b, UDP-glucose pyrophosphorylase 2b; ugp2, UDP-glucose pyrophosphorylase 2; wdpcp, WD repeat containing planar cell polarity effector; vti1b, vesicle transport through interaction with t-SNAREs 1B; rdh12, retinol dehydrogenase 12; zfyve26, zinc finger, FYVE domain containing 26; grp-c, galectin-related protein c; plek2, pleckstrin 2.

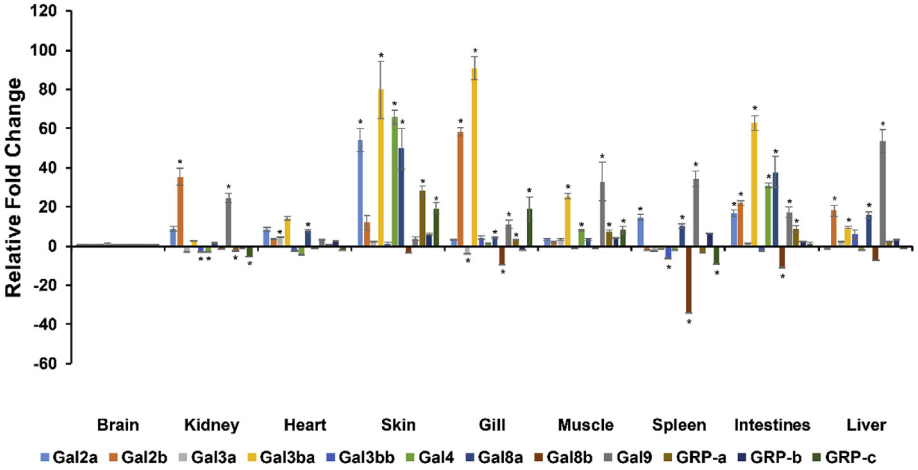

Fig. 3. Gene expression analysis of galectin genes in different healthy tissues of Japanese pufferfish. Expression levels were calibrated against those in the brain, and β-actin was used as the reference gene. Asterisks (*) indicated significant differences (p-value $<$ 0.05).

determined in healthy tissues and after bacterial infection.

Among all the galectin genes, only a single copy exists for each of the galectin genes in human and mouse, but multiple copies of galectin genes were found in Japanese pufferfish including Gal2 (2 copies), Gal3 (3 copies), Gal8 (2 copies), and GRP (3 copies). This might be caused by teleost-specific whole genome duplication. However, random genome loss after whole genome duplication in teleosts may account for the single copy of many galectin genes within teleost fish species [26]. The phylogenetic analysis provided insights into the evolution of galectin genes. Japanese pufferfish galectin genes were most closely related with the corresponding tetraodon genes, indicated its strongest relationships with tetraodon. The galectin genes were clustered into two clades, with Gal4, Gal8, Gal9, and GRP in one clade and Gal2 and Gal3 in the other clade. Gal4, Gal8, and Gal9 are tandem repeat-type galectins with two different CRDs interconnected by a linker peptide [27]. Even though Gal3 is the only chimera-type member in the galectin family, the Gal2 is the only prototype galectin in Japanese pufferfish. The other prototype galectin such as Gal-7, -10, −13, −14 and −15 were found to be absent from many kinds of teleost, including *Danio rerio*, *Ictalurus punctatus*, *Salmo salar*, *Astyanax mexicanus*, *Cynoglossus semilaevis*, *Oreochromis niloticus*, *Xiphophorus maculatus*. Both chimera-type and prototype galectin contains only one CRD domain, while all other galectin members belong to tandem repeat-type galectins, they contain two distinct CRDs domains. Therefore, all tandem repeat-type galectins were clustered together in the phylogenetic tree, while the two unique galectins with only one CRD domain were clustered more closely. Similar observation was also shown in the phylogenetic analysis of channel catfish [28], where the only prototype Gal1 and Gal3 clustered closely in the same clade. However, annotation of multiple copy galectins was inconclusive with only phylogenetic tree, therefore, syntenic analysis was also conducted to provide further support for annotations for these genes. Based on the conserved synteny, naming of Japanese pufferfish were confirmed following the gene nomenclature of zebrafish.

To provide insight into functions of galectin genes, expression profiles of galectin genes were determined in healthy Japanese pufferfish tissues. The results revealed different expression patterns among the galectin genes (Fig. 3). These galectin genes were constitutively expressed in all examined tissues, which was consistent with other reported galectins [29,30]. The different expression patterns of Japanese pufferfish galectins suggested that each galectin played different roles in different tissues [3]. Despite the wide tissue distribution, most of the galectins had much higher expression levels in mucosal tissues (skin, gill and intestine) than those in other tissues. Gal9 was highly expressed in classical immune tissues (kidney, spleen and liver), which was consistent with the expression profiles in *Labeo rohita* (Hamilton, 1822) [31]. In mammals, the mucosal epithelium has a high expression of Gal3 [32]. In channel catfish, gill, skin and intestine showed high level of expression in galectins [28]. The expression of Gal8 in Nile tilapia was highest in the skin and intestine [11], and in rock bream, Gal2 was expressed at the highest in the intestine [12]. In summary, the high expression levels of galectins in mucosal tissues might relate to the important roles they play in mucosal immune responses. Accordingly, we continued to examine the expression patterns of galectin genes in the Japanese pufferfish intestine after *V. harveyi* infection. In addition, after *V. harveyi* infection, naturally diseased and experimentally infected Japanese pufferfish both found prominent pathological changes in the spleen and liver [33]. Hence, in spite of intestine, spleen and liver were also collected for the expression analyses after *V. harveyi* infection.

The immune system protects the host against pathogen infection, but mucosal barriers are the first line of host defense [34]. Particularly for aquatic species, their mucosal surfaces (skin, gill and intestine) are constantly interacting with various pathogens in the aquatic environment [35]. During infection, pathogens must cross the surface mucosal barrier to successfully enter a host and establish efficient colonization and replication. Although disease control and prevention measures in aquaculture research require a comprehensive understanding of fish mucosal immunity [36], the knowledge of Japanese pufferfish mucosal immunity remains limited. Therefore, the expression patterns of galectin genes were detected in the intestine after *V. harveyi* infection. In *Sinonovacula constricta*, expression of Gal2 was significantly upregulated following challenge with *Vibrio anguillarum*, and recombinant Gal2 protein displayed strong agglutination activity toward gram-negative bacteria [37]. In this work, expression of Gal2a was significantly upregulated at 24 and 48 h after *V. harveyi* infection and then down-regulated in the following infection sample. The Gal2a gene tended to be upregulated early after infection but down-regulated at later stages of pathogenesis. This result suggested that Gal2a plays an indispensable role in innate immunity in Japanese pufferfish. In mammals, Gal3 plays a role in T cell-mediated inflammatory immunity [8], and Gal3 can regulate antibacterial autophagy through differential recognition of host glycans on damaged phagosomes [38]. It was primitively identified as a cell surface antigen expressed on murine peritoneal macrophages [8]. Gal3 works in multiple immune processes, such as inflammatory response, apoptosis, cell differentiation and cell proliferation [39–41]. Gal3 null mice demonstrated reduced T cell and macrophage responses to *Citrobacter rodentium* infection in the gut [42]. In this study, significantly upregulation in the intestine was observed in Gal3ba at three time points. In zebrafish, extracellular Gal3 interacted with the IHNV glycosylated envelope and glycans on the epithelial cell

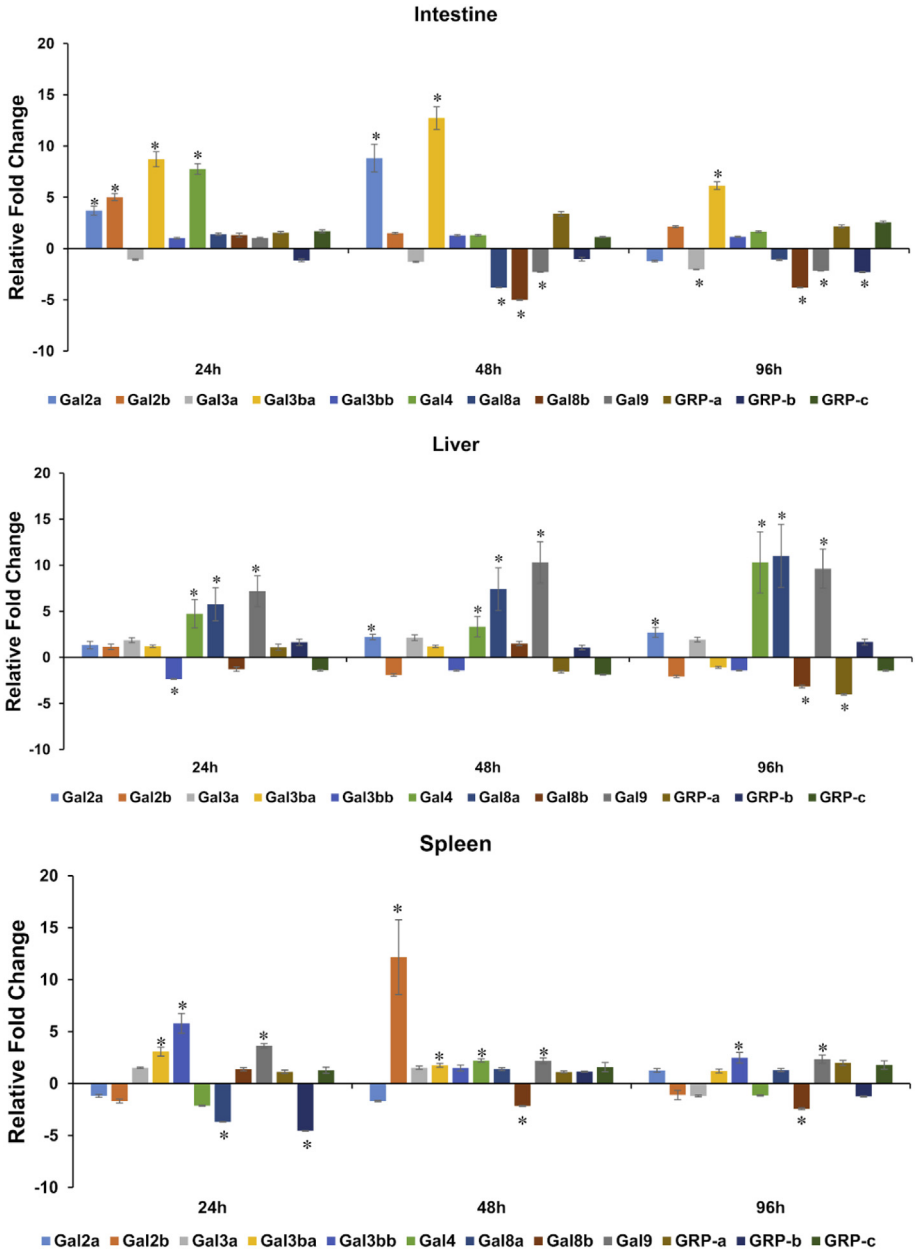

Fig. 4. Expression of galectin genes in the intestine, liver and spleen after *V. harveyi* infection. The expression level of galectin genes was presented as mean ± SE of fold change after *V. harveyi* challenge to the control as normalized with β-actin. Asterisks (*) indicated significant differences (p-value < 0.05).

surface, significantly reduced viral adhesion [43]. In mammals Gal4 was mostly expressed in the luminal epithelia of the gastrointestinal tract [44], which was consistent with our results. Fish Gal4 derived membrane binding peptide showed weak bactericidal activity to *V. harveyi* [45]. Here, Gal4 was significantly upregulated at 24 h after infection, although the upregulation was not significant in later time points. This result might be a manifestation of an acute response. However, Gal8a, Gal8b, Gal9 and GRP-b were downregulated at 48 and 96 h after infection, indicating host apoptosis process were induced by the infection [28]. Different from intestine, liver is an important organ to defend against microbial invasion by pathogen phagocytosis, bacterial agglutination, the production of reactive oxygen species and bacterial lysing via internal digestion [47]. Therefore, enough transcripts in hepatocytes must be needed to synthesize enough proteins to eliminate pathogens [31]. Significantly upregulation was observed in Gal9 at three time points in the liver challenged with *V. harveyi*, the result was consistent with study of *Labeo rohita* Gal9, its expression increased after infection with *Aeromonas hydrophila*, and reached to the maximum at 24 h post-injection [31]. Similar expression pattern was also seen in yellow catfish, where Gal9 increased remarkably at 24 h after challenge [46]. Gal2a mRNA in the liver showed a slight variation at 24 h after infection. However, it was significantly upregulated at 48 h and 96 h after infection. The slight change of Gal2a transcript in the liver at 24 h after infection might be due to the emergency response, large numbers of Gal2a mRNA were translated to protein for *V. harveyi* elimination. Gal3ba and Gal3bb were significantly upregulated to the highest in the spleen at 24 h after infection, the expression declined at 48 h and 96 h but still showed upregulation compared to that in control. This result might be a manifestation of an acute response. Gal9 elevated sharply to the highest at 24 h and remained high expression level in the later two time points in the spleen, this observation was consistent with the study conducted by Mushtaq et al. [31]. They revealed that Gal9 in *Labeo rohita* was significantly upregulated since 6 h post *A. hydrophila* injection, and maximum upregulation was seen at 12 h post-injection.

Even though galectins were ubiquitously distributed in each tissue, different expression patterns of galectins suggested that they performed distinct roles in normal and infected tissues.

In conclusion, in this work, 12 galectin genes in Japanese pufferfish were analyzed, characterized, their expression profiles were detected in healthy tissues, and then the expression profiles were determined after infection with *V. harveyi* in the intestine, liver and spleen. Our results suggested galectin genes played important roles in the immune response to bacterial infection in Japanese pufferfish. This work systematically proved the naming of Japanese pufferfish galectins genes and provided the foundation for further studies to understand mechanisms of galectins involved in the regulation of immune responses in Japanese pufferfish.

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Appendix A. Supplementary data

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