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Short communication

MicroRNA profile of immune response in gills of zebrafish (*Danio rerio*) upon *Staphylococcus aureus* infection

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ABSTRACT

The gills of fish are large mucosal surfaces that are very important portals for pathogen entry. Investigations have shown that microRNAs (miRNAs) are key regulators of immune response to bacterial infections in the gills of fish; however, how miRNA expression changes in response to infection by Gram-positive bacteria remains largely unknown. To further investigate the immunological role of miRNAs in fish gills under pathogen stress induced by Gram-positive bacterial infection, this study investigated *Staphylococcus aureus* (*SA*)-induced changes in the miRNAs levels in gills of adult zebrafish (*Danio rerio*). miRNA microarrays were used to analyze expression profiles of known miRNA in the gills of zebrafish in response to *SA* infection and compared these to uninfected control fish. A total of 30 differentially expressed miRNAs (DEMs) were identified. Target genes likely regulated by DEMs were predicted, and functional enrichment analyses were performed. The results indicated that DEM targets were primarily involved in innate immune processes, apoptosis, defense responses, and antibacterial responses. Pathways involving bacterial infection, innate immunity, metabolic process, disease, and apoptosis were mediated by DEMs. Furthermore, real-time quantitative PCR experiments for nine key *SA*-responsive DEMs that regulated the "*SA* infection" pathway validated the accuracy of microarray results. Dynamic variations in gene expression were surveyed in detail for these key *SA*-responsive DEMs for PBS control and at 6, 12, 24, and 48 h after *SA* challenge in detail. This study provides novel insight into the mechanisms underlying the miRNA regulation during the *SA*-induced immune response in zebrafish gills, and provides basic knowledge on the innate immune response against Gram-positive bacterial infection in bony fish.

1. Introduction

In fish, gills are large mucosal surfaces and play key roles in the immunologic response to pathogen invasion [1]. Fish gill tissue not only participates in water oxygen consumption, osmoregulation, and toxicological responses, but is also an important immune tissue [2]. In recent decades, the gene expression response under pathogen and pollutant challenge in fish gills has been investigated, and the gill immune response has been explored at different molecular levels. For example, Stavrum used a microarray approach to compare gene expression profiling in zebrafish gills treated by polluted water to that of controls maintained in clear freshwater, and found many differentially expressed genes (DEGs) that are involved in toxicant degradation and innate immunity [3]. Camposperez et al. investigated the expression level of a gene encoding inducible nitric oxide synthase (iNOS) in the gill of the rainbow trout, challenged with *Renibacterium salmoninarum*, and detected induced expression of this gene in comparison to that of control [1]. These investigations indicated that fish gills are not only a point of entry for pathogens but also a tissue capable of an immune response. Li et al. analyzed gill transcriptomes in the Japanese flounder (*Paralichthys olivaceus*) challenged with *Edwardsiella tarda* stress for different durations [4]. The authors identified many DEGs primarily related to leukocyte transendothelial migration, B cell receptor, Wnt signaling, and apoptosis signaling pathways. Moreover, several studies reported an expression alteration of immune-related genes in fish gills after parasite infection, e.g. in goldfish [5]. In addition, several investigations explored differentially expressed proteins (DEPs) between the gills of fish infected by pathogens or parasites and those of healthy

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controls. For example, Valdenegro-Vega et al. found 52 DEPs in gill mucus samples of the *Neoparamoeba perurans*-infected Atlantic salmon (*Salmo salar*) in comparison to normal control samples [6]. These DEPs primarily participated in the intracellular signaling transduction, the host response to parasites, inflammation, and innate immune pathways. Lü et al. analyzed gill proteins of zebrafish (*Danio rerio*) challenged by *Aeromonas hydrophila* using isobaric tags for relative and absolute quantitation (iTRAQ) technology [2]. Among the identified 82 DEPs, gene functional enrichment analysis indicated that ~33 DEPs were associated with stress and immune responses, complement and coagulation cascades, *Escherichia coli* infection, and phagosome signaling pathways [2]. Additionally, expression changes of microRNA (miRNA) transcriptomes have been analyzed in both the gills of control fish and those infected by parasites, such as *Larimichthys crocea* challenged by *Cryptocryon irritans* [7]. However, expression alterations of miRNA profiles in the gills of fish after acute pathogen challenge have not been investigated to date. Therefore, the regulatory mechanism of miRNA underlying the immune response to bacteria invasion remains largely unknown, and the genes and pathways potentially involved in this defense response still remain to be identified and analyzed.

Many reports that involve the effects of bacteria on fish have been published, most of which studied the effects of Gram-negative bacterial infection [8]. However, explorations of Gram-positive bacterial infection, such as *Staphylococcus aureus* (*SA*), in fish diseases are rare, particularly those that study the molecular response under bacterial infection [8]. To date, many studies report pathogenicity caused by *SA* in mammals and humans, while few studies investigate fish diseases [8]. Despite large-scale fish diseases caused by *Staphylococcus* have not been reported so far, partial occurrence of resulting fish death has been frequently found in China. For example, in Guangxi Province, China, researchers found that the main symptoms are head and anal swelling, and suppuration wounds in finless eels (*Monopterus albus*) infected by *Staphylococcus*; furthermore, hepatic congestion and hemorrhage, renal hemorrhage, gallbladder tumidness, cardiac congestion and hemorrhage, and intestinal congestion were detected after dissection. Similar symptoms caused by *Staphylococcus* were also detected in the golden arowana (*Scleropages formosus*) [8]. In addition, zebrafish presented obvious disease symptoms after *SA* infection, such as congestion and hemorrhage of gills, intestine fester, hepatorrhagia, and milky eyeball [8]. Due to a sensitive pathogenicity of *SA* in zebrafish, *SA* has been widely used to explore how Gram-positive bacteria affect the immune response of fish based on the zebrafish model [9,10].

miRNAs are a group of endogenous non-coding small RNAs (approximately 22 nt) that guide the RNA-induced silencing complex (RISC) and function as complementary sequences in the 3' untranslated regions (UTR) of mRNAs where they suppress gene expression and degrade mRNA [11,12]. Available research results indicate multiple biological functions of miRNAs in fish, mainly including organ formation, development, reproduction, osmotic regulation, metabolism, and immunity. For examples, Ivan et al. found that miR-204 regulated the formation of crystalline lens and retina by targeting the homeobox protein Meis2 (MEIS2) mRNA in *Oryzias latipes* [13]. Liu et al. demonstrated that miR-206 regulated gastrula formation by binding to the mitogen-activated protein kinase (MAPK) signaling system in zebrafish [14]. Abramov et al. found regulatory roles of miR-17a and miR-430b for the follicular development and ovocyte maturation in zebrafish [15]. A study on the Nile tilapia reported that miR-429 regulates plasma ion concentrations by regulating the expression of genes that encode the osmotic stress transcription factor 1 (OSTF1) that plays a key role in osmotic regulation of fish [16]. Furthermore, Tang et al. reported that eight miRNAs (such as miR-21) were highly expressed in the experimental groups treated with feeding vitamin E addition in the Nile tilapia, indicating that these eight miRNAs participated in the vitamin E metabolism [17]. Notably, research involving miRNA regulation in the immune and stimuli response of fish is most prevalent. Many studies have indicated miRNAs as key regulators of the response to antibacterial immunity, abiotic stress, and disease in fish [7,18,19]. Sha et al. reported 10 significantly differentially expressed miRNAs (DEMs) in the liver, head kidney, spleen, and intestine of *Cynoglossus semilaevis* infected with *Vibrio anguillarum* [20]. A similar pattern of differential expression was also reported for the grass carp (*Ctenopharyngodon idella*) infected by *Aeromonas hydrophila* [21], the Japanese flounder (*Paralichthys olivaceus*) infected with cytomegalovirus RBIV-C1 [22], and poly(I:C)-challenged large yellow croaker (*Larimichtly crocea*) [23]. Zhou et al. reviewed that miRNAs regulated TLR-signaling pathways by targeting multiple molecules in the antibacterial immunity of fish, including TLRs, TLR-related signaling adaptors, and TLR-induced cytokines [18].

Due to technological developments in high-throughput detection techniques, many miRNAs have recently been detected in response to pathogens, pathogenic mimics, and abiotic stress in aquatic animals. Relevant studies used the sequenced whole genomes and miRNA microarrays for investigations of e.g., amphioxus [24,25], zebrafish [19], and *Cynoglossus semilaevis* [26]. Despite the inability of microarray technology to obtain sequence information, this technique offers more advantages than deep sequencing when researchers only focus on the abundance of gene expression. miRNA microarrays are also generally less expensive, and most of the experimental and analytic processes can be conducted in general laboratories [27]. For model animals such as mice, zebrafish, and fruitflies, particularly known sequence information of full miRNAs and the sequenced genome are available; thus, probes used in microarray experiments adequately cover miRNAs across entire genomes [28]. Therefore, microarray analysis remains a suitable high-throughput technology for the detection of gene expression in fish, particularly in model fish (e.g. zebrafish).

The zebrafish has been widely considered as model bony fish, and it is used to investigate infectious disease and immune response [29]. For example, Lü et al. analyzed gene expression profiling in the skin of zebrafish infected by two different bacteria, *Citrobacter freundii* and *Staphylococcus chromogenes*, using the Affymetrix zebrafish microarray gene chip [30,31]. Rodríguez et al. detected the immune response of zebrafish host against the pathogenic bacteria (*Aeromonas hydrophila*) at the gene expression level [32]. The aim of this study was to explore miRNA expression changes in antibacterial immunity and to provide insights into the regulatory functions of miRNAs in the immune pathways of zebrafish. Immune-responsive miRNAs (DEMs) were identified in response to *SASA*-challenge in the gills of zebrafish using a combined approach of miRNA microarray and bioinformatics. Furthermore, quantitative real-time PCR (qRT-PCR) analyses of several key *SA*-responsive DEMs were implemented to confirm the obtained microarray results. To further understand the regulatory function of DEMs, the putative target genes of DEMs were Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) term enriched. These findings are useful for understanding the epigenetic regulatory mechanism of the immune response induced by Gram-positive bacteria in the gills of zebrafish.

2. Materials and methods

2.1. Staphylococcus aureus challenge and sampling of zebrafish

All zebrafish were treated in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Ethical Committee of Researches of the Nanjing University (NJU). Wild-type (AB strain) adult zebrafish (*Danio rerio*) were purchased from the China Zebrafish Resource Center (CZRC, http://en.zfish.cn/). All zebrafish were maintained in Beihai Marine Station at the Nanjing University at Beihai (Guangxi Province, China) according to methods reported in Wang et al. [19]. The experimental zebrafish were acclimatized for approximate five days to empty the contents of the digestive systems using filtering freshwater, prior to infection with *SA*. Next, 50 individuals were equally divided

into each of two groups in aquaria (25 fish per aquarium). For treated group, 30 μL (3×10^6 cfu/mL) [33] of live *SA* PBS suspensions were injected into each of 25 zebrafish enterocoelia. Another group, the control fish, was injected with equal sterile PBS only. Five individuals were collected randomly at each time-point, i.e., 6, 12, 24, and 48 h post-injection (hpi) after *SA* infection. Next, gill tissues of one control and four treated samples (6, 12, 24, and 48 hpi) were aseptically dissected and individually placed in centrifuge tubes. The five pooled gill samples were stored at −80 °C in an ultra-low temperature freezer (Sanyo, Japan). These experimental processes were independently conducted twice, as two biological replicates.

2.2. RNA extraction and miRNA microarray analysis

The total RNA of samples in each centrifuge tube was independently extracted using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocols. RNase-free DNase (Qiagen, Germany) was used to remove residual DNA and was applied according to the manufacturer's protocol. RNA structural integrity and concentration was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) (RNA integrity number ≥ 7, at a concentration ≥ 250 ng/μL). Part of the total RNA was used for miRNA chips microarray experiments, the other part was used for an expression survey of miRNA at multiple time points after *SA* challenge using quantitative real-time PCR (qRT-PCR) analysis. For the miRNA microarray, the RNAs of each sample collected at each time point after *SA* infection were diluted to the same concentration with RNase-free water, and samples were equally pooled to obtain the treatment group (two biological replicates) used for the further microarray experiment. While this may lead to a loss of information regarding the temporal expression of genes at each time point, the goal of this study was to obtain an overview of what genes exhibit an acute response to pathogen infection, rather than to detect dynamic changes in expression levels. The microarray processing of miRNAs was conducted using μParaflo®Microfluidic Chip Technology by LC Sciences technology (LC Sciences, USA) (https://www.lcsciences.com/). The miRNA microarray was designed to contain the full 364 transcript probes of zebrafish in the Sanger miRBase 21.0 (http://www.mirbase.org/). Briefly, total RNA received a poly(A) tail to the 3'-end using poly (A) polymerase. Next, the poly(A) tail was ligated with an oligonucleotide fragment for enable fluorescent staining. Hybridization was conducted for 12 hat a μParaflo®Microfluidic Chip platform. Hybridization was performed by using reaction system at 34 °C, including 100 L 6 × SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, 25% formamide, at pH 6.8). After RNA hybridization, tag-conjugating Cy5 dye was circulated through the microfluidic chip for dye staining. Subsequently, fluorescence images were collected and digitally transformed and then normalized using a LOWESS filter (locally-weighted regression). The miRNA microarray data was submitted to NCBI Gene Expression Omnibus (GEO) database.

2.3. Bioinformatics analysis process

To assess the reproducibility of two biological replicates per group, Pearson's correlations (r) of pairwise samples were calculated among the four microarray results based on the normalized values using IBM SPSS Statistics 22 software. DEMs were then identified using Agilent Genespring GX 12 software (Agilent Technology, USA), and the miRNAs with fold changes (FC) ≥ 2 (|log2 ratio| ≥ 1) and p-values (t-test) corrected via the Benjamini-Hochberg (BH) method (false discovery rate, FDR < 0.01) were considered as DEMs. All mRNAs from the zebrafish genome were downloaded from the Ensembl Genomes database FTP site (release-40, http://asia.ensembl.org/info/data/ftp/index.html), and their GO (http://www.geneontology.org/) and KEGG annotations (www.genome.ad.jp/kegg) were performed by hitting them to the corresponding databases. Target genes, likely regulated by DEMs, were predicted using RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/download/tools/rnahybrid.html) and miRanda (http://www.microrna.org/microrna/getDownloads.do) methods. The results (intersection) obtained by both software packages were retained as target genes of DEMs. Functional enrichment analysis, including GO and KEGG enrichment, was performed using both Blast2GO pipeline [34] and KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/) at default parameters and statistical options, respectively. The p-values (Fisher's exact test) obtained by both software packages were corrected by the BH method, and a threshold value of FDR of 0.05 was used. Significantly obtained redundancy of the GO terms was removed using GO trimming v2.0 (http://lucy.ceh.uvic.ca/go_trimming/cbr_go_trimming.py).

2.4. Validation of microarray results and expression survey of key DEMs that likely regulated the Staphylococcus aureus infection pathway enriched by KEGG analysis using qRT-PCR

Nine DEMs that likely regulated the *SA* infection signaling pathway enriched by KEGG analysis were considered as a set of key *SA*-responsive miRNAs (gene set 1, GS1) in this study. GS1 miRNAs were used for qRT-PCR analysis in the same RNA samples that were used for the microarray analysis to confirm the reliability of array technology. In addition, expression dynamics of these nine GS1 miRNAs among multiple time points post-injection (control, 6, 12, 24, and 48 hpi) were surveyed in a replicated experiment using the RNA samples obtained before. Specific primers of GS1 miRNAs are presented in Table S1. TransScript Green miRNA Two-Step qRT-PCR SuperMix (TransGen-Biotech, China) was used to conduct the reverse transcription of total RNA following the instruction guidelines. qRT-PCR analysis was implemented on an ABI 7300 Real-Time PCR System (Applied Biosystems, USA) with TransStart Tip Green qPCR SuperMix (TransGen Biotech, China) according to the manufacturer's manual. All reactions were conducted in triplicate in two biological replicates. A U6 was selected as reference gene. The normalization of miRNA expression was conducted based on the $2^{-\Delta\Delta CT}$ method [35]. All statistics analyses were implemented in IBM SPSS Statistics 22 and the results were exhibited as the means ± standard deviation (SD) of one-way analysis of ANOVA plus Duncan's multiple range tests.

3. Results and discussion

3.1. SA alters miRNA expression in zebrafish gills

A total of 30 miRNAs were found to be differentially expressed between PBS control and *SA*-treated groups. Expression levels of 18 DEMs were up-regulated, while those of the remaining 12 DEMs were down-regulated (Table 1). The FC cut-off for differential expression was set at two, which has been widely used in several previously published and relevant studies [19,36], indicating that DEMs were screened out following a reliable standard. Correlation r values were > 0.85 for pairwise comparisons between both biological replicates in similar treatments, while those between treatment and control samples only exhibited r values < 0.65. This indicated an effective immune challenge for treated fish sample in this study.

Previous studies have demonstrated that the loss of the miR-124 family decreased the axonal outgrowth and oligodendrocyte cell numbers in zebrafish [37]. In this study, the expression level of dre-miR-124 was found to be down-regulated after *SA* challenge, indicating that *SA* infection may lead to defects in the development of the central nervous system of fish. Additionally, differential expressions of dre-miR-10a and dre-miR-125 were detected in male juvenile zebrafish exposed to triclosan [19,38]. Furthermore, dre-miR-153a, dre-miR-725-3p, and dre-miR-7a showed an expression response to ethanol exposure [39]. These three miRNAs were also found in a list of DEMs in this study. In addition, the expressions of dre-miR-9 and dre-miR-124 could be significantly induced in zebrafish embryos through rapamycin challenge [40]. In the current study, dre-miR-124-3p expression was decreased in

Table 1
Differentially expressed miRNAs (DEMs) in the gills of zebrafish challenged with *Staphylococcus aureus* in comparison to the PBS control. FDR indicates false discovery rates used for *p*-value correction.

Sequence	miRNA id	log2 Ratio(G_t/G_c)	Up-Down-Regulation	FDR
UCCCUGAGACCCUUAACCUGUG	dre-miR-125a	−1.04	Down	0
UUUGGUCCCCUUCAACCAGCUA	dre-miR-133b-3p	3.14	Up	7.97E-09
UAAGGCACGCGGUGAAUGCCAA	dre-miR-124-3p	−1.01	Down	2.58E-40
UUGCAUAGUCACAAAAGUGAUC	dre-miR-153a-3p	−1.53	Down	8.20E-09
UGAUAUGUUUGAUAUAUUAGGU	dre-miR-190a	1.49	Up	0
AACUGGCCCGCAAAGUCCCGCU	dre-miR-193b-3p	−1.08	Down	0
UAACACUGUCUGGUAACGAUGU	dre-miR-200a-3p	7.78	Up	0
UAAUACUGCCUGGUAAUGAUGC	dre-miR-200c-3p	1.27	Up	0
AACAGUAAGAGUUUAUGUGCU	dre-miR-2184	1.46	Up	0
GCCGCGAUCAGCUGCACCAGC	dre-miR-2185-3p	2.71	Up	0
CGGUGCAGGACUCCGCGGCUC	dre-miR-2185-5p	−2.36	Down	0
UUACAGGCUAUGCUAAUCUAUG	dre-miR-2187-3p	2.46	Up	0
UUAAUUAGUAUAGCCUGUUUUA	dre-miR-2187-5p	−1.15	Down	1.61E-11
CUGUGUGAGGUUAGACCUAUC	dre-miR-2188-3p	−2.69	Down	0
UGAUUGUUUGUAUCAGCUGUGU	dre-miR-2189	−2.15	Down	0
UUGUGCUUGAUCUAACCAUGUG	dre-miR-218a	1.61	Up	9.40E-07
GGAGUUGUGGAUGGACAUCACGC	dre-miR-219-3p	−1.42	Down	1.40E-288
UACCCUGUAGAUCCGAAUUUGU	dre-miR-10a-5p	3.91	Up	9.33E-301
AGCUCGUGUCCCAAGGCGCCU	dre-miR-2198	1.85	Up	0
UGGCAGUGUCUUAGCUGGUUGU	dre-miR-34a	−2.29	Down	0
UAGGCAGUGUUGUUAGCUGAUUG	dre-miR-34b	3.6	Up	0
AAUCACUAACCUCACUACCAGG	dre-miR-34c-3p	−2.41	Down	0
AGGCAGUGCAGUUAGUUGAUUAC	dre-miR-34c-5p	1.35	Up	0
ACCCUCACAAAGGCACUGACU	dre-miR-430a-5p	4.52	Up	2.65E-105
AAAGUGCUAUCAAGUUGGGGUAG	dre-miR-430b-3p	2.49	Up	0
UUCAGUCAUUGUUUCUAGUAGU	dre-miR-725-3p	−3.45	Down	0
UGGAAGACUAGUGAUUUUGUUGU	dre-miR-7a	2.95	Up	0
AAAAGUGCUGUUUGUGCAGGUA	dre-miR-93	1.22	Up	0
UAAAGCUAGAUAACCGAAAGU	dre-miR-9-3p	2.19	Up	0
CAAUUAUGUGUAGUGCCAAUAU	dre-miR-96-3p	1.69	Up	0

response to *SA* infection in zebrafish gills. This difference may be attributed to differences in experimental tissues and stimuli. Zhang et al. reported that dre-miR-219 was involved in fish development, due to embryonic defects in a zebrafish lineage with knockdown or overexpression of this miRNA [41]. Guo et al. reported the regulated function of dre-miR-34a during zebrafish spermatogenesis [42]. In addition to embryonic and sperm development, dre-miR-219 and dre-miR-34a were found to participate in the antibacterial immune response in zebrafish gills in the current study. Interestingly, four members of the miR-34 family were identified DEMs in this study and showed divergent differential expression. Two DEMs (dre-miR-34a and dre-miR-34c-3p) were down-regulated, while both dre-miR-34b and dre-miR-34c-5p were up-regulated in response to *SA* challenge. This result suggests that different members that belong to the same miRNA family likely possess diverse and complex immune regulatory mechanisms. The DEMs identified in this study are an important resource of the antibacterial immunity of zebrafish gills, since they may be a set of useful molecular immune markers used to monitor the immune response.

3.2. Functional enrichment analysis of target genes

Via GO enrichment analysis, target genes that are likely regulated by DEMs, were enriched in 39 terms that belong to the three subcategories: biological processes, cellular component, and molecular function (Fig. 1). Among these, 19 biological process terms were primarily related to the innate immune response, cell death, biological function, signaling transduction, cell process, and antibacterial response. In addition, 11 cellular component GO terms were enriched, which were primarily involved in membrane part, molecular complex, and cell junction. Among the nine molecular functional terms enriched by GO analysis, terms involving molecular and transducer regulation, as well as molecular and transducer activity were overrepresented. In addition, 29 signaling pathways were found to be significantly regulated by DEMs through KEGG enrichment analysis in response to *SA* infection (Table S2). This indicates that these pathways primarily participated in the antibacterial immune response of zebrafish gills, and constitute a key genetic resource toward the understanding of the molecular mechanisms of gill immunity. The significantly enriched signaling pathways were primarily associated with apoptosis, bacterial disease, antibacterial immunity, cancer, innate immune signaling, stress tolerance, metabolic signaling, and parasitic diseases. Moreover, to macroscopically understand the changes of biological processes under *SA* challenge from a signaling system perspective, KEGG classification was implemented at higher levels according to annotated information (Fig. 2). The obtained results showed that target genes of DEMs were primarily included in pathways involving the immune system, signaling transduction, human disease, and metabolism. This indicates that DEMs regulate antibacterial immunity by affecting these signaling pathways at the genome-wide level after *SA* challenge. Furthermore, among significantly enriched KEGG pathways, 13 terms were detected that involve the immune response (Table 2), e.g., Th1 and Th2 cell differentiation, *SA* infection, NF-kappa B signaling pathway, and Toll-like receptor signaling pathway. Notably, both the Th1 and Th2 cell differentiation pathways are typical for cellular immune signaling, which was demonstrated as an important pathway through which macrophages activate Th1 cell responses and suppress Th2 cell responses [43]. This result suggests that Th1 and Th2 cells protect the zebrafish gills from invasive *SA* infection. This conclusion is further supported by a recent demonstration of Th1 cell roles for *SA* infection in mice [44]. The toll-like receptor signaling pathway and NF-kappa B signaling pathway are key signaling pathways in the innate immune response of deuterostomia. The toll-like receptor signaling pathway serves as a component that recognizes conserved structures in pathogens and that can activate both innate immune responses and prime antigen-specific adaptive immunity [45]; the activation of the NF-kappa B signaling pathway is a key step in the regulation of pro-inflammatory cytokine expression in innate immunity [46]. Moreover, the innate immune system is an activator of an adaptive immune response [47]. In recent

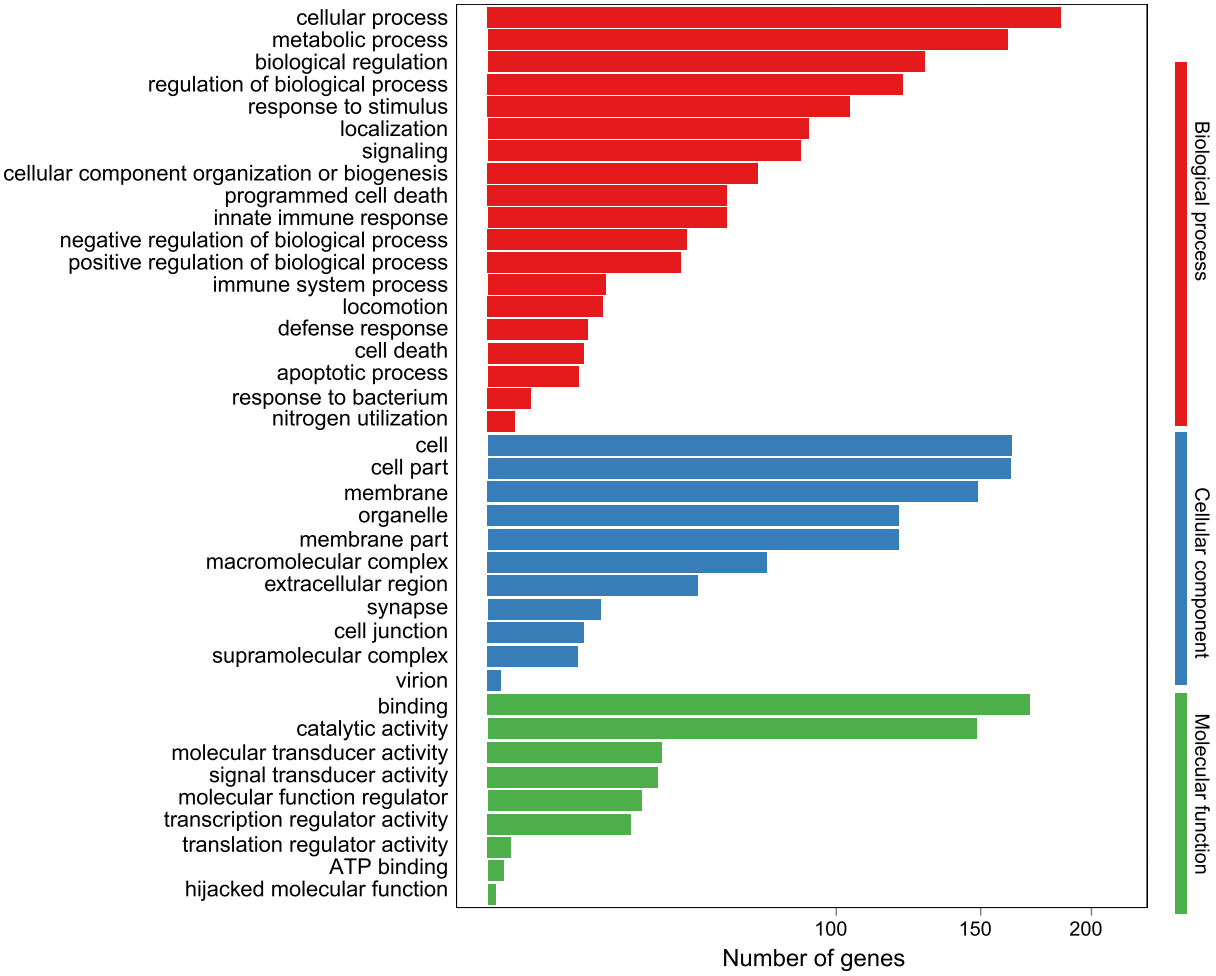

Fig. 1. List of GO terms enriched by DEM target genes. All terms listed here are significantly enriched (FDR < 0.05).

decades, this connection between the innate and the adaptive immune system has been widely investigated in fish [48,49]. Few adaptive immune pathways have been significantly enriched, which is mainly attributable to the short duration of *SA* challenge in this study. We therefore recommend that the expression alteration of gene involving adaptive immunity in response to bacterial infection needs to be further explored in future studies.

3.3. Expression analysis of key SA-responsive DEMs in response to Staphylococcus aureus challenge

The *SA* infection signaling pathway was indirectly enriched, and was included in the list of KEGG terms. A total of nine DEMs were identified as regulators of this signaling pathway using a bioinformatic approach; four of these DEMs were up-regulated and five were down-regulated in response to *SA* challenge in zebrafish gills. The DEMs that regulated the *SA* infection pathway were identified as key *SA*-responsive miRNAs (gene set 1, GS1) in the current study. In addition, qRT-PCR analysis for DEMs in GS1 showed a close correlation (r value = 0.921, $P < 0.01$) between expression changes in the microarray and qRT-PCR results, indicating quantitative accuracy of the microarray data (Fig. 3A).

Expression dynamics of the *SA*-responsive miRNAs identified above at multiple time points after *SA* challenge are shown in Fig. 3B in detail. Specifically, dre-miR-133b-3p, dre-miR-124-3p, and dre-miR-10a-5p were predicted to be optimal regulators (with a maximum predicted score) of complement factor H (*CFH*), complement component 2 (*C2*), and complement C1q (*C1q*) as members in the *S. aureus* infection pathway, respectively. *CFH* is responsible for the down-regulated activation of the complement pathway, and *C1q* is important for the classical pathway of the complement system in the innate immune response to pathogenic invasion (e.g. *S. pneumonia*) [50]. *C2* is central for the classical pathway of the complement system [51]. Furthermore, dre-miR-725-3p was also predicted as a potential regulator of the complement component 4 (*C4*) that can activate the complement system via its own cleavage [52]. The expression levels of dre-miR-133b-3p and dre-miR-10a-5p were significantly induced at all investigated time points after *SA* challenge in this study, namely 6, 12, 24, and 48 hpi. In contrast, the expressions of dre-miR-124-3p and dre-miR-725-3p were significantly decreased at 12, 24, and 48 hpi (an upregulation of the latter was also found at 6 hpi). Complement component *C3* was essential in the innate immune response against *S. pneumonia* [53], and *C3* degradation decreased the bacteria killing ability of the fish immune system [54]. In this study, *C3* was predicted to be an optimal target, regulated by dre-miR-193b-3p with down-regulated expression at 6, 12, 24, and 48 hpi. These results indicate that divergently regulated-expression of the miRNAs that regulated complement components in the antibacterial immune pathway contributes to the immune defense of zebrafish gills during *SA* challenge. With respect to the other DEMs, dre-miR-153a-3p can regulate gene encoding fibrinogen gamma chain (*FGG*) in the *SA* infection pathway, and *FGG* was suggested to play a key role in wound healing, as well as in disease and inflammatory responses [55]. In the present study, dre-miR-153a-3p was detected as a down-regulated miRNA at all time points following *SA* challenge within 48 hpi. The gene encoding mannan-binding lectin serine protease 1 (*MASP1*) was predicted as a potential target of dre-miR-2185-5p.

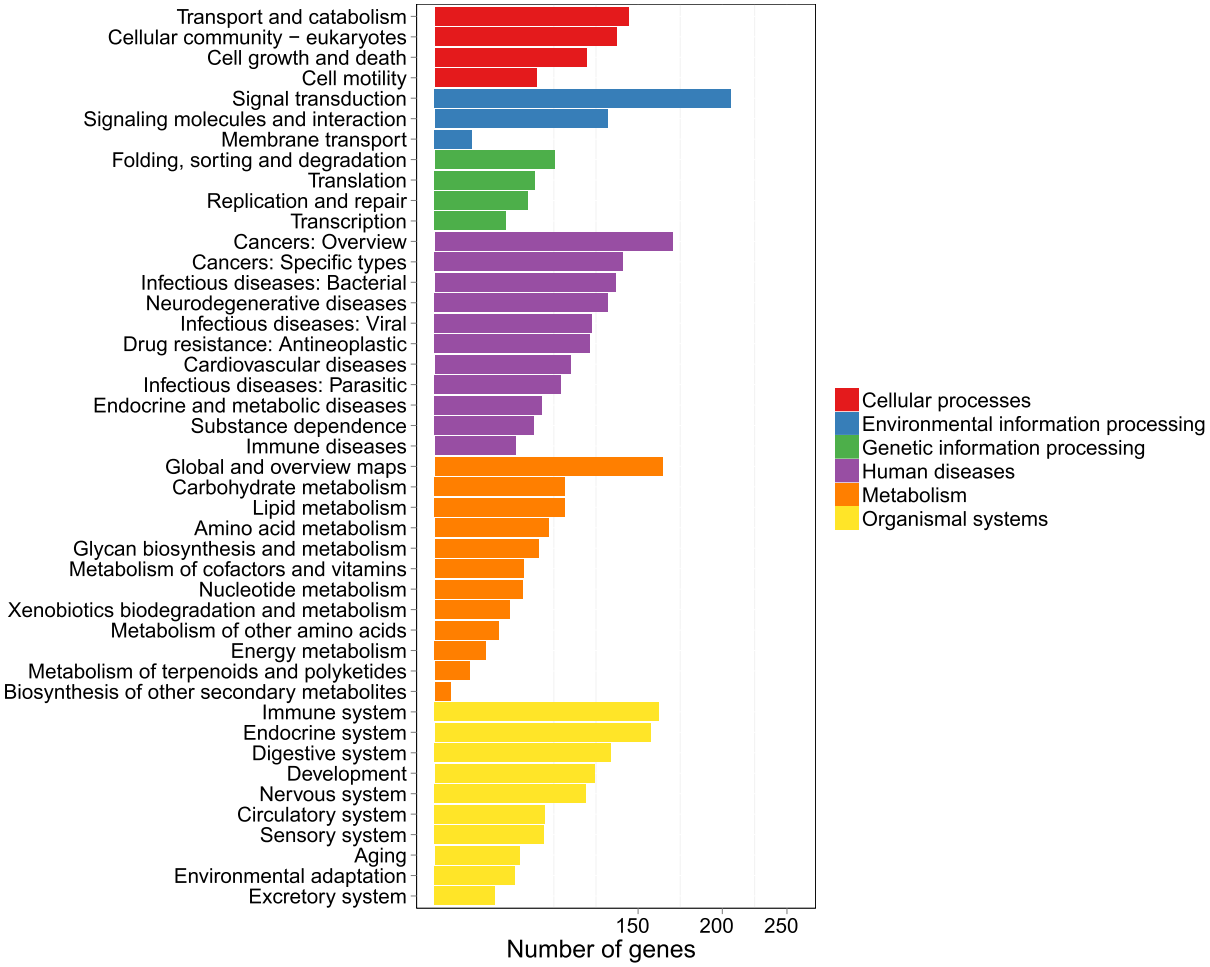

Fig. 2. KEGG classification enriched by DEM target genes. The x-axis indicates the number of DEM target genes, while the y-axis lists KEGG pathway terms at the second level.

Table 2
List of the primarily immune-related KEGG pathways enriched by DEM target genes between the gills of control zebrafish (PBS injection) and those challenged with *Staphylococcus aureus*.

Pathway	Pathway id	P-value	FDR
Apoptosis	ko04210	1.64E-12	5.32E-10
Pertussis	ko05133	7.26E-10	7.86E-08
Th1 and Th2 cell differentiation	ko04658	4.13E-08	1.68E-06
Staphylococcus aureus infection	ko05150	8.29E-07	1.71E-05
NF-kappa B signaling pathway	ko04064	8.46E-07	1.71E-05
Complement and coagulation cascades	ko04610	5.90E-06	8.34E-05
Cytokine-cytokine receptor interaction	ko04060	4.90E-05	5.13E-04
Toll-like receptor signaling pathway	ko04620	1.00E-04	8.59E-04
NOD-like receptor signaling pathway	ko04621	1.97E-04	1.56E-03
Pathways in cancer	ko05200	1.75E-03	9.79E-03
Lysosome	ko04142	2.77E-03	1.45E-02
Primary immunodeficiency	ko05340	7.83E-03	3.22E-02
Leishmaniasis	ko05140	1.28E-02	4.79E-02

Takahashi et al. reported that activation of the alternative complement pathway (AP) was extensively decreased in *MASP1* knockout mice, indicating a key role of *MASP1* in AP activation [56]. In this study, dre-miR-2185-5p presented down-regulated expression at 12, 24, and 48 hpi relative to the PBS control, suggesting that AP activation played a key role in the antibacterial immune response in zebrafish gills. Singh et al. reported that binding between bacteria and plasminogen was converted to plasmin that degrades complementary component (e.g., *C3b* and *C5*), which consequently led to significantly reduced bacterial (*Moraxella catarrhalis*) killing, and evasion due to innate host immunity [54]. qRT-PCR analysis indicated that the expression of dre-miR-93 (that likely regulated gene encoding plasminogen) was induced at 24 and 48 hpi, indicating that up-regulation of dre-miR-93 likely suppressed plasminogen abundance and promoted the killing of *SA*. Gene encoding P-selectin protein (*SELP*) was predicted as a target regulated by dre-miR-34c-5p with up-regulated expression at 6, 12, and 24 hpi in this study. Previous studies demonstrated a key role of *SELP* in antigen-induced immune responses in the microvasculature [57]. This indicates that dre-miR-34c-5p participated in the *SA*-induced immune response by regulating the *SELP* member of the *SA* infection pathway.

In conclusion, this investigation showed that *SA* challenge altered the expression of miRNAs that are primarily involved in antibacterial immunity, cell death, cellular immunity, bacterial disease, cancer, innate immune and stress response, signal transduction, and the translation regulator in zebrafish gills. Moreover, several significant pathways that are involved in antibacterial immunity were identified in zebrafish gills in response to *SA* infection. The present work contributes towards understanding the molecular mechanism of the gill immune response in fish. However, due to the high complexity and the lack of knowledge about the defense systems that responds to *SA* invasion, several results that cannot be reasonably explained were obtained and require further investigation in future studies.

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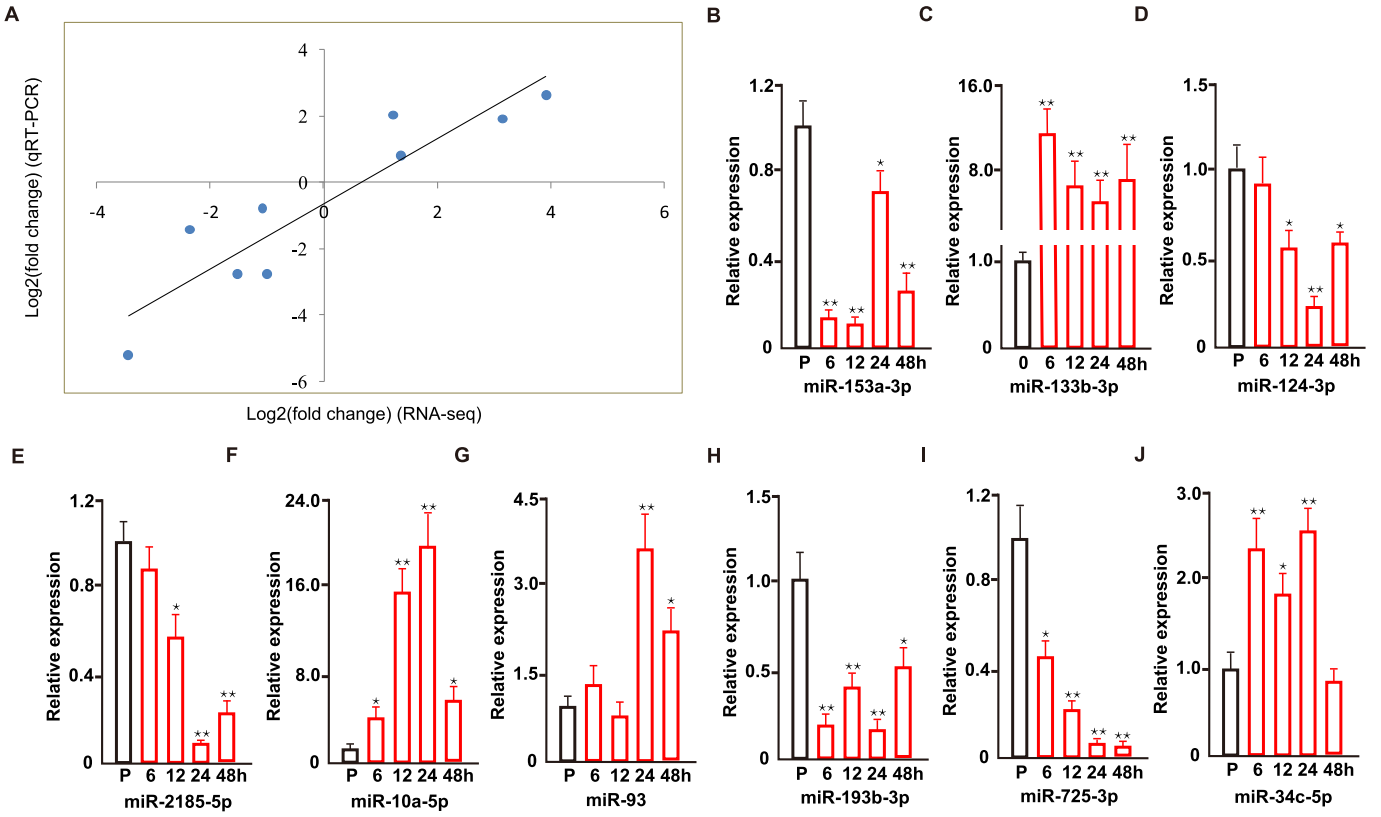

Fig. 3. qRT-PCR analyses of key responding miRNAs in gill after immune challenge. (A) Correlation between relative fold changes in expression based on both microarray and qRT-PCR analyses. Three technical replicates were performed for each of the three biological replicates. (B–J) Expression survey of nine key *SA*-responsive miRNAs after immune challenge at multiple time points. Data are presented as the mean ± SD (n = 6).*$p < 0.05$, **$p < 0.01$ relative to control; one-way ANOVA plus Bonferroni post-tests.

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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.2019.01.026.

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