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Short communication

Identification of circular RNAs and their altered expression under poly(I:C) challenge in key antiviral immune pathways in amphioxus

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ABSTRACT

Amphioxus is a key model for studying comparative immunity of vertebrates. Circular RNA (circRNA), as RNAs with a circular structure, has received little attention until recently, where several studies have reported that circRNA expression changes are involved in the immune response in animals. However, circRNA and its immune role in amphioxus have not been previously studied. Here, circRNAs in Chinese amphioxus (*Branchiostoma belcheri*) were sequenced, and 1859 circRNAs were identified using two algorithms (find_circ and CIRI). The analysis of miRNA target sites on circRNAs showed that 332 circRNAs may function as miRNA sponges. Furthermore, we identified circRNAs that were conserved between *B. belcheri* and vertebrates, tracing the origin of these circRNAs within chordates. Additionally, in combination with several key antiviral immune (poly(I:C), pIC) pathways identified in our previous *B. belcheri* studies, nine circRNAs potentially involved in these pathways were identified using bioinformatic predictions. Among these nine circRNAs, eight were selected to examine their expression response in *B. belcheri* challenged by pIC in comparison to control using real-time quantitative PCR. The results showed that four circRNAs were induced as part of the antiviral response against pIC, while expression of two circRNAs was decreased, and the expression levels of the remaining two were not significantly altered after pIC challenge. This work is the first to identify circRNAs and reveal their antiviral role in amphioxus. Therefore, it opens a new window to explore the comparative immunology of circRNAs in chordates and the regulatory roles of circRNAs in antiviral immunity in amphioxus.

1. Introduction

The existing Cephalochordata subphyla only includes amphioxus as a sister clade to Urochorda and Vertebratain in the Chordata [1]. Due to proximal genomic and morphological features with the living chordate ancestor ~520 million years ago, amphioxus has been widely considered as a proxy for the last common ancestor of chordates, and is used as a key animal model in comparative immunology and evolutionary developmental biology to understand the evolution and origin of immune-related genes, cells and tissues of vertebrates [2–4]. Moreover, several pathogens and their mimics, such as *Vibrio parahaemolyticus*, lipopolysaccharide (LPS) and poly(I:C) (pIC; a double-stranded RNA viral mimic) have been used to construct amphioxus models of viral or bacteiral infections [5–8]. This success has led to the use of amphioxus to investigate the roles of immune-related genes, and this model has been used to identify various key antiviral immune-related DNA and RNA molecules.

Recently, with the prevalence of high-throughput RNA sequencing (RNA-seq) and sequencing of Chinese amphioxus (*Branchiostoma belcheri*) whole genome [9], various types of RNA molecules, including microRNAs (miRNAs) and mRNA were identified, and their genome-wide expression patterns were identified in *B. belcheri* under immune challenge [8,10,11]. For example, Zhang et al. identified 1999 differently expressed genes between the gills of *B. belcheri* challenged by pIC and that of a control using digital expression profiling based on RNA-seq and then identified 12 typical antiviral immune and viral disease-related signaling pathways [10], such as RIG-I (retinoic-acid-inducible protein 1)-like receptor signaling pathway, Influenza A and Hepatitis C. Furthermore, Zhang et al. screened a total of 77 differentially expressed miRNAs (DEMs) in *B. belcheri* infected by pIC relative to the control, further finding these DEMs were likely linked to 14 important antiviral immune-related signaling pathways. These investigations provide

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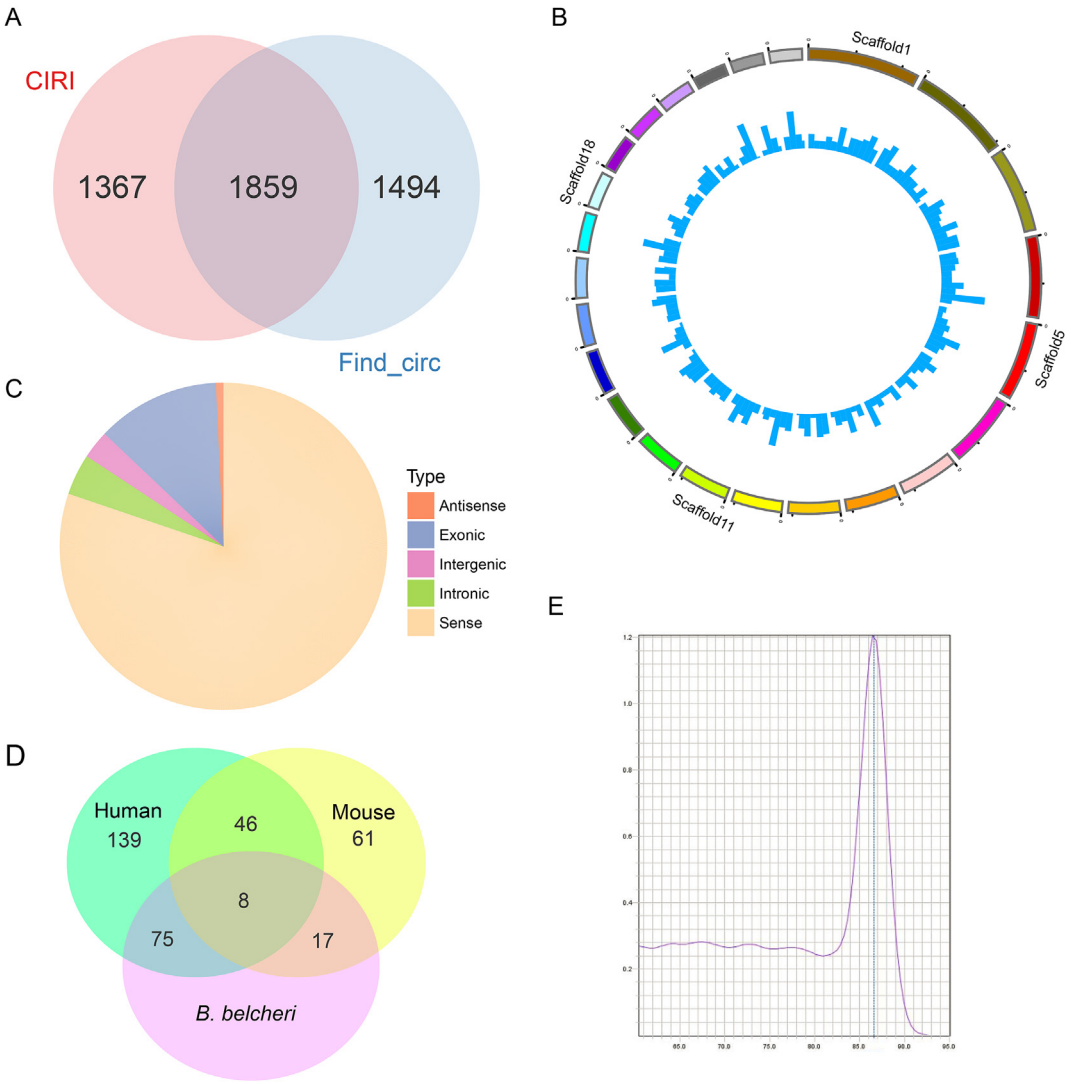

Fig. 1. Identification and characteristics of *B.belcheri* circRNAs. (A) The circRNAs were identified by two algorithms (find_circ and CIRI). (B) Circular map of circRNA distribution in the whole genome of *B. belcheri*. The top 24 scaffolds with the longest length were used in analysis. (C) The percentage of five types of circRNAs. (D) *B. belcheri* circRNAs shared homology between humans and mice. (E) A dissociation curve example of scaffold275:78397|121920 in qRT-PCR.

valuable information for a comprehensive understanding of the molecular mechanisms of the immune response in amphioxus and represents a set of useful genetic resource for further exploration of the function of immune-related genes in future studies. However, to the best of our knowledge, any research involving circular RNAs (circRNAs) in amphioxus has not been reported.

CircRNAs are a type of non-coding RNA that covalently forms a closed loop in eukaryotes [12]. CircRNAs are not easily degraded by the nucleic acid exoenzyme RNase R, particularly in body fluid (i.e. blood and saliva) [13]. Compared to linear RNA, circRNAs have higher stability [14]. Moreover, circRNAs are widely considered as efficient miRNA sponges and can competitively liberate expression of their target mRNAs that have been inhibited by miRNAs [13]. In addition to constraining miRNAs, circRNAs also directly participate in the regulation of the alternative splicing and gene expression [15]. Currently, many studies have reported that changes in circRNA expression could be detected in response to various immune stimuli or pathological conditions in a variety of animals, including male *Xenopus laevis* [13] and *Bombyx mori* [15].

To identify circRNAs in amphioxus and perform a preliminary exploration of their expression in key antiviral immune signaling pathways in response to viral challenge in amphioxus, we first attempted a genome-wide identification of circRNAs in *B. belcheri* using RNA-seq. *B. belcheri* circRNAs were characterized in detail. Next, based on these identified circRNAs, and combined with several key anti-pIC immune pathways found in our previous *B. belcheri* studies, we identified potentially regulated circRNAs for these pathways. Most of these circRNAs were chosen to examine their expression in response to pIC challenge in *B. belcheri* using real-time quantitative PCR (qRT-PCR). This study provides a basis for further investigation of the regulatory roles of circRNAs in antiviral immunity in amphioxus.

2. Materials and methods

2.1. B. belcheri samples and RNA extraction

Healthy adult *B. belcheri* were supplied by Beihai Marine Station of Nanjing University at Beihai, Guangxi Province, China. We maintained them following our previous methods [16,17]. Experimental animals were acclimatized for ~6 days to empty the contents of the amphioxus bodies. Fifteen male and fifteen female individuals were randomly selected from the amphioxus population. To obtain amphioxus circRNAs, various tissues were collected separately, including the nerve cord, notochord, skin, hepatic cecum, gill, intestines, skin, muscle, ovary and

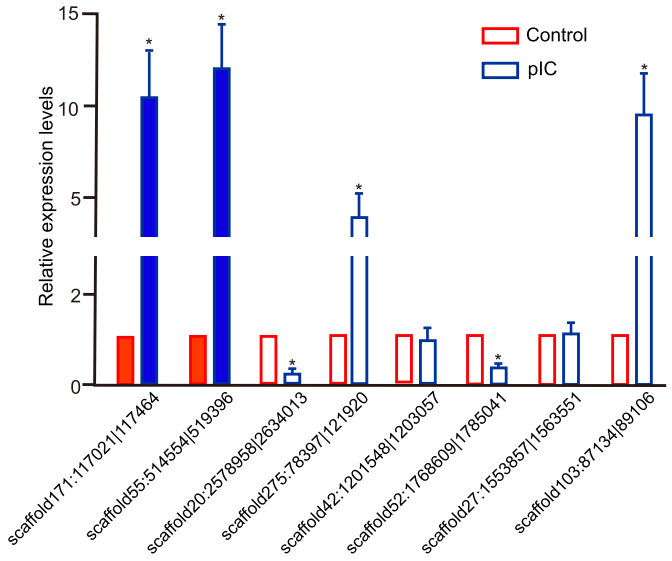

Fig. 2. qRT-PCR analysis of eight circRNAs that regulated key antiviral immune signaling pathways in response to pIC challenge. Data are presented as the mean ± SD (n = 9). *$p < 0.05$ relative to control; one-way ANOVA plus Bonferroni post-tests. Solid columns indicate circRNAs that regulated ko04622, and hollow columns indicate circRNAs that regulated ko04060 signaling pathways.

testis, and total RNA was extracted from each tissue type. Residual genomic DNA was removed using RNase-free DNase (Qiagen, Germany). The RNA concentration was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, USA). RNA purity was assessed using the OD_{260}/D_{280} absorbance ratio with expected values between 1.8 and 2.0. RNA structural integrity was verified by gel electrophoresis using a 1.5% agarose gel and analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Ten types of RNA samples were pooled equally after adjusting them to the same concentration using RNase-free water. We used a CircRNA Enrichment Kit (Cloud-seq Biotech, USA) to enrich for circRNAs and then linear RNAs were removed by digestion with RNase R (20 U/ml, Epicentre, USA) for 20 min at 37 °C, following removal of rRNA from the total RNA using Ribo-Zero rRNA Removal Kit (Illumina, USA), following the manufacturer's instructions.

2.2. RNA library construction and circRNA sequencing

The obtained circRNAs were used to construct RNA libraries with the TruSeq Stranded Total RNA Library Prep Kit (Illumina, USA) according to the manufacturer's instructions. Briefly, we added buffer to randomly interrupt circRNAs into 140–160 nt followed by reverse transcription with random primers. Magnetic beads were then used to capture the amplified cDNA fragments. Finally, library quality was assessed using a BioAnalyzer 2100 system (Agilent Technologies, USA) and ABI StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The prepared circRNA library was sequenced and raw reads were generated on a HiSeq4000 platform (Illumina, USA) at Beijing Genomics Institute (BGI, China).

2.3. Identification of circRNAs

Paired-end raw reads was generated by sequencer. After quality control using SOAPnuke software (https://github.com/BGI-flexlab/SOAPnuke), the high quality clean reads were retained for downstream analysis. The clean reads were mapped to the *B. belcheri* reference genome (http://mosas.sysu.edu.cn/, v18h27.r3, early available) using TopHat2 (version 2.1.1, http://ccb.jhu.edu/software/tophat/index.shtml). Two bioinformatics approach were used to identify circRNA with default parameters suggested by software manual, including Find_circ [18], CIRI [19]. Predicted circRNAs identified by both software programs were considered as the final targets. CircRNAs were assigned into five groups according to their transcriptional location in the genome, including exonic, intronic, sense, antisense and intergenic circRNAs [12].

2.4. Target prediction and bioinformatic analysis

For the circRNAs identified above, RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/download/tools/rnahybrid.html) and miRanda (http://www.microrna.org/microrna/getDownloads.do), were employed to predict miRNA sites in circRNAs using the default parameters, and the five miRNAs with the highest binding probabilities were retained as circRNA targets [12,13]. In addition, mRNA targets of miRNA were also predicted using RNAhybrid and miRanda. CircRNA homologs among *B. belcheri*, humans and mice were identified using local blastn tool (e-value threshold = $1e^{-5}$, ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). The circRNA sequences of human and mouse were obtained from circBase database (http://www.circbase.org).

2.5. Identification of circRNAs that regulated potentially key anti-pIC immune signaling pathways

In our previous studies, we sequenced the transcriptomes of *B. belcheri* in response to pIC challenge [20], and also investigated genome-wide gene expression profiling in the gills using RNA-seq [10]. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differentially expressed genes (DEGs) between pIC-treated and control groups, we identified a set of key anti-pIC immune signaling pathways in the whole body of adult *B. belcheri* and their gills in our previous studies [10,20]. Among these, cytokine-cytokine receptor interaction (id: ko04060) and the RIG-I-like receptor signaling pathway (id: ko04622) were shared by these two groups. In the current study, these two KEGG terms were selected as potentially key antiviral immune signaling pathways in *B. belcheri* exposed to pIC challenge. Based on the circRNA-miRNA-mRNA interactions predicted above by RNAhybrid and miRanda, circRNAs that regulated likely gene members within the ko04060 and ko04622 pathways were extracted as circRNA set 1 (CS1).

2.6. Expression analysis of circRNAs in response to pIC challenge

The pIC-treated and control groups used in this study were constructed according to our previous studies [10,20]. Extraction and treatment of total RNA was implemented as in section 2.1. Notably, linear RNAs were not removed from the portion of total RNA used for quantifying the expression of the reference genes. Then, cDNA synthesis was performed using the Invitrogen Superscript cDNA Synthesis kit (Invitrogen, USA). All reactions were performed according to the manufacturer's instructions. Specific primers were designed using the "Out-facing" strategy to guarantee amplification of the head-tail joint sites of the circular templates [21]. All primer sequences are shown in Table S1. The expression analyses for circRNAs were conducted on an ABI 7300 Real-Time PCR System (Applied Biosystems, USA) with SYBR green master mix (Applied Biosystems, USA). Elongation factor 1A (*EF1A*) was selected as a reference gene [17]. The expression level of each circRNA relative to that of *EF1A* was normalized using the $2^{-\triangle\triangle CT}$ method [22]. Three biological replicates were performed for each qRT-PCR reaction. One PCR product from each circRNA was validated by Sanger sequencing. The results are presented as mean ± standard deviation (SD). All statistical values were calculated using the IBM SPSS Statistics 22 software.

3. Results and discussion

3.1. Identification of circRNAs in B. belcheri

In this study, we obtained 23,967,358 clean reads, which have been submitted to NCBI Sequence Read Archive (SRA) repository with the accession number SRR6731120. The total RNA used for preparation of circRNAs was collected from each tissue and circRNA abundance was improved by enrichment, ensuring accurate and full circRNA sequencing. A total of 1859 circRNA candidates were identified using both CIRI and Find_circ (Fig. 1A and Table S2). All potential circRNAs were mainly equally distributed in the *B. belcheri* genome, while several regions presented a higher circRNA density (Fig. 1B), such as those in scaffold 4, 10, 17, 22, and 24. The results indicated that circRNA distribution did not show an obvious regional preference. In the *B. belcheri* genome, exonic, intronic, sense, antisense and intergenic circRNAs occupied 27.12%, 4.52%, 74.82%, 3.33% and 4.15%, respectively (Fig. 1C). Shen et al. found that these exonic, intronic, sense, antisense and intergenic circRNAs occupied 24.48%, 19.62%, 39.30%, 3.18% and 13.42% in zebrafish (*Danio rerio*), respectively. We observed that the percentage ranking of each type in amphioxus was similar with that of zebrafish, while the relative abundance percentage of sense circRNA is dramatically reduced, and an obvious increase of both intronic and intergenic types of circRNAs in zebrafish was detected in comparison to that of amphioxus. The analysis suggested that alteration of relative abundance of types is a key feature in circRNA evolution.

3.2. The target miRNAs of circRNAs

Previous studies found that circRNA formation affected miRNA regulation of mRNA through a sponge mechanism [23]. CircRNAs function as miRNA sponges that regulate target gene expression by binding to miRNAs. In this study, 332 circRNAs were predicted to be miRNA sponges. The circRNA with the largest number of regulated miRNAs was scaffold26:1110556 (10 miRNAs), followed by scaffold370:76261|76786 (9 miRNAs) and scaffold103:87134|89106 (8 miRNAs). The top 5 miRNA targets based on their binding capacity are listed in Table S3. Among these, bbe-miR-129b-5p is bound by the largest number of circRNAs (34), followed by bbe-miR-2066-3p (28). Previously, multiple miRNAs were found to be bound by circHIPK3 [23]. Conversely, five miRNAs, including bbe-let-7a-2-3p, bbe-miR-1-5p, bbe-miR-2061-3p, bbe-miR-29a-5p, and bbe-miR-96-3p, appear to bind potentially to only one circRNA based on bioinformatic analysis. Similarly, Sai et al. reported that many circRNAs can act as a sponge for single miRNAs [13]. Chen et al. showed that circRNA_100290 is a competing endogenous RNA that binds miR-29 family members in human oral squamous cell carcinomas development [24]. Despite limited exploration of the regulatory roles of circRNAs in amphioxus in response to immune challenge, all of the analyses suggest that circRNAs may have diverse functions in immunity and disease in amphioxus. In addition to circRNAs as miRNA sponges, most circRNAs are unable to bind miRNAs, as reported in many studies [12,25]. Interestingly, Zhang et al. reported the immune response of several known miRNAs in *B. belcheri* in response to pIC [11], of which two miRNAs (bbe-miR-10c-5p and bbe-miR-210-3p) were found to bind to circRNAs (caffold27:1553857|1563551 and scaffold103:87134|89106) in this study, respectively. These results indicated that *B. belcheri* circRNAs may participate in the regulation of antiviral immunity. Certainly, the circRNAs as miRNA sponge were only predicted by bioinformatics, thus the binding of these circRNAs to miRNAs is required to be further determined.

3.3. Conservative analysis of circRNAs

Amphioxus is a key model for tracing the origins of vertebrate RNA molecules, particularly those involving in the immune system. Compared with circRNA sequences of representative vertebrates (human and mouse), 75 (4.03%) *B. belcheri* circRNAs were homologous to those in humans, 17 (0.91%) to those in mice and 8 (0.43%) were homologous to those in both humans and mice. Meanwhile, the majority of circRNAs homologous to those in *B. belcheri* could not be found in these two vertebrate species. In this study, a total of 100 (5.38%) *B. belcheri* circRNA homologs were conserved among all three chordates (Fig. 1D). Previous studies reported that most circRNAs were conserved among vertebrates [12,26]. However, a few vertebrate circRNAs were found to be homologous to those in *B. belcheri*, indicating that most ancestral circRNAs from vertebrates have been lost and many new circRNAs have evolved during vertebrate evolution.

3.4. Expression changes of circRNAs that potentially regulated key antiviral-immune signaling pathways in B. belcheri under pIC challenge

In total, 12 circRNAs that regulated potentially key anti-pIC immune signaling pathways were identified. Among these, eight circRNAs were selected to survey their expression in response to pIC challenge using qRT-PCR (Fig. 1E). Products with the expected size were confirmed by Sanger sequencing (Fig. S1) and their corresponding circRNA sequences are presented in Table S4. In the RIG-I-like receptor signaling pathway (ko04622), qRT-PCR analysis of two circRNAs (scaffold171:117021|117464 and scaffold55:514554|519396) showed significantly up-regulated expression following pIC challenge in *B. belcheri* (Fig. 2). Recently, Wang et al. found that the delivery of circRNAs activates RIG-I-mediated antiviral immune signaling *in vitro* and provides protection against viral infection [27]. For the cytokine-cytokine receptor interaction (ko04060) pathway, qRT-PCR analysis of six circRNAs showed that 2 circRNAs (scaffold275:78397|121920 and scaffold103:87134|89106) were down-regulated, while 2 circRNAs (scaffold20:2578958|2634013 and scaffold52:1768609|1785041) were up-regulated. However, 2 circRNAs (scaffold42:1201548|1203057 and scaffold27:1553857|1563551) in the ko04060 pathway did not exhibit any changes in expression in response to pIC challenge. Molecular targets of miRNAs sponged by these eight circRNAs in two key antiviral-immune signaling pathways (ko04622 and ko04060) were presented in detail in Table S5. These results showed several circRNAs potentially participated in antiviral immunity of amphioxus by binding miRNAs that regulate the expression of associated genes belonging to key antiviral immune-related pathways. Notably, according to bioinformatic and qRT-PCR analysis, we detected circRNA that potentially regulated key antiviral-immune signaling pathways for the first time in amphioxus. However, regulated relationships of these circRNAs to key antiviral pathways need to be further determined by confirming that the circRNAs bind to miRNAs using *in vitro* experiments in the future.

In summary, 1859 *B. belcheri* circRNAs were identified using two algorithms, of which the 17.86% of all identified circRNAs were predicted to bind miRNAs. In addition, several circRNAs identified in this study were well conserved with those in vertebrates. Based on the circRNA set of *B. belcheri*, we identified circRNAs that potentially regulated key antiviral immune signaling pathways using a bioinformatic approach for the first time, and some of these were detected to be responsive to pIC challenge. This work provides a useful circRNA resource for the future studies of antiviral immunity, as well as novel insights into molecular immune mechanisms under virus infection from a circRNA perspective in amphioxus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.061.

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