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MicroRNA profiling of Atlantic salmon challenged with Infectious Pancreatic Necrosis virus: Comparison between resistant and susceptible fish

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ABSTRACT: Resistance to Infectious Pancreatic Necrosis Virus (IPNV) is a heritable trait largely under the control of a single genomic region, yet the underlying functional mechanisms remains unclear. We recently developed a microRNA (miRNA) resource for Atlantic salmon using high-throughput sequencing. In this study, the aim was to characterize the miRNA profile in IPNV-challenged and control Atlantic salmon, and to compare resistant and susceptible fish. 36 Atlantic salmon fry were sequenced on the Illumina platform resulting in the discovery of ~900 putative miRNAs. One miRNA was identified which showed highly significant differential expression between fry homozygous for the resistant allele at the major IPN resistance QTL and those homozygous for the susceptible allele. miR-4792 was overexpressed 3.6 fold in resistant homozygotes ($P < 0.0006$) and its predicted target genes may play a role in the differential host response to IPN infection underlying the major QTL.
Keywords: Atlantic salmon; miRNA; disease resistance; IPN; QTL

Introduction

Aquaculture is the fastest growing industry in the animal-food-producing sector, and is essential for meeting the increasing global requirement for fish. However, for key farmed species such as Atlantic salmon (*S. salar*), there are several viral diseases that pose a serious threat to fish production. Infections with the Infectious Pancreatic Necrosis virus (IPNV) can cause mortality to farmed populations in both the freshwater and seawater stages of production Vaccines are only partially effective but a genetic basis of resistance to IPNV has been demonstrated with moderately high heritability (0.31 and 0.38 in freshwater and seawater respectively), and a single QTL conferring resistance has been discovered (Houston et al. (2008; 2010); Moen at al. (2009)). Large-scale gene expression differences between resistant and susceptible genomes have been shown (Bekaert et al. (2012)), yet the causative mechanism of this major QTL remains unknown.

MicroRNAs (miRNAs) have been shown to play a major role in regulating many biological processes in a diverse range of eukaryotic organisms. miRNAs are short (18-26 nt) non-coding RNAs which act as post-transcriptional repressors of gene expression by degrading mRNA or inhibiting translation. They are transcribed from miRNA genes by Pol II, sequentially producing pri-miRNA and pre-miRNA, before the final mature sequence is formed (Ambros et al. (2003); Zhang et al. (2007)). It is becoming increasingly clear that miRNAs contribute to the complexity of eukaryotic systems, including the immune system. Whilst most non coding RNA research has focused on mammalian species, studies have also demonstrated their importance in other vertebrates such as fish (Chen et al. (2005); Schier and Giraldez (2006); Mennigen (2014)). Teleost fish miRNAs have already been highlighted as potential molecular markers for important harvest traits such as egg quality (Ma et al. (2012)) and, additionally, have been shown have an immunoregulatory role (Wu (2012)).

The aim of this study was to characterize the miRNA profile of IPNV-challenged Atlantic salmon fry, and to identify any differences in miRNA expression between resistant and susceptible animals.

Materials and Methods

Viral challenge: The three families used for this study were a subset of those described in Houston et al. (2010) and all parents were heterozygous for the IPN QTL. Full siblings from each of these three families were given an IPNV bath challenge in family-specific tanks (described in Houston et al. (2010)) and sampled at 0 day (i.e. pre-challenge) and 1 day post-infection. Samples were snap-frozen and stored at -80^{o}C. Six fry from each family were utilized at both timepoints; three of which were homozygous for the resistance QTL allele and three homozygous for the susceptible QTL allele (total n = 36).

Small RNA library construction and sequencing Total RNA was isolated from homogenised whole fry, and checked for integrity. Library preparation was performed according to the Illumina Truseq Small RNA preparation guide (Illumina, Inc., USA) using total RNA as starting material. All 36 barcoded libraries were pooled and sequenced across two lanes of the Illumina HiSeq 2000 (37 base paired-end run).

Characterization of miRNA candidates and target sites: Putative miRNAs were predicted using MiRanalyzer v. 0.3 (beta) (Hackenberg et al., 2011), and sequences were aligned with the Atlantic salmon draft genome assembly (NCBI Assembly GCA_000233375.1). Candidate miRNAs that were represented by fewer than 10

reads per sample were removed. All remaining candidate miRNAs were filtered using RepeatMasker and aligned using BLASTN to a set of known miRNAs from miRBase (v19; Kozomara and Griffiths-Jones (2011)). To predict the putative target genes of the miRNA, full-length transcript sequences were downloaded from the Centre for Biomedical Research (University of Victoria) website (Koop et al. (2008)). To predict the target genes of the miRNAs on the 3' UTR regions of the transcripts, both TargetSpy v 1.0 (Sturm et al. (2010)) and RNAHybrid v 2.1 (Rehmsmeier et al. (2004)) were used. These predictions were filtered to make sure the binding site presented a seed of at least 7 nt and started at the first or second position of the miRNA.

Identification of miRNAs with a potential role in IPNV resistance Expression levels of both infected and uninfected individuals were calculated and compared between QTL genotypes using DESeq in R (Anders and Huber, 2010). Differential expression between fry of alternate QTL genotypes was compared in each of the three families individually and in a combined analysis across both timepoints (i.e. pre- and post-infection).

Results and Discussion

Identification of miRNAs The main aims of this study were to examine the microRNA repertoire of IPNV-challenged and control salmon fry and to compare the expression levels of these miRNAs between alternate homozygotes at the major IPN resistance QTL. A total of ~355 M paired-ended reads (37 nucleotides long) were obtained from the 18 uninfected fry (data made available through the NCBI BioProject accession number SRP017393). Following the filtering procedures, a total of 888 robust pri-miRNAs were discovered and retained for further analysis (Bekaert et al. (2013)). Two particular miRNA families were highly abundant across all samples (miR-181 and miR-10); together accounting for >72 % of total expressed miRNAs (Figure 1).

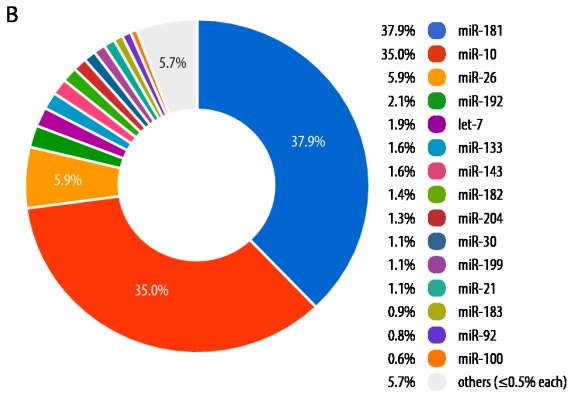

Figure 1. Distribution of miRNA abundance by miRNA family.
The top 15 miRNA families represent 94.3% of the expressed miRNA. All the 'others' (miRNA representing individually ≤ 0.5% of the global abundance) represents only 5.7% of the overall expressed miRNA.

Identification of miRNAs with a potential role in IPN resistance A cross-family comparison of the miRNA expression levels of infected fry homozygous for the QTL resistance allele and those homozygous for the QTL susceptible allele demonstrated that miR-4792 had a significantly higher expression (3.62 fold; $p<0.0006$) in resistant fish compared to susceptible fish during IPNV challenge.

Putative targets of differentially expressed miRNAs The miRNA exhibiting the most significant differential expression (miRNA-4792), was found to have two predicted target sequences. Alignment of these target sequences to the draft Atlantic salmon reference genome resulted in significant similarity to Contig_319304 and Contig_200509. A putative target gene of miRNA-4792 is *BNIP3* (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3), which encodes a pro-apoptotic protein. Bnip3 has been shown to regulate apoptosis in effector cytotoxic T lymphocytes (Wan et al. (2003)). Further, it has been demonstrated that Bnip3 can prompt apoptosis on viral entry (Cai et al. (2003)), and can therefore prevent viral proliferation. It is therefore possible that miR-4792 may play a role in IPNV resistance in salmon, possibly by regulating infection-induced apoptosis. Additional studies to further characterize this miRNA are underway.

Table 1. Details for the differentially expressed miRNA and its putative target gene.

ID	GE	DE	TGE	PG
miR-4792	Contig_061743	p<0.0006	Contig_319304	BNIP3

Details of the sequence and putative target of the miRNA demonstrated to be differentially expressed between IPN-resistant and –susceptible Atlantic salmon fry.
ID: miRNA identification
GE: Genome contig containing the miRNA gene sequence
DE: P Value of differential expression of the miRNA between QTL susceptible fry and QTL resistant fry
TGC: Target genome contig
PG: Putative target gene

Conclusion

Deep sequencing of IPNV-challenged and control fry has yielded an extensive resource of putative miRNA, their expression levels and putative targets. One miRNA (miR-4792) showed highly significant differential expression between fry homozygous for the resistance allele at the IPN QTL, and those homozygous for the susceptibility allele. The pattern of expression of this miRNA, and the biological role of its putative target gene, suggest it may form a component of the underlying functional mechanism of the QTL.

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