Small non-coding RNA expression status in animals faced with highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV)

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Summary

The commercial pig industry has long suffered economic losses due to infections from porcine reproductive and respiratory syndrome virus (PRRSV). The virus is easily spread among herds and in its lentogenic (low pathogenic) form leads to respiratory illness, reduced birthweights, stillborn piglets, sterility, and in its velogenic (high pathogenic) form death. It has been established that reduced susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV) has a genetic component that may take the form of small non-coding RNA (sncRNA) molecules that function as regulators of host and viral gene expression. In order to identify differences in sncRNA expression between healthy and highly pathogenic PRRSV (HP-PRRSV) challenged pigs, a transcriptomic analysis of porcine whole blood from control and infected pigs was examined for changes in expression profiles associated with the virus. The results revealed multiple classes of sncRNA were both present and differentially expressed during HP-PRRSV infection. This included classes, such as, small nucleolar and transfer RNAs previously only seen during other viral respiratory infections and cancer. By assessment of the expression changes in sncRNA during infections, researchers can move closer to a discernment of how the PRRSV virus disrupts host homeostasis and possibly uncover additional channels of detecting or destroying the virus.

Keywords: small non-coding, expression, viral, PRRSV, pigs

Introduction

The commercial pig industry has long suffered economic losses due to infections from porcine reproductive and respiratory syndrome virus (PRRSV) (Dietze, 2011; Lunney, Benfield, & Rowland, 2010; Neumann et al., 2005). The virus is easily spread among herds and in its lentogenic (low pathogenic) form leads to respiratory illness, reduced birthweights, stillborn piglets, and can cause sterility for those who recover. However, in its velogenic (highly pathogenic) form, infection often leads to death. The highly pathogenic form of PRRSV was first discovered in Chinese viral isolates where it is endemic and can be distinguished from the low pathogenic by a large deletion of non-structural protein 2 (NSP2) (Dietze, 2011; Kappes & Faaberg, 2015). Over time, researchers have made strides in the uncovering of differentially expressed host mRNAs present during the increased pathogenicity of the Chinese isolate (HP-
PRRSV) that show similar innate immune system invasion as the lentogenic strains, but a much more far-reaching effect on genes related to antiviral defense and pro-inflammatory signaling (Kommadath et al., 2017; Lunney et al., 2010; Miller et al., 2012; Miller et al., 2017; Xiao et al., 2010). Currently there is still very little known about how the virus interacts with the host immune system and the changes in transcriptional profiles accompanied with infection. One possible method HP-PRRSV may employ to boost its viral infectivity and replication is suppression of host immune functions through the dysregulation of small non-coding RNA (sncRNA) homeostasis. Recent PRRSV studies have shown that sncRNAs, in the form of miRNAs (Liu, Du, & Feng, 2017), effect the host immune response to PRRSV infection, in some cases helping the host respond (Li et al., 2015; C. Wang et al., 2016; J. Wang et al., 2016; Zhang et al., 2014) and in others, facilitating the virus (Xiao et al., 2016). Therefore, a better understanding of the role sncRNAs play in host-virus interactions could provide alternative means of supporting homeostasis and recovery of HP-PRRSV infected pigs. In general, sncRNAs are considered to be non-protein coding genomic sequences shorter than 200nt in length (Bartel, 2004; Chan & Lowe, 2016; Kozomara & Griffiths-Jones, 2014; Mattick, 2009; Ro-Choi, 1997; Tuck & Tollervey, 2011). They are best represented by their most abundant classes in eukaryotes, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and micro RNAs (miRNAs), but also include smaller groups such as small nucleolar RNAs (snoRNAs) and the fragments of tRNAs (tRFs) and snoRNAs (sdRNAs) (Bartel, 2004; Massirer & Pasquinelli, 2006; Mattick, 2009; Phizicky & Hopper, 2010; Tuck & Tollervey, 2011). Most studies of sncRNA involvement in host-viral interactions has mainly examined the roles played by miRNA because of their ubiquitous role as silencers of gene expression and their ability to behave in an antiviral nature by altering critical viral processes like replication (Bartel, 2004; Liu et al., 2017; Mattick, 2009; Samir & Pessler, 2016; Slonchak, Shannon, Pali, & Khromykh, 2015). Other sncRNA classes such as small nucleolar (snoRNA) and transfer RNA (tRNA) gene expression has been largely ignored as classes of RNA with potential immune functions. This lack of inclusion may be due to the nature of their copy numbers which causes them to be redundantly encoded and produced from multiple genomic locations (Chan & Lowe, 2016; Iben & Marais, 2014). However, other classes of sncRNA like tRNAs and snoRNAs can be fragmented into miRNA-like molecules capable of affecting host immune functions. Therefore, expression profiling of other sncRNA classes is a necessary step in understanding how some sncRNAs can act as possible “agents of change” that can cause silencing or enhancement by their binding (Anthon et al., 2014; Gebetsberger & Polacek, 2013; Martens-Uzunova, Olvedy, & Jenster, 2013; Massirer & Pasquinelli, 2006; Venkatesh, Suresh, & Tsutsumi, 2016). By studying and comparing the expression changes in non-coding RNA during infections, we can move closer to a discernment of how the PRRSV pathogen disrupts host homeostasis and possibly uncover additional means of detecting or destroying the virus.

Material and methods

Animals and sample collection

The experimental design called for the collection of whole blood samples (~2.5 ml/pig) at 3 time points (1, 3, 8 dpi) by jugular venipuncture from 9-week old male pigs. Control animals were given a sham inoculation (2ml/pig) while animals challenged with HP-PRRSV received isolate rJXwn06 (10^4 TCID_{50}/ml, 2 ml/pig). Samples were randomized and 12 infected
and 12 non-infected samples were chosen for total RNA extraction and sncRNA library creation.

**RNA isolation and sequencing**

Total RNA was extracted from 2.5 ml whole blood samples using a modified miRNA extraction kit protocol optimized according to Taxis *et al.*, 2017. Extracted RNA was globin depleted to account for high levels of globin transcripts using porcine specific hemoglobin A and B (HBA and HBB) oligonucleotides based on the procedure from Choi *et al.*, 2014. Prior to library creation sample quality was checked using the Agilent Bioanalyzer 1000 which showed the total RNA quality ranged from a RIN # of 6.5 – 9.2 and 260/280 nm concentrations ratios were at or above 2 for all samples after globin reduction prior to library preparation. The globin reduced total RNA samples were then used for library preparation and 100 bp single-end sncRNA sequence generation using the illumina Hiseq 3000.

**Transcriptomic analysis**

Prior to differential expression (DE) analysis all reads were quality assessed using FastQC and TrimGalore ("www.bioinformatics.babraham.ac.uk,")) to remove adaptors and barcodes from multiplexing. Reads with a quality score below 38 and length less than 18 nucleotides were discarded. A total of 24 sequences were generated for downstream analysis. The sequenced reads were mapped to the S.scrofa 10.2 reference genome using the Hisat2 (Kim, Langmead, & Salzberg, 2015) package and annotated using FeatureCounts (Liao, Smyth, & Shi, 2014) and an in-house created sncRNA GTF file. Differential expression analysis of the data was implemented using DeSeq2 (Love, Huber, & Anders, 2014) in which the main effects of treatment (control/HP-PRRSV) and time (1,3,8 dpi) were examined along with their interaction. Statistical significance was based on a multiple test corrected q-value of ≤ 0.1 (10% FDR) with no fold change threshold applied. All software package analyses were performed using default parameters. Final results are based on the reduced model of ~Treatment

\[\text{Control} + \text{Time}_{1,3,8}\]  and the values (logFC) solely represent the difference between treatments in the presence of time. We chose to only present this level of results for the proceedings, however the experiment did examine the effects of the interaction of treatment and time, as well as, examined the change in expression within the infected samples across each dpi.

**Results**

Of the contrast of the 2 treatments (infected vs. control), only 76 sncRNAs met the statistical threshold for significance (FDR 0.1). The largest downregulated fold changes were recorded for trna1637-AlaCGC (-1.26) and ssc-mir-532 (-1.16) and the largest upregulated expression changes were recorded for the sncRNAs trna19-AlaAGC (1.16) and trna1021-GlyCCC (1.14). By class, the largest represented groups were the miRNA(n=28), tRNA(n=25), and the snoRNAs (n=20). The snoRNA results include 3 distinct groups (C/D box, H/ACA, and small cajal RNAs) grouped into one classification in which the C/D box snoRNAs (n=10) were the most abundant within the class. Of the 10 largest up or down regulated fold changes, none were greater than 1.5 logfc. This result is likely due to the expression being normalized across all 3 dpi in order to observe the overall change based on infection status. Table one shows the 10 genes that were most differentially expressed between the contrast of the HP-PRRSV infected
pigs and the controls. Though many of the functions or targets of these sncRNA are unknown, some such as ssc-mir-222, ssc-mir-532, and trna1021-GlyCCC, have been implicated in host-viral interactions (Table 1.). The interaction of treatment and time was the most significant between groups by day, however those will be presented in a later manuscript.

**Discussion**

Although further examination is needed to understand the course of action that ties sncRNA DE to the host immune response to HP-PRRSV, the results do lend insight into some possible mechanisms. The downregulated miRNAs ssc-mir-222 and ssc-mir-532 both have pivotal roles in battling viral infection by acting as suppressors of inflammatory cytokines and viral replication respectively. Because of the ability of HP-PRRSV to subvert host innate immune functions, downregulation of ssc-mir-222 within the host may be an attempt to initiate an inflammatory response against HP-PRRSV. Downregulation of ssc-mir-532 seems to favor HP-PRRSV infection in this case by allowing for less regulation of viral proliferation. This downregulation may be essential for HP-PRRSV survival and if so, overexpression of this miRNA might help to stimulate an immune response. In regards to the tRNA, expression may vary at the anticodon position and also appear redundant in the amino acid isotype being expressed to allow for more diversity of the anticodon being expressed. It is also possible that the host is trying to prevent the virus from using certain tRNAs to modify translation and promote viral protein synthesis. Lastly, the examination of DE snoRNAs proved to have no current standing in the host response to PRRSV infections, however their abundance and variable expression may be linked to perturbations in the homeostasis of the mRNA genes that generate them. Overall, the DE sncRNAs that were detected were mostly downregulated across all of the represented classes of non-coding RNA. This propensity towards downregulation could point to markers of homeostatic dysregulation of the porcine immune system.

**Table 1. List of differentially expressed sncRNAs during HP-PRRSV infection.**

<table>
<thead>
<tr>
<th>Class id</th>
<th>Gene id</th>
<th>Logfc</th>
<th>Immune involvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA</td>
<td>trna19-AlaAGC</td>
<td>1.16</td>
<td>Possible marker of cellular stress response</td>
<td>Maraiya &amp; Arimbasseri <em>et al.</em>, 2017</td>
</tr>
<tr>
<td>tRNA</td>
<td>trna1021-GlyCCC</td>
<td>1.14</td>
<td>Possible involvement in viral respiratory infections</td>
<td><em>Zhou et al.</em>, 2017; <em>Samhita et al.</em>, 2013</td>
</tr>
<tr>
<td>C/D box snoRNA</td>
<td>SNORD14 (U14)</td>
<td>1.11</td>
<td>Involved in heat stress response in rats</td>
<td><em>Stallings et al.</em>, 2014</td>
</tr>
<tr>
<td>tRNA</td>
<td>trna750-ThrCGT</td>
<td>1.10</td>
<td>Possible viral sequence, Intronic tRNA</td>
<td><em>Chan &amp; Lowe et al.</em>, 2016</td>
</tr>
<tr>
<td>miRNA</td>
<td>ssc-mir-374a</td>
<td>0.97</td>
<td>Expression enhances cancer cell proliferation</td>
<td><em>Xu et al.</em>, 2015</td>
</tr>
<tr>
<td>C/D box snoRNA</td>
<td>miRNA ssc-mir-222</td>
<td>tRNA trna1635-AlaAGC</td>
<td>tRNA trna1647-AlaCGC</td>
<td>tRNA trna1700-ValAAC</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>SNORD60 (U60)</td>
<td>ssc-mir-222</td>
<td>trna1635-AlaAGC</td>
<td>trna1647-AlaCGC</td>
<td>trna1700-ValAAC</td>
</tr>
<tr>
<td>0.93</td>
<td>-1.07</td>
<td>-1.09</td>
<td>-1.13</td>
<td>-1.15</td>
</tr>
</tbody>
</table>

- **Cholesterol trafficking in cells**
  - Brandis et al., 2013

- **Negative regulation of cytokine production involved in inflammatory response (GO:1900016)**
  - Negative regulation by host of viral genome replication (GO:0044828)
  - Zhu et al., 2011; http://amigo.geneontology.org/

- **Possible marker of cellular stress response**
  - Maraia & Arimbasseri et al., 2017

- **Possible marker of cellular stress response**
  - Maraia & Arimbasseri et al., 2017

- **Possible marker of cellular stress response**
  - Maraia & Arimbasseri et al., 2017

- **Possible antiviral response**
  - Slonchak et al., 2015

### List of References


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