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MicroRNAomes of Porcine Arterial and Venous Blood

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Abstract: Blood plays an important role in many biological processes and circulating microRNAs (miRNAs) in the blood have been reported to be biomarkers for various physiological and pathological states. However, few studies have examined porcine blood miRNAs, especially arterial blood miRNAs. Here, researchers sequenced the microRNAomes of porcine arterial and venous blood samples in which we identified 277 and 294 known porcine miRNAs, respectively. In both blood samples, highly expressed miRNAs were implicated in erythropoiesis and angiogenesis. A total of 57 miRNAs showed significantly different expression levels ($p<0.01$) between arterial and venous blood of which seven were enriched in arterial blood and five were enriched in venous blood by more than a 1.5 fold change. Interestingly, functional enrichment analysis of the genes predicted to be targeted by the enriched miRNAs indicated that arterial blood was mainly associated with the immune response and venous blood was primarily associated with apoptosis and the hypoxia response. The results suggest that the 12 miRNAs with differential expression should be considered as biomarkers for physiological differences between arterial and venous blood and that they may help promote the further molecular diagnosis of blood.

Key words: Arterial blood, miRNAs, pig, small RNA-sequencing, venous blood

INTRODUCTION

Blood is a vital animal body fluid that delivers essential substances such as nutrients and oxygen and removes carbon dioxide and other metabolic waste products from the cells of the body. In addition, the blood can also transport hormones and signals, regulate core body temperature and help to fight infection in open wounds. According to differences in oxygen content, blood can be divided into two kinds, venous blood and arterial blood. Venous blood is typically colder than arterial blood and has a lower pH, a lower concentration of glucose and other nutrients but a higher concentration of urea and other waste products (Forster *et al*., 1972).

Various nucleic acids that are readily accessible in the blood play a critical role in a wide range of physiologic and pathologic processes (Bremnes *et al*., 2005). MicroRNAs (miRNAs), small non-coding nucleic acids, present in the blood have spar (Wu *et al*., 2007) ked interest recently because of their potential use as biomarkers. Increasing evidence has shown that the bioinformatic analysis of miRNA profiles enables discrimination between whole blood samples of cancer patients and healthy controls with >70% accuracy (Hausler *et al*., 2010; Huang *et al*., 2010; Roth *et al*., 2011). However, little is known of the miRNA expression profile in arterial blood and the differences between arterial and venous blood have yet to be analyzed (Dai *et al*., 2007; Di Stefano *et al*., 2011; Keller *et al*., 2011). The identification of differentially expressed miRNAs between arterial and venous blood would however be beneficial for further molecular diagnosis based on blood.

Here, researchers present a comprehensive analysis of miRNA expression profiles between pig arterial and venous blood based on small RNA-sequencing. Researchers identified specific miRNAs associated with different functions between arterial and venous blood which could be considered as biomarkers to distinguish the two blood types.

MATERIALS AND METHODS

Blood samples collection and RNA isolation: Arterial and venous blood samples (5-10 mL per piglet) were collected from three healthy female piglets aged at 30 days. After been anticoagulant treatment, all blood samples were aliquoted into centrifuge tubes and stored at -80°C. Total RNA was isolated from arterial and venous blood samples using TRIzol Reagent (Takara, Dalian, China) according to the manufacturer's protocol. The RNA quality was determined using formaldehyde denaturing gel electrophoresis.

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Small RNA libraries construction and sequencing: Qualified RNA was prepared for sequencing samples as follows: equal amounts (5 μg) of total RNA was isolated from three individual arterial blood samples were pooled, approximately 15 μg of total RNA was used for library construction, the same as venous blood sample. A pair of Solexa adaptor was ligated to the 5' and 3' ends of the small RNA fraction (10-40 nt) which was isolated by 15% Polyacrylamide Gel Electrophoresis (PAGE). The small RNA was then converted to cDNA and amplified by RT-PCR. Subsequently, the enriched cDNA was sequenced on Genome Analyzer Instrument (GAI, Illumina, San Diego, CA, USA).

Sequencing data analysis and identification of miRNAs: According to the method describe by Li *et al.* (2010), the approach to process raw data reads were modified after processing with Illumina's Genome Analyzer Pipeline Software. The raw data reads were generated after applying a series of additional filters: reads without the 3' adaptor, 5' adaptor-contaminant; being longer than 16 nt and shorter than 29 nt; not be junk reads (<2N, <7A, <8C, <6G, <7T, <10 dimer, <6 trimer or <5 tetramer); not matching to porcine known classes of RNAs in the NCBI (Pruitt *et al.*, 2012), Rfam (Gardner *et al.*, 2009) and Repbase database (Kohany *et al.*, 2006); been observed at least two times. The reads passed the criteria were called high-quality reads. The high-quality reads were then mapped to the pig genome (Sscrofa 10.2) using the NCBI local BLAST package (http://blast.ncbi.nlm.nih.gov/). First, map the high-quality reads to porcine known miRNAs and pre-miRNAs in miRBase 19.0. The mappable reads were then mapped to pig genome to obtain their genomic locations and annotations.

Prediction and functional annotation of miRNA target genes: Due to the highly functional conservation of miRNAs and the absence of the porcine mRNA-miRNA interactive algorithm in current version, miRNA target predictions were performed by the online databases of PicTar and TargetScan human 6.2 based on human mRNA-miRNA interactions. The overlaps of results from two databases composed the final predicted targets. The Gene Ontology Biological Process (GO-BP) terms and KEGG pathway terms enriched in predicted target genes were determined using the online DAVID bioinformatics resources, the terms and pathways giving statistically significant values ($p<0.05$) were chosen for further analysis.

Q-PCR validation: All 12 different expressed miRNAs were selected to validate the sequencing results by q-PCR on the CFX96™ Real-time PCR Detection System (Bio-Rad, CA, USA). For q-PCR experiments, three biological replicates mentioned above were used and each RNA sample was analyzed in triplicate. RNA was converted to cDNA by using SYBR® PrimeScript™ miRNA RT-PCR kit (Takara, Dalian, China) and amplified according to the manufacturer's instructions. Porcine U6 snRNA, 5S rRNA and 18S rRNA were simultaneously used as endogenous control genes. Relative expression levels of objective miRNAs were calculated using the ΔΔCt Method.

RESULTS AND DISCUSSION

Description of small RNA sequencing data: A total of 5.21 Million (M) and 8.61 M counts of sequenced sequences were obtained from pig arterial and venous blood, respectively. The >78% of counts met the accepted criteria to be considered high-quality reads). The length distribution of high-quality reads peaked at 22 nucleotides (nt) (arterial blood: 36%; venous blood: 31%) followed by 21 and 23 nt (Fig. 1a) which is consistent with

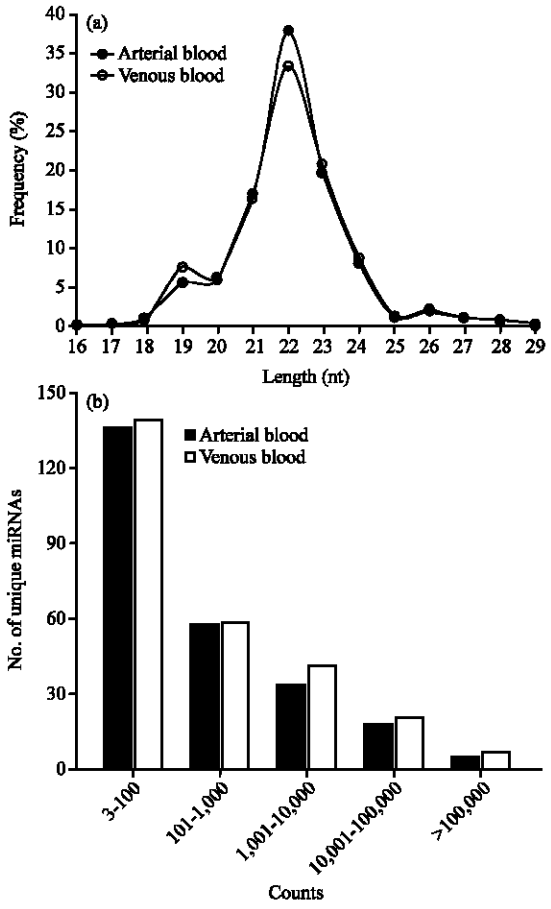

Fig. 1: The length and count distribution of small RNA sequences; a) The length distribution and frequency percentage of high-quality reads; b) Distribution of read counts of known porcine miRNAs in both libraries

the known 21-23 nt range for porcine miRNAs (Li *et al*., 2010). Hence, the data sets obtained from small RNA-sequencing of arterial and venous blood were deemed reliable for further analysis.

After collating and mapping sequences to the miRBase 19.0 and pig reference genome, researchers identified 250 and 267 known porcine miRNAs corresponding to 277 and 294 precursor miRNAs (pre-miRNAs) in arterial and venous blood, respectively. To date, the miRBase 19.0 has documented 306 known porcine miRNAs corresponding to 271 pre-miRNAs. This illustrates that the arterial and venous blood encompass almost the entire repertoire of previously known miRNAs (arterial blood: 90.52%; venous blood: 96.08%) which is as expected when the blood participates in various biochemistry pathways and material metabolism. These results add to the growing evidence that miRNAs are not only found in solid tissues but also in body fluids such as blood which have been regarded as sources of free circulating nucleic acids (Mitchell *et al*., 2008; Takeshita *et al*., 2013; Tan *et al*., 2009).

Highly expressed miRNAs were implicated in erythropoiesis and angiogenesis: The miRNAs showed a broad range of expression levels in this study although, only a few were highly abundant (Fig. 1b). The top 20 unique miRNAs with high expression levels accounted for 85.35 and 84.75% of the total counts in arterial and venous blood, respectively. Among the top 20 most abundant miRNAs, 17 were present in both libraries (Fig. 2a).

Because of their very high abundance, the 17 miRNAs might be blood-specific and be of a housekeeping cellular role. The miR-451 and miR-144 well-characterized and blood-specific miRNAs in human and zebrafish are known to be essential for erythropoiesis (Dore *et al*., 2008). Moreover, the miRNA-144/451 locus enhances erythroid differentiation, modulates the rate of erythrocyte maturation (Bruchova-Votavova *et al*., 2010; Pase *et al*., 2009), protects erythrocytes against oxidant stress (Yu *et al*., 2010) and maintains erythroid homeostasis (Rasmussen *et al*., 2010). With the exception of miRNA-144/451, miR-15b (Lawrie, 2010), miR-16 (Bruchova *et al*., 2007), miR-191 (Ji *et al*., 2011; Zhang *et al*., 2011) and miR-142 (Chen *et al*., 2004; Sun *et al*., 2010a, b) have all been implicated in erythropoiesis. In human erythroid cells, miR-486 regulates γ-globin expression and might contribute to fetal Hemoglobin (HbF) modulation. MiR-92a, a member of the miR-17-92 cluster, regulates vascular endothelial cell hyperplasia *in vivo* and is necessary for stabilizing blood vessels (Fang and Davies, 2012; Kuhnert and Kuo, 2010) while the let-7 family are attractive targets fo modulating angiogenesis (Ding *et al*., 2013; Kuehbacher *et al*., 2007). Furthermore, four of the top 20 miRNAs accounting for 7.30 and 6.79% of all unique reads in arterial and venous blood, respectively are from the let-7-family (let-7a-5p, let-7f-5p, let-7g-5p and let-7i-5p). This is consistent with previous reports that show the let-7-family are both highly and ubiquitously expressed (Reddy *et al*., 2009; Roush and Slack, 2008) and might be

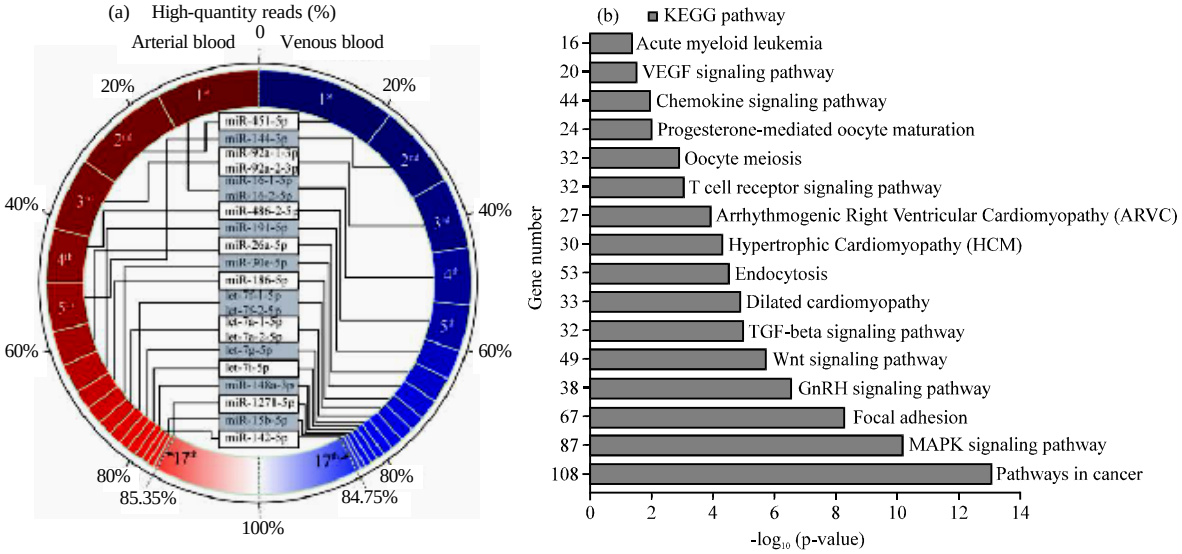

Fig. 2: The top 20 miRNAs and KEGG pathways enriched for miRNA targets; a) Plot of the top 20 miRNAs with highest read counts versus their percentage total counts of high-quality reads; b) KEGG enrichment analysis using targets of 17 most abundant miRNAs in both libraries

the most important miRNA regulators of fundamental biological processes (Abbott *et al*., 2005; Ason *et al*., 2006).

To better understand the function of the most abundant miRNAs, the target genes of the 17 miRNAs were predicted using PicTar (Krek *et al*., 2005) and TargetScan human 6.2 databases (Lewis *et al*., 2003), the overlap of results from both databases was used tocompose the final predicted targets. The predicted target genes were analyzed using DAVID Software (Huang *et al*., 2008) to determine whether they were enriched in specific pathways. According to KEGG pathway annotation, the enriched pathways, i.e., focal adhesion, VEGF signaling, MAPK signaling, Wnt signaling and TGF-beta signaling were previously reported to be related to erythropoiesis (Geest and Coffer, 2009; Nostro *et al*., 2008; Vemula *et al*., 2010) and angiogenesis (Goodwin and D'Amore, 2002; He *et al*., 2006; Shen *et al*., 2005), highlighting the implication of these most abundant miRNAs in the two processes. The GnRH signaling, progesterone-mediated oocyte maturation and oocyte meiosis pathways but not spermatogenesis were also enriched (Fig. 2b) which is consistent with the gender characteristics of the research objects (female piglets). This indicates that these abundant miRNAs may participate in sexual maturation (Tesfaye *et al*., 2009).

Furthermore, endocytosis, T cell receptor signaling, Fc epsilon RI signaling and chemokine signaling pathways were also enriched, conforming to the defense function of the blood. The data thus highlighted the pathways related to cancer and cardiovascular diseases, indicating that abnormal miRNA expression in the blood could accurately reflect a pathological state. Taken together, the results and those of previous studies support a relationship between a high abundance of miRNAs and blood physiology.

Differentially expressed miRNAs between arterial and venous blood: To further reveal the differences between arterial and venous blood, the IDEG6 Program (Romualdi *et al*., 2003) was used to test the significance of differences in miRNA expression between arterial and venous blood. A unique miRNA was considered to be Differentially Expressed (DE) when it obtained $p<0.001$ simultaneously following three statistical tests (Audic-Claverie test, Fisher's exact test and the Chi-squared 2×2 test) with the Bonferroni correction.

By applying this criterion, 57 miRNAs were identified to be DE between arterial and venous blood but only five (miR-18a, miR-21, miR-99a, miR-144 and miR-7) (Fig. 3a) and seven (miR-423, miR-16, miR-181a, miR-425, miR-151, miR-192 and miR-142) (Fig. 3b) were significantly enriched in venous and arterial blood, respectively meeting the 1.5 fold changes. All 12 DE miRNAs were selected and validated using q-PCR and the comparison between q-PCR and sequencing results showed a significant positive correlation (Person's $r = 0.881$, $p<10^{-3}$), again highlighting the reliability of the small RNA-sequencing approach. Moreover, the three biological replicates were highly correlated with a variable coefficient <0.2, suggesting that the q-PCR approach has high repeatability and reliability, making it possible to pool the samples during the sequencing process. The enrichment of miR-21 and miR-7 in venous blood probably resulted from the low oxygen pressure in venous blood both miRNAs are hypoxia-regulated and are up-regulated in hypoxic cancer cells (Kulshreshtha *et al*., 2008; Sun *et al*., 2010a, b). Of the seven miRNAs enriched in arterial blood, three were shown to be involved in immune function and inflammation. MiR-181a is implicated in T cell receptor signaling by augmenting T cell receptor sensitivity (Li *et al*., 2007; Wu *et al*., 2007) as well as establishing and maintaining the fate of immune cells (Chen *et al*., 2004). MiR-16 and miR-142 are involved in myeloid or lymphoid differentiation and ectopic expression of miR-142 was found to substantially alter lineage differentiation within the T cell compartment (Chen *et al*., 2004). Moreover, the down-regulation of miR-16 during T cell apoptosis was required for the increased proliferation of activated T cells (Wu *et al*., 2007).

To further highlight the functional features of arterial and venous blood, the target genes of the 12 DE miRNAs were predicted and analyzed by DAVID. The GO terms transcription regulation, intracellular signaling cascade, macromolecule biosynthetic processes, cellular biosynthetic process and phosphorus metabolic process were enriched in both blood (Fig. 3c and d). Interestingly, the regulation of cell death, regulation of programmed cell death and regulation of apoptosis terms were only enriched in venous blood (Fig. 3c) which conforms to its function in removing unwanted harmful and abnormal cells from tissues. The target genes of venous blood-enriched miRNAs were enriched in hypoxia-regulated adipocytokine signaling, TGF-beta signaling and mTOR signaling pathways, highlighting the lower oxygen level in venous blood. Since, low oxygen is one of the reasons for cells to undergo apoptosis, researchers speculate that venous blood miRNAs play a role in apoptosis through their involvement in the low oxygen response. However, the target genes of arterial blood-enriched miRNAs were strongly associated with immune response pathways such as T cell receptor signaling, B cell receptor signaling, MAPK signaling and insulin signaling (Fig. 3d).

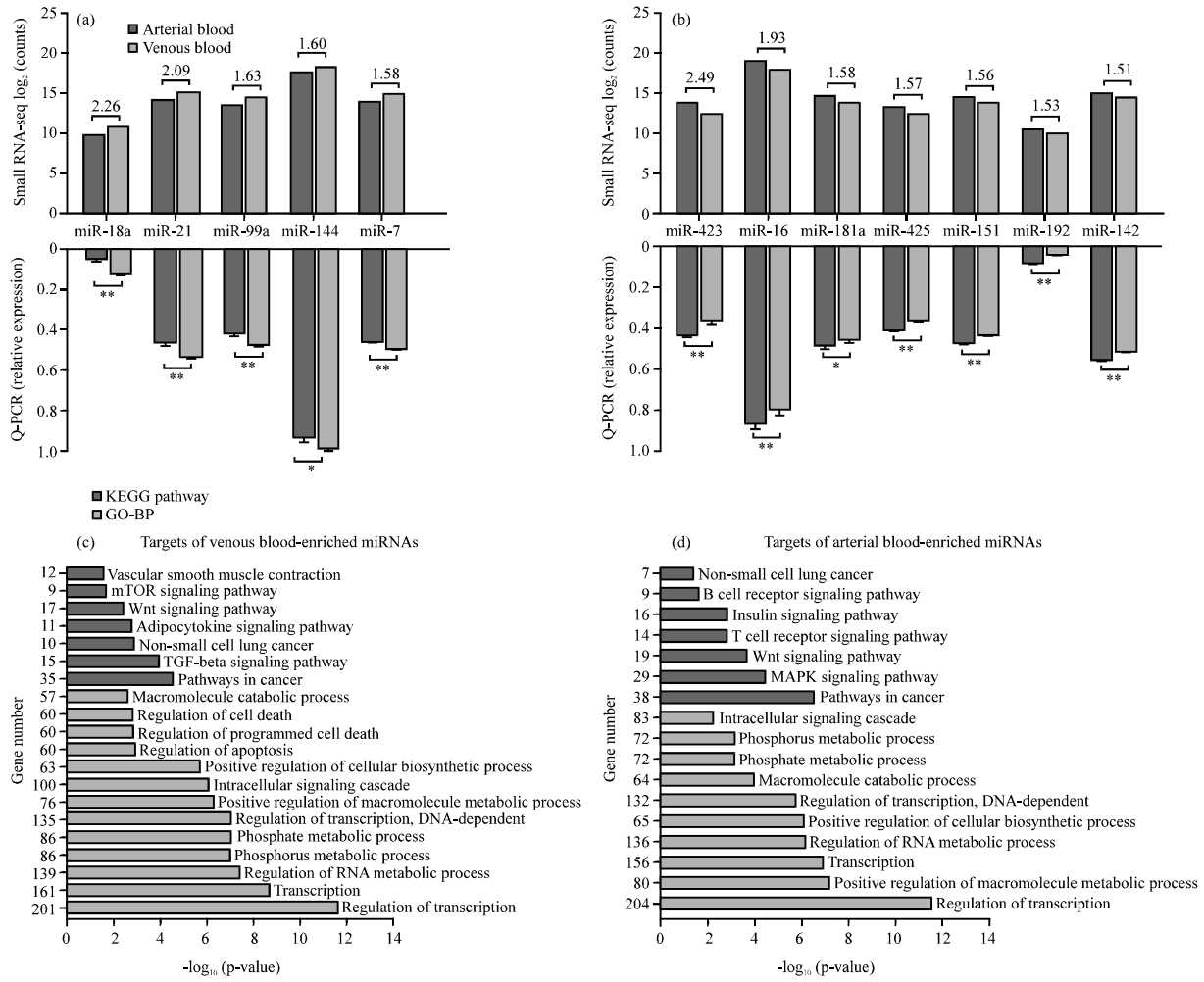

Fig. 3: Expression levels of 12 different expression miRNAs and the KEGG pathway and Gene Ontology Biological Process (GO-BP) categories enriched in the target genes of the miRNAs; a) Q-PCR validation and small RNA-seq results of the five venous blood-enriched miRNAs; b) seven arterial blood-enriched miRNAs; c) Q-PCR results are expressed as mean±SD. *p<0.05; **p<0.01. KEGG pathways (gray) and GO-BP (black) categories enriched for the target genes of the five venous blood-enriched miRNAs and d) seven arterial blood-enriched miRNAs

CONCLUSION

The annotations for the predicted targets indicated that physiological and functional differences between porcine arterial and venous blood were regulated by miRNA regulators.

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