Plasma MicroRNA, a Potential Biomarker for Acute Rejection After Liver Transplantation

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Background. Acute rejection (AR) of an organ transplant is a life-threatening complication. Currently, there are few diagnostic biomarkers suitable for clinical application. We aim to determine the potential of plasma microRNAs as biomarkers for AR.

Methods. Using rat orthotopic liver transplantation model and microarrays, we compared the difference in the spectrum and levels of microRNAs in both plasma and grafts between AR rats and control. AR-related plasma microRNAs were selected and validated using real-time quantification polymerase chain reaction. To clarify the origin of AR-related plasma microRNAs, drug-induced liver damage rat model were performed and in situ hybridization was used to detect and localize the specific microRNA in allografts.

Results. We found that plasma miR-122, miR-192, and miR-146a was significantly up-regulated when AR occur (fold change=22.126; P=0.002) and the elevation could be repressed by immunosuppression. In liver injury rat model, up-regulated plasma miR-122 (fold change=22.126; P=0.002) and miR-192 (fold change=8.833; P=0.001) rather than miR-146a (fold change=1.181; P=0.594) were observed. Further study demonstrated that miR-146a was up-regulated by sifoxin in microvesicles isolated from AR plasma, whereas miR-122 and miR-192 showed no distinct change. In situ hybridization revealed that the portal areas of the AR graft were brimming with lymphocytes, which showed highly intense staining for miR-146a.

Conclusions. Our study provides the global fingerprint of plasma microRNAs in AR rats and suggests that plasma miR-122 and miR-192 reflect liver injury, whereas miR-146a may associate with cellular rejection.

Keywords: MicroRNAs, Plasma, Acute rejection, Liver transplantation, Rat model.

Orthotopic liver transplantation (OLT) is the first and best choice for patients with end-stage liver disease. Acute rejection (AR) is one of the main factors that cause allograft dysfunction, although patients receive lifelong treatment with multiple nonspecific and toxic immunosuppressive drugs (4, 5). The early detection of AR and subsequent timely intervention are critical for saving the graft and increasing the organ lifespan.

Currently, liver biopsy is the most frequently used technique to evaluate allograft status and is the gold standard for the diagnosis of AR after OLT. Although liver biopsy is generally safe, rare complications, sampling error, and significant patient anxiety do occur. Furthermore, histomorphologic changes do not occur early in AR. A timeline model of allograft rejection clearly demonstrated that the development of

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30972949 and 81172277), the Shanghai Key-Tech Research & Development Program (Grant No. 09411951700), the National Science Foundation for Distinguished Young Scholars of China (Grant No. 81225019), and the Program of Shanghai Excellent Subject Leaders (Grant No. 10XD1401200).

The authors declare no conflicts of interest.

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AR is a continuum, with the initial events being molecular perturbations after histomorphologic changes and clinical manifestations occurring relatively late in the disease timeline (6). Nevertheless, treatment efficiency may decrease if initiated later in the course of the disease (see Figure S1, SDC, http://links.lww.com/TP/A787). The theory that early intervention is efficacious is the rationale for the development of molecular surveillance strategies that can anticipate histologic and clinical rejection (7). Therefore, studies that focus on circulating molecular biomarkers associated with AR are urgently needed.

MicroRNAs, small regulatory noncoding RNAs, can affect the stability of mRNA and the initiation and progression of protein translation (8, 9). MicroRNAs regulate the expression of a vast array of genes, including those involved in adaptive immunity, and play important roles in the mammalian immune system (10). Anglicheau et al. (5) screened 33 renal biopsies by microRNA arrays and real-time quantification polymerase chain reaction (RT-qPCR) and found that AR and renal allograft function can be predicted with a high level of precision by microRNA levels in graft. Wang et al. (11) examined the plasma microRNA expression profiles in a drug-induced liver injury mouse model, and their findings suggest the potential use of specific circulating microRNAs as sensitive and informative biomarkers of liver injury. Wang et al.’s result was later validated with human samples in another study (12).

In the current study, we first adopted a rat OLT model using Brown Norway (BN) rats that received OLT with liver graft from Lewis rats (Lewis-to-BN), OLT with BN rats as the donors and recipients were performed and used as control (nonrejection [NR] group). Then, global microRNA expression profiles of the plasma and graft were evaluated and validated with high-throughput microarray and RT-qPCR. Another AR model system (OLT with ACI-to-Lewis and OLT with BN-to-Lewis) were used to validate our findings. Furthermore, we conducted a preliminary and exploratory study on the origin of AR-related plasma microRNA.

**RESULTS**

**Global MicroRNA Expression in OLT Rat**

We first analyzed the global microRNA expression in the plasma and graft from AR (Lewis-to-BN) and NR (BN-to-BN) donors and recipients were performed and used as control (nonrejection [NR] group). Then, global microRNA expression profiles of the plasma and graft were evaluated and validated with high-throughput microarray and RT-qPCR. Another AR model system (OLT with ACI-to-Lewis and OLT with BN-to-Lewis) were used to validate our findings. Furthermore, we conducted a preliminary and exploratory study on the origin of AR-related plasma microRNA.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Liver graft</th>
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<tbody>
<tr>
<td>microRNAs</td>
<td>FC (log₂)</td>
</tr>
<tr>
<td>rno-miR-128</td>
<td>−3.93</td>
</tr>
<tr>
<td>rno-miR-328</td>
<td>−3.80</td>
</tr>
<tr>
<td>rno-miR-126*</td>
<td>−3.24</td>
</tr>
<tr>
<td>rno-miR-223</td>
<td>−3.07</td>
</tr>
<tr>
<td>rno-miR-92a</td>
<td>−2.24</td>
</tr>
<tr>
<td>rno-miR-20a</td>
<td>−2.22</td>
</tr>
<tr>
<td>rno-miR-23a</td>
<td>−1.88</td>
</tr>
<tr>
<td>rno-miR-130b</td>
<td>−1.67</td>
</tr>
<tr>
<td>rno-miR-27a</td>
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</tr>
<tr>
<td>rno-miR-484</td>
<td>−1.58</td>
</tr>
<tr>
<td>rno-miR-19a</td>
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<tr>
<td>rno-miR-26a</td>
<td>−1.26</td>
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<tr>
<td>rno-miR-24</td>
<td>−1.16</td>
</tr>
<tr>
<td>rno-miR-19b</td>
<td>−1.06</td>
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<tr>
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<tr>
<td>rno-miR-146a</td>
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</tr>
<tr>
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<td>rno-miR-139-3p</td>
<td>2.10</td>
</tr>
<tr>
<td>rno-miR-192</td>
<td>3.20</td>
</tr>
</tbody>
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The common microRNAs that changed in both tissue and plasma are boldfaced.

FC, fold change, NR, nonrejection.
rators. All microarray data were registered into the National Center for Biotechnology Information’s Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/) with the accession number of GSE36798. Agilent microRNA microarrays detected 133 expressed microRNAs in the plasma samples (Fig. 1A).

**Discovery of Candidate MicroRNAs and Clustering Analysis**

Microarray analysis indicated that 24 microRNAs (fold change>2; \(P<0.05\)) were differentially expressed in the plasma of AR (Lewis-to-BN) versus NR (BN-to-BN) rats. Of the 24 differential microRNAs, 9 were up-regulated and 15 were down-regulated in the AR group compared with the NR group. On the contrary, 54 microRNAs (fold change>2; \(P<0.05\)) were differentially expressed in the graft between the two groups. Of these, 33 were up-regulated and 21 were down-regulated in the AR group compared with the NR rats (Table 1). Hierarchical clustering analysis indicated that the selected differentially expressed microRNAs could clearly separate AR rats from NR rats by both the plasma and graft results (Fig. 1B,C). Inspection of the differentially expressed microRNAs in the plasma and grafts revealed nine microRNAs that changed significantly by AR in both the plasma and graft (Table 1; Fig. 1D). Of the nine differentially expressed microRNAs, eight showed reciprocal changes in the graft and plasma (Fig. 1D). miR-122 and miR-192 were up-regulated in the plasma but down-regulated in the graft, whereas miR-223, miR-23a, miR-27a, miR-328, miR-128, and miR-130b were down-regulated in the plasma but up-regulated in the graft. miR-146a was the only microRNA up-regulated in both the plasma and the liver graft. We selected three microRNAs (miR-122, miR-192, and miR-146a) that were elevated in the plasma of AR rats for further study.

**Target Genes of miR-122, miR-192, and miR-146a**

The changes of microRNA in the graft/plasma could be due to AR-induced cellular responses. We searched for target genes of miR-122, miR-192, and miR-146a in gateway miRecords (http://mirecords.biolead.org/) and mapped the validated target genes in the KEGG pathway database (http://www.genome.jp/kegg/tool/search_pathway.html). We found that the target genes of miR-122 and miR-192 exert important modulatory effects on metabolism-related signal transduction pathways and associated factors, including vascular endothelial growth factor signaling, osteoclast differentiation, metabolic pathways, carbohydrate digestion and absorption, tight junctions, insulin signaling, and cell adhesion molecules. On the contrary, the targets of miR-146a played an important role in immune-related pathways, including cytokine-cytokine receptor interactions, Toll-like receptor signaling, chemokine signaling, and leukocyte transendothelial migration (see Table S1, SDC, http://links.lww.com/TP/A787).

**Dynamic Changes of miR-122, miR-192, and miR-146a in the Plasma**

To assess the dynamic changes of miR-122, miR-192, and miR-146a in the plasma, we evaluated the expression of the microRNAs on the 3rd, 7th, and 10th days after OLT. miR-122 and miR-192 continued to show an upward trend from the 3rd to 10th days after OLT in the AR group, whereas miR-146a levels peaked on the 3rd day after OLT (Fig. 2). All three microRNAs were significantly overexpressed in the plasma of rats with AR compared with that of NR rats on the 3rd, 7th, and 10th days after OLT (Fig. 2). The levels of miR-122 and miR-192 demonstrated the same trend as alanine aminotransferase (ALT) in the AR group (Fig. 3A,B), whereas the trend of miR-146a was different from that of ALT (Fig. 3C). In the AR+FK506 group, the plasma level of miR-146a showed no statistical differences compared with the NR group. In the AR+delay FK506 group, the elevated plasma miR-146a could be promptly repressed at the 7th day and then decreased to low level at the 10th day after OLT (Fig. 3L), whereas miR-122 and miR-192 started to decrease at the 10th day after OLT (Fig. 3J,K). The results were similar in another AR rat model (ACI-to-Lewis). Please see the SDC file (http://links.lww.com/TP/A787). In BN-to-Lewis group, miR-122 and miR-192 peaked on the 7th day after OLT and slightly decreased on the 10th day after OLT (see Figure S2a and S2b, SDC, http://links.lww.com/TP/A787), whereas miR-146a was reduced significantly to a level that showed no significant difference from Lewis-to-Lewis group on the 10th day after OLT (see Figure S2c, SDC, http://links.lww.com/TP/A787).

**Origin of Plasma MicroRNAs**

Many different cell types and tissues might contribute to the composition of the microRNAs found in plasma. To clarify the origin of the overexpressed plasma microRNAs in AR rats, we quantified the expression of miR-122, miR-192, and miR-146a in the brain, thymus, lung, heart, spleen, kidney, liver and peripheral blood mononuclear cells. The results demonstrated that the only difference in the expression of the three microRNAs between AR rats (Lewis-to-BN) and NR rats (BN-to-BN) were found in the liver graft (\(P<0.001\) for miR-122, \(P=0.002\) for miR-192, and \(P=0.014\) for miR-146a; see Table S3, SDC, http://links.lww.com/TP/A787). We further isolated microvesicles from plasma of AR and NR rats (Fig. 4A) and quantified the expression of the three microRNAs. Interestingly, the expression of miR-122 and miR-192 in microvesicles showed no significant change when AR occurred (fold change=1.769; \(P=0.106\) for miR-122; fold change=1.247; \(P=0.787\) for miR-192), whereas miR-146a showed significant elevation (fold change=6.425; \(P<0.001\); Fig. 4B).

**miR-146a In Situ Hybridization**

Graft from the AR (Lewis-to-BN) and NR (BN-to-BN) rats were used to perform miR-146a in situ hybridization (ISH) analysis. miR-146a expression was observed in both the cytoplasm and the nucleus. The expression intensity of miR-146a in hepatocytes showed no difference between graft from the AR and NR groups. The portal areas of the AR graft were brimming with lymphocytes, which showed highly intense staining for miR-146a (Fig. 4F–H).

**DISCUSSION**

Liver transplantation is currently the definitive treatment for end-stage liver disease. Although the prevalence of AR is
FIGURE 1. Microarray analysis. (A) 133 detectable microRNAs and their expression level in plasma. (B) Hierarchical clustering of 24 differential plasma microRNAs (fold change $>2$; $P<0.05$). (C) Hierarchical clustering of 54 differential microRNAs in the liver graft (fold change $>2$; $P<0.05$). The rows and columns represent genes and samples, respectively. (D) Fold change of nine differentially expressed microRNAs after AR. The nine microRNAs were changed significantly in both the plasma and the liver graft. Eight of the nine microRNAs showed reciprocal changes in the plasma and liver graft, whereas miR-146a was elevated in both the plasma and the grafts in the AR group. AR, acute rejection.
declining, 20–40% of patients still have at least one episode of AR that requires treatment with additional immunosuppression (13). Clinical assessment and the pathologic diagnosis of liver biopsies have been the norm for measuring allograft function and diagnosing AR in OLT patients. According to the AR timeline model, molecular biomarkers are superior to histomorphologic data for the early diagnosis of AR. Unfortunately, there are few reliable molecular biomarkers for the diagnosis of AR after OLT in the clinics. Serum ALT is used for the evaluation of AR in OLT patients, but ALT is also elevated in patients with liver injury caused by hepatotoxic drugs, hepatitis viruses, and bile duct injury.

MicroRNAs are produced at high concentrations within cells in a tissue-specific manner (14, 15) and have been recently reported to be remarkably stable in plasma (11, 16). Laterza et al. (17) found that increases of plasma miR-122, miR-133a, and miR-124 correspond to injuries in the liver, muscle, and brain, respectively. In the immune system, microRNAs have emerged as important regulatory elements involved in the highly specific control of cellular development, homeostasis, and immune responses. Ectopic or overexpression of individual microRNAs can have severe physiologic consequences in the immune system (10). Limited studies suggest that microRNAs can be used as biomarkers for AR. Sotolongo et al. (18) studied microRNA profiles in paraffin-embedded mucosal biopsy tissue from small bowel transplantsations and suggested that microRNAs may serve as targets for intervention and be useful in the monitoring of allograft status. Anglicheau et al. (5) found that miR-142-5p, miR-155, and miR-223 overexpressed in renal AR biopsies and may serve as indications of human renal allograft status. However, most of these studies were restricted to intragraft level. Detection of biomarkers from peripheral blood is more convenient and more applicable for AR prediction than invasive biopsy.

Farid et al. (19) selected 15 microRNAs based on the literature and tested the expression in the serum of patients after OLT. Their results suggested hepatocyte-derived microRNAs are promising candidates as early, stable, and sensitive biomarkers of rejection and hepatic injury after liver transplantation. In the present study, we investigated the global plasma microRNA expression profiles in OLT rat model using microarrays. The expression of nine microRNAs changed significantly in both the plasma and the liver graft by AR. The ideal serum/plasma biomarkers for monitoring tissue injury are those up-regulating in patients but maintaining at relatively low levels in healthy individuals (17), which makes them more suitable for clinical application. Therefore, we selected three up-regulated microRNAs (miR-122, miR-192, and miR-146a) for further study. We found that the elevation of the three plasma microRNA could be repressed by immunosuppression.

Plasma miR-122 and miR-192 increased in AR rats and have the same trend with ALT. We also observed this phenomenon in CCL4-induced liver injury. Wang et al.'s study had the same result in acetaminophen-induced liver injury (11). The possible reason that may account for this phenomenon is that miR-122 and miR-192 are the two most abundantly expressed microRNAs in human liver (20). Our microarray data also showed that miR-122 and miR-192 were among the most abundant microRNAs in rat liver. The cellular damage in the liver graft may result in the transport and release of cellular miR-122 and miR-192 into the plasma. Other studies reported that circulating miR-122 and miR-192 levels were also up-regulated in the plasma of viral-, alcohol-, and chemical-related hepatic diseases (12, 21). The target genes of miR-122 and miR-192 were widely involved in the metabolism-related signal pathway, suggesting their important roles in the physiology function of hepatocytes. Therefore, plasma miR-122 and miR-192 may be true biomarkers that reflect liver injury but have low specificity for AR.

miR-146a was the only microRNA that showed significant increases in both the plasma and the liver graft. miR-146a was first identified as an immune system regulator that influences the mammalian response to microbial infection (22). AR mainly occurs through T-cell–dependent immune pathways. The expression of miR-146a is low in naïve T lymphocytes. However, after T-cell receptor stimulation, the miR-146a level gradually increases. The induction of miR-146a expression appears to be important for immune cell maturation and activation (23). Forced miR-146a expression can protect T lymphocytes from Fas-mediated apoptosis (24). Further
study suggested that miR-146a may be involved in the maintenance of lineage identity in lymphocytes and is considered a Th1-specific microRNA (25). Th1-predominant infiltration is one of the features of rejection status and leads to cytotoxic T lymphocytes activation. Our previous study and other studies revealed that the intragraft expression level of a Th1 transcription factor was significantly higher in AR graft compared with NR control, especially in the early stage of rejection (26, 27).

These results suggested that miR-146a plays an important role in the initiation of AR. The dynamic monitoring results in this study showed that plasma miR-146a reached a high level early in AR group and the changes did not parallel those of ALT. In CCl₄-induced liver injury rats, we did not observe the elevation of plasma miR-146a. The result was similar to that from acetaminophen-overdosed mouse (11). These results indicated that the elevated plasma miR-146a might not originate from...
damaged hepatocytes. Nevertheless, after evaluating the expression of miR-146a in the brain, thymus, lung, heart, spleen, kidney, liver and peripheral blood mononuclear cells, we found that the only significant difference between the AR group and NR group was observed in the liver graft. Further ISH analysis found that the high staining intensity of miR-146a was concentrated in the portal area of AR graft, a location where there was an abundance of inflammatory cells and lymphocytes. There showed no difference in hepatocyte staining intensity between AR and NR rats.

To validate our findings, we adopted another AR rat model (ACI-to-Lewis) and we got similar results. OLT from BN-to-Lewis rats displayed a long-term graft survival and was considered as spontaneous liver tolerance model. In

**FIGURE 4.** Microvesicles (MVs) and ISH. Transmission electron microscopy micrographs of the microvesicles isolated from rat plasma (A). miR-122 and miR-192 from MVs showed no significant difference when AR occurred (B), whereas miR-146a showed significant elevation. Allograft miR-146a ISH indicated that the expression of miR-146a was concentrated in lymphocytes in the portal area (F–H) and that liver cells showed low-intensity staining in the cytoplasm. In the NR graft (L–N), miR-146a showed low-intensity staining. (C–E) H&E staining of the same AR graft. (I–K) H&E staining of the same NR graft. AR, acute rejection; H&E, hematoxylin-eosin; ISH, in situ hybridization; NR, nonrejection.
BN-to-Lewis rats, the allograft showed a lower proportion and activity of cytotoxic cells (28). We found that plasma miR-146a displayed significant low level in BN-to-Lewis model when compared with that from ACI-to-Lewis model.

Microvesicles are small vesicles that are shed from many cell types under both normal and pathologic conditions (29, 30). These vesicles play an important role in cell-to-cell communication and also function as hormones through transfer microRNA, mRNA, and protein (31). We further explored the expression of microRNAs in plasma microvesicles and found that miR-122 and miR-192 showed no significant change when AR occurred. On the contrary, miR-146a demonstrated a six-fold increase when compared with that from NR rats. Previous study revealed that miR-146a is among the microRNA species that often delivered by microvesicles and exosomes (32, 33). These results suggested that, when AR occurred, miR-122 and miR-192 may leak from damaged liver cells, whereas miR-146a may be packaged into microvesicles and actively secreted into blood circulation by stimulated lymphocytes that infiltrated into the portal area of the allograft. The high expression of miR-146a in the internal environment might reflect a shift in the Th1/Th2 balance toward Th1 during AR.

In summary, our study provides the global fingerprint of plasma microRNAs in AR rats. We found that an increase in plasma miR-146a may associate with AR in rat OLT models and that miR-122 and miR-192 levels can reflect liver injury. Detection of a combination of the three microRNAs may increase the specificity and sensitivity of diagnosing AR after OLT. However, the precise mechanism by which plasma microRNAs are associated with AR remains to be clarified and investigated and our results need to be further studied in human samples.

MATERIALS AND METHODS

Animals and OLT

Inbred male Lewis, BN, and ACI rats were used in this study. Lewis-to-BN (AR) and BN-to-BN (control) OLT were carried out. OLT was performed as described previously (34) without reconstruction of the hepatic artery. All procedures in this study were performed according to the guidelines of the Council of Animal Care at Fudan University. In the FK506 (tacrolimus)–treated group (AR+FK506 group; n=10), 1 mg/kg per day FK506 was applied by intramuscular injection from the first day after OLT until the day of sacrifice. In the delay FK506-treated group (AR+delay FK506 group; n=10), FK506 was applied by intramuscular injection from the first day after OLT until the day of sacrifice. In CCL4-induced liver injury rat model (n=6), 50% CCL4 was given intraperitoneally to BN rats at a dose of 1 ml/kg of body weight, whereas olive oil was injected to control group (n=6). Another AR model, ACI-to-Lewis (n=6), was used to confirm our finding (see details in Supplemental Information). Furthermore, a spontaneous liver tolerance model (BN-to-Lewis OLT model) was adopted to evaluate the plasma microRNA expression (see Supplemental Information).

Microarrays and RT-qPCR

A rat microRNA microarray from Agilent Technologies (Santa Clara, CA) was used in this study. The microarray contains probes for 350 rat microRNAs found in the Sanger miRBase database version 10.1. Plasma and graft samples from AR (n=4) and NR rats (n=4) were subjected to microarray analysis. All eight rats were sacrificed on the seventh day after OLT. Rejection status (positive in the AR group and negative in the NR rats) was confirmed in all eight rats by three pathologists according to Banff scheme (35).

Independent cohorts (sham operation, AR, NR, AR+FK506, and AR+delay FK506, 10 rats in each group; for histomorphologic graph, see Figure S4, SDC, http://links.lww.com/TP/A787) were used to further evaluate candidate microRNAs selected from the microarray results.

Plasma Microvesicle Isolation and MicroRNA Quantification

Please see Supplemental Information.

miR-146a ISH

We used ISH to localize the expression of miR-146a in allografts (see Supplemental Information).

ACKNOWLEDGMENTS

The authors thank Shanghai Biochip Co. for microarray hybridizations and Yi Zhang, M.S., for statistical assistance.

REFERENCES


