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An Ectopically Expressed Serum miRNA Signature Is Prognostic, Diagnostic, and Biologically Related to Liver Allograft Rejection

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The ability to noninvasively diagnose acute cellular rejection (ACR) with high specificity and sensitivity would significantly advance personalized liver transplant recipient care and management of immunosuppression. We performed microRNA (miRNA) profiling in 318 serum samples from 69 liver transplant recipients enrolled in the Immune Tolerance Network immunosuppression withdrawal (ITN030ST) and Clinical Trials in Organ Transplantation (CTOT-03) studies. We quantified serum miRNA at clinically indicated and/or protocol biopsy events (n = 130). The trajectory of ACR diagnostic miRNAs during immunosuppression withdrawal were also evaluated in sera taken at predetermined intervals during immunosuppression minimization before and at clinically indicated liver biopsy (n = 119). Levels of 31 miRNAs were significantly associated with ACR diagnosis with two miRNAs differentiating ACR from non-ACR (area under the receiver operating characteristic curve = 90%, 95% confidence interval = 82%-96%) and predicted ACR events up to 40 days before biopsy-proven rejection. The most differentially expressed miRNAs were low or absent in the blood of healthy individuals but highly expressed in liver tissue, indicating an ectopic origin from the liver allograft. Pathway analyses of rejection-associated miRNAs and their target messenger RNAs (mRNAs) showed induction of proinflammatory and cell death-related pathways. Integration of differentially expressed serum miRNA with concordant liver biopsy mRNA demonstrates interaction between molecules with a known role in transplant rejection. *Conclusion:* Distinct miRNA levels profiled from sera at the time of clinical allograft dysfunction can be used to noninvasively diagnose ACR. Predictive trajectories of the same profile during supervised immunosuppression minimization diagnosed rejection up to 40 days prior to clinical expression. The rejection-associated miRNAs in sera appear to be ectopically expressed liver and specific immune cell miRNAs that are biologically related, and the consequences of immune-mediated damage to the allograft. (HEPATOLOGY 2017;65:269-280).

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Liver transplantation is currently the only treatment option for end-stage liver failure, with patient survival rates of approximately 88% observed in the first year after transplantation.[1] Standard immunosuppression entails calcineurin inhibitor-based therapies, and for most patients is aimed at achieving and maintaining therapeutic trough levels. Recipients who have clinical evidence of allograft dysfunction, usually indicated by elevated bilirubin and/or

Abbreviations: ACR, acute cellular rejection; AUC, area under the receiver operating characteristic curve; CI, confidence interval; CTOT, Clinical Trials in Organ Transplantation; FDR, false discovery rate; IPA, Ingenuity Pathway Analysis; ITN, Immune Tolerance Network; miRNA, microRNA; mRNA, messenger RNA; NPV, negative predictive value; PPV, positive predictive value.

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liver enzymes, must undergo invasive liver biopsy to confirm the diagnosis of acute rejection.[2]

Long-term exposure to immunosuppressive drugs puts transplant patients at significantly higher risk of developing chronic kidney failure, new onset of diabetes after transplantation, and cardiovascular morbidities.[3-5] Therefore, reducing the dose of immunosuppression to a level sufficient to prevent allograft rejection would be a major milestone in the prevention of immunosuppression-related adverse effects. However, no method currently exists to personalize immunosuppression on a patient-to-patient basis and thus minimize exposure to the toxic side effects of such drugs. Empiric minimization of immunosuppression that is guided by clinical monitoring risks the development of acute rejection and allograft dysfunction, and the need to intensify immunosuppression when ACR is diagnosed. Consequently, personalizing immunosuppression would greatly benefit from biomarkers that are informative of alloimmune activation, are predictive of minimization outcomes, and are diagnostic for rejection without the need for invasive liver biopsy. Such biomarkers were recently explored in other organ transplant settings, including sequential monitoring of urinary cell messenger RNA (mRNA) and metabolites that can predict and diagnose renal allograft rejection.[6,7] Furthermore, cell-free donor-specific genomic DNA from sera can be used to monitor and diagnose cardiac and pulmonary allograft rejection.[8,9]

MicroRNAs (miRNAs) are attractive potential biomarkers for the management of Immunosuppression therapy (IST) and diagnosis of rejection. These small noncoding RNA sequences, typically 22 nucleotides in length, function as transcriptional and posttranscriptional regulators of gene expression and can act as translational inhibitors or by degrading mRNA transcripts.[10] Altered miRNA expression and polymorphisms in miRNA coding and binding sites have been shown to associate with inflammatory diseases and with the regulation of immune responses in a number of tissues.[10,11] They are observed to leak into the periphery circulatory system from solid organs and therefore may reflect pattern of injury or recovery in a disease process. miRNA studies in the human transplant setting to date have been mostly restricted to intragraft expression in human kidney allografts, where subsets of mature miRNAs have been observed to discriminate between acutely rejecting kidney allografts from normal allografts[12] or as markers of ischemia/reperfusion injury after transplantation.[13] A recent biomarker study identified four miRNAs that were significantly differentially expressed between rejecting and nonrejecting biopsies and sera samples in heart allograft recipients.[14]

This study explores serum miRNA levels as biomarkers of alloimmune status at the time of allograft dysfunction wherein rejection is suspected and diagnostic liver biopsy is performed to rule out rejection. In addition, serum miRNA trajectories were followed in a subgroup of recipients undergoing minimization of immunosuppression when patients are at risk for developing rejection to determine whether rejection is predicted prior to the expression of clinical allograft dysfunction and damage. Clinical biomarker findings were subjected to network analyses to determine whether elevated miRNAs are ectopically expressed liver and blood cell transcripts, and to explore their biological association with intrahepatic pathways of rejection and consequent liver damage.

Materials and Methods

STUDY DESIGN

Liver transplant recipients with nonviral nonimmune end-stage liver diseases (n = 231) were recruited

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for participation in the prospective Immune Tolerance Network immunosuppression withdrawal (ITN030 ST) and Clinical Trials in Organ Transplantation (CTOT-03) studies. Posttransplant immunosuppression management included tacrolimus and steroids with/without the addition of mycophenolate mofetil, with the aim of minimizing to tacrolimus monotherapy within the first year after transplantation. Recipients in both studies were followed from transplantation for up to 48 months after surgery. All recipients had scheduled blood draws at predetermined intervals and at any event of allograft dysfunction when liver biopsy was performed. Allograft dysfunction was defined as elevation of liver function test results (including aspartate aminotransferase, alanine aminotransferase, and/or bilirubin) to higher than twice the upper limit of normal. After 1 year of follow-up after surgery, a subset of recipients in the ITN030ST study (n = 48) underwent gradual minimization of immunosuppression starting at a mean of 17.7 ± 4.7 months from transplantation and was followed up to 48 months after completion of the study. Blood samples were drawn at predetermined intervals during minimization of immunosuppression, and at the event of allograft dysfunction. Withdrawal of immunosuppression was deemed successful when the recipient became tolerant to the allograft. Withdrawal of immunosuppression was discontinued if any event of allograft dysfunction occurred, at which point patients were returned to the previous immunosuppression dose.

A data coordinating center sponsored by the National Institutes of Health was responsible for data management, and study specimens were collected and stored at the ITN030ST and CTOT-03 biorepositories. A single central core pathologist independently confirmed the histopathology diagnoses using the 1997 Banff liver allograft rejection criteria. The institutional review board at each site approved the study, and all patients provided written informed consent.

GENOMIC ASSAYS

To determine acute rejection signature, sera samples (n = 130) were analyzed both at the time of clinically indicated liver biopsy and at the 12-month posttransplantation protocol biopsy and were assayed for miRNA levels; of the 130 samples, 37 had a diagnosis of ACR and 93 had no ACR. Sequential sera samples (n = 119) were obtained during minimization of immunosuppression; serum miRNA profiles obtained before the clinical development of liver dysfunction were tested for their capacity to predict biopsy-proven ACR (n = 19) versus non-ACR (n = 17). A unique feature of this study is that at the time of clinically indicated liver biopsy, the enrolled participants agreed to donate an additional liver biopsy and whole blood for genomic studies, providing the opportunity to correlate miRNA findings with intragraft and peripheral pathways that may be impacted and/or regulated by miRNAs that are detected in the serum. Consequently, liver biopsy samples (ACR, n = 18; non-ACR, n = 41) and blood samples (ACR, n = 18; non-ACR, n = 41) were profiled for their global mRNA gene expression levels (Supporting Fig. S2). Detailed methods area available in the Supporting Information.

Total RNA, including small RNAs, was purified from 200 μL of patient serum (miRCURY RNA Isolation kit-Biofluids; Exiqon, Woburn, MA) and assessed for purity and quality. Levels of miRNA were quantified following complementary DNA synthesis and amplification-enhanced quantitative polymerase chain reaction assays using primers for 752 different human miRNA targets and six reference gene assays (miRCURY LNA Universal RT miRNA PCR; Exiqon, Vedbaek, Denmark). Liver biopsies were collected in RNAlater (Qiagen, Valencia, CA) and stored overnight at 4°C after which they were transferred to −80°C until further processing was required. Total RNA was extracted from the liver biopsies using Trizol (Invitrogen, Carlsbad, CA). The RNA was then further purified using the RNeasy kit (Qiagen), according to the manufacturer's instructions. Blood samples were collected in PAXgene Blood RNA tubes (Qiagen) for transportation and storage and RNA was purified from blood using PAXgene Blood RNA Kit (Qiagen). Affymetrix Human Gene 1.0 ST Arrays were used to quantify global gene expression levels for liver biopsy and blood RNA samples. When done locally, the turnover time from extraction to miRNA determination is done within 48 hours. Further details are outlined in the Supporting Information.

ANALYSIS

miRNA normalization was performed using the exogenous spiked miRNAs, while mRNAs were normalized using robust multichip average normalization. Outliers implicated by both principal components analysis clustering and mean absolute deviation scores were excluded. Details of expression data normalization and statistical analysis can be found in the Supporting Information. The associations between ACR status with individual miRNA

or mRNA expression levels were tested using a generalized linear model, adjusting for the potential confounder of time since transplantation. Standard false discovery rate (FDR; Benjamini-Hochberg method) adjustment was applied for multiple testing corrections. In addition to identifying individual miRNAs that differentiate comparison groups, the "glmnet" package (https://cran.r-project.org/web/packages/glmnet/glmnet.pdf) was employed to identify a multimarker panel to maximize the discrimination ability from the combination of potential biomarkers. Leave-one-out cross-validation resampling was performed for training the model, and the performance of the resulting best model was estimated from the test sets. The regression estimates from the best "glmnet" model defined a diagnosis or prediction signature. Model performance was evaluated using the area under the receiver operating characteristic curve (AUC). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and their 95% confidence intervals (CIs) were calculated with 2000-bootstrap resampling. Locally estimated scatterplot smoothing (LOESS) curves with corresponding 95% CIs were obtained for the retrospective trajectories of the diagnostic signature, looking backward from the time of biopsy for ACR and non-ACR episodes. All statistical analyses were performed in Array Studio software (www.omicsoft.com), NCSS version 8.0.14 (www.ncss.com), and R (http://cran.r-project.org).

Serum miRNA showing association with ACR (FDR $q < 0.05$) were hierarchically clustered, and expression heat maps were created using Gene Cluster 3.0 and Java Treeview.[15,16] Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) was used to identify experimentally verified and high-confidence-predicted miRNA-mRNA target interactions to define miRNA-mRNA pairs for input into pathway enrichment analysis. Data from the miRNA and mRNA experimental platforms were also integrated and subjected to network analysis via IPA to identify plausible associations and potential regulatory networks relating to alloimmune activation and known intrahepatic rejection pathways. The IPA strategy is summarized in Supporting Fig. S1.

Results

PATIENT CHARACTERISTICS, IMMUNOSUPPRESSION, AND BIOPSY FINDINGS

The study included adult patients with a mean age of 54.8 ± 8.9 years. Sixty-five percent of the patients were male, and 35% were female. The patients were predominantly white (91%), with 4% African Americans, 1% Asians, and 3% other races or mixed races. Pretransplantation liver disease diagnosis and time from transplantation to liver dysfunction and liver biopsy were similar in the rejection versus no rejection groups (Table 1). Rejection episodes were more common in patients who were managed with tacrolimus monotherapy ($P = 0.02$). Biopsy results were placed in categories of rejection (n = 37) or nonrejection (n = 93) diagnosis. The grade of rejection (RA 1-3) and histopathology findings in the no rejection group are shown in Table 1.

TABLE 1. Clinical Characteristics, Biopsy Findings, and Immunosuppression

	Rejection (n = 37)	No rejection (n = 93)	*P*
Pretransplantation diagnosis			0.32
Alcoholic cirrhosis	53%	51%	
Nonalcoholic steatohepatitis	17%	24%	
Cryptogenic cirrhosis	11%	16%	
Metabolic disease	6%	4%	
Other	14%	4%	
Time from transplantation to biopsy, days	552 ± 370.4	643.7 ± 320.9	0.16
Immunosuppression			0.02
Tacrolimus monotherapy	56%	80%	
Tacrolimus with MMF	36%	14%	
Others	8%	6%	
Primary diagnosis at time of biopsy			
RAI1	31%	–	
RAI1	58%	–	
RAI3	11%	–	
Steatohepatitis	–	40%	
Nonspecific change	–	21%	
Nonspecific inflammation	–	13%	
Nodular regenerative hyperplasia	–	4%	
Chronic hepatitis	–	3%	
Other	–	18%	

Abbreviations: MMF, mycophelolate mofetil; RAI, rejection activity index.

DEVELOPMENT OF A LIVER ALLOGRAFT ACR miRNA DIAGNOSTIC TEST

In order to test for miRNAs associated with ACR, we measured serum miRNA levels from 69 patients who had one or more episodes of allograft dysfunction at any time during the first year after transplantation or during withdrawal of immunosuppression. All patients underwent clinically indicated liver biopsy demonstrating rejection (n = 37) or no rejection (n = 93) (Supporting Fig. S2, left panel). The samples (n = 130) were randomized to a training set (ACR, n = 18; non-ACR, n = 45) and testing set (ACR, n = 19;

TABLE 2. Predictive Performance Parameters of the Multi-miRNA Panels Identified by "glmnet"

Regression Model	Data Set	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
Signature = 3.6494831 - 0.5886157*miR-483-3p - 0.6086077*miR-885-5p	Train	90.25% (80.61%-97.53%)	83.33% (66.67%-100%)	88.89% (80%-97.78%)	0.75	0.93
	Test	89.25% (78.51%-97.59%)	84.21% (68.42%-100%)	85.42% (75%-93.75%)	0.70	0.93
	Train + Test	89.54% (82.07%-95.67%)	83.78% (72.97%-94.59%)	87.10% (79.57%-93.55%)	0.72	0.93

A 2000 bootstrap resampling estimated performance matrix was incorporated. ACR probability = 1 − (1/(1 + exp(−Signature))).

non-ACR, n = 48), stratifying by ACR status and study. A total of 43 miRNAs were found to be significant at greater than two-fold absolute change and a nominal P value < 0.05 in the training set; of these, 37 were replicated in the test set with a nominal P value < 0.05. After multiple testing corrections, 31 miRNAs were significantly associated with ACR diagnosis in the combined training and test sets (FDR adjusted P value < 0.001, Supporting Table S1).

To build a multiple marker panel signature capable of differentiating ACR from non-ACR, we included the 31 ACR-associated miRNAs in a variable selection model that was built with GLMNET. The final ACR diagnostic test included two miRNAs, hsa-miR-483-3p and hsa-miR-885-5p, that differentiated ACR from non-ACR with an AUC of 90% (95% CI = 81%-98%), with 88.9% sensitivity and 83.3% specificity in the training set (P = 0.0001) (Table 2). This model was replicated in the test set with an AUC of 89% (95% CI = 79%-98%), with 84.2% sensitivity and 85.4% specificity. For the combined training and test set, the AUC was 89.5% (95% CI = 82%-96%), with 83.8% sensitivity, 87.1% specificity, 0.72 PPV, and 0.93 NPV (Fig. 1). Variables including time from transplantation to liver dysfunction event, or whether ACR was found at routine follow-up or induced by intentional minimization of immunosuppression, were not observed to affect the model performance.

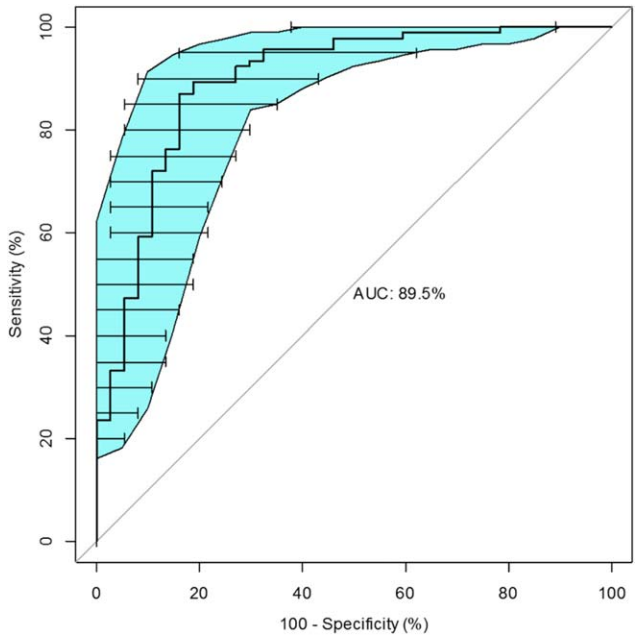

FIG. 1. Receiver operating characteristic curve for the two-miRNA ACR prediction signature in combined training and test sets of 37 ACR and 93 non-ACR events. The 95% CIs of the specificity for every 5% increase in sensitivity are indicated by horizontal bars. The blue-shaded regions indicated the 95% CIs of the sensitivity for every 5% increase in specificity.

TRAJECTORY OF ACR DIAGNOSTIC PANEL DURING IMMUNOSUPPRESSION WITHDRAWAL

We tested the retrospective trajectory of our ACR diagnostic test in ITN030ST recipients who underwent minimization of immunosuppression (n = 27). Sera samples were taken at predetermined intervals during immunosuppression minimization before and at the time of clinically indicated liver biopsy (n = 119; Supporting Fig. S2, right panel). A total of 64 serum samples were associated with ACR diagnosis, 45 of which were available before ACR diagnosis and 19 of which were available at biopsy-proven ACR diagnosis. A total of 55 samples were associated with non-ACR diagnosis, 38 of which were available prior to biopsy and 17 of which were available at non-ACR biopsy diagnosis. At least two samples for each biopsy event were available before the biopsy event. The LOESS-smoothed plot (Fig. 2) demonstrates that the miRNA ACR diagnostic test separated ACR events

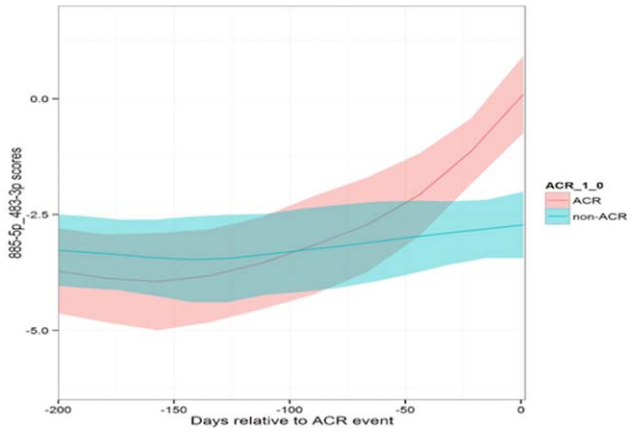

FIG. 2. Retrospective longitudinal trajectory of two-miRNA ACR signature as a function of time before biopsy. The LOESS-smoothed averages and 95% confidence bands of the two-miRNA ACR signature for the ACR group (red) and the non-ACR group (green) are shown, looking backward from the time of biopsy (day 0). The *y* axis denotes the scores from ACR prediction model, and the *x* axis denotes days relative to biopsy.

from non-ACR events, with 95% CIs of the smoothed means for these two groups separating at approximately 40 days prior to the biopsy-proven rejection. This indicates that molecular events initiating pathways of acute rejection can be recognized before clinical allograft injury.

HIERARCHICAL CLUSTERING AND ANALYSES OF THE PUTATIVE ORIGIN OF THE LIVER ALLOGRAFT REJECTION SERA miRNA SIGNATURE

Global assessment of serum miRNA at a time of liver dysfunction demonstrated significant differences between patients having biopsy-proven rejection versus those who had nonspecific inflammatory changes. The two-gene model of miRNA hsa-miR-483-3p and hsa-miR-885-5p was the best-fitting model yielding the diagnostic signature. Our assay revealed a total of 95 miRNAs that were detected at the time of liver rejection. We hypothesize that these miRNAs were leaking from hepatocytes and immune response cells that were injured at the time of rejection. We believe that these miRNAs may provide insight into intragraft biological pathways of rejection. Hierarchical clustering was performed on the 95 serum miRNAs showing significant association with ACR diagnosis (FDR, $q < 0.05$) using the gene cluster tool (Supporting Fig. S3). In order to detect biological pathways associated with rejection, we employed a less stringent significant level (FDR, $P < 0.05$) to include wider miRNA panels versus those used in the initial inclusion criteria (FDR, $P < 0.001$) in multimarker models of rejection diagnosis. Two sample groups were evident (sample groups A and B). Sample group A predominantly contained serum samples from subjects with no rejection, with 10 ACR samples and 68 non-ACR samples (12.8% ACR samples). Sample group B contained a high number of samples taken at the time of rejection, with 27 ACR samples and 15 non-ACR samples (64% ACR samples). The proportion of ACR samples in group B was significantly higher compared with the proportion of ACR samples in group A ($P = 1.3 \times 10^{-8}$). The differential expression Log FDR q value and previously published baseline miRNA expression in healthy liver and blood (GSE69825) are shown on the right side of Supporting Fig. S3. miRNA expression was also seen to cluster into two groups. miRNA cluster 1 was notably up-regulated in sample group B, containing most of the rejection samples. The miRNAs in cluster 1 showed a trend toward stronger differential expression and also tended to show higher expression in normal liver and lower expression in normal blood, indicating that the miRNAs in cluster 1 represent ectopically expressed liver miRNA. miRNA cluster 2 showed a trend toward lower differential expression and similar expression in both liver and blood or in some cases predominant expression in blood. Association results annotated with reference panel data are presented in Supporting Table S2.

DECONVOLUTION OF IMMUNE-SPECIFIC CELL TYPES IN SERUM miRNA EXPRESSION

We hypothesized that serum miRNA at the time of allograft dysfunction originate from immune and proinflammatory cells that are injured at the time of rejection. We next sought to deconvolute the putative cell type origins of the 95 miRNA differentially expressed (FDR, $q < 0.05$) in serum of ACR patients by comparison against immune cell miRNA expression reference data sets (GSE28492).[17] The reference data set consisted of 54 miRNAs showing enriched expression (average log2 signal value >7 in at least one cell type) in neutrophils, eosinophils, monocytes, CD4+ T cells, CD8+ T cells, natural killer cells, and B cells. In total, 10 of 95 ACR-associated miRNAs were of

putative immune cell origin. In cluster 1, two of 38 miRNAs were immune cell enriched: miR-378a-3p (monocytes) and miR-29a-3p (B-cells, T-cells). In cluster 2, eight of 57 miRNA were immune cell enriched: miR-146b-5p and miR-342-3p (T-cells, natural killer cells); miR-532-5p and miR-106a-5p (monocytes); miR-145-5p (neutrophils); miR-25-3p (eosinophils, neutrophils); and miR-93-5p and miR-425-3p (monocytes, eosinophils, neutrophils).

miRNA TARGET ANALYSIS

Next, we attempted to determine the possible relations between the significantly elevated serum miRNA to liver biopsy mRNA expression that were available at the same time points. Because all 95 ACR-associated miRNAs showed up-regulated expression, and because miRNAs function in gene silencing, miRNA target analysis was restricted to 311 mRNA transcripts showing down-regulated differential expression ($P < 0.05$) in ACR liver biopsies. Down-regulated mRNAs were evaluated for experimentally confirmed and high-confidence miRNA targets of the miRNA contained in cluster 1 and 2 (using IPA miRNA target filter analysis). The serum miRNA contained in cluster 1 and 2 and their confirmed liver mRNA target transcripts were used as combined input for IPA (Supporting Table S3). The same process of miRNA-mRNA target pairing was repeated for 484 mRNA transcripts showing down-regulated differential expression ($P < 0.05$) in ACR blood samples (Supporting Table S4).

INTEGRATIVE PATHWAY ANALYSIS

As a first step toward identifying the biological mechanisms underpinning our ACR-associated serum miRNAs, we performed an IPA (Qiagen) for diseases and biological functions on the combined serum miRNA and their liver mRNA targets for clusters 1 and 2 (Fig. 3 and Supporting Table S5). We excluded cancer-related functions from the results due to the overrepresentation of miRNA-related cancer annotations. Pathway enrichment appeared to reflect the putative miRNA tissue origin for each cluster. In the case of cluster 1, enrichment is seen in a number of liver specific functions, including nonalcoholic fatty liver disease and hepatic steatosis; cell movement and migration; cell death and apoptosis; and cell migration and proliferation. Cluster 2 is dominated by inflammatory processes, including lupus nephritis and fibrosis. A similar enrichment analysis was also performed on the combined serum miRNA and respective blood mRNA targets for clusters 1 and 2 (Supporting Table S6). A similar trend in biological processes was seen between both clusters.

HYPOTHESIS-DRIVEN ASSESSMENT OF KNOWN HEPATIC REJECTION PATHWAYS

IPA was used to integrate differentially expressed miRNA and mRNA with known genes and drugs involved in transplant rejection. A network was created in a stepwise manner. First, a custom network of genes and drugs with a known role in transplant rejection was created using a Medline subject headings (MESH) query. The query identified 76 molecules (Supporting Table S7) that were used to populate a core transplant rejection network. Fifteen liver mRNA targets of miRNA cluster 1 showed direct interaction with known transplant interactors. Six liver mRNA targets of cluster 2 also showed direct interactions. All noninteracting genes were discarded, and a core network was created (Supporting Fig. S4). ACR liver biopsy expression ($P < 0.05$) for all mRNA molecules is shown, and ACR blood mRNA are also shown, which included two additional known transplant rejection genes (*NR3C1* and *PPP3CA*). Four known transplant rejection genes were significantly up-regulated in liver biopsy mRNA (*B2M*, *C3AR1*, *TIMP1*, and *MTOR*).

Discussion

In this study, we asked whether a noninvasive miRNA biomarker signature can predict and diagnose liver allograft rejection both in the setting of individuals undergoing routine postoperative care, and for patients in whom immunosuppression was minimized, and whether these sera-derived miRNA are biologically related to immune pathways of allograft rejection. The recipient population for this study includes nonviral, nonimmune liver disease etiologies; consequently, this biomarker's diagnostic and prognostic signature may be relevant to >75% transplant recipients, including those treated for HCV infection and who have sustained viral response. We identified two miRNAs that, when combined together in a signature, could diagnose and predict rejection with high accuracy. The current gold standard for diagnosis of ACR is liver biopsy, a

Diseases and Bio Functions	Catagories	enrichment logp cluster1	enrichment logp cluster2	cluster1&2 logp	
nonalcoholic fatty liver disease	Endocrine System Disorders, Gastrointestinal Disease, Hepatic	6.84		7.79	Top 10 Cluster 1
endometriosis	Organismal Injury and Abnormalities, Reproductive System Disease	6.32	3.21	6.77	
T-cell lymphoproliferative disorder	Hematological Disease, Immunological Disease	6.31	9.15	12.7	
hepatic steatosis	Gastrointestinal Disease, Hepatic System Disease, Metabolic Disease	6.19	3.03	6.84	
cell movement	Cellular Movement	5.71	3.48	5.21	
cell death	Cell Death and Survival	5.71	5.43	6.94	
apoptosis	Cell Death and Survival	5.65	4.32	6.06	
migration of cells	Cellular Movement	5.63	2.95	4.31	
growth of skin	Hair and Skin Development and Function, Organ Development	5.43	4.5	4.3	
proliferation of dermal cells	Cellular Development, Cellular Growth and Proliferation, Hair and Skin	5.4	4.56	4.48	
inflammation of body region	Inflammatory Response	3.74	12.3	13.1	Top 10 Cluster 2
class II lupus nephritis	Inflammatory Disease, Inflammatory Response, Organismal Injury and	4.01	11	13.2	
inflammation of body cavity	Inflammatory Response	3.09	10.8	12.6	
idiopathic pulmonary fibrosis	Connective Tissue Disorders, Inflammatory Disease, Inflammatory		9.31	11.4	
inflammation of organ	Inflammatory Response	2.69	9.21	9.63	
fibrosis of lung	Inflammatory Disease, Organismal Injury and Abnormalities, Respiratory	2.43	9.16	10.4	
stenosis of artery	Cardiovascular Disease, Organismal Injury and Abnormalities	3.11	8.59	7.67	
glomerulonephritis	Inflammatory Disease, Inflammatory Response, Organismal Injury and	3.27	7.84	8.86	
Fibrosis	Organismal Injury and Abnormalities	2.46	7.65	9.54	
azoospermia	Organismal Injury and Abnormalities, Reproductive System Disease		7.52	9.92	

FIG. 3. IPA for diseases and biological functions on the combined serum miRNA and their liver mRNA targets.

costly and invasive procedure that is associated with a number of well-described complications. This highly sensitive and specific miRNA signature is selected with cutoffs of miRNA levels that are aimed at PPV and NPV that add significant patient safety margins when applied in the clinical setting. Importantly, the

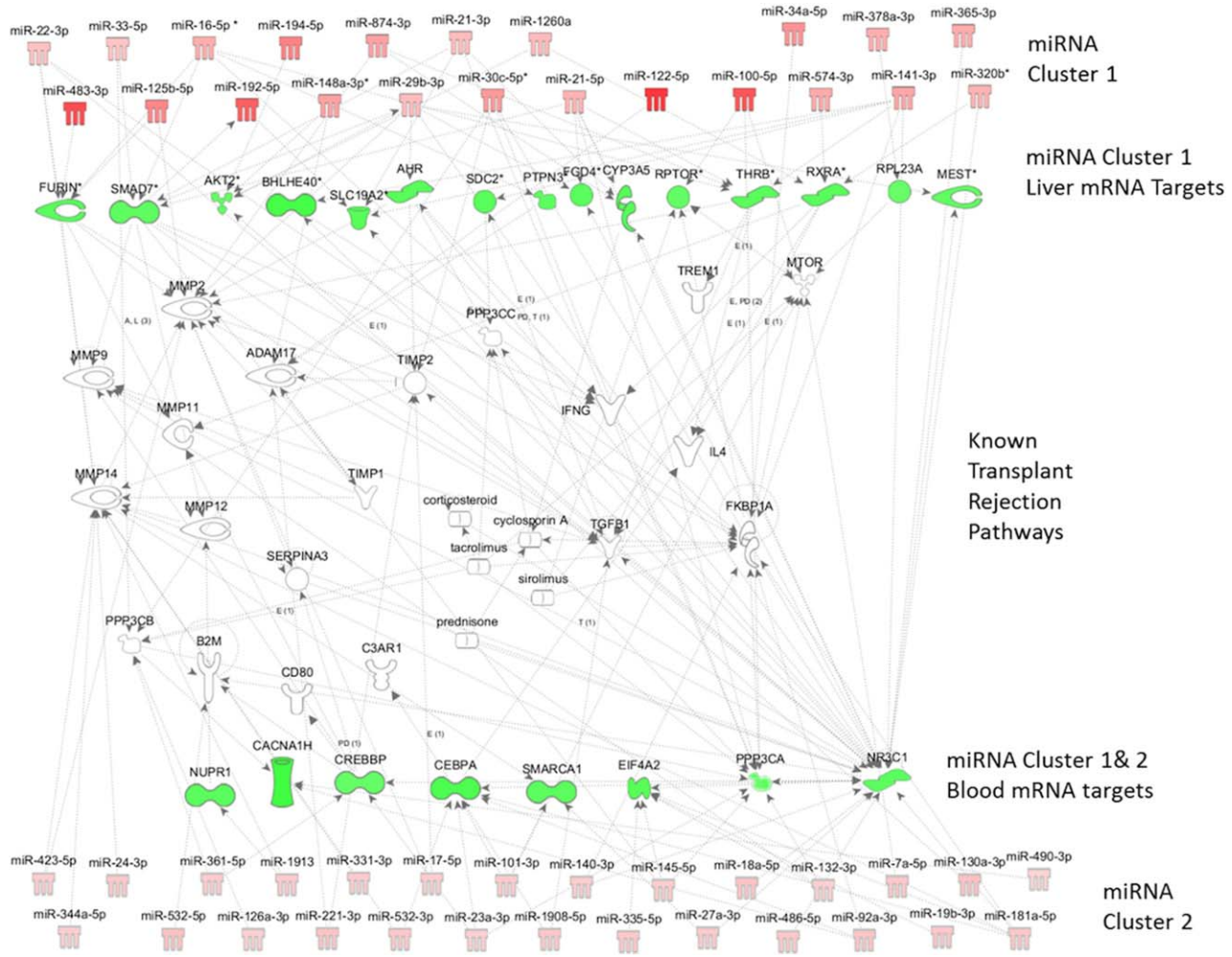

FIG. 4. IPA of differentially expressed miRNA and mRNA in ACR patients.

rejection signature is as sensitive as the histology diagnosis. Moreover, biopsy histopathology findings may be contested, because interpretation is subjective and is known to have intra- and interpathology observational differences, and graft damage may occur by the time the patient presents with clinical symptoms.[18] The advantages of serum biomarkers for the diagnosis and prediction of liver allograft rejection are obvious; the signature allows more frequent as well as a significantly more precise, less invasive, and less costly method to determine allograft status and management of immunosuppression. A reliable laboratory assay that is available within 48 hours of blood withdrawal will have a substantial effect on the management of immunosuppression and will provide objective diagnostics of allograft dysfunction, ideally in a manner that avoids continuous damage to the organ. The test is best used when recipients are diagnosed with allograft dysfunction >30 days from transplantation to avoid the effect of residual ischemia reperfusion injury on miRNA levels.

Our study demonstrates a two-miRNA ACR test in serum obtained at the time of allograft dysfunction and clinically indicated liver biopsy is highly diagnostic for ACR versus non-ACR events. The unique findings in this study add to the growing literature describing peripheral genomic profiles that can reliably inform allograft alloimmune status, including our recently described urinary cell mRNA signature that is diagnostic of kidney allograft ACR, and others have reported

that circulating cell-free, donor specific genomic DNA enables noninvasive diagnoses of heart allograft rejection.[6,8,14]

The same two-miRNA ACR signature is detectable in serum up to 40 days before biopsy-proven rejection in patients undergoing supervised immunosuppression minimization. Its trajectory is predictive of rejection with high accuracy and is indicative of molecular rejection prior to clinical expression of allograft dysfunction. These data demonstrate significant potential for personalizing immunosuppression through facilitating minimization of immunosuppression dosing monitoring alloimmune response and allowing preemptive adjustment of drug levels before clinical allograft injury. Interestingly, the ability to diagnose molecular rejection prior to clinical allograft dysfunction was also demonstrated in our previous observational kidney transplant study in which mRNA urinary cell ACR profile is up-regulated up to 20 days before biopsy-proven rejection.[6] The clinical implications are considerable, because minimization of drug dose would likely benefit the recipient by reducing drug-related toxicities, which are major issues over the lifetime of the recipient.

Taken together, routine clinical test and miRNA profiles may be used to establish clinical pathways for precise diagnosis of liver allograft dysfunction and long-term management of immunosuppression. We envisioned a pathway in which a routine biochemical assay that indicates liver allograft dysfunction could be followed by immediate miRNA testing for the diagnosis of ACR, and a positive result should be followed by appropriate antirejection therapy. Return of liver biochemistry and miRNA tests to baseline would indicate successful treatment of ACR. Patients who are considered for minimization of immunosuppression, usually 6-12 months after transplantation, would undergo supervised dose reduction, followed by routine blood biochemistry and miRNA ACR tests taken at predetermined intervals. The finding of an miRNA test that is predictive of imminent ACR should trigger adjustment in immunosuppression drug dose to former levels, with the aim of controlling molecular rejection before the development of clinical allograft dysfunction. Consequently, miRNA biomarker-guided minimization will allow clinicians to determine the optimal minimal dose that is necessary to control alloimmune response and avoid clinical allograft dysfunction. Such an approach will avoid higher rates of ACR when minimization is empiric.

The investigations were extended to determine whether ectopically expressed miRNAs are directly associated with pathways of immune-mediated injury to the allograft and are informing of nodes that may be targeted for preemptive intervention. Hierarchical clustering was based on preexisting literature related to the putative origins and targets of the ectopically expressed ACR miRNA. Furthermore, the unique setting in these prospective clinical studies. in which liver tissue, blood, and serum samples were collected at the same time point, allowed putative biological pathways of immune activation to be investigated. We confirmed enrichment of immune activation pathways by correlating ACR miRNA expression in the serum with liver mRNA expression from the same timepoints.

The application of hierarchical clustering of miRNA expression appears to be an effective classifier of the 95 miRNAs showing association with ACR. Clustering highlights two putative groups, clusters 1 and 2, which were largely segregated into biological processes related to liver and immune function, respectively. We postulate that the miRNAs in cluster 1 were ectopically expressed, originating from rejecting hepatic graft tissue and reflecting acute liver injury. A pathway analysis of cluster 1 was also strongly supportive of a predominant role in liver function for this cluster. Cluster 1 included 5/10 top-ranked liver-expressed miRNAs in the human reference panel (GSE69825), including miR-122-5p, the most highly expressed miRNA in healthy liver, which has also been proposed as a serum biomarker of primary biliary cirrhosis[19] and nonalcoholic fatty liver disease.[20] Other serum miRNAs seen in cluster 1 are specifically associated with liver function and are not observed in healthy sera, for example, miR-885-5p is significantly elevated in the sera of patients with liver pathologies[21] and after acute HCV infection.[22] Although cluster 2 also contains several miRNAs that are most highly expressed in the liver, the miRNAs in this cluster generally appear to originate from the blood and immune cells and may more closely represent immune cellular rejection pathways. We identified 10 of 95 ACR-associated miRNAs that are strongly enriched in immune cell subtypes; these miRNAs were disproportionately distributed (8/57 miRNA) in cluster 2. Only two of 38 immune-enriched miRNAs were seen in cluster 1. A pathway analysis of the putative mRNA targets of cluster 2 also strongly supported an immune-inflammatory role for the miRNAs in cluster 2.

Our integrated pathway analysis focused on experimentally confirmed or high-confidence mRNA targets of the 95 differentially expressed miRNAs, restricted to mRNA that showed differential down-regulated

expression in ACR liver biopsies. We focused on a concordant up-regulation of miRNA and down-regulation of mRNA based on the widely observed phenomenon of mRNA target degradation after miRNA binding.[23] The miRNA-mRNA target pairing identified in our analysis is supported by the results of the mRNA pathway analysis, with clear enrichment of liver-related pathways in the targets of miRNA cluster 1 and immune pathways in miRNA cluster 2.

This mechanistic analysis integrates peripheral biomarkers (miRNA) with molecular pathways of rejection (mRNA) and the consequent progression to organ injury. Our results indicate that ACR-associated serum miRNAs are the products of activated immune and hepatic cells at the time of rejection and suggest that these miRNAs in turn regulate mRNA expression and the production of proinflammatory mediators. This approach also demonstrates that the search for peripheral biomarkers for management of immunosuppression presents unique opportunities for enhancing our mechanistic understanding of immune activation and trajectories of rejection from molecular inception to clinical expression. Ultimately, we expect these mechanistic insights to inform strategies for immune monitoring and intervention, dramatically enhancing our ability to care for patients in the periods immediately after transplant, and over the longer term.

A limitation of our study was that the primary immunosuppression used in the study populations was tacrolimus, a calcineurin inhibitor that is most commonly used in >75% of liver transplant recipients in the United States. While this makes our results more widely applicable to current clinical practice, it is possible that other immunosuppression agents differentially alter gene expression in the allograft and the periphery, and consequently, alter the miRNA diagnostic profiles. It remains to be determined whether our miRNA ACR diagnostic signatures are only relevant for liver transplant recipients who are suppressed with tacrolimus or include those who are treated with mammalian target of rapamycin inhibitors such as sirolimus or everolimus.

To rigorously assess the clinical use of these findings, interventional randomized clinical trials using the ACR diagnostic and predictive profile should be conducted to compare standard-of-care versus miRNA-guided patient management at the time of allograft dysfunction, and could be further extended for miRNA-guided reductions in immunosuppression dosing. These studies must allow sufficient follow-up periods to demonstrate the superiority of miRNA-guided approaches for ACR related outcomes, and to demonstrate that immunosuppression minimization is beneficial for a range of comorbidities. Due to sample sizes, the miRNA profiles in the immunosuppression drug minimization component of this study should be considered exploratory and should be tested in larger group of recipients prior to clinical application.

In conclusion, the predictive and diagnostic miRNA ACR test and baseline miRNA profiles in sera prior to reduction in immunosuppression dosing represent a minimally invasive approach for diagnosis of acute rejection and a potential guide to personalizing tacrolimus regimes. These miRNA tests are highly sensitive for the diagnosis of ACR in the setting of liver allograft dysfunction. Future efforts may refine the diagnostic to an outpatient point-of-care application and help establish the cost/benefit for recipients to determine the extent by which personalizing immunosuppression reduces drug toxicity while preserving immune and organ function.

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