Gene Expression Profiling for the Identification and Classification of Antibody-Mediated Heart Rejection

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European Heart Journal (2014) **35**, 3194–3202
doi:10.1093/eurheartj/ehu346

MicroRNAs as non-invasive biomarkers of heart transplant rejection

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Received 10 July 2014; revised 31 July 2014; accepted 5 August 2014; online publish-ahead-of-print 31 August 2014

See page 3150 for the editorial comment on this article (doi:10.1093/eurheartj/ehu376)

Aim Rejection is one of the major causes of late cardiac allograft failure and at present can only be diagnosed by invasive endomyocardial biopsies. We sought to determine whether microRNA profiling could serve as a non-invasive biomarker of cardiac allograft rejection.

Methods We included 113 heart transplant recipients from four referral French institutions (test cohort, $n = 60$, validation cohort, $n = 53$). In the test cohort, we compared patients with acute biopsy-proven allograft rejection ($n = 30$) to matched control patients without rejection ($n = 30$), by assessing microRNAs expression in the heart allograft tissue and patients concomitant serum using RNA extraction and qPCR analysis. Fourteen miRNAs were selected on the basis of their implication in allograft rejection, endothelial activation, and inflammation and tissue specificity.

Results We identified seven miRNAs that were differentially expressed between normal and rejecting heart allografts: miR-10a, miR-21, miR-31, miR-92a, miR-142-3p miR-155, and miR-451 ($P < 0.0001$ for all comparisons). Four out of seven miRNAs also showed differential serological expression (miR-10a, miR-31, miR-92a, and miR-155) with strong correlation with their tissular expression. The receiver-operating characteristic analysis showed that these four circulating miRNAs strongly discriminated patients with allograft rejection from patients without rejection: miR-10a (AUC = 0.975), miR-31 (AUC = 0.932), miR-92a (AUC = 0.989), and miR-155 (AUC = 0.998, $P < 0.0001$ for all comparisons). We confirmed in the external validation set that these four miRNAs highly discriminated patients with rejection from those without. The discrimination capability of the four miRNAs remained significant when stratified by rejection diagnosis (T-cell-mediated rejection or antibody-mediated rejection) and time post-transplant.

Conclusion This study demonstrates that a differential expression of miRNA occurs in rejecting allograft patients, not only at the tissue level but also in the serum, suggesting their potential relevance as non-invasive biomarkers in heart transplant rejection.

Keywords Transplant rejection • Biomarker • MicroRNA • Non-invasive • Heart transplantation • Molecular medicine • Personalized medicine

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Translational perspective

Rejection still represents a major threat to heart allografts. The current gold standard for heart rejection monitoring relies on repeated heart transplant biopsies that are invasive procedures and represent a serious burden in the clinical management of heart transplant recipients. Here, we studied in heart transplant tissue and in the concomitant serum the expression of relevant micro-RNAs involved in immune response and relevant biology related to allograft rejection. We demonstrate that the assessment of these four miRNAs (miR-10a, miR-31, miR-92a, and miR-155) in sera discriminates with a very high accuracy between patients with allograft rejection and those without. Taken together, our results support that miRNAs represent relevant and non-invasive biomarkers that may serve to better investigate heart transplant rejection and guide the clinical management of heart recipients.

Introduction

Heart transplantation is a life-saving treatment for patients with end-stage heart failure, which represents a severe burden worldwide.[1] Despite considerable advances in transplantation, allograft rejection remains a major issue leading to allograft loss and mortality.[1]

Currently, the gold standard for diagnosing and monitoring acute heart rejection relies on multiple and repeated endomyocardial biopsies (EMB) performed for acute clinical indications (heart failure, decrease in left ventricular function) or carried out on a regular basis in patients in a steady state[2] in order to screen for subclinical rejection.[3,4] Despite their usefulness, EMB remains an invasive procedure associated with rare but potentially serious complications, discomfort for the patients and increased cost for the community.

Identifying non-invasive and reliable biomarkers for screening heart transplant rejection is one of the major challenges of solid organ transplantation.[5] While attempts have been made to isolate such biomarkers using peripheral blood gene expression in low-risk heart transplant recipients, this question is still unanswered.[6,7]

A recent breakthrough has occurred with the discovery of small and non-coding RNAs called microRNAs that regulate gene expression.[8] Though miRNAs are known to be involved in many biological processes[9] such as development,[10] cell proliferation,[11,12] differentiation,[13] apoptosis,[14] and oncogenesis;[15] emerging data suggest that they may play a critical role in the regulation of immune cell development and in the modulation of innate and adaptive immune responses.[16–20] Consequently, miRNAs have become a potential interest in the field of organ transplant rejection.[19,20]

In the present study, we hypothesized that specific miRNAs could be used as relevant biomarkers for heart transplant rejection. We sought to identify a miRNA signature in rejecting heart allografts and to determine whether assessment of miRNAs post-transplant could serve as non-invasive biomarkers of heart transplant rejection. Such information could have a major impact on the clinical management of heart transplant recipients.

Methods

Study design

This study included heart transplant recipients from Pompidou Hospital (Paris, France) between January 2004 and October 2010 who had a diagnosis of biopsy-proven allograft rejection ($n = 43$). Thirteen patients were excluded because of a lack of suitable material for miRNA biopsy assessment, leaving 30 rejecting heart allograft patients as the study sample. This group of patients was compared with a matched control group of 30 patients transplanted during the same period of time but without allograft rejection. These patients were matched based on the following criteria: recipient age, donor age, cold ischaemia time, time from transplantation to index biopsy and maintenance immunosuppressive regimen. All the patients had conventional graft histopathology together with concomitant assessment of microRNA expression in the allograft and serum taken at the time of biopsy.

We used an additional independent validation sample of 53 patients from three heart transplant centres [Necker ($n = 25$), Rouen ($n = 19$), and Pitié-Salpétrière hospitals ($n = 9$)].

All of the transplants were ABO compatible and had current negative IgG T cell and B cell complement-dependent cytotoxicity cross-matching at the time of transplantation. The transplantation allocation system was identical for all four centres and followed the rules of the French national agency for organ procurement (Agence de la Biomédecine).

Clinical data

Clinical data for the donors and recipients in the development and validation cohorts were obtained from reviews of the patients' charts. We recorded the data for all the patients regarding donor age, donor gender, recipient age, recipient gender, primary heart disease, date of transplantation, follow-up, severe bacterial infection, CMV-related disease, cold ischaemia time, and immunosuppressive drug regimen.

Histology and immunohistochemistry

Endomyocardial biopsies were formalin-fixed, paraffin-embedded, and routinely stained with haematoxylin-eosin. Immunohistochemistry was performed on tissue sections with specific antibodies: rabbit monoclonal anti-C4d (DB Biotech, Kosice, Slovac Republic) and monoclonal anti-CD68 (DakoCytomation, Glostrup, Denmark) using an immunoperoxidase method as previously described.[4]

Definition of heart allograft rejection

Endomyocardial biopsies were carefully examined for the presence of rejection by three trained pathologists (P.B., P.R., and J.P.D.V.H.) according to updated international classification criteria. T-cell-mediated rejection was defined according to the International Society for Heart and Lung transplantation (ISHLT) 2004 classification.[21] Antibody-mediated rejection was defined according to the last recommendation of the pathology task force of the ISHLT[22] as follows: pAMR0: no features of ABMR; pAMR1: suspicious ABMR subdivided into pAMR1(H+) with histopathology positive and immunohistochemistry negative and pAMR1(I+) with histopathology negative and immunohistochemistry positive; pAMR2: histopathology and immunohistochemistry both positive; pAMR3: severe ABMR.[22] The rejection episodes were considered in both test and validation cohorts as early (occurring before 1-year post-transplant) and late (occurring after 1-year post-transplant), Supplementary material online, *Table S1*.

Detection of antibodies against donor-specific HLA molecules

Patients with available serum at the time of biopsy were screened for the presence of circulating anti-HLA antibodies. Antibodies against the HLA-A, HLA-B, HLA-DR, HLA-DQ, and HLA-DP epitopes were tested using single-antigen flow bead assays (One Lambda, Inc., Canoga Park, CA, USA) on a Luminex platform as previously described.[23,24]

MicroRNA analysis

Extraction of total RNA from frozen EMBs and serum was conducted anonymously. Sample information was replaced by numbers, and the technician was blinded to clinical information regarding allograft rejection status.

Extractions were performed with the Ambion Extraction Kit (Ambion, Austin, TX, USA) and the Qiagen miRNeasy Serum/Plasma kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations. The yield and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer. RNAs were then individually retrotranscribed using the microRNA reverse transcription kit (Applied Biosystems). Each reverse transcription was performed with 5 μL of a 1 ng/μL RNA solution, 7 μL of master mix [containing 100 U of transcriptase Superscript II (Invitrogen, Carlsbad, CA, USA) and a mix of dNTP], 3 μL of specific miRNAs probes (Ambion, Austin, TX, USA) in a BioRad Thermal Cycler using the following parameter values: 16°C for 30 min, 42°C for 30 and 85°C for 5 min. MicroRNAs were then quantified by real-time PCR using specific probes of the cDNA obtained from RT. Real-time PCR was performed with 2,5 μl of RT product mixed with 15 μl of ABsoluteTM QPCR Mix (ABgene, Epsom, UK) in a 385-well plate in a ABI 7500FAST real-time PCR system. miRNA with more than half of the cycle threshold (Ct) values >35 per group were excluded from the analysis.[25]

MicroRNA copy numbers were normalized using RNU48 small nucleolar RNA copy numbers to obtain ΔCt values. ΔΔCt was then obtained by subtracting the average of ΔCt to each ΔCt values. Finally, we determined fold values for each sample ($2^{(-\Delta\Delta Ct)}$).[26,27]

Selection strategy of relevant miRNAs related to allograft rejection

Selection of miRNAs was first conducted by careful *in silico* analysis, studying the literature for relevant miRNAs associated with allograft rejection. We also screened for miRNAs related to cardiovascular pathogenesis including endothelial injury, endothelial activation, and vascular inflammation.

We then performed a database screening (TargetScan.org, miRBase.org, microRNA.org) to determine relevant miRNA biomarker candidates according to their predicted interactions with molecules of interest (VCAM, ICAM, eNOS, heparan sulfate, CD68, and CD40) and signalling pathways (inflammatory, ischaemic and endothelial activation pathways, such as mTOR or NFkappaB).

Following this analysis, we identified 14 miRNAs of interest for heart transplant rejection (Supplementary material online, *Table S2*): (i) miRNAs predominantly expressed in endothelium and associated with endothelial activation (miR-92a, miR-126, miR-221, and miR-296);[28–32] (ii) miRNAs expressed in cardiomyocytes and associated with cardiovascular tissue remodelling (miR-21, mi-R31, and miR-208);[29,30,33,34] (iii) miRNAs related with inflammation (miR-10a, miR-142-3p, miR-155, miR-181a, miR-181b, miR-182, and miR-451).[26,35–41]

In situ hybridization

MicroRNA*in situ* hybridization (ISH) was performed on FFPE tissue as previously described.[31] After deparaffinization of the tissue sections, the tissue was incubated with PFA 4%, washed with PBS-DEPC and bathed with the acetylation solution. Sections were then washed and incubated with proteinase K (5 μg/mL) at 37°C. After washing, saturation followed with the incubation of sections with the hybridization buffer for 5 h. The probe for each miRNA (miRCURY LNA, microRNA detection probes from ExiKon, Vedbaek, Denmark) was then added to a preparation containing the hybridization buffer, CHAPS 10% and Tween 20%. Sections were incubated with this solution overnight at 56°C and rinsed afterward with SSC buffers. Sections were then incubated with B1 solution and with the blocking solution. Anti-DIG was added to the blocking solution and sections were incubated overnight. Sections were then washed with B1 solution and a NTMT/Levamisole solution. Sections were then revealed with NBT/BCIP mix for 3 h to 5 days depending on the specific miRNA. Sections were washed, incubated with PFA 4% and mounted. The negative control of ISH was performed using scrambled probe control (Supplementary material online, *Figure S1*).

Statistical methods

Continuous variables are reported as median, min max, and inter-quartile range. We compared means and proportions with Student's *t*-test and the χ^2 test (or Fisher's exact test if appropriate). Non-parametric analysis (Kruskal–Wallis and Mann–Whitney tests) was performed when appropriate. We used a conventional receiver-operating characteristic (ROC) curve to analyse miRNA levels to determine the cut-off points that yielded the highest combined sensitivity and specificity with respect to distinguishing subjects with acute rejection from subjects with normal biopsy results. We calculated the area under the curve (AUC) and 95% confidence intervals for the AUC. The association between intragraft miRNAs and serum levels of miRNAs was analysed using Pearson correlation coefficient.

The association of miRNAs of interest with rejection patterns was investigated using unsupervised methods such as hierarchical cluster analysis and principal component analysis based on the combination of the expression of the 14 miRNAs [fold values ($2^{(-\Delta\Delta Ct)}$] obtained from the ΔΔCt value for each patient and each miRNA (ΔCt of the miRNA for a patient − mean ΔCt of the population)]. Hierarchical cluster analysis and dendrograms were performed with the hcluster module of the amap package of the R software, while principal component analysis was carried out using the dudi.pca module of 12 the ade4 package of the R software (version 2.10.1). Other statistical analyses were performed using the STATA 11.0 software (Stata Corporation, College Station, TX, USA) and the Graphpad PRISM 5 software.

Results

Patient's characteristics

The main analysis included 60 heart transplant patients: heart recipients with a biopsy-proven acute rejection (acute rejection group, $n = 30$) and 30 matched control recipients with a normal heart allograft biopsy (normal allograft group). The two groups were similar with regard to recipient age, gender, primary heart disease, donor age, cold ischaemia time and maintenance immunosuppression. The median time since transplantation and index biopsy was also similar between the two groups: 22 months in the rejection group ($_{25-75}$IQR: 12–49) and 24 months ($_{25-75}$IQR: 10–52). No patient had ongoing sepsis or CMV infection at the time of index biopsy. Anti-HLA donor-specific antibodies assessment at the time of index EMB revealed that 2 out of 30 (7%) patients from the control group had DSA when compared with 21/30 in the rejection group

Table 1 Baseline patient's characteristics in the principal cohort

	Normal allografts ($n = 30$)	Rejecting allografts ($n = 30$)	*P*-value
Recipient age, [median, $_{IQR}$ (25–75)]	43 (33–51)	41 (32–48)	0.6
Recipient gender, male, *n* (%)	21 (70)	17 (57)	0.4
Primary heart disease, *n* (%)			
Congenital cardiopathy	4 (13)	5 (17)	1.0
Non-ischaemic cardiopathy	11 (37)	15 (50)	0.4
Ischaemic cardiopathy	10 (33)	6 (20)	0.4
Valvular cardiomyopathy	3 (10)	2 (7)	1.0
Retransplant	0 (0)	1 (3)	1.0
Miscellaneous	2 (7)	1 (3)	1.0
Donor age [median, $_{IQR}$ (25–75)]	44 (31–49)	42 (25–52)	0.6
Donor gender, male, *n* (%)	24 (80)	20 (67)	0.4
Cold ischaemia time, *min* [median, $_{IQR}$ (25–75)]	210 (147–235)	208 (120–242)	0.8
Maintenance immunosuppression *n* (%)			
Steroids	28 (93)	29 (97)	1.0
Calcineurin inhibitors	28 (93)	30 (100)	0.5
Mycofenolate acid	25 (83)	22 (73)	0.5
m-TOR inhibitors	15 (50)	14 (47)	1.0
Azathioprine	5 (17)	2 (7)	0.4
Circulating donor specific anti-HLA antibodies (DSA) at time of index EMB *n* (%)	2/30 (7)	TCMR ($n = 2/11,18$) ABMR[a] ($n = 19/19,100$)	<0.0001*
Time between Tx and Index EMB, months $_{IQR}$ (25–75)	22 (12–49)	24 (10–52)	0.9

TCMR, T-cell-mediated rejection; ABMR, antibody-mediated rejection.
**P*-value comparing the % of patients with circulating donor-specific anti-HLA antibodies (DSA) in the normal allograft group vs. rejecting allograft group.
[a]$P < 0.0001$: *P*-value comparing the % of patients with circulating donor-specific anti-HLA antibodies (DSA) in the TCMR group vs. ABMR group.

(70%, $P < 0.0001$). The baseline patient characteristics are detailed in *Table 1*. The acute rejection group included 11 cases of T-cell-mediated rejection and 19 cases of AMR. Both groups received similar maintenance therapy. The detail of rejection histopathological grade is given in Supplementary material online, *Table S3*.

MicroRNAs expression in heart allograft biopsies

The dendrogram and unsupervised principal component analysis were examined in all heart transplant biopsies and the respective expression of the 14 miRNAs identified in primary analysis (Supplementary material online, *Figure S2*). Among the 14 miRNAs, we identified 7 miRNAs that were highly ($P < 0.0001$) differentially expressed (over or under expressed) between normal and rejecting heart allografts: miR-10a, miR-21, miR-31, miR-92a, miR-142-3p miR-155, and miR-451 (*Figure 1A*). Four miRNAs (miR-126, miR-181b, miR-182, and miR-296) showed some association with rejection, but at a lower significance ($0.001 < P < 0.05$). Three remaining miRNAs were not found to be associated with rejection (miR-181a, miR-208, and miR-221).

In situ hybridization performed in heart allograft biopsy showed that miR-10a and MiR-92a were expressed in endothelial cells, while MiR-31 was localized in interstitial cells (*Figure 1B*). *In situ* hybridization analysis was not available for the remaining four miRNAs of interest.

Circulating microRNAs predict cardiac allograft rejection

We tested whether the seven miRNAs differentially expressed in EMBs showed distinct expression in the concomitant patient's serum. Three miRNAs (miR-21, miR-142-3p, and miR-451) were not amenable to qPCR amplification in the serum (CT value >35, see Methods), leaving four miRNAs (miR-10a, miR-31, miR-92a, and miR-155) that demonstrated distinct expression with regard to the heart allograft status; normal vs. rejection (*Figure 2*). Levels of miR-31, miR-92a, and miR-155 were significantly higher in the sera of patients with rejecting allografts compared with patients without rejection ($P < 0.0001$ for all comparisons). The level of miR-10a was significantly lower in the sera of patients with rejecting allografts compared with patients without rejection ($P < 0.0001$).

The ROC analysis showed that these four circulating miRNAs strongly discriminated patients with allograft rejection from patients without rejection [miR-10a: AUC = 0.975, $CI_{95\%}$ = (0.946–1.005); miR-31: AUC = 0.932, $CI_{95\%}$ = (0.876–0.989), miR-92a: AUC =

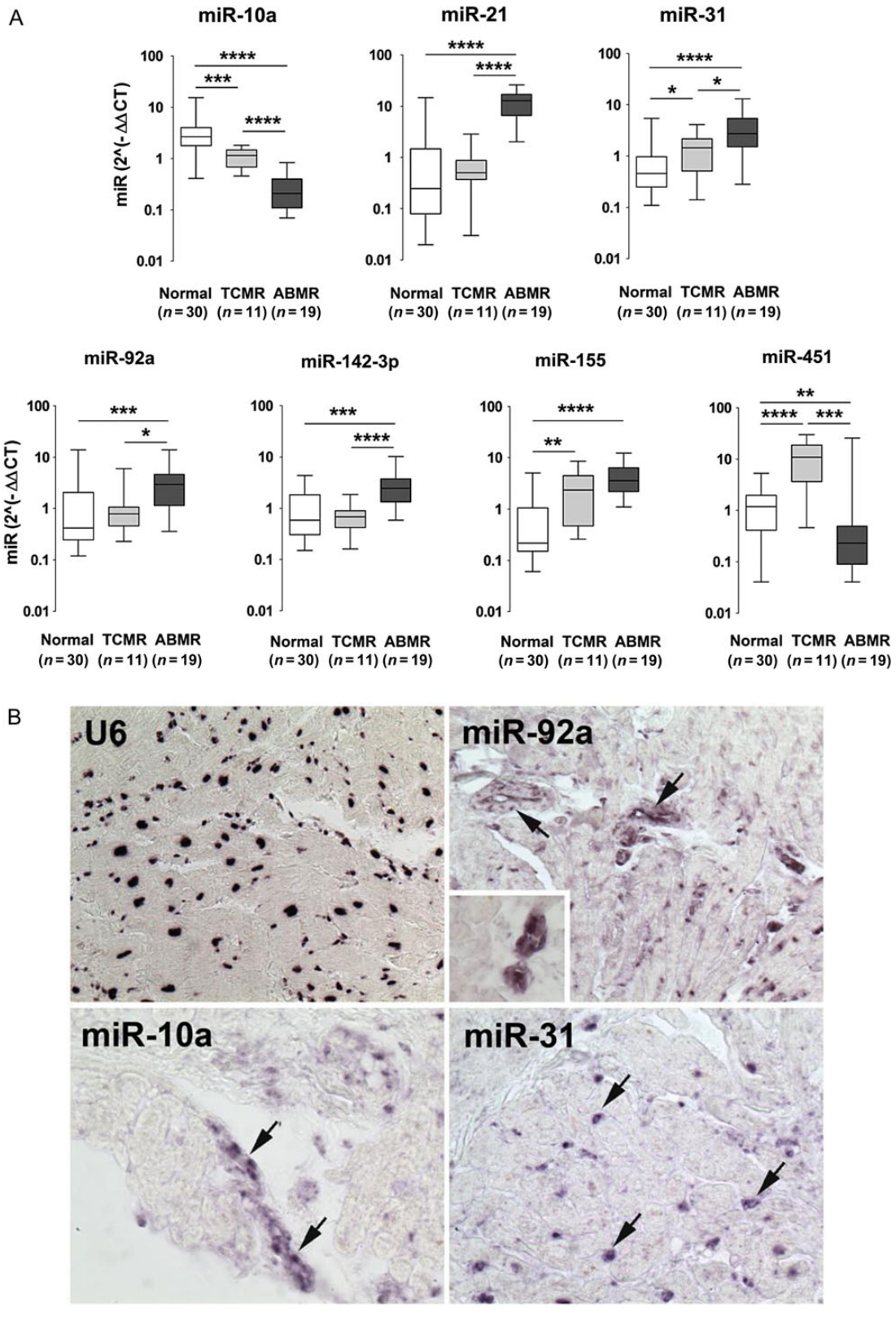

Figure 1 (*A*) miRNAs differentially expressed (over- or underexpressed) between normal and rejecting heart allografts. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$0.01 < P < 0.05$. (*B*) Localization of miR-92a, miR-10a and miR-31 in heart allograft by *in situ* hybridization. miR-92a and miR-10a are localized in the endothelium of the microcirculation (arrows and insert). miR-31 probe stained the nuclei of interstitial cells (arrows). U6 probe stained all nuclei (positive control).

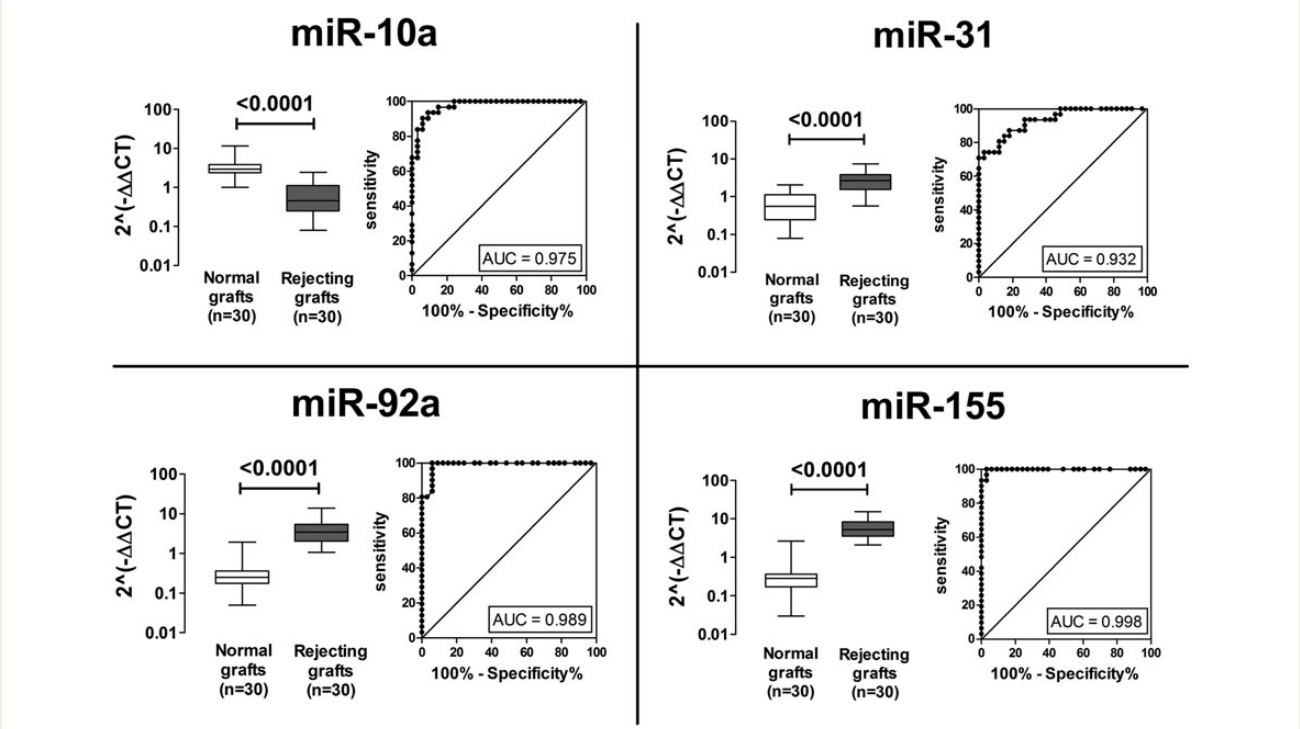

Figure 2 Diagnostic accuracy of miRNA levels in the patient's serum from the test cohort.

0.989, $CI_{95\%}$ = (0.971–1.007), and miR-155: AUC = 0.998, $CI_{95\%}$ = (0.993–1.003), $P < 0.0001$ for all comparisons] (*Figure 2*).

Finally, there was a high correlation between tissue and serological expression of the four miRNAs (Pearson analysis, miR-10a: $R^2 = 0.369$, $P = 0.0006$; miR-31: $R^2 = 0.207$, $P = 0.015$; miR-92a: $R^2 = 0.326$, $P = 0.0015$; miR-155: $R^2 = 0.4702$, $P < 0.0001$).

External validation of the circulating microRNA rejection signature

The external validation set was composed of 53 heart transplant recipients from three different centres randomly selected on the basis of the heart allograft status: biopsy-proven acute rejection or normal allograft biopsy, all having serum available at the time of biopsy. The baseline characteristics of the validation set are shown in Supplementary material online, *Table S4*.

Of the 53 patients included, 31 had allograft rejection (T-cell-mediated rejection $n = 14$ and AMR, $n = 17$), while 22 had normal allograft EMB (Supplementary material online, *Table S3*).

We confirmed that the four relevant miRNAs found in the primary analyses highly discriminated patients with rejection from those without (*Figure 3*): miR-10a [AUC = 0.981, $CI_{95\%}$ = (0.947–1.015), miR-31 (AUC = 0.867, $CI_{95\%}$ = (0.771–0.963)), miR-92a (AUC = 0.959, $CI_{95\%}$ = (0.908–1.009)), and miR-155 (AUC = 0.974, $CI_{95\%}$ = (0.940–1.007)] ($P < 0.0001$ for all comparisons).

Sensitivity analysis

The sensitivity analysis was conducted to confirm that the miRNA rejection signature was robust across the two rejection entities (i.e. T-cell-mediated rejection and antibody-mediated rejection). We stratified the ROC analysis by rejection diagnosis and found that all four miRNAs were associated with T-cell-mediated rejection [miR-10a (AUC = 0.981), miR-31 (AUC = 0.902), miR-92a (AUC = 0.977), and miR-155 (AUC = 0.984, Supplementary material online, *Figure S3*) and also discriminated patients with antibody-mediated rejection (miR-10a (AUC = 0.969), miR-31 (AUC = 0.903), miR-92a (AUC = 0.984), and miR-155 (AUC = 0.986), (Supplementary material online, *Figure S4* $P < 0.0001$ for all comparisons between normal and rejection biopsies]. We also confirmed that the four miRNAs are differentially expressed in normal allografts vs. rejecting allografts in both early (before 1 year) and late (after 1 year) rejection cases (Supplementary material online, *Figure S5*).

Discussion

In this study, we demonstrated that four miRNAs, miR-10a, miR-31, miR-92a, and miR-155, showed differential tissue expression between rejecting and normal heart allografts. We also showed that strong correlations exist between tissue and serological expression of these four miRNAs and that their assessment in patients' sera permits discrimination with very high accuracy between patients with allograft rejection and those without. Taken together, our results suggest that these miRNAs are of potential clinical interest as non-invasive biomarkers of heart transplant rejection.

Personalized medicine[42] has gained momentum in the transplantation field.[5,43] The improvement of knowledge regarding rejection pathophysiology together with the implementation of technologies

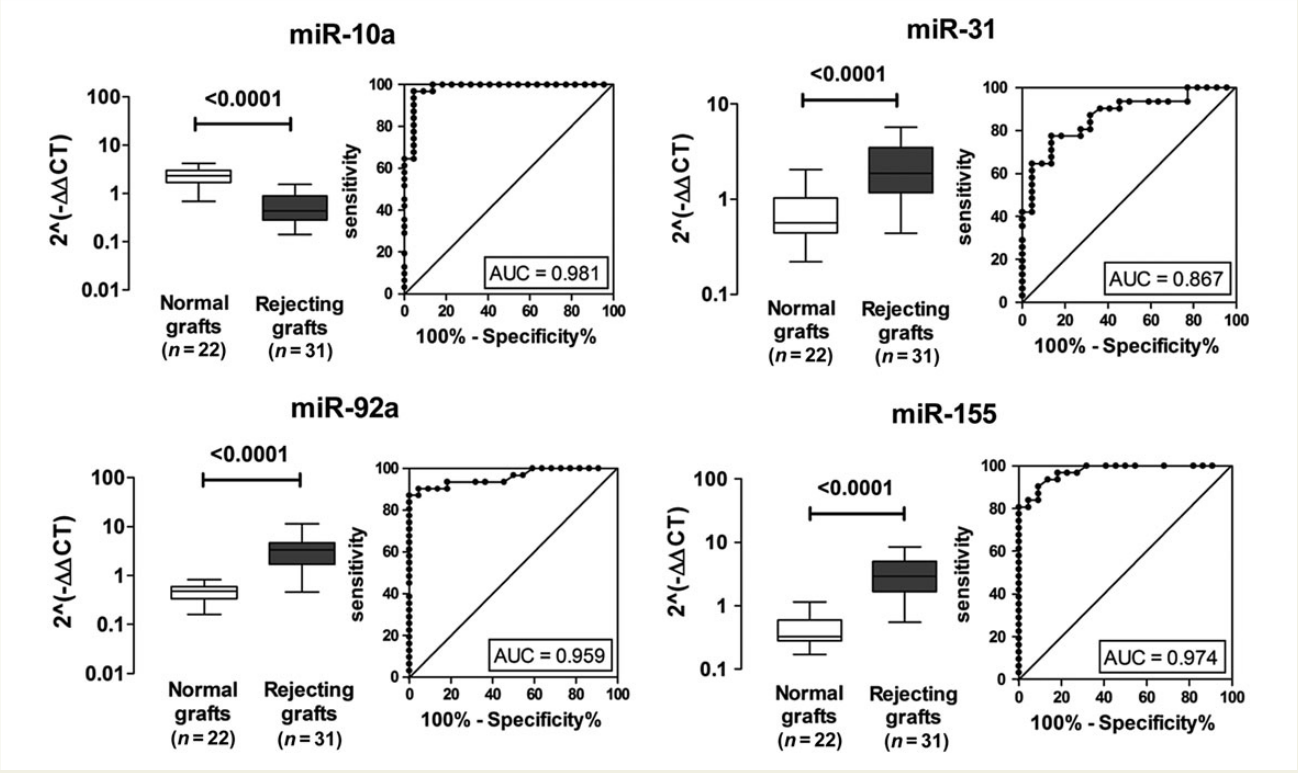

Figure 3 Diagnostic accuracy of miRNA levels in the patient's serum from the validation cohort.

has led to the emergence of new potential biomarker candidates for predicting allograft rejection.[19,44] In recent years, the field of kidney transplantation has moved to molecular medicine with growing evidence that molecular techniques applied to biopsies[45] and urine analysis[46,47] could be useful for both investigating biopsies and screening patients at risk for allograft rejection in a non-invasive manner.

In heart transplantation, defining non-invasive and relevant biomarkers for allograft rejection is an unmet need. This is particularly true in the context of current clinical practice that includes repeated and routine EMBs for screening allograft rejection, a procedure that is not without consequences for the patient.[2] An important study published in 2006 provided proof that a peripheral blood gene-expression profiling strategy could be used in heart transplantation without an increased risk of serious adverse outcomes.[6] However, this study was limited by several factors including the low immunological risk of the population studied as well as the exclusive attention to T-cell-mediated rejection without assessment of antibody-mediated rejection,[7] which today represents a serious threat to heart allografts.[3,4] Moreover, this approach was limited to the genes expressed in leucocytes, potentially ignoring significant genes expressed in other cell types or heart tissue specific genes. Finally, in this study, the gene signature found in the peripheral blood was not tested in allograft biopsies.[7]

MicroRNAs have recently emerged as relevant candidates in the field of organ rejection because of their capacity to regulate thousands of genes that are key elements of both innate and adaptive immune responses.[16–18] The potential interest of microRNAs lies in the fact that their expression is regulated by several inflammatory stimuli. Further, some miRNAs have tissue specificity and are involved in a number of processes such the regulation of adhesion and inflammation or angiogenesis,[29,48] making them markers of choice for microvascular inflammation observed the humoral rejection in heart transplantation.[22] These properties were the basis for selecting potentially relevant miRNAs and for testing whether these were differentially expressed in rejecting allografts in both tissue and peripheral blood.

The four miRNAs identified in our analysis (miR-10a, miR-155, miR-31, and miR-92a) were specific for inflammatory burdens in endothelial cells, inflammatory pathways, cardiomyocytes/interstitial cells, and endothelial cells, respectively. MiR-10a has been shown to inhibit NFkB signalling pathway and consequently is involved in increasing the pro-inflammatory markers MCP-1, IL -6, IL -8, -1, and VCAM in endothelial cells.[35] Lind *et al.*[36] described several inflammatory functions of miR-155 that include its increased expression following the activation of the T-cell receptor, the repression of the expression of the IFN receptor and the contribution to Ig class switching in B cells. MiR-31 mainly regulates the expression of E-selectin and ICAM-1 when induced by the TNF pathway[49] as well as integrins (α2, α5, and β1 subunit), indicating its major role in the regulation of inter-/intra-cell adhesion. Finally, miR-92a targets the integrin α5, S1P1, MKK4, and eNOS, demonstrating an endothelial tropism and a potentially important role in the vascular inflammatory response.[50] All of these functions have been linked to processes implicated in both humoral and cellular rejection in solid organ transplantation. In support of this possibility, we demonstrated that miR-10a and MiR-92a are mainly expressed in heart allograft

endothelial cells, which are the primary target for the alloimmune response, while MiR-31 is localized in heart allograft interstitial cells (note that we were not able to obtain a reliable and robust signal for miR-155 reaching our standards for ISH).

Strengths and limitations

In this paper, we employed a unique tissue and serological approach using relevant pathogenesis-based miRNAs that reflected immune response and heart tissue specificity. We also used an extensive allograft phenotyping with contemporary tools to diagnose cases of T-cell and antibody-mediated rejection. Our study also includes rejection episodes occurring in the first year post-transplant (41%) as well as rejection occurring after 1-year post-transplant (59%), most of late cases antibody-mediated rejection (67% of late rejections). Therefore, this represents stereotypical clinical situations and the contemporary picture of allograft rejection encountered in the modern era of heart transplantation. The important differences that we found in miRNA expression between normal and rejecting allografts could partly be explained by the 'sick vs. well' strategy as part of our case selection criteria. While this approach is relevant to isolate relevant biomarkers as part of discovery sets (and we confirmed the associations in independent validation set), our results need to be tested in unselected prospective cohorts of heart recipients. Moreover, we used in the present study a pathogenesis-based strategy of miRNA selection. Despite our results are robust, we cannot exclude that other potentially relevant miRNAs could be involved in heart allograft rejection. We believe our results will serve as a basis for future investigations regarding the clinical relevance of applying a non-invasive miRNA strategy to detect normal or rejecting heart allografts, to establish thresholds and decisions based on the miRNA values and to determine the kinetics of miRNA as well as response to treatment.

Finally, we are not seeking to compare the clinical relevance of a peripheral miRNA signature to histopathology assessment, which still represent the gold standard for the diagnosis of heart allograft rejection. In current practice, a heart biopsy remains mandatory in clinical situations. However, we believe that the peripheral profiling of miRNAs could guide clinicians in the setting of stable heart allografts and aid in monitoring and determining in which cases a screening biopsy would be mandatory.

Conclusion

In this study, we demonstrated that regulation in the expression of miRNAs occurs during heart transplant rejection, not only at the tissue level but also in the patient's serum. We also showed that the assessment of miRNAs miR-10a, miR-31, miR-92a, and miR-155 in patients' sera discriminates with a very high accuracy between patients with allograft rejection and those without. This suggests that miRNAs may represent relevant and non-invasive biomarkers that may serve to better investigate heart transplant rejection and guide the clinical management of heart recipients.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Funding

This research has been supported by funding and/or resources from Novartis Pharma France and Astellas France.

Conflict of interest: The authors declare no financial relationships with any organizations that might have an interest in the submitted work in the previous three years. There are no other relationships or activities that could appear to have influenced the submitted work. All authors, external and internal, had full access to all of the study data (including statistical reports and tables) and take full responsibility for the integrity of the data and the accuracy of the data analysis.

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