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Alpha-cardiac Actin Serum Expression Levels Detect Acute Cellular Rejection in Heart Transplant Patients

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Background. Given the central role of sarcomeric dysfunction in cardiomyocyte biology and sarcomere alterations described in endomyocardial biopsies of transplant patients with rejection, we hypothesized that the serum expression levels of genes encoding sarcomeric proteins were altered in acute cellular rejection (ACR). The aim of this study is to identify altered sarcomere-related molecules in serum and to evaluate their diagnostic accuracy for detecting rejection episodes. **Methods.** Serum samples from transplant recipients undergoing routine endomyocardial biopsies were included in an RNA sequencing analysis (n = 40). Protein concentrations of alpha-cardiac actin were determined using a specific enzyme-linked immunoassay (n = 80). **Results.** We identified 17 sarcomeric genes differentially expressed in patients with clinically relevant rejection (grade ≥2R ACR). A receiver operating characteristic curve was done to assess their accuracy for ACR detection and found that 6 relevant actins, myosins, and other sarcomere-related genes showed great diagnostic capacity with an area under the curve (AUC) > 0.800. Specifically, the gene encoding alpha-cardiac actin (*ACTC1*) showed the best results (AUC = 1.000, $P < 0.0001$). We determine ACTC1 protein levels in a larger patient cohort, corroborating its overexpression and obtaining a significant diagnostic capacity for clinically relevant rejection (AUC = 0.702, $P < 0.05$). **Conclusions.** Sarcomeric alterations are reflected in peripheral blood of patients with allograft rejection. Because of their precision to detect ACR, we propose sarcomere ACTC1 serum expression levels as potential candidate for to be included in the development of molecular panel testing for noninvasive ACR detection.
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INTRODUCTION

Allograft rejection is among the main causes of death in the first year after cardiac transplantation.[1] Currently, the gold standard for diagnosing and monitoring cardiac allograft rejection is based on the multiple and serial collection of endomyocardial biopsies (EMBs). However, this technique presents considerable limitations. EMBs are an invasive process associated with infrequent but potentially

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serious complications and are prone to sampling error and interobserver variability.[2]

Therefore, noninvasive methods and reliable biomarkers are actively sought to screen for heart transplant rejection and to better understand the pathophysiology of this posttransplant complication.[3] The omics sciences have a great potential to identify biomarkers in the body fluid samples of patients undergoing heart transplantation.[4-6] Furthermore, studies previously conducted on EMB point to the existence of gene and protein dysregulation during cardiac rejection.[7-10]

Cardiac contractility is regulated through calcium homeostasis, cell signaling, and the maintenance of the sarcomere, the smallest contractile unit of the cardiac muscle.[11] The proper functioning of the sarcomere is determined by the expression levels of different sarcomeric genes.[12,13] Specifically, alterations have been observed in the cytoskeletal and sarcomeric genes; this suggests the existence of structural changes in the cardiomyocytes.[14,15] Alterations in the structure of the sarcomere, such as loss of myosin filaments and anomalies in the Z-lines, have also been described in EMB of patients with acute cellular rejection (ACR).[16,17] The basic components of sarcomere are actin and myosin; they are associated with other structural and regulatory proteins.[18] However, the studies based on the detection of these molecules in the peripheral blood from patients with cardiac allograft rejection are limited and are focused only on specific proteins such as troponin T or I.[19-21]

Taking into account this background, we performed a large-scale expression profiling and explored the link between histological indicators of ACR in the transplanted human heart and the serum expression levels of genes encoding sarcomeric proteins.

MATERIALS AND METHODS

Sample Collection

A total of 40 consecutive EMB and blood samples were collected from heart transplant patients (>18 y) who were referred for EMB for routine surveillance before the availability of the gene expression profiling analysis at the University and Polytechnic Hospital La Fe (October 2016 to April 2017). At the time of EMB, blood samples were collected for laboratory analysis. Rejection episodes were assessed according to the International Society for Heart and Lung Transplantation consensus report.[22] First, a preliminary RNA sequencing study included 40 samples from 24 heart transplant patients (grade 0R, n = 12, grade 1R, n = 16; and grade ≥2R, n = 12). Next, we used an additional cohort of 80 consecutive samples from 30 heart transplant patients (grade 0R, n = 41; grade 1R, n = 28; and grade ≥2R, n = 11) for protein analysis. The associated clinical data were also collected at the time of each biopsy (Table 1). Experimenters were blinded to group allocation and outcome assessment.

This study was approved by the Ethics Committee (Biomedical Investigation Ethics Committee of the University and Polytechnic Hospital La Fe of Valencia, Spain) and was conducted in accordance with the principles outlined in the Declaration of Helsinki.[23] Informed consent was obtained from each patient before sample collection.

RNA Sequencing Study

RNA sequencing analysis has been extensively described by Tarazón et al.[24] Briefly, RNA extraction was performed using NucleoSpin miRNA Plasma of Macherey Nagel, following the protocol and instructions provided by the manufacturer. RNA quantification was performed using a NanoDrop 1000 spectrophotometer and the Qubit 3.0 fluorometer (Thermo Fisher Scientific). cDNA libraries were obtained following Illumina's recommendations. The quality and quantity of cDNA libraries were analyzed using the High-Sensitivity D1000 ScreenTape Assay and the 4200 TapeStation System (Agilent Technologies). cDNA libraries were then pooled and sequenced by two lanes of 100 bp paired-end sequencing using an Illumina HiSeq 2500 sequencer.

Quality control of the raw sequence data was performed using FastQC software. The raw paired-end reads were mapped against the human hg38 genome using the bowtie algorithm.[25] Insufficient quality reads, with a phred score ≤20, were eliminated using the SAMtools method.[26] RNA quantification was then estimated using HTSeq software (version 0.6.0).[27] Lastly, differential expression analysis between conditions were assessed using the DESeq2 method (version 3.4).[28]

Enzyme-linked Immunoassay

ACTC1 was determined using a specific sandwich enzyme-linked immunoassay (ELISA) (Human Actin, Alpha Cardiac Muscle 1 [ACTC1] ELISA kit, MyBioSource, CA). The ACTC1 test has a limit of detection of 2 ng/mL. The intra-assay and interassay coefficients of variation were 9% and 11%, respectively. No significant cross-reactivity or interference between ACTC1 and analogues was observed. The tests were quantified at 450 nm in a dual-wavelength microplate reader (Sunrise; Tecan, Switzerland) using Magellan version 2.5 software (Tecan, Switzerland).

Statistical Analysis

Clinical characteristics were expressed as mean ± SD for continuous variables and percentages for discrete variables. Results for each variable were assessed for normality using the Kolmogorov–Smirnov test. Continuous variables not following a normal distribution were compared using the Mann–Whitney test, and variables with a normal distribution were compared using the Student *t* test. Fisher exact test was used to compare discrete variables. The diagnostic capability of serum markers for the presence of transplant rejection was assessed by the construction of receiver operating characteristic (ROC) curves. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0; SPSS Inc, IL).

For RNA sequencing analysis, we used the false discovery rate method for adjusts the original *P* value using the number of tests. Differentially expressed RNAs with fold change values ±1.5, and with false discovery rate adjusted $P \leq 0.05$ were included to avoid identification of false positives across the differential expression data.[28]

RESULTS

Clinical Characteristics of the Patients

Only patients with grade ≥2R ACR displayed significant differences between specific clinical characteristics

TABLE 1.

Patient characteristics at the time of biopsy and blood sample extraction

	RNA sequencing study		ELISA	
	Non-ACR (n = 12)	ACR ≥2R (n = 12)	Non-ACR (n = 41)	ACR ≥2R (n = 11)
Age, y	48 ± 15	42 ± 15	50 ± 13	46 ± 13
Male sex, %	75	75	88	82
Time between transplantation and study enrollment, mo	8.1 ± 3.8	3.8 ± 4.2[a]	7.2 ± 4.1	3.9 ± 3.7[a]
Body mass index, kg/m^2	25 ± 5	24 ± 3	25 ± 4	26 ± 5
Hypertension, %	58	42	59	36
Diabetes, %	58	50	29	36
Dyslipemia, %	42	25	49	27
Echo-Doppler study				
Ejection fraction, %	72 ± 8	70 ± 10	71 ± 7	70 ± 6
LV end-systolic diameter, mm	25 ± 3	31 ± 3[a]	28 ± 6	27 ± 6
LV end-diastolic diameter, mm	41 ± 3	45 ± 4	44 ± 5	42 ± 3
Hemodynamic parameters				
Mean right atrial pressure, mm Hg	3.6 ± 1.8	7.7 ± 1.5[a]	4.3 ± 2.4	8.5 ± 0.7[a]
Systolic right ventricular pressure, mm Hg	33 ± 5	42 ± 4[a]	37 ± 7	44 ± 2
Diastolic right ventricular pressure, mm Hg	4.2 ± 1.6	8.7 ± 3.8	4.6 ± 3.1	9.5 ± 4.9
Immunosuppressive therapy				
Tacrolimus, %	100	100	100	100
Mycophenolic acid, %	100	100	100	100
Steroids, %	100	100	100	100
Induction therapy				
Basiliximab, %	100	100	100	100
Neutrophils, thousands/mm^3	4.8 ± 4.1	8.2 ± 6.3	5.1 ± 4.3	6.5 ± 5.3
Leukocytes, thousands/mm^3	7.1 ± 3.8	11.0 ± 6.3	7.5 ± 5.3	9.3 ± 5.7
Lymphocytes, thousands/mm^3	1.5 ± 0.53	2.0 ± 0.8	1.5 ± 0.9	1.8 ± 0.9
Hemoglobin, mg/dL	11.6 ± 2.4	11.9 ± 1.7	11.8 ± 2.0	11.4 ± 1.9
Hematocrit, %	37 ± 8	37 ± 4	37 ± 6	36 ± 5
NT-proBNP, pg/mL	152 (113–467)	1209 (736–2382)[a]	378 (154–1120)	2043 (868–5445)[b]
Troponin T, ng/L	19 (11–66)	25 (13–40)	21 (10–51)	33 (26–77)

[a]$P < 0.05$.
[b]$P < 0.0001$.
ACR, acute cellular rejection; LV, left ventricular; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

when we compared with the nonrejection group. Patients with grade ≥2R ACR showed worse hemodynamic function. We found higher values in the mean right atrial pressure, systolic right ventricular pressure, and left ventricular end-systolic diameter in grade ≥2R ACR compared with nonrejection patients. In addition, we found an increase in N-terminal pro-B-type natriuretic peptide levels when we compared with the nonrejection group (Table 1).

Differentially Expressed Sarcomere-related Genes

This study focused on the expression analysis of sarcomere-associated genes. We classified these molecules according to the main sarcomeric gene families: actins, myosins (light chain myosins and heavy chain myosins), actinins, troponins, tropomyosins, and other sarcomeric genes. We identified 59 sarcomeric genes in the serum of posttransplant patients, 17 of which were differentially expressed in patients with clinically relevant rejection (grade ≥2R ACR), compared with the case in patients without rejection (**Table S1, SDC**, http://links.lww.com/TP/C500). We found an upregulation of alpha-cardiac actin (*ACTC1*) (Figure 1A) and also observed the significant dysregulation of several myosins: light chain myosins such as *MYL3* (Figure 1B) and heavy chain myosins such as *MYH1* (Figure 1C). We also found changes in the expression levels of some actinins (Figure 2A), troponins, tropomyosins such as *TPM4* (Figure 2B), and other known sarcomere-related genes, such as *DES* and *CAPZA2* (Figure 2C).

Next, we analyzed the diagnostic capacity of the expression of the main structural genes of the sarcomere to detect heart transplant rejection. As shown in Table 2, the ROC curves of these molecules were obtained. Specifically, the best diagnostic capacity for the detection of patients with moderate or severe degree of rejection (area under the curve [AUC] >0.800) correspond to *ACTC1* (AUC = 1.000, $P < 0.0001$), *MYL3* (AUC = 0.889, $P = 0.001$), *MYH1* (AUC = 0.813, $P = 0.009$), *TPM4* (AUC = 0.840, $P = 0.005$), *DES* (AUC = 0.958, $P < 0.0001$), and *CAPZA2* (AUC = 0.931, $P < 0.0001$). The sensitivities, specificities, and predictive values are also included in Table 2.

We represented fold change over the relative mRNA expression levels in the nonrejection group of the seven structural sarcomere genes with the best diagnostic capacity. The mRNA levels of central sarcomere-related genes, such as *MYH1* and *TPM4*, were significantly decreased in the samples from subjects with rejection grade ≥2R, whereas those of other genes, *DES, ACTC1, CAPZA2,*

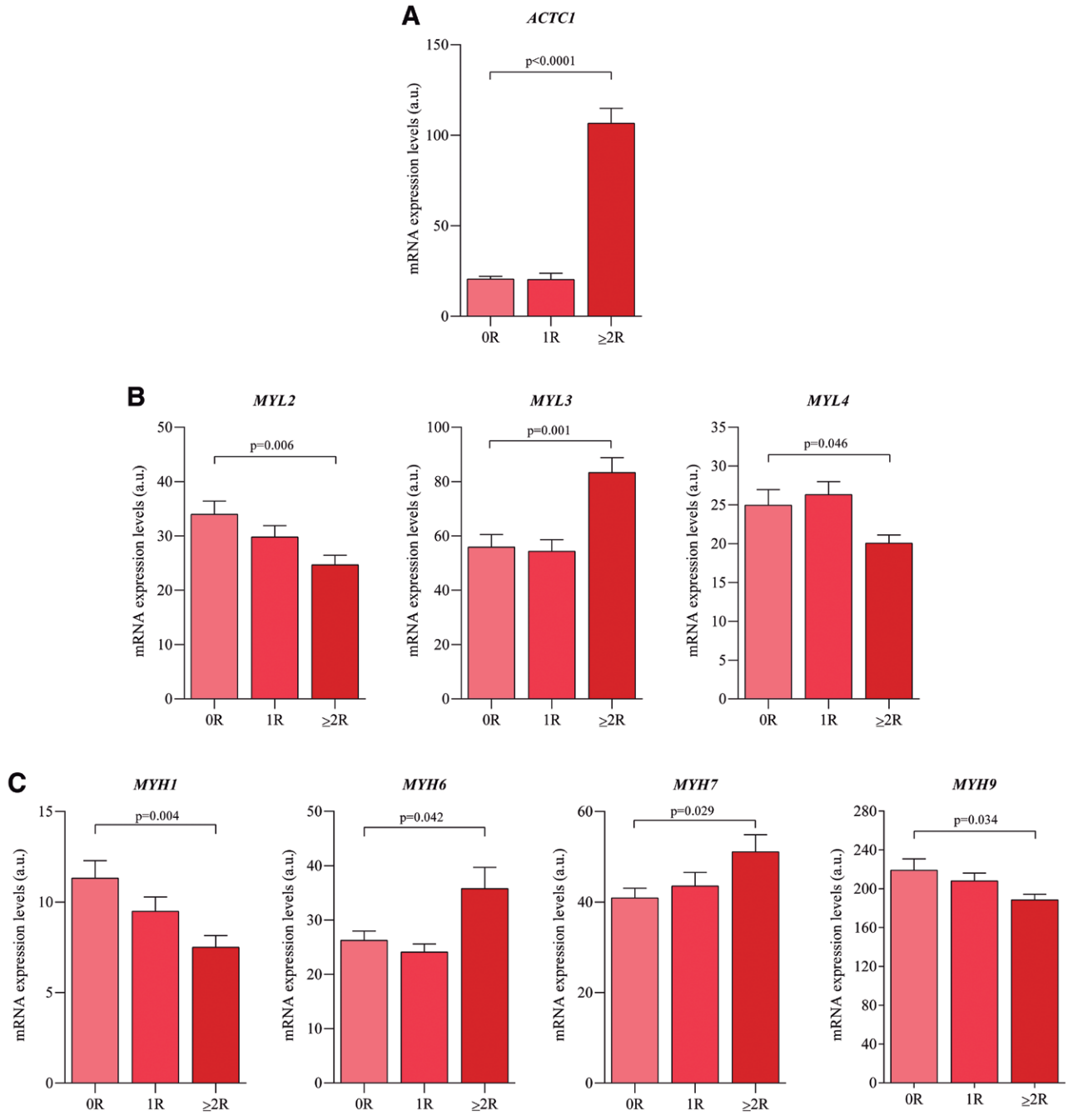

FIGURE 1. Circulating expression levels of mRNA encoding alpha-cardiac actin (*ACTC1*) and different altered myosins. Comparison between nonrejection and the different grades of acute rejection of heart allografts (acute cellular rejection [ACR] grade 1R and grade ≥2R). Bar graph of mRNA expression levels ± SEM of *ACTC1* (A), light chain myosins (B), and heavy chain myosins (C). Values were obtained by Illumina HiSeq 2500 sequencing. a.u., arbitrary units.

and *MYL3*, were increased (Figure 3A). In addition, those were represented in a heat map and hierarchical clustering based on the fold change values. Notably, this analysis identified two divergent gene expression profiles, showing a clear demarcation between the rejection grade ≥2R and the nonrejection group (Figure 3B).

Alpha-cardiac Actin Protein Levels

Taking into account the excellent diagnostic value observed in *ACTC1*, we decided to verify the overexpression in alpha-cardiac actin also occurred at the protein level in a larger patient cohort. ACTC1 protein levels were increased in patients with clinically relevant rejection (grade ≥2R ACR) (39.87 [27.65–67.48] ng/mL versus 71.43 [36.72–440.88] ng/mL P = 0.041; Figure 4A). We observed a correlation between mRNA and protein serum levels (r = 0.486, P = 0.019). However, we did not observe differences in expression between patients without rejection and patients with mild rejection. Furthermore, we confirmed that circulating ACTC1 protein levels, as in the primary analysis at mRNA level, discriminates between patients with rejection from those without (Figure 4B). ROC curves confirm the capability of ACTC1 protein levels for detecting heart transplant rejection obtaining a

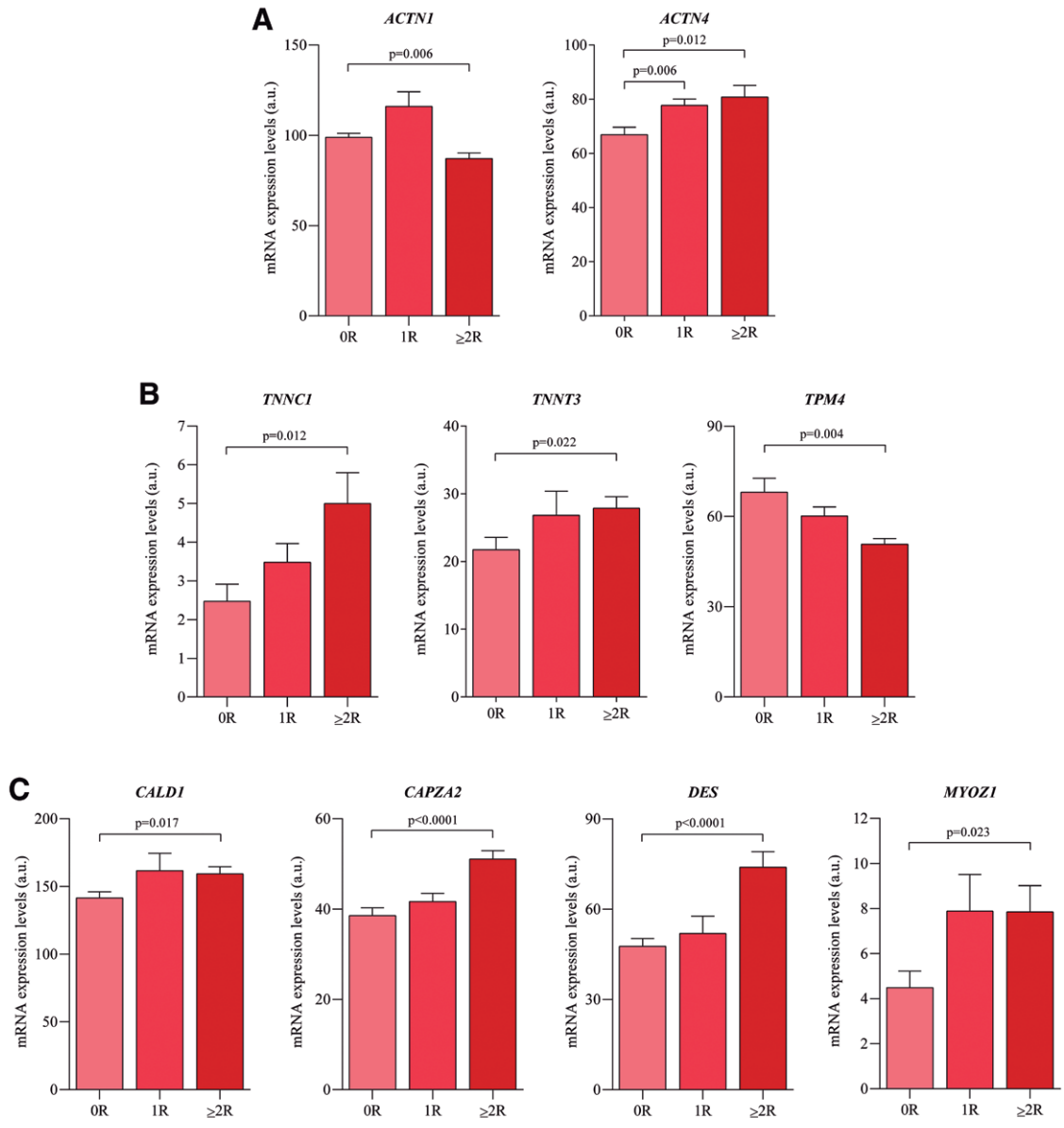

FIGURE 2. Circulating expression levels of mRNA encoding altered actinins, troponins, tropomyosins, and other sarcomeric genes. Comparison between nonrejection and the different grades of acute rejection of heart allografts (acute cellular rejection [ACR] grade 1R and grade ≥2R). Bar graph of mRNA expression levels ± SEM of actinins (A), troponins and tropomyosins (B), and other sarcomeric genes (C). Values were obtained by Illumina HiSeq 2500 sequencing. a.u., arbitrary units.

significant area under the curve (AUC = 0.702; $P = 0.041$). The sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis of rejection were 36%, 100%, 36%, and 100%, respectively (optimum cutoff point 264.57 ng/mL obtained from the ROC curve).

DISCUSSION

Acute rejection continues to represent a challenge in the heart transplant area because immune injury contributes to graft failure[29] and cardiac graft vasculopathy,[30] being one of the main causes of death. Thus, an understanding of the mechanisms involved in rejection and a better diagnosis are required to improve the survival of transplant patients. Despite the important body of evidence highlighting the preponderant role of the sarcomere dysfunction in cardiac pathologies and in the rejection process, a large-scale study has never been performed to detect potential sarcomere-associated molecules altered in the peripheral blood of transplant patients. This study focused on the detection and evaluation of circulating mRNA levels of sarcomeric structure-associated genes in patients with cardiac ACR. Our results showed a relevant dysregulation of the main genes related to the sarcomeric cytoskeleton, such as the alpha-cardiac actin *ACTC1* and its protein expression.

Previous studies on EMB showed dysregulation of sarcomeric genes and abnormalities in the structure of the sarcomere in patients with rejection.[14-17] Specifically, an increase in the protein levels of the beta heavy chain myosin has been previously reported.[15] This is a cardiac sarcomere-specific protein encoded by the *MYH7* gene. In concordance with these results, we detected *MYH7* mRNA levels in serum and the expression of this gene was increased in patients with clinically relevant rejection. The

TABLE 2.

ROC curve of circulating altered sarcomere mRNA for detecting heart transplant rejection (ACR grade ≥2R)

Gene name	AUC	*P*	95% CI	SS	SP	PPV	NPV
ACTC1	1.000	<0.0001	1.000-1.000	100	83	86	100
MYL3	0.889	0.001	0.748-1.000	75	83	83	83
MYH1	0.813	0.009	0.641-0.984	67	83	78	67
TPM4	0.840	0.005	0.664-1.000	58	92	80	58
DES	0.958	<0.0001	0.887-1.000	83	100	100	86
CAPZA2	0.931	<0.0001	0.817-1.000	58	92	89	73

Sensitivities, specificities, and predictive values (%) for the diagnosis of cardiac rejection (cutoff point FC ≥ 1.3).
ACR, acute cellular rejection; AUC, area under the curve; CI, confidence interval; FC, fold change; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; SP, specificity; SS, sensitivity.

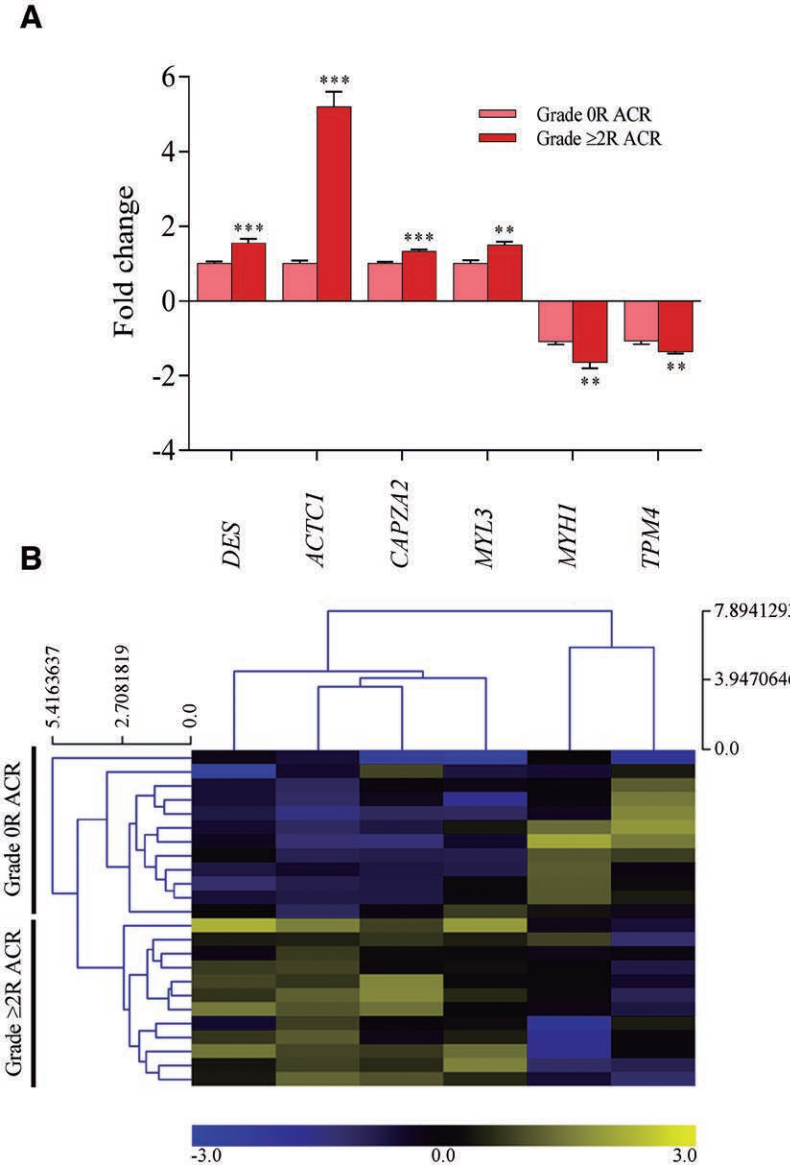

FIGURE 3. Relative expression levels of altered circulating genes encoding sarcomeric proteins in acute rejection grade ≥2R. The bars indicate the fold change (FC) ± SEM (A). The controls values were set to 1. The FC units represent the FC over the control RNA relative expression levels (nonrejection group). $^{**}P < 0.01$ and $^{***}P < 0.0001$ vs the nonrejection group. Hierarchical clustering heat map based on the relative expression levels of genes between the normal and rejected heart allografts (B). The hierarchical clustering heat map analyses show the separation of the ≥2R rejection and nonrejection groups based on the expression of circulating sarcomeric genes. The genes are listed in columns and samples in the rows. The colors depict the relative expression level of each molecule, with blue being the lowest and yellow the highest.

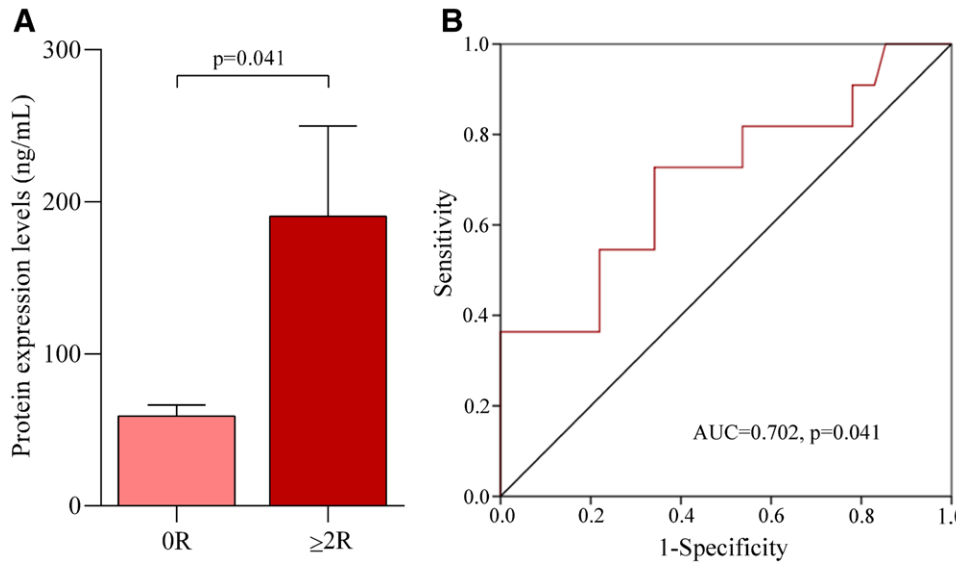

FIGURE 4. Circulating alpha-cardiac actin (ACTC1) protein levels. Comparison between nonrejection and acute rejection (acute cellular rejection [ACR] grade ≥2R). Bar graph of the ACTC1 protein levels ± SEM (SEM) (A). Receiver operating characteristic (ROC) curves of circulating ACTC1 for the detection of cardiac allograft rejection (ACR grade ≥2R) (B).

loss of myosin filaments and alterations at the Z-line level have also been described previously.[16,17] We have observed the dysregulation of genes related to these sarcomeric components, such as several myosins (*MYH1* and *MYL3*) in patients with ACR. On the other hand, different molecules related to the structure of the sarcomere have been previously described as potential markers of cardiac rejection. The alterations in the serum protein levels of troponin T and I have been related to the development of allograft rejection, but their ability to screen acute rejection is widely debated.[19-21] We have detected the mRNA encoding each of the subunits of both troponins in the serum. We showed that only *TNNT3* gene was differentially expressed in patients with clinically relevant rejection; however, based on the sensitivity analysis, this gene did not meet the criteria to be considered a good marker of rejection. Therefore, to delve into the study of new circulating molecules related to the structure of the sarcomere is of significant interest because of the close relationship between ACR and sarcomere dysfunction described.

In the present study, we observed alterations in the main components of the sarcomeric cytoskeleton. We found dysregulation in the expression of different genes that make up the sarcomeric actin and myosin filaments. In murine and nonhuman primate models, alterations in the expression of several actins and myosins have been observed in the context of cardiac rejection.[31-34] In addition, an increase in the levels of anti-actin and anti-myosin antibodies has been observed in EMB and serum from rejection patients.[35-38] These results highlight the key role of these molecules in the pathophysiology of cardiac rejection. In our study, *ACTC1* and its protein levels were overexpressed in the serum of patients with ACR. This gene encodes alpha-cardiac actin, which is the main protein of the thin filaments of the cardiac sarcomere.[39] Alterations in the expression of *ACTC1* have been related to the development of different cardiomyopathies.[40,41] The serum levels of *ACTC1* were able to discriminate with great accuracy between patients with and without clinically relevant rejection.

On the other hand, we found a dysregulation in different sarcomeric accessory genes, such as *DES*, gene encoding desmin protein. Desmin is a cytoskeleton intermediate filament protein that connects the Z-disks in adjacent myofibrils and the myofibrils to nuclear envelope and sarcolemma.[42] Furthermore, desmin is found at the Z-line of the sarcomere and is necessary for sarcomere integrity,[43] together with CapZ, which is an actin capping protein that binds and anchors the barbed ends of the thin filaments to the Z-disc.[44] Therefore, they are critical for the structural integrity of cardiomyocytes. Alterations in these molecules have been related to the development of heart failure.[44,45] Specifically, increase in expression of the *DES* is a typical characteristic of the failing heart, which could indicate a potentially adaptive compensation for a specific dysfunction in cardiac muscle contractility.[46,47] We observed *DES* and *CAPZA2* overexpression in the serum of patients with ACR and showed these molecules to have a high capability to detect clinically relevant rejection.

Currently, different methods for the noninvasive diagnosis of acute rejection have been proposed. AlloMap is a commercial gene expression assay, which is the only noninvasive analytical test included in International Society for Heart and Lung Transplantation guidelines to diagnosis ACR. AlloMap test is based on qPCR, but first it is necessary to isolate peripheral blood mononuclear cells, a complex process. In addition, it presents some limitations: it has not been shown to distinguish between antibody-mediated rejection (AMR) and cellular rejection, and it can only be used to rule out the presence of clinically relevant rejection.[48] The percent donor-derived cell-free DNA (%ddcfDNA) is a promising technique for the identification of acute rejection. %ddcfDNA has demonstrated excellent performance characteristics for both ACR and AMR and correlates with severity of acute rejection grades and allograft dysfunction.[49] Despite these advantages, the performance of the %ddcfDNA assay may be limited by its labor intensity and long processing time. We propose alpha-cardiac actin determination, a relatively simpler

and cheaper technique, as a potential biomarker in the detection of acute cardiac cellular rejection. It presents significant capability for diagnosis of cellular rejection comparable with AlloMap and %ddcfDNA. In addition, alpha-cardiac actin determination based on ELISA assay from free blood proteins is methodologically effortless and substantially inexpensive.

We acknowledge several limitations to this study. Our study involved a single center focused on ACR diagnostic and has not specifically evaluated AMR. However, the results obtained are a very interesting preliminary approach. In addition, the results in our work are compared with the EMB pathological analysis, which is a poor gold standard. Thus, future research in larger cohorts and in-depth analysis of the pathophysiological implications of sarcomeric dysregulation are needed.

In conclusion, this transcriptomic study showed that ACR is related to relevant changes in key components of the sarcomere. We identified serum mRNA levels of sarcomere-associated gene *ACTC1* and its protein levels as potential candidate to be included in the development of molecular panel testing for noninvasive ACR detection. Our findings provide the basis for further studies to focus on the role of these molecules in the pathophysiology of ACR.

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