Association between *ACR1* gene product expression and cardiomyopathy in children

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Abstract. Cardiomyopathy is a heterogeneous heart disease. Although morbidity of pediatric cardiomyopathy has been on the increase, effective treatments have not been identified. The aim of the study was to examine the expression of *ACR1* gene products in association with cardiomyopathy in children. In total, 73 patients and 76 healthy subjects were enrolled in the study, from April, 2013 to April, 2015. The relative expression of ACR1 mRNA and protein were quantified in all cases, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), ELISA and western blot analysis. Immunohistochemistry was used to stain cardiac tissue samples to reveal differences between the patients and the control group. The results showed that the level of ACR1 mRNA by RT-qPCR was not different between the two study groups. However, ELISA and western blot analysis showed a significant difference, with patients expressing lower levels of ACR1. Additionally, immunohistochemistry revealed the levels of ACR1 were reduced in patients as the time course of disease increased. Thus, there is an association between the inhibition of ACR1 expression and the development of the disease. These findings are useful in the elucidation of the pathogenesis of pediatric cardiomyopathy, a severe disease with few effective treatment options available.

Introduction

Cardiomyopathy is a heterogeneous heart disease. The European Society of Cardiology has defined cardiomyopathy in children as 'a heart disease generated by an abnormal cardiac structure and function caused by non-coronary heart disease, hypertension, valvular heart disease or congenital heart disease' (1,2). Statistics show the morbidity of pediatric cardiomyopathy has been on the increase annually probably due to the increasing number of immunocompromised infants and the aggravation of environmental pollution and other environmental problems (3). Relevant research results show that the current incidence of cardiomyopathy in children is ~0.001% (4) while postoperative rehabilitation is extremely poor for children with cardiomyopathy (5), the mortality rate after surgical procedures is approximately 30% and there is a high need for cardiac transplantation (6). According to the published results from the National Child Health Survey Statistics released in 2104, the current incidence of cardiomyopathy of children in China is approximately 30% higher than the incidence in the United States (7). In spite of the bleak prognosis for pediatric cardiomyopathy, investigations have not produced significant advances (7), and the etiology and pathogenesis of the disease remain uncertain providing a weak basis for educated treatment approaches (8).

Previous findings show the *ACR1* gene is important for the growth and repair of myocardial cells in mice, where it is involved in the promotion of the rehabilitation of dysfunctional cardiomyocytes (9).

The aim of the present study was to examine the association between the expression of the *ACR1* gene and cardiomyopathy in children, providing theoretical and practical preliminary references that may be useful in the elucidation of the pathogenesis of cardiomyopathy in children.

Materials and methods

General data. In total, 73 children with cardiomyopathy treated at the Xuzhou Children's Hospital (Jiangsu, China) from April, 2013 to April, 2015 were enrolled in the study as the observational group. The patients comprised 38 male and 35 female children, with an average age of 6.2±3 years. During the same period, 76 healthy children were selected for the control group. The healthy children included 34 males and 32 females, with an average age of 5.8±3.2 years. The study subjects were evaluated in accordance with the relevant standards for children with cardiomyopathy and were found to suffer no other additional diseases.

Approval for the study was provided by the ethics committee of Xuzhou Children's Hospital. Written informed consent was provided by the children's guardians.

Methods. Venous blood samples (6 ml each) were drawn from each study subject. Samples were centriguged at 2,000 x g

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Table I. RT-qPCR primers.

Gene	Primer sequence	Fragment length
AVR1	F: 5'-ACGGTCGATGCAGGTCAGC-3'	138 bp
	R: 5'-TGCTCGGACCTAGCATGCAG-3'	
GAPDH	F: 5'-GAAGGTGAAGGTCGGAGTC-3'	226 bp
	R: 5'-GAAGATGGTGATGGGATTTC-3'	

PCR, polymerase chain reaction.

for 10 min, and the supernatant serum was stored at -80˚C. The cell pellets were resuspended in frozen stock solution (Biosharp Co., Hefei, China), and then cryopreserved at -80˚C. ACR1 antibody used in the present study was purchased from Roche (Mannheim, Germany). RNA extraction kits were purchased from Axygen (Union City, CA, USA) and their associated molecular reagents were purchased from Takara Bio (Dalian, China). RT-qPCR primers were produced by Shanghai Biological Engineering Co., Ltd. (Shanghai, China).

RT-PCR

RNA extraction. RNA was extracted as per the instructions of the Axygen kit (10). Briefly, frozen pelleted cells were mixed with RNA Plus (KeyGen BioTech, Nanjing, China) to separate the cells and extract the RNA by sequential centrifugation steps. The purified RNA was resuspended in H_2O prior to verifying the quality of the RNA via spectroscopy. An aliquot from each sample was used for a reverse transcription reaction.

RT-qPCR. The RT-qPCR kit was purchased from Takara Bio. A three-step method was used in the experimental protocol, and the specific scheme was carried out as per the manufacturer's instructions. GAPDH was used as an internal control. The primers used are shown in Table I.

Enzyme-linked immunosorbent assay (ELISA). An antibody-sandwich method was used to detect the protein expression level of ACR1 (11), and the specific methods used were: i) Coating: The protein was diluted with phosphate-buffered saline buffer solution (pH 9.0) to a concentration of ~5 μg/ml. Then, 0.1 ml of the sample were added to a well in 96-well plates, and stored at 4˚C overnight. Once the protein was sufficiently incubated to coat the plate, the solution in the 96-well plates was discarded, and the plate was washed with scrubbing solution five times for 2 min at a time. ii) Sample addition: Each 0.1 ml serum sample was added to a well in the abovementioned 96-well plates, and then incubated at 37˚C for 1 h. Five washes with scrubbing solution for 2 min at a time were then performed. Blank, negative control and positive control plates were set up. ii) Secondary antibody addition: After washing, 0.1 ml secondary antibody solution [HRP-labeled goat anti-rabbit IgG (H+L) monoclonal antibody, dilution: 1:5000; Suzhou Alpha BioTech Co., Ltd., Suzhou, China, cat no.: 456213] were added to the 96-well plates, and the samples were incubated at 37˚C for 0.5-1.2 h. The plates were then washed with buffer solution 5 times for 2 min at a time. iv) Chromogenic substrate addition: 0.1 ml of the freshly prepared chromogenic substrate solution were added to each well, and 96-well plates were incubated at 37˚C for 30 min. v) Stop buffer addition: 0.005 ml of 0.2 M sulfuric acid were added to each well to stop the reaction. vi) Reading: The 96-well plates were placed on top of a blank paper to conduct qualitative observation by comparing the color depth. The darker the color, the higher the protein content of ACR1. The negative plate control was colorless. The 96-well plates were placed on a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) for quantitative detection and the wavelength was set to 450 nm. A blank plate was used to zero the reader and the OD values for each well were determined. A sample optical density (OD) value greater than that of the control by >1.2-fold was considered as positive.

Western blotting. The Roche animal cell protein extraction kit was used to extract the total protein of samples according to the manufacturer's intructions (12). Antibody dilution was in accordance with the manual and the final dilution ratio was 5,000:1. The remaining related operations were operated according to the manufacturer's instructions.

Immunohistochemical detection of AVR1 in myocardial tissue. Conventional antibody incubation and staining with cardiomyopathy tissue samples from children using streptomycin and enzymes (S-P) was performed. A published immunohistochemical standard was used (9): Briefly, membrane staining of <10% was considered negative, and membrane staining >10% was considered positive (+, for weak; ++, for moderate; and +++, for strong staining).

Data processing. SPSS 20.2 software (IBM SPSS, Armonk, NY, USA) was used for the statistical analysis on experimental data. Measurement results were presented as mean ± standard deviation and tested using the χ^2 test. $P<0.05$ was considered statistically significant.

Results

Relative ACR1 mRNA expression measurements. The ACR1 mRNA expression in the samples of the control and observation groups was quantified using fluorescence methods. The differences between the two groups were not significant ($P>0.05$; Fig. 1).

ELISA relative ACR1 protein expression. The ACR1 protein expression in the samples of the control and observation groups was quantified using ELISA. The observational group showed a lower protein level than that in the control group, and the difference was significant ($P<0.05$; Fig. 2).

Western blotting of ACR1 proteins. Western blotting was used to verify the results obtained by ELISA in the serum protein samples of the two groups. The results confirmed the former findings, and the ACR1 protein content in the sera of children with cardiomyopathy was lower than that in the control group (Fig. 3).

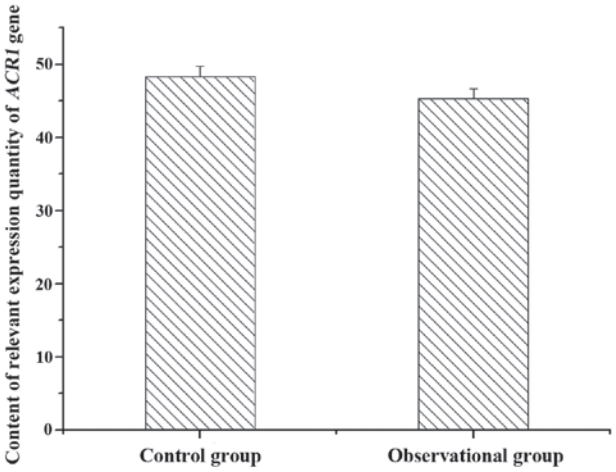

Figure 1. ACR1 mRNA relative expression in plasma from patients in the control and observational groups.

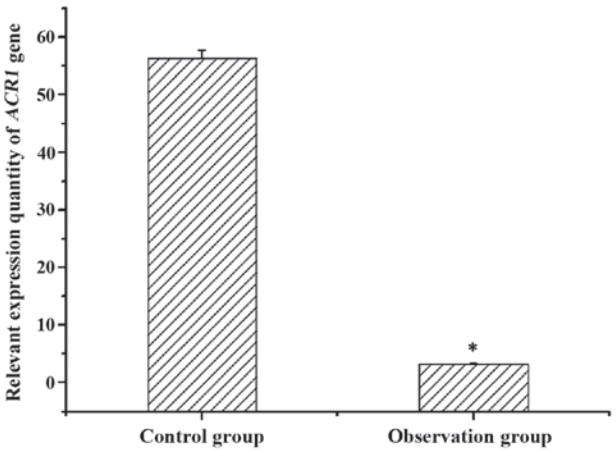

Figure 2. Relative expression of *ACR1* Protein in plasma from patients in the control and observation groups (ELISA measurements). P<0.05, statistically significant.

Relative ACR1 protein expression in patients at different disease duration times. Western blotting was used to assess

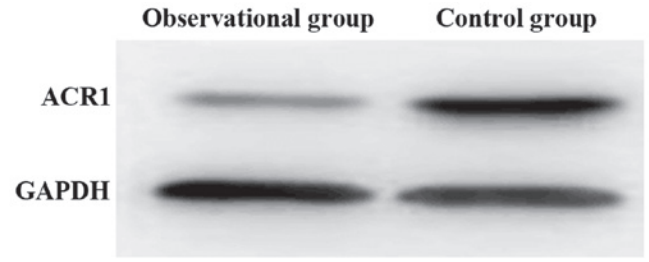

Figure 3. Western blotting showing relative expression of ACR1 protein from plasma of patients in the control and observational groups.

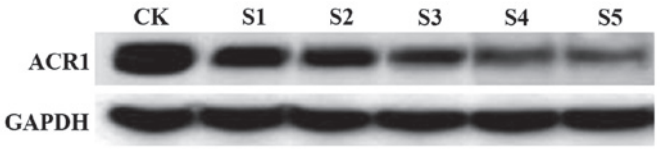

Figure 4. Western blotting showing the relative expression of ACR1 protein from plasma of patients in the control and observational groups (CK, sample from the control group; S1-S5, samples from the observation group at different time points during the couse of disease: S1: 8 months, S2: 17 months, S3: 24 months S4: 37 months, S5: 48 months).

the expression of ACR1 protein in the sera of children with cardiomyopathy, at different time points of the disease course. The levels of ACR1 protein decreased with a longer disease course (Fig. 4).

ACR1 in myocardial tissue tested by immunohistochemistry. ACR1 staining in cardiomyopathy tissues was mainly concentrated on the membranes (brown-yellow small particles of heterogeneous size). The normal staining observed in healthy cardiomyocytes was not present in the cardiocytes of patients (Fig. 5).

Discussion

Previous findings have shown that cardiomyopathy is often associated with disorders of mechanical and electrical

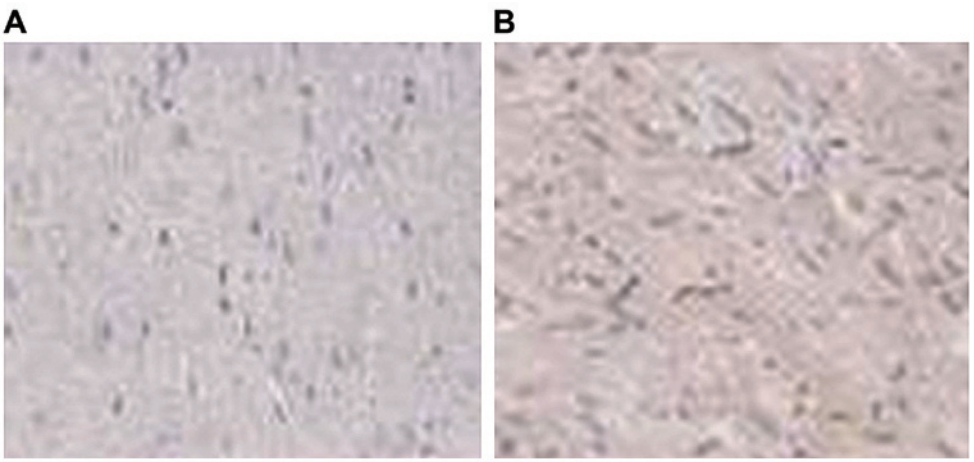

Figure 5. Immunohistochemical staining showing ACR1 in myocardial tissue. (A, the observational group; B, the control group).

activity of the heart (13,14). Its main clinical manifestations are ventricular dysplasia, cardiac hypertrophy and ventricular dilatation abnormalities, and these ultimately lead to heterogeneous myocardial disease. Pediatric cardiomyopathy is a major cause of cardiac failure during childhood, and it severely impairs an affected child's life (15). Cardiomyopathy is divided into dilated, hypertrophic, restrictive and arrhythmogenic cardiomyopathy (16), but the pathogenesis of the different types of disease is not clear, and there is no good basis for effective treatment approaches in children (17). At present, studies on the etiological agent of cardiomyopathy in children suggest genetic defects (such as the deletion of *LMNA* gene), congenital metabolic disorders (such as cardiac hypertrophy caused by fatty acid metabolism), congenital anomalies (for example, *TAZ* gene mutation during the embryonic period) and relevant post-inflammatory responses as the main causes (18-20).

In the present study, after measuring the expression quantity of *AVR1* gene products in the observation and control groups, significant differences were found at the protein levels, but not at the RNA levels, indicating that regulation of the expression occurs post-transcriptionally. The levels of ACR1 protein in affected individuals was reduced with longer disease time courses, possibly correlating with the deterioration of the patient's status. However, other relevant genes and factors are involved in the process and these remain to be determined.

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