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Echocardiography allows for analysis of pulmonary arterial flow in mice with congenital diaphragmatic hernia

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ABSTRACT

Background: Congenital diaphragmatic hernia (CDH) is a structural birth defect associated with pulmonary hypoplasia and pulmonary arterial hypertension (PAH). We hypothesize that echocardiography provides a method to assess real-time right ventricle (RV) function, remodeling, and pulmonary artery (PA) flow.

Materials and methods: Slit3 wild-type (WT) ($n = 6$) and knockout (KO) ($n = 5$) mice were analyzed at 2-3 months of age. Mice were anesthetized using isoflurane. Echocardiography was performed to analyze left and right ventricular wall thickness, internal diameter (ID), and function. Color Doppler was used to analyze flow in the PA and across the tricuspid valve.

Results: There was significant RV dilation in the KO mice *versus* WT, with an average RVID of 1.99 mm *versus* 1.26 mm, respectively ($P = 0.007$). Flow in the PA of KO mice was altered compared to WT, with elevated PA velocity time indices, 30.68 mm *versus* 22.13 mm ($P = 0.012$), elevated PA peak velocities, 952.61 mm/s *versus* 628.73 mm/s ($P = 0.003$), and decreased pulmonary acceleration times, 8.94 ms *versus* 16.18 ms ($P = 0.002$), respectively. Pulmonary vascular resistance, calculated by measuring tricuspid regurgitation peak velocity and right ventricular outflow tract velocity time index, was increased in KO *versus* WT mice, 17.61 mm^2/s *versus* 8.91 mm^2/s ($P = 0.003$), respectively.

Conclusions: Slit3 KO mice with CDH show evidence of PAH and resultant RV dilation. Using direct cardiac puncture, elevated RV systolic pressures have been demonstrated in KO mice as evidence of PAH. Echocardiography allows direct analysis of the PA and real-time RV function without sacrifice of the mouse. This mode of evaluation allows longitudinal study in mice with PAH and CDH.

Introduction

Congenital diaphragmatic hernia (CDH) is a relatively common congenital disorder affecting approximately 1 in 3000 children. There is significant morbidity and mortality related to CDH, and 10%-35% of all CDH patients do not survive beyond the neonatal period. Of the survivors, 50% will have long-term morbidity.[1] Morbidity and mortality in CDH are

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largely due to the pulmonary arterial hypertension (PAH) and pulmonary hypoplasia that affects most CDH patients. Studies have shown that more severe PAH is associated with worse survival.[2] No therapy exists for PAH in CDH because the mechanisms leading to the development of PAH in CDH have yet to be determined. Thus, CDH patients are managed with ventilator strategies and pharmacologic measures that are only supportive in nature.

Slit3 is part of the Slit family of genes with a number of functions, including axonal guidance, angiogenesis, axon attraction and repulsion, and cell chemotaxis. It is expressed in the mesothelium of the diaphragm during embryonic development. Slit3 knockout (KO) mice have been found to develop a diaphragmatic hernia at birth similar to the central (septum transversum) CDH in humans.[3] Previous studies in humans with CDH have shown pathologic changes in the pulmonary arteries and veins, known as pulmonary vascular remodeling, that lead to the development of PAH.[4] The Slit3 KO mouse is a unique mouse model for CDH because the mice are viable, which provides the unique opportunity to measure for changes consistent with PAH *in vivo*.

Current methods to evaluate PAH in mouse models include right heart catheterization and measurement of the right ventricular systolic pressure (RVSP), which in the absence of pulmonary stenosis allows for an estimation of PAH.[5] However, right heart catheterization is a terminal procedure in mice, which precludes longitudinal study. Furthermore, right heart catheterization is difficult in Slit3 KO mice with CDH that often have distorted thoracic anatomy as a result of the diaphragmatic hernia. Therefore, assessment of RVSP by direct cardiac puncture has been performed in our laboratory to estimate PAH. However, this too results in sacrifice of the mouse. The aforementioned limitations result in an inability to serially assess PAH in mouse models and prevent the longitudinal assessment of mice with PAH before and after potential treatment modalities, inhibiting the investigation of the mechanisms leading to the development of PAH in CDH.

In humans, although right heart catheterization is required to confirm the diagnosis of PAH, echocardiography is a commonly used screening test, and the only noninvasive technique to follow the course of the disease.[6] Furthermore, studies have shown that echocardiography can successfully assess pulmonary hypertension in rat and mouse models in a noninvasive manner.[7,8] We hypothesize that transthoracic echocardiography provides a method to assess real-time right ventricle (RV) function, remodeling, and pulmonary artery (PA) flow in the Slit3 KO mouse model for CDH.

Methods

Slit3 WT and KO mice were bred and housed at our institutional animal facility under Institutional Animal Care and Use Committee protocol. Six WT and five KO mice were analyzed at 2-3 months (adult) of age, of which six mice were males and five mice were females. To perform transthoracic echocardiography, the mice were lightly anesthetized using isoflurane anesthesia. Inhaled isoflurane was administered at 3% induction and 1%-1.5% maintenance. Once anesthetized, each mouse was placed in the supine position on a temperature-controlled mouse pad. Isoflurane was administered with a goal heart rate of 450-500 beats per minute. The mouse was restrained by taping all four limbs to the temperature-controlled mouse pad. A depilatory agent was then applied to the anterior chest to remove any hair. The mice were observed continuously while anesthetized and restrained during the echocardiography procedure. Clinical signs of deterioration included a dramatic slowing of heart rate less than 400 bpm, abnormal breathing patterns, or absent response to toe-pinch, any of which resulted in reduction of the isoflurane anesthesia.

Cardiac function was analyzed via echocardiography using the VisualSonics Vevo 2100 ultrasound machine (FUJIFILM VisualSonics, Inc, Toronto, Ontario, Canada). Aquasonic Gel (Parker Laboratories, Inc, Fairfield, NJ), kept in a gel heater, was applied to the anterior chest. The transducer probe (40 MHz, VisualSonics Model MS550D) was applied to the anterior chest first in a parasternal long axis view, with an approximately 30° angle counterclockwise, left of the parasternal line with the transducer notch pointing caudally[8] (Fig. 1A). In this position, using the B mode setting of the ultrasound machine, a full view of the left ventricle (LV) in the parasternal long axis view can be obtained (Fig. 2). Once the left ventricle is clearly visualized, the ultrasound can be switched to M mode, the mode representing the movement of structures over time, with the indicator line positioned to go through the widest portion of the LV chamber, using the aorta as a landmark. This view can saved via cine store on the ultrasound machine to be used to perform off-line calculations of heart rate, LV anterior and posterior wall thickness, LV internal diameter, LV ejection fraction, and LV fractional shortening (Fig. 2).

Next, the parasternal short axis view is obtained. The ultrasound is placed back in B mode, and the transducer probe is rotated 90° clockwise from the parasternal long axis view, to obtain the parasternal short axis view (Fig. 1B). The probe can be tilted gently toward the cranium to obtain a cross-sectional view of the heart at the level of the aortic valve. In this view, the right atrium can be seen, separated from the RV by the tricuspid valve (Fig. 3). Once this view is obtained, the ultrasound can be switched to the color Doppler mode. The pulse wave (PW)-line of the ultrasound, the line placed in the direction of flow, can be placed over the tricuspid valve to measure flow through the tricuspid valve. This can be used for off-line calculations of pulmonary vascular resistance (PVR), using the equation,

$$PVR = TR_{\text{max velocity}} / VTI_{RVOT}$$

where $TR_{\text{max velocity}}$ is the maximum velocity of tricuspid regurgitation (Fig. 4), divided by the velocity time integral of the right ventricular outflow tract, VTI_{RVOT}.[9]

A modified parasternal long axis view can be obtained to visualize blood flow through the PA. Beginning in the parasternal long axis view position, in B mode of the ultrasound, the probe can be gently rotated clockwise, until the probe is parallel to the sternum on the left hemithorax of the mouse. The probe can then gently be tilted laterally to obtain a view of the PA crossing over the aorta. Once this view is obtained in B