Clinica Chimica Acta 462 (2016) 71–76

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Lipocalin 2 as a new biomarker for fetal lung hypoplasia in congenital diaphragmatic hernia

Hiroyuki Tsuda [a,*], Tomomi Kotani [a], Tomoko Nakano [a], Kenji Imai [a], Shima Hirako [a], Hua Li [b], Fumitaka Kikkawa [a]

[a] Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[b] Bell Research Center for Reproductive Health and Center, Nagoya, Japan

ARTICLE INFO

Article history:
Received 12 November 2015
Received in revised form 25 May 2016
Accepted 30 August 2016
Available online 31 August 2016

Keywords:
Amniotic fluid
Congenital diaphragmatic hernia
Lipocalin-2
Lung hypoplasia
Observed/expected lung-to-head ratio

ABSTRACT

Background: Congenital diaphragmatic hernia (CDH) causes pulmonary hypoplasia, which are often fatal. We established a new biomarker for fetal lung hypoplasia in CDH.
Methods: We collected newborn lung tissue specimens at E21 from normal and nitrofen-induced CDH rats (administered 100 mg orally at E9) and performed a microarray analysis and real-time PCR (RT-PCR). Sixty-three human amniotic fluid (AF) samples, including samples from isolated CDH cases (n = 33) and Cesarean section (CS) cases without fetal complications (controls) (n = 30), were obtained. All AF samples were obtained at the time of CS, which was performed after 35–38 gestational weeks, from April 2007 to January 2016.
Results: A microarray analysis and RT-PCR showed decreased gene expression levels of lipocalin 2 (LCN2) in the nitrofen-induced CDH lungs ($p < 0.05$). We next examined the LCN2 levels in human AF samples using ELISA and the levels were significantly lower in the CDH cases than in controls (73.7 ng/ml vs 163.8 ng/ml; $p < 0.05$). A significant positive correlation was observed between the amniotic LCN2 level and the observed/expected lung-to-head ratio ($p < 0.001$).
Conclusions: LCN2 may be a potentially useful biomarker for lung hypoplasia in a rat and human CDH.

1. Introduction

Congenital diaphragmatic hernia (CDH) is a defect of the diaphragm that allows the abdominal contents to ascend into the thoracic cavity, causing lung hypoplasia and pulmonary hypertension [1]. This condition has a mean incidence of 1 in 2500 newborn children, and its etiology remains poorly understood [2]. Despite recent progress in postnatal care, CDH remains associated with a high rate of mortality (approximately 20–30%) [3]. In order to improve clinical care and counseling, reliable prenatal parameters predicting the fetal outcome in cases of CDH are urgently needed.

Prenatal predictors of the fetal outcome in CDH patients have been reported to be focused mainly on assessing the fetal lung volume (FLV). Ultrasound and MRI have been used to measure the antenatal FLV and evaluate fetal pulmonary hypoplasia [4–7]. However, these modalities have certain disadvantages, such as image resolution, cost and data variability among examiners [2].

In order to examine the underlying pathogenesis of CDH, a rat model of nitrofen-induced CDH was established, which is remarkably similar to human CDH [1,2].

2. Materials and methods

2.1. Ethics statement

This study using animals was carried out in accordance with the Nagoya University institutional guidelines for animal care, which conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All surgical procedures were performed under anesthesia with isoflurane inhalation, and all efforts were made to minimize suffering.

2.2. Experimental design and animal model

Timed pregnant Sprague-Dawley rats were purchased from Chubu Kagaku Shizai. The rats were exposed to nitrofen (Sigma-Aldrich) on day 9 of pregnancy (E9), as previously described [2]. Nitrofen (100 mg) was dissolved in 1 ml of olive oil, and the control animals were treated with 1 ml of olive oil. At term (E21), the fetuses were collected via Cesarean section, thoracolaparotomy was performed to inspect the diaphragm and the lungs were harvested and kept at −80 °C

* Corresponding author at: Hiroyuki Tsuda, MD, Department of Obstetrics and Gynecology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.
E-mail address: hiro-t@med.nagoya-u.ac.jp (H. Tsuda).

http://dx.doi.org/10.1016/j.cca.2016.08.023
0009-8981/

for the analysis. Due to the fact that not all the fetuses developed CDH, only those that developed CDH were included in our analysis. We defined the 2 groups as follows: normal rats (N, 20 fetuses from 8 dams), and nitrofen-induced CDH with olive oil rats (CDH, 23 fetuses from 9 dams). In this study, the CDH rate of the pups was 47.8% (44/92).

2.3. Microarray analysis

To screen for possible target genes associated with lung hypoplasia in CDH, the gene expression profiles of the rat lungs with and without CDH were compared using a CodeLink Rat Whole Genome Bioassay (Applied Microarray Inc.), according to the manufacturer's standard protocols. Candidate genes were selected based on the following criteria: only those genes whose transcription had changed >3-fold in an equidirectional manner (i.e., whose transcription was significantly up- or downregulated). The mRNA expression of the selected genes in the rat lung with and without CDH was confirmed by a real-time quantitative PCR analysis.

2.4. Real-time quantitative PCR

Total RNA isolation was performed using the RNeasy kit, as suggested by the manufacturer (Qiagen Inc.). Total RNA (1 μg) was reversed transcribed in a 20 μl reaction volume using ReverTra Ace (Toyobo), according to the manufacturer's standard protocols. Quantitative real-time PCR was performed using the Thermal Cycler Dice Real Time System and SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio), according to the manufacturer's standard protocols. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The level of expression of each mRNA was determined in arbitrary units in comparison with that of an external DNA standard (β-actin cDNA). The primers used in this study are listed in Table 1. We examined 5 lung samples from pups from different dams in each group for the target genes.

2.5. Case registration and amniotic fluid collection in the clinical CDH cases

Data were collected from April 2007 to January 2016 at Nagoya University Hospital, Nagoya, Japan. All amniotic fluid (AF) samples were obtained at the time of Cesarean section (CS) performed at 35 to 38 gestational weeks. Sixty-three amniotic fluid samples comprising 33 cases of isolated CDH and 30 cases involving uncomplicated pregnancies were obtained with signed informed consent. We collected amniotic fluid via centesis while looking directly at the amniotic bag after creating an incision in the myometrium during CS, as previously described [8]. This study was approved by the ethics committee of Nagoya University Hospital.

Maternal and neonatal data, including maternal age, parity, gestational age at delivery, birth weight, sex and umbilical artery pH, were extracted. We also examined the management of the CDH infants, such as extracorporeal membrane oxygen (ECMO), high-frequency oscillation (HFO), nitric oxide (NO), patch insertion at surgery, and oxygen therapy at 1 y. Next, in the CDH cases, we calculated the observed to expected lung area to head circumference ratio (o/e LHR) in order to predict the severity of the disease [7]. We used the o/e LHR data which was measured within one week before delivery.

2.6. Measurement of the amniotic lipocalin 2 (LCN2) levels

The AF was centrifuged and stored at −80 °C until the analysis. LCN2 concentrations were measured using a Quantikine ELISA Kit according to the manufacturer's protocol. Duplicate samples were used to determine the concentrations. The detectable range was as follows: 5–300 ng/ml for LCN2.

2.7. Measurement of the lipocalin 2 (LCN2) levels in rat lungs

The rat lungs were stored at −80 °C until the analysis. The LCN2 concentrations were measured using a CircuLex Rat NGAL/Lipocalin-2 ELISA Kit (CycLex Co.) in accordance with the manufacturer's instructions. Duplicate samples were used to determine the concentrations. The detectable range was 31.3–2000 pg/ml for LCN2.

2.8. Statistical analysis

The statistical analysis of the data was performed with the SPSS for Windows (ver.19.0) and Excel (Microsoft). The values are expressed as the mean ± SD, and the normality of the data was assessed with the Shapiro-Wilk test. The chi-square test was used for comparisons of categorical variables. Differences between the means were assessed employing the Mann-Whitney test for unpaired samples. A receiver-operating characteristic curve was generated to characterize the ability of the LCN2 levels in AF and o/e LHR to predict the HFO use in human CDH cases, and the area under the curve (AUC) was applied as a measure of the diagnostic accuracy. A $p < 0.05$ was considered to be significant.

3. Results

3.1. LCN2 gene expression was significantly decreased in the rat CDH lung

The microarray analysis revealed that *thioredoxin 2* (4.8-fold), *IGFBP 1* (3.7-fold) and *cathepsin E* (3.5-fold) were significantly upregulated and that *LCN2* (9-fold) and *GATA 2* (3.3-fold) were significantly downregulated in the nitrofen-induced CDH lungs (Table 2). Next, the mRNA expression of these selected genes in the rat lungs was examined using real-time quantitative PCR. As a result, the *LCN2* gene expression was found to be significantly decreased ($p < 0.05$), whereas that other genes was not significantly different (Fig. 1A–E). The LCN2 levels in the rat lung were significantly lower in the nitrofen-induced CDH rat

Table 1
Primer sequences for quantitative real-time RT-PCR.

Gene	Sequence (5′–3′)
β-actin	
Forward	CGA GTA CAA CCT TCT TGC AGC
Reverse	ATA CCC ACC ATC ACA CCC TGG
Thioredoxin 2	
Forward	GAC ACC AGT TGT CGT GGA CT
Reverse	CAC AGC AGA CAC CTC GTA CT
IGFBP 1	
Forward	GAA GCT TTT CTC ATC TCC ATA CAT GT
Reverse	AAG GCC CCT ACC TCA GAC TGA
Cathepsin E	
Forward	ATG CGG AGT TTG ACG GGA TT
Reverse	CCT TGT GGG TCA CTG CTC AA
Lipocalin 2	
Forward	TCT GGG CCT CAA GGA TAA CAA C
Reverse	AGA CAG GTG GGA CCT GAA CCA
GATA 2	
Forward	CCA GCA AAT CCA AGA AGA GC
Reverse	CGT TGG CGT AGG TAG GAT GT

IGFBP 1, insulin-like growth factor binding protein 1.

Table 2
Significantly changed genes (>3-fold) in the neonatal nitrofen-induced CDH rat lungs.

	Fold change (vs. normal lung)
Thioredoxin 2	4.8
IGFBP 1	3.7
Cathepsin E	3.5
Lipocalin 2	−9
GATA 2	−3.3

IGFBP 1, insulin-like growth factor binding protein 1.