Gene 588 (2016) 86–94

Contents lists available at ScienceDirect

Gene

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

Research paper

Mutations of *NKX2.5* and *GATA4* genes in the development of congenital heart disease

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ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 27 April 2016
Accepted 29 April 2016
Available online 3 May 2016

Keywords:
Transcription factors
NKX2.5
GATA4
Gene mutation
Congenital heart disease

ABSTRACT

Objective: To investigate the mutations of *NKX2.5* and *GATA4* genes in the development of CHD in Chinese population.
Methods: Between December 2010 and December 2014, 185 cases of CHD patients and 210 cases of healthy people were enrolled. *NKX2.5* and *GATA4* gene mutations and gene expression were detected via DNA sequencing and real-time PCR (RT-PCR), respectively. BMSCs were transfected with pCMV-HA-NKX2.5 and pCMV-Myc-GATA4 plasmids. Cardiac troponin T (cTnT) and connexin 43 (Cx43) and *β-myosin heavy chain* (*β-MHC*) and *myosin light chain-2* (*MLC-2*) expressions were detected. Co-immunoprecipitation assay was used to detect the interaction of NKX2.5 and GATA4 and luciferase to detect their effect on *B-type natriuretic peptide* (*BNP*) gene promoter.
Results: NKX2.5 and *GATA4* gene mutations were found in the CHD group, but not in the normal control group, and *NKX2.5* and *GATA4* gene expressions were significantly lower in the case group compared with the control group (both $P < 0.05$). Compared to the control and empty vector groups, cTnT and Cx43 expressions were significantly higher in the pCMV-HA-NKX2.5 and pCMV-Myc-GATA4 plasmid groups; *β-MHC* at 1–4 weeks and *MLC-2* at 2–4 weeks were higher in the pCMV-HA-NKX2.5 plasmid group; and *β-MHC* at 2–3 weeks and *MLC-2* at 3–4 weeks were higher in the pCMV-Myc-GATA4 plasmid group (all $P < 0.05$). NKX2.5 and GATA4 interacted in the BMSCs and co-transfected pCMV-Myc-GATA4 and pCMV-HA-NKX2.5 significantly enhanced the fluorescence intensity of BNP.
Conclusion: NKX2.5 and *GATA4* gene mutations might participate in the development of CHD and can promote BMSCs differentiate into cardiomyocytes.

1. Introduction

Congenital heart disease (CHD) is an important cause of morbidity and mortality during infancy and childhood and is characterized by abnormalities in the structure or function of cardiocirculation (Celik et al., 2015). CHD varies in severity and requires several treatment procedures, surgery or catheterization (Pinto Junior et al., 2015). Neonates with significant CHD had higher gestational ages and birth weights compared with infants born before and CHD infants were twice to be delivered late-term in 2010 than in 2004–2008 (Dadlez et al., 2014). With significant medical advances, >1.000,000 adults with CHD were found in the U.S. and the number is expected to continue growing by 5% per year (Jackson et al., 2015). CHD is a multifactorial disorder associated with both genetic and environmental factors and approximately 30% of CHD was related to genetic syndromes (Ko, 2015). The etiology of CHD has a strong genetic component and CHD occurs with some congenital anomalies within a single gene disorder, sporadic malformative complex, or chromosomal abnormality (Granados-Riveron et al., 2012).

The mammalian *NKX2.5* gene was discovered to be essential for cardiac development and the NKX2.5 protein has been categorized as a Class I NK-2 homeodomain protein (Granados-Riveron et al., 2012). The cardiac specific homeobox protein NKX2.5, a critical GATA4 cofactor, is essential for heart development and mutations in the *NKX2.5* gene are associated with CHD, including septal defects and abnormal conduction system (Kinnunen et al., 2015). As a family member of the evolutionarily conserved GATA proteins, GATA has emerged as a critical regulator of cardiogenesis either directly in cardiac precursors or in the adjacent endoderm by modulating cardiogenic factors (Gallagher et al., 2012). Besides, *GATA4* is necessary for preventing myocardial damage caused by ischemia/reperfusion injury and also promotes the survival of cardiomyocytes by regulating

Abbreviations: CHD, congenital heart disease; BMSCs, bone marrow derived stroma cells; cTnT, cardiac troponin T; Cx43, connexin 43; *β-MHC*, *β-myosin heavy chain*; *MLC-2*, *myosin light chain-2*; *BNP*, *B-type natriuretic peptide*; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; PS, pulmonary stenosis; TOF, tetralogy of Fallot; AS, aortic stenosis; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; PBS, phosphate buffered saline; RIPA, radio immunoprecipitation assay; TBS, Tris buffer saline; ANOVA, analysis of variance.

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http://dx.doi.org/10.1016/j.gene.2016.04.061
0378-1119/

pathways (Luu et al., 2015). Mutations in *GATA4* and *NKX2.5* have been implicated in heart diseases, indicating their roles in the normal cardiac function (Carter et al., 2014). Previous study also demonstrated that the genes, which when mutated, predispose to heritable neoplasias, in particular, have been viewed as providing useful models for clinical cancer genetics practice, which includes genetic counseling (Erlic et al., 2010). However, the synergic roles of *NKX2.5* and *GATA4* genes in Chinese CHD were rarely studied.

Our study aims to investigate the mutations of *NKX2.5* and *GATA4* genes in the development of CHD in Chinese population. We detected the *NKX2.5* and *GATA4* gene mutations in vivo and studied the synergic roles of NKX2.5 and GATA4 in vitro. Our study could provide better understanding on the development of CHD in the genetic level.

2. Materials and methods

2.1. Study participants

Between December 2010 and December 2014, 185 cases of children with CHD hospitalized at the department of cardiology in Nanchang First Hospital were enrolled as the CHD group. Among them, there were 120 cases of males and 65 cases of females with a mean age of 5.5 ± 1.6 years (ranges, 1 month to 15 years old); and there were 26 cases of atrial septal defect (ASD), 112 cases of ventricular septal defect (VSD), 9 cases of patent ductus arteriosus (PDA), 21 cases of pulmonary stenosis (PS), 15 cases of tetralogy of Fallot (TOF) and 2 cases of aortic stenosis (AS). The inclusion criteria of the CHD group were: 1) patients had the age <16 years old; 2) patients were confirmed by cardiac color Doppler echocardiography or cardiac intervention and surgery. The exclusion criteria were: 1) patients had deformities out of the heart vessels; 2) patients did not suffer from hypertension, severe renal insufficiency, or malignant tumor; 3) patients combined with chromosome diseases; 4) patients suffering from Eisen Mange's syndrome. A total of 210 cases of gender- and age-matched healthy volunteers were chosen as normal control group, and among them, there were 136 cases of males and 74 cases of females at a mean age of 5.8 ± 1.5 years (ranges, 1–16 years old). All the healthy participants were excluded from CHD by the cardiac color Doppler echocardiography. After the agreement of the patients and their families, venous blood of all patients (5 ml) and their family members (5 ml) was extracted, ethylene diamine tetraacetic acid (EDTA) was added as an anticoagulant, and the blood sample was stored at −70 °C and prepared for the next steps. Besides, myocardial tissues of 27 patients undergoing cardiac surgery were also collected. The study was approved by the Ethics Committee of Nanchang First Hospital and the ethical approval for this study conformed to the standards of the Declaration of Helsinki (M,, 2014), and the study participants or their guardians signed informed consent.

2.2. Selection conditions and pedigree

Six CHD patients were selected based on the diagnostic criteria of *Practical Cardiology* edited by Chen haozhu (Chen, 2012) and *Pediatrics Cardiology* edited by Yang siyuan (Yang, 2007). Cyrillic2.02 software was used for drawing the pedigree of the collected six CHD families (Fig. 1).

2.3. Polymerase chain reaction (PCR) reactions

Genomic DNA was extracted using blood genomic DNA extraction kit (Biomarket Box company, S hanghai, China) and the concentration and purity of DNA were detected by an ultraviolet spectrophotometer. PCR reaction was carried out by a MJR-PT200DNA PCR instrument (MJ company, US) and the primers were synthesized by Shanghai Generay Biotech Co., Ltd (Table 1). The reaction system (50 μl) was as follows: genomic DNA 2 μl, 10 × reaction buffer ($MgCl_2$ 25 mmol/l) 5 μl, dNTP (2.5 mmol/l) 3 μl, upstream and downstream primer (100 μmol/l) 1 μl for each, Taq enzyme (Takara, Dalian, China) 3 μl, and ddH_2O 35 μl. PCR conditions were: *NKX2.5* gene: pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 53–64 °C for 45 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min, and the product was preserved at 4 °C; *GATA4* gene: pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 51–59 °C for 45 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min, and the product was preserved at 4 °C. After the PCR amplification, the reaction product (4 μl) was taken and mixed with bromophenol blue loading buffer (1 μl) sufficiently, and was stained by ethidium bromide (EB). The stained samples were loaded on the wells of 2% agarose gel electrophoresis, and 5 μl of 1000 bp Marker was loaded in a 2% agarose gel well as a control. The samples were subjected to agarose gel electrophoresis at 40 V for 45 min. After the electrophoresis, the products were observed by an ultraviolet light and photographed and preserved.

2.4. DNA sequencing

An ABI 3130 genetic analyzer (ABI company, US) was used to conduct capillary electrophoresis and 96-wells were placed into the analyzer to conduct automatically sequencing according to the operating procedures. After sequencing, GENERUNR and VNTI software programs were used for analysis and the sequences were compared with the standard sequence of *NKX2.5* and *GATA4* gene published by Gene Bank and each peak of sequence was observed. The sequencing results with relative more miscellaneous bands were analyzed to exclude the impact factors and then a second positive sequencing was conducted, and the abnormal sequencing results were verified by reverse sequencing and repeated PCR amplification sequencing.

2.5. Real-time PCR (RT-PCR) to detect NKX2.5 and GATA4 gene expressions

Approved by the Ethics Committee and the consent of patients and their families, another 25 cases of pregnancy terminated fetal heart tissues due to pure CHD were enrolled as case group (group A), the myocardial tissues taken from 27 patients who underwent cardiac surgery were selected as case group (group B), and 25 cases of normal fetus heart tissues obtained by induced abortion at middle and late stages due to unplanned pregnancy with matched age and gender were enrolled as the control group. The total RNA in the myocardial tissue was extracted and was reverse transcribed into cDNA. The primers were designed according to the principle of primer design: *NKX2.5*: upstream: 5′-TGACCGATCCCACCTCAA-3′ and downstream: 5′-ATCGCCGCCACAAACTCT-3′; *GATA4*: upstream: 5′-GCCTGTCATCTC ACTACGG-3′ and downstream: 5′-GGAAGAGGGAAGATYACG-3′; and glyceraldehyde phosphate dehydrogenase (GAPDH): upstream: 5′-CATCTTCCAGGAGCGAGA-3′ and downstream: 5′-TGTTGTCATACTTCTC AT-3′. The PCR reaction conditions were: denaturation at 94 °C for 3 min, a 40 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 45 s, and extension at 72 °C for 7 min. RT-PCR amplification products (10 μl) were subjected to 2% agarose gel electrophoresis and automated imaging was conducted by gel imaging system. The relative content of each brand was calculated using GAPDH as an internal reference and semi-quantitative analysis of gene expression levels was conducted.

2.6. Isolation and culture of Sprague–Dawley (SD) rat bone marrow derived stroma cells (BMSCs)

SD rats at 3 weeks old were provided by the Experimental Animal Center of China Medical University. The rats (male and female rats) were obtained regardless of gender and weighed 20–25 g, were killed by cervical dislocation, and soaked in 75% ethanol for 5 min. Humerus, femur and tibia were separated under strict sterile conditions and washed with 0.01 mol/l sterile phosphate buffered saline (PBS) solution