



# Association between *RNF41* gene c.-206 T > A genetic polymorphism and risk of congenital heart diseases in the Chinese Mongolian population

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**ABSTRACT.** This study aimed to explore the association between ring finger protein 41 (*RNF41*) c.-206 T > A variant and susceptibility to congenital heart disease (CHD) in the Chinese Mongolian population. The association between *RNF41* gene c.-206 T > A polymorphism and CHD was examined in two independent case-control studies consisting of 219 CHD patients and 208 healthy controls. Genotype was determined by direct sequencing of PCR products. We found that the genotype frequencies of *RNF41* c.-206 T > A differ significantly

between the two groups ( $P < 0.05$ ). The TT and TA genotypes in the CHD group were 80.67 and 19.33%, respectively. On the other hand, the frequencies of TT and TA in the control group were 94.44 and 5.56%, respectively. Furthermore, the allelic frequencies of CHD patients (T, 90.34%; A, 9.66%) were significantly different as compared with those of non-CHD controls (T, 97.22%; A, 2.78%;  $\chi^2 = 4.031$ ,  $P = 0.041$ ). Our study demonstrates that the *RNF41* c.-206 T > A polymorphism may be a risk factor for congenital heart disease in the Chinese Mongolian population.

**Key words:** Congenital heart diseases; E3 ubiquitin ligase; Genetic variant; *RNF41*; Mongolian

## INTRODUCTION

Congenital heart disease (CHD) is one of the most common birth defects, and accounts for approximately 28% of the major congenital birth defects. It is generally agreed that the occurrence of CHD is due to complex interactions between environmental and genetic factors, but the latter plays a more important role in the development of CHD (van der Linde et al., 2011). *RNF41* is a RING finger-type E3 ubiquitin ligase, which belongs to the family of RING finger-containing proteins. It is primarily expressed in the heart, skeletal muscle, and brain (Abdullah et al., 2001). *RNF41* has been reported to be involved in growth regulation, tumor suppression, and inflammatory responses by promoting the ubiquitination of several proteins such as ErbB3, BRUCE/apollon, and MyD88 (Qiu and Goldberg, 2002; Qiu et al., 2004; Wang et al., 2009). We have previously shown that overexpression of *RNF41* in the heart tissue induced rapid inactivation of cardiac ErbB3, elevated myocardial apoptosis, inflammation, as well as tissue infarction in a rat model of heart I/R injury (Zhang et al., 2011). *RNF41* has been shown to interact with ErbB3, and down regulate its expression via proteasome-mediated degradation (Yen et al., 2006; Chen et al., 2010). Targeted deletion of ErbB receptors is embryonically lethal due to cardiovascular defects (Fuller et al., 2008). For example, ErbB3- null mice exhibit hypoplastic endocardial cushions, and often die during mid-gestation (Riethmacher et al., 1997). It is therefore possible that *RNF41* may play a role in cardiac pathology, however, the exact mechanism by which *RNF41* contributes to the pathogenesis of CHD remains elusive. In this study, we investigated the association between *RNF41* polymorphisms and CHD in the Chinese Mongolian population.

## MATERIAL AND METHODS

### Study population

This study was approved by the Capital Medical University Beijing Anzhen Hospital and the Baotou Medical College Ethics Committee. Clinical and laboratory data were obtained, and informed consent was given by all subjects prior to peripheral blood sample collection. CHD patients (123 males, 96 females) were recruited between June 2007 and December 2014 from the Department of Pediatric Cardiology, Beijing Anzhen Hospital. All patients were of Chinese Mongolian descent originating from the Inner Mongolia autonomous region. Clinical details of the patients are summarized in Table 1. CHD was confirmed by color Doppler

ultrasound or magnetic resonance imaging. A total of 208 non-CHD controls (119 males, 89 females) were recruited from the First Affiliated Hospital of Baotou Medical College, and were frequency-matched to CHD patients by gender and age. None of the control subjects had family history of CHD or other syndromes, and all were unrelated. Informed consent was obtained from all subjects.

**Table 1.** Clinical summary of CHD patients and non-CHD control subjects.

	CHD	non-CHD
Number of subjects	219	208
Male (%)	123 (56.30)	119 (57.40)
Current age (years)	2.15 ± 1.23	2.44 ± 1.69
Height (cm)	86.48 ± 7.53	89.32 ± 6.49
Weight (kg)	11.86 ± 2.14	12.71 ± 2.72
Pulse (rpm)	118 ± 9.83	116 ± 8.87
Respiratory frequency (rpm)	28 ± 5.23	26 ± 4.17
Blood pressure (systolic/diastolic, mmHg)		
Left upper limb	91/57	92/56
Right upper limb	91/58	91/59
Left lower limb	106/67	110/71
Right lower limb	107/66	109/70
Complications (N, %)		
Pneumonia	41 (34.45)	
Heart failure	3 (2.52)	
Aneurysm (N, %)	39 (32.77)	
VSD size (mm)	8.54 ± 1.27	

## Genotyping

Blood (2 mL) was drawn into EDTA-containing tubes, and white blood cells were isolated via centrifugation at 4000 rpm/min for 10 min. Genomic DNA was extracted using the DNA Extraction Kit from Qiagen (Hilden, Germany). The human *RNF41* gene (GenBank accession No. NM\_005785) was amplified with specific primers designed using the Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA). PCRs were performed in a 20- $\mu$ L reaction mixture containing 50 ng template DNA, 100 nM Tris-HCl (pH 8.3), 500 mM KCl, 0.25 mM primers, 2.0 mM MgCl<sub>2</sub>, 0.25 mM dNTPs (Biotek Corporation, Beijing, China), and 0.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The PCR amplification protocol was as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58.5°C for 32 s, 72°C for 35 s, and final extension was carried out at 72°C for 5 min. The genotypes of *RNF41* c.-206T > A were investigated via DNA sequencing (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA).

## Statistical analysis

Statistical analyses were conducted using the SPSS 15.0 statistical software. Hardy-Weinberg equilibrium for the c.-206 T > A *RNF41* variants were analyzed, and  $\chi^2$  tests were performed to compare the allelic and genotypic distributions between patients and controls.  $P < 0.05$  was considered statistically significant.

## RESULTS

### General characteristics

The CHD patients were adequately matched for with non-CHD controls by gender and age, as suggested by  $\chi^2$  tests ( $\chi^2 = 1.9158$ ,  $P = 0.1934$  for gender;  $\chi^2 = 1.9571$ ,  $P = 0.1628$  for age). The mean age of CHD patients was comparable to that of non-CHD controls ( $2.15 \pm 1.23$  vs  $2.44 \pm 1.69$ ).

### Analysis of *RNF41* gene polymorphism

We analyzed the allelic and genotypic distributions of c.-206 T > A polymorphism in all 219 CHD patients and 208 non-CHD controls. Sequence analyses suggested that this genetic polymorphism was a non-synonymous mutation resulting from a T to A transition in exon 2 of the *RNF41* gene. Table 2 summarizes all allelic and genotypic frequencies in the studied populations. The allelic frequencies of CHD patients (T, 90.41%; A, 9.59%) differed significantly from that of non-CHD controls (T, 97.12%; A, 2.88%;  $\chi^2 = 4.031$ ,  $P = 0.041$ , Table 2). The genotypic frequencies of CHD patients (TT, 80.82%; TA, 19.18%) were also different from that of non-CHD controls (TT, 94.23%; TA, 5.77%;  $\chi^2 = 7.726$ ,  $P = 0.009$ , Table 2). In both CHD subjects and non-CHD controls, the genotypic distributions of the c.-206 T > A polymorphism were in Hardy-Weinberg equilibrium.

**Table 2.** Allelic and genotypic distribution frequencies for c.-206 T > A genetic polymorphism of *RNF41* gene in the CHD patients and non-CHD control subjects.

Allele or genotype	CHD [N (%)]	non-CHD [N (%)]	$\chi^2$	P value
Allele A	42 (9.59%)	12 (2.88%)		
Allele T	396 (90.41%)	404 (97.12%)	4.031	0.041
Genotype TA	42 (19.18%)	12 (5.77%)		
Genotype TT	177 (80.82%)	196 (94.23%)	7.726	0.009

Genotypic and allelic data are reported as number with percent in parentheses, respectively. The  $\chi^2$  tests were used for statistical analyses.

## DISCUSSION

In this study, we examined whether sequence variations in the *RNF41* gene contribute to CHD development. In the cardiovascular research, more focus has been placed on finding genes that are activated during pathogenesis of heart diseases. However, the removal of damaged proteins and organelles has been underappreciated in this field. Cardiac proteotoxicity has been increasingly recognized as an important contributor to the development of various congenital and acquired human heart diseases (Sandri and Robbins, 2014). The ubiquitin-proteasome system (UPS) is involved in the maintenance of protein homeostasis (Egeler et al., 2011; Schlossarek et al., 2014). Impairment of the UPS is characterized by reduced levels of ubiquitinated proteins and proteasome activities, which has been reported in inherited cardiomyopathies. Altered UPS activity may contribute to cardiac dysfunction due to dysregulated turnover of cardiac proteins, changes in transcriptional activation of stress response proteins, reduced degradation of damaged proteins, and onset of stress responses (Weekes et al., 2003; Sarikas et al., 2005; Vang et al., 2005; Liu et al., 2006; Patterson et

al., 2007; Rajasekaran et al., 2007). The ubiquitin ligases UBR1 and UBR2 appear to be indistinguishable in their recognition of N-degrons, and are essential for proper cardiac development. Studies showed that knockdown of UBR1 and UBR2 resulted in a wide range of cardiovascular abnormalities in mice (Kwon et al., 2003; Varshavsky, 2004). In addition, mutation analysis in regions of homozygosity identified a conserved homozygous missense mutation in the *TRIM32* gene. *TRIM32* also encodes for an E3 ubiquitin ligase, and mutations in this gene contributes to cardiac abnormalities (Chiang et al., 2006).

*RNF41* is an E3 ligase that dictates the spatial, temporal, and substrate specificity of ubiquitination. It promotes the ubiquitination of ErbB3, BRUCE/apollon, TBK1, Parkin, and C/EBP $\beta$ , thereby regulating cell growth, apoptosis, and inflammation (Qiu and Goldberg, 2002; Qiu et al., 2004; Yu and Zhou, 2008; Wang et al., 2009; Ingalla et al., 2010; Tan et al., 2011; Zhou et al., 2011; Ye et al., 2012). Accumulating evidence suggests that *RNF41* plays an important role in heart diseases. The E3 ubiquitin ligase *RNF41* has been shown to interact with the N terminal of ErbB3, and catalyzed degradation of ErbB3 via the ubiquitin-proteasome pathway (Qiu and Goldberg, 2002). Our previous study showed that *RNF41* mediated ErbB3 protein degradation to augment ischemia/reperfusion heart injury (Zhang et al., 2011).

The ErbB receptor tyrosine kinase family is comprised of 4 members, namely EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. Gene targeting strategies in mice have highlighted the significance of the ErbB receptors in cardiac function and development (Chen et al., 2010). Cardiac-specific ErbB2-knockout mice were able to survive to adulthood, but showed dilated cardiomyopathy at 3 months of age. Mice lacking the ErbB4 gene die during mid-embryogenesis from aborted development of the myocardial trabeculae in heart ventricles (Chen et al., 2010). More importantly, ErbB3-null mice often die during mid-gestation due to the presence of hypoplastic endocardial cushions. Recent studies have reported that *RNF41* specifically binds to ErbB3 to induce proteasome-dependent degradation of ErbB3 and cell death (Yen et al., 2006; Cao et al., 2007), suggesting that *RNF41* may contribute to heart development via regulation of ErbB3 expression.

Mo et al. (2010) identified two genetic variations (c.-206 T > A and c.-208-8 A > G) in the Han Chinese population with and without Parkinson disease. The c.-206 T > A polymorphism was identified in 6.52% of the 302 controls, but c.-208-8 A > G was not found in those subjects. However, they failed to show a positive association between c.-206 T > A polymorphism and Parkinson disease. Considering the influence of genetic heterogeneity, the contribution of *RNF41* to the pathogenesis of disease may be markedly influenced by ethnic background and geographic origin of the patients. It is therefore necessary to screen mutations in *RNF41* in other ethnic populations. Since c.-206 T > A is located in exon 2 of the *RNF41* gene, we investigate the potential association between c.-206 T > A genetic polymorphism in the *RNF41* gene and risk of CHD. Our findings indicated that allele-A and genotype-TA may be associated with increased risk for CHD. Our results suggested that the allelic and genotypic frequencies of *RNF41* gene c.-206 T > A polymorphism are associated with CHD in the Mongolian Chinese population.

In summary, to the best of our knowledge, this study was the first to evaluate the genetic variants of the *RNF41* gene. We found that variant c.-206 T > A polymorphism was significantly associated with risk of CHD in the Chinese Mongolian population. Therefore, it may be a potential molecular biomarker for evaluating CHD risk. Our results accentuated the importance of E3 ligase during embryo heart development. This study provided new insights for risk assessments of common birth defects, and shed some light on the roles of genetics

and ethnic backgrounds in congenital heart diseases. Future studies should aim to determine whether RNF41 is involved the degradation of ErbB3 or other substrates via the ubiquitin-proteasome pathway in CHD patients.

### Conflicts of interest

The authors declare no conflicts of interest.

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