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Short Communication

The *NFKB1* -94 ATTG insertion/deletion polymorphism (rs28362491) contributes to the susceptibility of congenital heart disease in a Chinese population

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ABSTRACT

Congenital heart disease (CHD) is the most frequently occurring congenital disorder in newborns and is the most frequent cause of infant death from birth defects. Human genetic studies have identified that numerous genes encoding transcription factors that regulate specific events in heart development are responsible for inherited and sporadic CHD. Nuclear factor-kappa B (NF-κB) is a major transcription regulator of immune response, apoptosis and cell-growth control genes. The aim of this study was to investigate whether the functional -94 insertion/deletion ATTG polymorphism (rs28362491) in the promoter of nuclear factor κB gene (*NFKB1*) is associated with susceptibility to CHD. Polymerase chain reaction (PCR)-polyacrylamide gel electrophoresis (PAGE) method was used to genotype rs28362491 in 122 atrial septal defect (ASD) patients, 114 ventricular septal defect (VSD) patients, and 412 controls. The frequencies of II (Insertion/Insertion) genotype in the ASD and VSD patients were significantly higher than that of controls ($p = 0.004$ for ASD Vs. controls, and $p = 0.009$ for VSD Vs. controls, respectively), and the frequencies for I allele in CHD patients were also significantly higher than that in controls ($p = 0.01$ for ASD Vs. controls, and $p = 0.009$ for VSD Vs. controls, respectively). This study suggests that the functional -94 insertion/deletion ATTG polymorphism in the promoter of *NFKB1* is associated with CHD.

1. Introduction

Congenital heart disease (CHD), usually refers to abnormalities in the heart's structure or function that arise before birth, affects 19–75 of every 1000 live births, depending on which types of defect are included, and is the leading cause of death in infants under 1 year of age (Hoffman and Kaplan, 2002). The incidence is higher when fetuses that do not survive to term are included (Hoffman, 1995). Despite of exciting advances in surgical therapy, mortality and morbidity remain high for CHD. Great strides have been made in understanding the morphological contortions that accompany the progressive development of the mammalian heart, while only in recent years has a detailed understanding emerged of the molecular pathways that regulate this important process (Srivastava, 2006). Because dysregulation of heart development is at the root of CHD, a clear picture of how the heart forms is crucial for understanding the genesis of this disease. Although the major underlying defects that cause CHD are mutations in regulators of heart development during embryogenesis, environmental factors also contribute to this disease (Jenkins et al., 2007; Pierpont et al., 2007).

By the recurrence of CHD in families, and by studies showing an association of CHD with inherited microdeletion syndromes, in which a chromosomal region containing many genes is deleted, the genetic component for CHD has been implicated. Numerous genes responsible for inherited and sporadic CHD have been identified. Most of these genes encode transcription factors regulate specific events in heart development, such as ventricular septation or outflow tract morphogenesis (Bruneau, 2008). The T-box transcription factor gene *TBX5*, which is the causative gene in Holt–Oram syndrome (HOS), and HOS predominantly includes ASDs, VSDs and conduction-system defects, is the first identified single-gene mutation giving rise to an inherited CHD (Li et al., 1997; Schott et al., 1998). Soon after that, more and more mutations in different genes encode transcription factors were identified in different CHD. To date, mutations within many cardiac transcription factors have been identified as genetic causes for CHD in humans: *ZIC3*, *NKX2.5*, *TBX5*, *GATA4*, *TFAP2B*, *TBX1*, and *FOG2* (Clark et al., 2006). Because of their important role in the orchestration of cardiac development, mutations in these genes result in significant

Abbreviations: CHD, congenital heart disease; NF-κB, nuclear factor-kappa B; PCR-PAGE, polymerase chain reaction-polyacrylamide gel electrophoresis; ASD, atrial septal defect; VSD, ventricular septal defect; I, insertion; D, deletion; OR, odds ratios; CI, confidence intervals; HWE, Hardy–Weinberg equilibrium.

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disruption/dysregulation of downstream gene expression, and thus lead to cardiovascular malformations.

Nuclear factor-κB (NF-κB) was originally discovered in 1986 by David Baltimore's laboratory (Sen and Baltimore, 1986). The NF-κB family consists of five members: p65 (*RelA*), RelB (*RelB*), c-Rel (*Rel*), p50/p105 (*NFKB1*) and p52/p100 (*NFKB2*). Although many dimeric forms of NF-κB have been detected, the major form of NF-κB is a heterodimer of the p50 and p65 subunits (Chen et al., 1999). As a major transcription regulator in a large array of biological processes including immunity, inflammation, proliferation, tumorigenesis, cardiac hypertrophy, and cell survival, defects in NF-κB signaling have been associated with a variety of human diseases, such as cancers, rheumatoid arthritis, inflammatory bowel disease, AIDS, Alzheimer's disease, and so on (Baeuerle and Baltimore, 1996; Demaria et al., 2010; Dhingra et al., 2010). NF-κB has also been shown to influence numerous cardiovascular diseases including myocardial ischemia/reperfusion injury, ischemic preconditioning, vein graft disease, cardiac hypertrophy, atherosclerosis and heart failure (Dhingra et al., 2010; Van der Heiden et al., 2010). Blockade of NF-κB signaling during early cardiac morphogenesis induces apoptosis and is associated with multiple congenital cardiac alterations of outflow tract (Hernandez-Gutierrez et al., 2006). Although it remains unknown which members of the Rel family are relevant in this process, these findings indicate that NF-κB may be associated with CHD.

A common insertion/deletion polymorphism (-94 insertion/deletion ATTG, rs28362491), which seems to be the first functional *NFKB1* genetic variation, has been identified located between two putative key promoter regulatory elements in the *NFKB1* gene. The presence of ATTG deletion (allele D) resulted in the loss of binding to nuclear protein, leading to reduced promoter activity (Karban et al., 2004). This functional variation has been extensively investigated for association to cancer, ulcerative colitis, dilated cardiomyopathy, endometriosis, rheumatoid arthritis, systemic lupus erythematosus, and so on (Gao et al., 2012; Karban et al., 2004; Lopez-Mejias et al., 2012; Wang et al., 2011; Zhou et al., 2009, 2010; Zou et al., 2011). However, its association with CHD is still unclear. We hypothesized that this functional variation in NFKB1 is involved in the development of CHD and we genotyped this polymorphism in ASD and VSD, two common types of CHD, respectively, to test our hypothesis.

2. Materials and methods

2.1. Subjects

This study was approved by the institute ethics committee and all subjects gave written informed consent to participate. One hundred and twenty two patients with ASD (male: 47, female: 75) and 114 patients with VSD (male: 58, female: 56) diagnosed by echocardiogram and cardiac catheterization between May 2011 and June 2012 were enrolled into the present study. The control group consisted of 412 healthy subjects (male: 198, female: 214) from a routine health survey. Subjects with any personal or family history of CHD or other serious disease were intentionally excluded. All subjects were Han population living in Chongqing of southwest China.

2.2. Genotyping

Genomic DNA of each individual was extracted from 200 μl EDTA-anticoagulated peripheral blood samples by a DNA isolation kit from Bioteke (Peking, China) and the procedure was performed according to the manufacturer's instructions. The polymerase chain reaction (PCR)-polyacrylamide gel electrophoresis (PAGE) method was used to genotype the -94 insertion/deletion ATTG polymorphisms of *NFKB1* (Zhou et al., 2009). The primer sequences were: F 5′-tggaccgcatgactctatca-3′, R 5′-ggctctggcttcctagcag-3′, and DNA fragments containing the polymorphism were amplified in a total volume of 25 μl, including 2.5 μl 10× PCR buffer, 1.5 mmol/l $MgCl_2$, 0.15 mmol/l dNTPs, 0.4 μmol/l each primer, 100 ng of genomic DNA and 1.5 U of *Taq* DNA polymerase. The PCR conditions were 94 °C for 4 min, followed by 32 cycles of 30 s at 94 °C, 30 s at 64 °C and 30 s at 72 °C, with a final elongation at 72 °C for 7 min. 3 μl PCR products were separated by a 6% polyacrylamide gel and staining with 1.5 g/l argent nitrate. Allele D yields a 154 bp band and allele I yields a 158 bp band (Fig. 1). The genotypes were confirmed by the DNA sequencing analysis (BigDye®Terminator v3.1 Cycle Sequencing Kits, Applied Biosystems, Foster City, CA). About 10% of the samples were randomly selected to perform the repeated assays and the results were 100% concordant.

2.3. Statistical analysis

Genotype frequencies of rs28362491 within the *NFKB1* gene promoter in ASD, VSD and controls were obtained by directed counting. Hardy–Weinberg equilibrium was evaluated by chi-square test. All data analyses were carried out using SPSS 13.0 statistical software. Odds ratio (OR) and respective 95% confidence intervals were reported to evaluate the effects of any difference between allelic and genotype distribution. Probability values of 0.05 or less were regarded as statistically significant in patients compared to control subjects.

3. Results

Three genotypes of rs28362491 within *NFKB1* were successfully identified. Genotype distributions had no deviation from Hardy–Weinberg equilibrium in ASD, VSD and controls. Differences in allelic and genotype distribution of rs28362491 were tested between ASD patients and control subjects, as well as between VSD patients and control subjects, and observed differences are presented in Table 1.

As shown in Table 1, both of the frequencies for allele I in ASD and VSD patients were significantly higher than that in control subjects (68.4% vs. 59.3%, $p=0.010$, OR = 1.486, 95% CI = 1.097–2.013 for ASD vs. controls; 68.9% vs. 59.3%, $p=0.009$, OR = 1.515, 95% CI = 1.108–2.071 for ASD vs. controls). No significant difference was observed between VSD patients and controls in ID Vs. DD comparison ($p=0.065$), while ID genotype frequency for ASD patients was higher than that of controls in ID vs. DD comparison ($p=0.013$). Subjects carrying allele I (ID/II genotypes) of rs28362491 had a significantly increased risk for ASD and VSD compared with that without allele I (DD genotype) in a dominant genetic model ($p=0.004$, OR = 2.704, 95% CI = 1.310–5.579 for ASD VS. controls, and $p=0.020$, OR = 2.240, 95% CI = 1.116–4.494 for VSD vs. controls, respectively). The frequency for II homozygous was significantly overrepresented in both ASD and VSD patients ($p=0.004$, OR = 2.920, 95% CI = 1.367–6.238 for II vs. DD between ASD and controls; $p=0.009$, OR = 2.579, 95% CI = 1.241–5.360 for II vs. DD between VSD and controls, respectively).

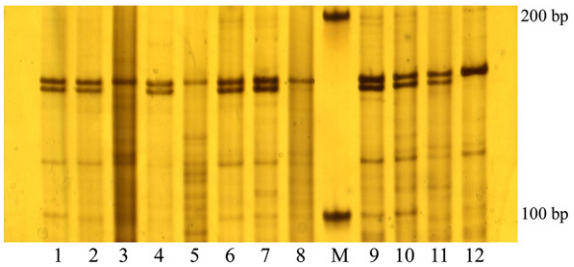

Fig. 1. PCR-PAGE analysis of the -94 insertion/deletion ATTG polymorphism in the *NFKB1* gene. Lanes 1, 2, 4, 6, 7, 9, 10, and 11: heterozygous (ID genotype); Lanes 3, 5, 8, and 12: homozygous (II genotype); M: DNA marker.