ASSOCIATION OF NFKB1, NKX2-5, GATA4 AND RANKL GENE POLYMORPHISMS WITH SPORADIC CONGENITAL HEART DISEASE IN GREEK PATIENTS

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ABSTRACT

Congenital heart disease (CHD) is a group of structural defects of the heart and the great vessels, and one of the leading causes of death among infants and young adults. Several gene variants are involved in diverse mechanisms of cardiac and vessel development and could thus be considered candidate mutated genes for a congenital heart defect or a specific variant could predispose a person to CHD. In the present study, variants in four such genes are investigated for the first time in a group of young Greek CHD patients: the NFKB1 gene polymorphism (–94ins/delATTG), rs28362491, NKX2-5 gene polymorphism rs2277923, GATA4 gene polymorphism rs11785481 and RANKL gene polymorphism rs4531631. A total of 43 CHD patients and 100 healthy adults were included in the study. The polymerase chain reaction-restriction fragment length polymorphism (PRC-RFLP) method was used to genotype the aforementioned polymorphisms of NFKB1, NKX2-5, GATA4 and RANKL. The association analysis identified that there was a protective association between CHD and the A allele of rs2277923 polymorphism (p = 0.004). The D allele of the rs28362491 polymorphism is also a likely risk factor for causing CHD (p = 0.006). The differences of the rs4531631 and rs11785481 variant contribution had no statistical significance between the groups (p >0.05). In conclusion, our results revealed that the rs28362491 and rs2277923 gene polymorphisms, but not the rs4531631 and rs11785481 polymorphisms, may contribute to CHD risk in a cohort of Greek CHD patients.

Keywords: Congenital heart disease (CHD); GATA4; NFKB1 (–94ins/delATTG); NKX2-5; RANKL

INTRODUCTION

Congenital heart disease (CHD), is a structural defect of the heart and the great vessels, and is an important cause of mortality in newborn infants, with an estimated prevalence rate of 1.0% in live births [1,2]. As a multifactorial disease, both genetics and environmental factors contribute to its development and approximately 30.0% of CHD present as part of a genetic syndrome or chromosomal abnormality [3]. Multiple environmental factors, such as viral infections with rubella, febrile illness and influenza can affect the fetal development and increase the incidence of CHD. Other implicated maternal factors include diabetes, systemic lupus erythematosus, exposure to therapeutic drugs, alcohol consumption and cigarette smoking [1,4]. Nonetheless, to date, the molecular mechanisms that lead to the occurrence of CHD are largely unknown. Up to the present, more than 40 genes encoding transcription factors, signal transduction and structural proteins, have been revealed to be involved in congenital heart defects. Any mutation in these genes may lead to abnormal heart structure and CHD [5,6]. The NKX2-5 and GATA4 genes have been associated with CHD discovered by linkage analysis and candidate gene studies in highly affected families [6].

The NKX2-5 is a member of the NKX2 family located on chromosome 5134 and consists of two exons that encode a 324-amino acid transcription factor. The genetic polymorphism rs2277923 is a synonymous mutation 63A>G leading to an amino acid substitution of glutamine by glutamine at position 21 (Glu21Glu) and was reported as a genetic predictor of CHD [7]. In ad-
dition, *GATA4* interacts with *NKKX2-5*, and it is known that mutations in transcription factors can result in severe cardiac defects [3]. For these reasons, the *GATA4* gene polymorphism, an important transcription factor that is involved in the morphogenesis of the heart, was chosen to be studied in order to further validate its pivotal role in modulating CHD risk. Transcription factor *GATA4* is a 442-amino acid protein, which is mapped to chromosome 8q22-23 [6]. Consistent with these findings, a common variant +1158C>T (rs11785481) in the 3’ untranslated region (3’UTR) of the *GATA4* gene, was confirmed to be associated with CHD susceptibility, possibly by altering microRNA (miRNA) posttranscriptional regulation [8].

Another transcription factor implicated in the pathogenesis of CHD is the *NFKB1* (nuclear factor-κB1) gene. The NF-κB family is a member of transcription factors that regulate numerous biological processes such as inflammation, proliferation and cell survival. The NF-κB family has five subunits: p65(RelA), RelB(RelB), c-Rel(Rel), p50/p105 (NFKB1) and p52/p100 (NFKB2) [9].

The human *NFKB1* gene is located on chromosome 4q24 and encodes a 50 kDa DNA-binding peptide (p50). Interestingly, a common –94ins/delATTG variant (rs2277923, rs4531631 gene polymorphism, the A and G insertion allele) fragments were then digested using *PfI* (restriction enzyme) for the –94ins/delATTG polymorphism of *NFKB1*. The wild type allele D yields a 281 bp band and allele I yielded two fragments of 240 and 45 bp, respectively. The A and G alleles of the *NKKX2-5* gene polymorphism produced DNA fragments of 264+37 and 213+51+37 bp, respectively, using *BpmI* restriction endonuclease. For the *GATA4* gene polymorphism, the C and T alleles produced DNA fragments of 261 and 174+87 bp, respectively, using *HpyCH4III* restriction endonuclease. For the *RANKL* gene polymorphism, the A and G alleles produced DNA fragments of 149+61 and 210 bp, respectively, using *EcoRV* restriction endonuclease.

### Table 1. Primer sequences of *NFKB1*, *NKKX2-5*, *GATA4*, and *RANKL* in CHD

| Locus         | Primer Sequences (5’>3’)                                                                 | Products (bp) | Annealing Tm (°C) | Endonuclease |
|---------------|-------------------------------------------------------------------------------------------|---------------|-------------------|--------------|
| rs28362491    | F: TGG GCA CAA GTC GTT TAT GA R: CTG GAG CCG GTA GGG AAG                                  | 281           | 62                | PfI/MI       |
|               | F: CTG CCC GGA CAC ATC CAG R: GTA GGC CTC TGG CTT GAA GG                                  | 301           | 62                | BpmI         |
| rs11785481    | F: TCC TCT CTA GCC CTT GGT CA R: ATC AGG GGC AGA AAC AGC A                                  | 261           | 60                | HpyCH4III    |
| rs4531631     | F: CTG GAA GGC TGG CAG TAC TTT R: TTC CTG CAC ATA GTA GGC TCT T                            | 210           | 62                | EcoRV        |

Tm: melting temperature.
Table 2. Model of analysis of NKX2-5 rs28362491, NKKX2-5 rs2277923, GATA4 rs111785481 and RANXL rs4531631 gene polymorphisms in patients with congenital heart disease and control subjects.

| Locus                  | Model of Analysis | Cases (n=43) | Controls (n=100) | OR (95% CI) | p Value | Corrected p Value |
|------------------------|-------------------|--------------|------------------|-------------|---------|-------------------|
| rs4531631 Reference: G | 0; 1; 2           | RR (%)       | 33 (77.0)        | 68 (68.0)   | 0.61    | 0.208             |
|                        |                   | RV (%)       | 10 (23.0)        | 29 (29.0)   | 0.29    | 0.550             |
|                        |                   | VV (%)       | 0 (0.0)          | 3 (3.0)     | 0.71    | 0.554             |
|                        | VV vs. RR         | RR           | 33              | 68          | 0.32    | 0.554             |
|                        |                   | RV           | 0               | 3           | 0.64    | 0.554             |
|                        | RV vs. RR         | RR           | 33              | 68          | 0.32    | 0.554             |
|                        |                   | RV+VV        | 33              | 68          | 0.32    | 0.554             |
|                        | VV vs. RV+VV      | RR           | 43              | 97          | 0.62    | 0.211             |
|                        |                   | VV           | 76              | 165         | 0.62    | 0.554             |
| rs2277923 Reference: G | 0; 1; 2           | RR (%)       | 7 (16.0)         | 7 (7.0)     | 0.42    | 0.004             |
|                        |                   | RV (%)       | 26 (61.0)       | 44 (44.0)   | 0.59    | 0.369             |
|                        |                   | VV (%)       | 10 (23.0)       | 49 (49.0)   | 0.39    | 0.123             |
|                        | VV vs. RR         | RR           | 7               | 7           | 0.32    | 0.004             |
|                        |                   | RV           | 10              | 49          | 0.47    | 0.004             |
|                        | RV vs. RR         | RR           | 7               | 7           | 0.32    | 0.004             |
|                        |                   | RV+VV        | 7               | 7           | 0.32    | 0.004             |
|                        | VV vs. RV+VV      | RR           | 33              | 51          | 0       | 0.47             |
|                        |                   | VV           | 40              | 58          | 0.47    | 0.004             |
| rs11785481 Reference: C| 0; 1; 2           | RR (%)       | 36 (84.0)       | 77 (77.0)   | 0.65    | 0.365             |
|                        |                   | RV (%)       | 7 (16.0)        | 23 (23.0)   | 0.65    | 0.365             |
|                        |                   | VV (%)       | 0 (0.0)         | 0 (0.0)     | 0.65    | 0.365             |
|                        | VV vs. RR         | RR           | 36              | 77          | 0       | 0.65             |
|                        |                   | RV           | 7               | 77          | 0       | 0.65             |
|                        | RV vs. RR         | RR           | 36              | 77          | 0       | 0.65             |
|                        |                   | RV+VV        | 36              | 77          | 0       | 0.65             |
|                        | VV vs. RV+VV      | RR           | 43              | 100         | 0       | 0.68             |
|                        |                   | VV           | 79              | 177         | 0.68    | 0.395             |
| rs29362491 Reference: D| 0; 1; 2           | RR (%)       | 11 (26.0)       | 9 (9.0)     | 0.37    | 0.003             |
|                        |                   | RV (%)       | 27 (63.0)       | 61 (61.0)   | 0.37    | 0.003             |
|                        |                   | VV (%)       | 5 (12.0)        | 30 (30.0)   | 0.37    | 0.003             |
|                        | VV vs. RR         | RR           | 11              | 9           | 0.14    | 0.001             |
|                        |                   | RV           | 5               | 30          | 0.001   | 0.006             |
|                        | RV vs. RR         | RR           | 11              | 9           | 0.04    | 0.040             |
|                        |                   | RV+VV        | 11              | 9           | 0.29    | 0.009             |
|                        | VV vs. RV+VV      | RR           | 38              | 70          | 0.31    | 0.019             |
|                        |                   | VV           | 49              | 79          | 0.49    | 0.012             |

OR: odds ratio; 95% CI: 95% confidence interval; RR genotype: homozygosity for the wild-type allele; RV genotype: heterozygosity for the wild-type and non wild-type alleles; VV genotype: homozygosity for the non wild-type allele; NA: not assigned.

The p value was corrected for multiple comparisons.

The reference wild-type allele.

The variant non wild-type allele.
Statistical Package for the Social Sciences (SPSS) version 24.0 software (https://www.ibm.com/product/spss-statistics) and Project for Statistical Computing (R) (version 3.6.1) software (https://www.r-project.org/), to identify the differences in variant contributions between the two groups under the six models of genetic association. Haldane Anscombe correction (0.5 is added to all cells of the contingency table) was used when we had cells in the contingency table with zero counts. In addition, the false discovery rate (FDR) method was applied to correct for bias caused by multiple comparisons [14]. The odds ratio (OR) and 95% confidence interval (95% CI) was estimated, and a p value of <0.05 was considered to be statistically significant.

RESULTS

The association of rs28362491, rs2277923, rs4531631 and rs11785481 polymorphisms are shown in Table 2. The genotype frequencies of the above variants in CHD patients and controls were in HWE. The polymorphism rs28362491 was associated with decreased risk of CHD with the additive [risk per allele RR genotype (homozygosity for the wild-type allele); RV genotype (heterozygosity for the wild-type and non wild-type alleles); VV genotype (homozygosity for non wild-type allele); p = 0.003], homozygous (VV vs. RR: p = 0.001), heterozygous (RV vs. RR: p = 0.040), dominant (RV+VV vs. RR: p = 0.009), recessive (VV vs. RV+RR: p = 0.019) and allelic model (V vs. R: p = 0.006). Also, decreased risk was found with the additive (risk per allele RR genotype) of rs4531631 and rs11785481 polymorphism distribution between the two groups (p >0.05).

DISCUSSION

To date, several studies have reported that genetic and epigenetic variations play a crucial role in CHD susceptibility, however, the pathogenesis of disease is highly complex and largely unknown. Mutations in genes controlling cardiac development and regulation, such as GATA4, NKX2-5, TBX5 [15], NOTCH1, TBX1, TBX20, CFC1, CITED2, CRELD1, are associated with non syndromic CHD [1]. In this case-control study, we examined the association of variants in NFKB1, RANKL, GATA4 and NKX2-5 genes with risk of CHD in patients of Greek origin.

Up to the present, polymorphisms in NF-κB have been implicated in the pathogenesis of CHD. The NFKB1 (–94ins/delATTG) polymorphism (rs28362491) has been broadly studied in cardiovascular diseases as a regulator of various biological processes, such as inflammation, proliferation and apoptosis. The NFKB1 gene encodes both p50 and p105 subunits of NF-κB protein. Deletion of one ATTG repeat in the promoter region of NFKB1 may result in lower levels of p50 homodimer by reduced p50 synthesis [16]. Partial depletion of p50 may decrease the anti-inflammatory response because the formation of the pro inflammatory p65/p50 heterodimer depends on the concentration of p50. Consequently, it may be that individuals with carrying allele D are genetically determined toward a higher inflammatory response [17]. A previous study [17] recognized that D-allele carriers of NFKB1 (–94ins/delATTG) are at increased risk of coronary heart disease, and may therefore show that inflammation is a potential mechanism for CHD development. Thus, it could reflect a positive association between human carrying allele D of rs28362491 and congenital cardiovascular anomalies.

In our study, we recognized a strong association between rs28362491 with the susceptibility to CHD. Individuals carrying allele D (ID/DD) of rs28362491, are at higher risk for CHD compared to those without allele D (II genotype) according to all six genetic models of analysis. In contrast to our results, positive association of the allele I with increased risk for development of an atrial septal defect (ASD), as well as with a ventricular septal defect (VSD), were demonstrated [1]. The discrepancy of results among diverse populations may be related to the racial and geographic differences.

In line with NFKB1, the genetic variant RANKL (rs45311631) was also shown to be a significant variant in predisposition to CHD in a Chinese population [10]. The RANKL gene is a member of TNF family is a type II trans-membrane peptide. Furthermore, it is identified in numerous cell types, such as T and B cells, mammary ligands, vascular endothelial cells and synovial fibroblasts [18]. Decreased production of a RANKL significantly inhibited the NF-κB signaling pathway and thus, may affect CHD risk [10,11]. Consistent with these findings, RANKL may also modulate CHD risk [10]. In the present study, no association was detected between the variant rs45311631 of the RANKL gene and CHD in the patients of Greek origin.

There are connections between normal heart structure and multiple transcription factors, therefore, any mutation in transcription factor may lead to abnormal cardiac development and CHD. Transcription factor GATA4 contains a zinc finger domain that binds to specific DNA. The GATA4 gene is highly conserved during evolution and plays a critical role in cardiac stem cell differentiation and cardio-
genesis [6]. In addition, mutations in the GATA4 gene can cause congenital cardiac anomalies and thus, deletions or duplications and point mutations of the gene, play a role in CHD susceptibility [8]. Mutations throughout the GATA4 gene have been associated with many types of abnormal heart structure such as VSDs, ASDs and pulmonary stenosis, and different alterations in the same gene can cause different CHD subgroups [6]. A previous study identified association between the +1158 T allele in the 3' UTR of the GATA4 gene (rs11785481) with a reduced risk for CHD in a group of 146 Caucasian patients [8]. In our results, no positive association was found, either in genotype or allele frequencies of the GATA4 rs11785481 polymorphism, indicating low mutation frequency of GATA4 in the Greek population. A potential explanation for this discrepancy may be related to the restricted group of CHD patients and the geographic differences.

Furthermore, the GATA4 gene expression has a direct impact on the embryonic and postnatal development of heart and acts synergistically with other transcription factors, such as NKX2-5 during cardiogenesis [8,19]. Cardiac transcription factor NKX2-5 acts in combination with GATA4 in genetic, transcriptional and biochemical processes during formation of the heart [3]. The NKX2-5 gene mutations regulate heart morphogenesis and function and were detected in patients suffering from tetralogy of Fallot. Also, GATA4 alterations were shown to impair interactions with NKX2-5, causing CHD [3]. NKX2-5 is an evolutionarily conserved homeobox protein gene and was first identified to be implicated in diverse manifestations of heart defects [5]. In the present study, our results showed that a significant statistical association exists between rs2277923 and CHD. Specially, the A allele of rs2277923 in the NKX2-5 gene was found to be protective between rs2277923 and CHD. Association between two genetic variants in NKX2-5 and GATA4 genes contribute to the genetic risk of congenital heart disease. Pediatr Cardiol. 2016; 37(8): 1548-1561.

In conclusion, our findings suggest the NFKB1 (rs28362491) and NKX2-5 (rs2277923) gene polymorphism as potential biomarkers of CHD in a Greek population. However, further studies in more CHD patients from different populations are needed to explore the association between rs28362491, rs2277923, rs4531631 and rs11785481 polymorphisms and CHD occurrence.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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