Combined effects of AKT serine/threonine kinase 1 polymorphisms and environment on congenital heart disease risk
A case-control study
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Abstract
This study aimed to explore the combined association between AKT serine/threonine kinase 1 (AKT1) polymorphisms and congenital heart disease (CHD) risk, meanwhile, the role of AKT1 single polymorphism on CHD was also analyzed.

In the first, AKT1 polymorphisms were genotyped in 130 CHD patients and 145 healthy people with the way of polymerase chain reaction-direct sequencing. The clinical data and genotypes, alleles between 2 groups were compared by \( \chi^2 \) test and the genotype distributions in the control group were checked by Hardy-Weinberg equilibrium. The relative risk strength of disease based on genetic variant was revealed using odds ratio (OR) with 95% confidence interval (95%CI).

In 3 polymorphisms of AKT1 (rs1130214, rs2494732, rs3803300), the GT/TT genotype of rs1130214 in cases and controls had a significant frequency difference \( (P = .04) \) and was 1.71 times risk developing CHD, compared with AA (OR = 1.71, 95%CI = 1.02–2.86), and T allele had 1.63 times risk for carriers (OR = 1.63, 95%CI = 1.05–2.54). Similarly, both rs3803300 GG genotype and G allele had obvious differences between case and control groups \( (P < .05) \) and it was closely associated with CHD susceptibility. At the same time, the combined effects of rs1130214, rs3803300 and family history, smoking were found in our study.

AKT1 rs1130214, rs3803300 polymorphisms are associated with the increased susceptibility to CHD. Environmental factors are found the interaction with AKT1 polymorphisms. Further study is needed to verify this conclusion.

Abbreviations: 95%CI = 95% confidence interval, AKT1 = AKT serine/threonine kinase 1, CHD = congenital heart disease, OR = odds ratio, SNP = single nucleotide polymorphism.

Keywords: AKT serine/threonine kinase 1, congenital heart disease, interaction, polymorphism

1. Introduction
Congenital heart disease (CHD) is a structure defect of heart or great vessels at birth, which is caused by the abnormal development of heart’s arteries in fetus period.\textsuperscript{[1,2]} It is the most common congenital disease and the leading cause of miscarriage, infant death.\textsuperscript{[3,4]} The incidence of CHD is 0.8% to 1% in newborns and it explains about 10% of infants death, which gives heavy economic and life burden for the whole family.\textsuperscript{[5–7]} In clinical, the common types of CHD include ventricular septal defect, pulmonary stenosis and tetralogy of Fallot. So far, scholars at home and abroad take a large effort to explore the pathogenesis of CHD, but the result is unsatisfactory.\textsuperscript{[8]} Clinical data and epidemiologic research report that CHD, as a complicated disease, is influenced by genetic and environmental factors.\textsuperscript{[9]} Recently, single nucleotide polymorphism (SNP) come into sight as an important tool studying the ethology of disease.

AKT serine/threonine kinase 1 (AKT), also called protein kinase B, is a serine/threonine-specific protein kinase which involves in various cellular progresses including metabolism, apoptosis and development as well as several cancers and cardiovascular disease.\textsuperscript{[10,11]} AKT kinase comprises 3 isoforms in humans, namely AKT1, AKT2, AKT3 and they have the similar structure but not function. All AKT kinase contain 3 conserved domains: an N-terminal pleckstrin homology domain, a serine/threonine specific center kinase domain and an C-terminal regulatory domain.\textsuperscript{[12,13]} The distinct functions may derive from their tissue-specific expression and AKT1 is expressed widely in various tissues.\textsuperscript{[14]} Embryos and newborns of AKT1-deficient mice are found with heart defects and heart function also decrease, which indicates AKT1 is a indispensable for heart development and function.\textsuperscript{[15,16]} The genetic variant of AKT1 may alter the expression of protein,\textsuperscript{[17]} but the the roles of AKT1 SNPs in CHD were studied rarely in the past.
In this study, the association of AKT1 polymorphism with CHD susceptibility were researched and 3 SNPs (rs1130214, rs2494732, rs3803300) were selected. At the same time, the interaction of gene polymorphism and environmental factor was also analyzed to clarify the mechanism of CHD and provide basis for genetic intervention.

2. Materials and methods

2.1. Study subjects selection

In this study, a case-control design was adopted. The cases experienced the clinical and pathological diagnosis and were confirmed in cardial surgery department of West China Hospital, Sichuan University. It included 130 CHD patients, consisting of 71 boys and 59 girls with the mean age of 7.23 ± 2.92 and only 3 to 15 years old children were selected. The controls were healthy children from the same region with the cases without any the symptoms of diseases. Their age range was 4 to 17 years old with the average age of 7.75 ± 2.75. 80 boys and 65 girls were selected without blood relationship each other at the same time with the cases. Both the case and control groups were Chinese Han population and this study was supported by the Ethics Committee of the above hospitals. We informed all subjects’ parents and gained the written consents before collecting blood sample. The detailed clinical information of all subjects were recorded, including age, gender, birth weight, premature birth, family history of CHD, mother’s pregnant age, smoking, drinking, childbearing history and made into excel form. It is considered as the premature birth that the gestation was terminated at 28 weeks to 37 weeks. Family history was defined that 1 or more than 1 direct relatives had suffered from CHD once or nowadays. Smoking is that pregnant woman had a smoking history or exposed to secondhand smoke in a long-term. Drinking was defined that pregnant woman had a alcohol consumption history, that is, she drank more than 1 times every week.

2.2. DNA extraction and genotyping of AKT1 polymorphisms

2mL peripheral venous blood was collected from every participant conformed to the ethics criteria of human genome research and put into blood collection tube with EDTA-2Na anticoagulation. The blood genomic DNA was extracted with the blood DNA extraction Kit from TIANGEN Biotech (Beijing) Co., Ltd and stored at −20°C. The genotyping of AKT1 rs1130214, rs2494732, rs3803300 polymorphisms was done by the way of polymerase chain reaction (PCR)-direct sequencing. PCR primer sequences referred the report of Wang et al.18 and were synthesized by Shanghai Sangon Biotech Co., Ltd (Table 1). The PCR system was a volume of 25 μL solution and the reaction program was as follows: initial denaturation at 95°C for 3 minutes, and then 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 30 seconds, extension at72°C for 30 seconds, and then 72°C extension for 7 minutes. The PCR products were checked the quality and purity with 1% agarose gel electrophoresis and NanoDrop 2000 NanoVue Plus. The eligible PCR products were directly sequenced in Shanghai Sangon Biotech Co., Ltd to confirm the genotype of every subject based on these 3 polymorphisms.

Table 1

| SNP       | Primer sequence         | Location |
|-----------|-------------------------|----------|
| rs1130214 | 5′-ACGTTGATGATG666TTTCTCCAGGAGG-3′ | Intron   |
| rs2494732 | 5′-ACGTTGATGATG666TTTCTCCAGGAGG-3′ | Intron   |
| rs3803300 | 5′-ACGTTGATGATG666TTTCTCCAGGAGG-3′ | Intron   |

AKT1 = AKT serine/threonine kinase 1, SNP = single nucleotide polymorphism, UTR = untranslated region.

2.3. Statistical analysis

The genotype distributions of AKT1 polymorphisms in the control group were tested by χ² to determine whether conformed to Hardy-Weinberg equilibrium (HWE). The age difference was compared with t-test and the other indexes, including genotype and allele were compared with χ² test between the case and control groups. The relative risk intensity of disease was displayed by odds ratio (OR) with 95% confidence interval (95%CI). All statistical analyses were conducted in PASW statistics 18.0 software and P < 0.05 represented a significant meaning. The data were showed with ± s or %.

3. Results

3.1. The basic characteristics of subjects

A total of 275 subjects were enrolled in this study, including 130 cases and 145 controls. As was shown in Table 2, there was no significant difference between the cases and control in age and gender (P > 0.05). Mother’s pregnant age, childbearing history and drinking history didn’t show significant difference between 2 groups, either (P > 0.05). However, birth weight less than 2500g was more frequently discovered in CHD patients (10.00%) than in healthy subjects (3.45%) (P < 0.05). Premature birth and family

Table 2

| Index                   | Case, n=130 (%) | Control, n=145 (%) | P value |
|-------------------------|----------------|--------------------|---------|
| Children Age            |                |                    |         |
| Mean age                | 7.23 ± 2.92    | 7.75 ± 2.75        | >.05    |
| Gender Boys             | 71 (54.62)     | 80 (55.17)         | >.05    |
| Girls                   | 59 (45.38)     | 65 (44.83)         |         |
| Birth weight/g           |                |                    |         |
| <2500                   | 13 (10.00)     | 5 (3.45)           | <.05    |
| ≥2500                   | 117 (90.00)    | 140 (96.55)        |         |
| Premature birth          |                |                    | <.01    |
| Yes                     | 18 (13.85)     | 7 (4.83)           |         |
| No                      | 112 (86.15)    | 138 (95.17)        |         |
| Family history           |                |                    | <.05    |
| Yes                     | 21 (16.15)     | 11 (7.59)          |         |
| No                      | 109 (83.85)    | 134 (92.41)        |         |
| Mothers Pregnant age     |                |                    | >.05    |
| ≤25                     | 79 (60.77)     | 97 (66.90)         |         |
| >25                     | 51 (39.23)     | 48 (33.10)         |         |
| Childbearing history     |                |                    | >.05    |
| Yes                     | 54 (41.54)     | 49 (33.79)         |         |
| No                      | 76 (58.46)     | 96 (66.21)         |         |
| Smoking                 |                |                    | >.05    |
| Yes                     | 48 (36.92)     | 36 (24.83)         |         |
| No                      | 82 (63.08)     | 109 (75.17)        |         |
| Drinking                |                |                    | >.05    |
| Yes                     | 34 (26.15)     | 29 (20.00)         |         |
| No                      | 96 (73.85)     | 116 (80.00)        |         |
history of CHD were also the influence factors for CHD occurrence ($P < .01$, $P < .05$). Besides, mother’s smoking might increase risk which her child suffered from CHD ($P < .05$).

The HWE of 3 polymorphisms in the control group was checked. The results were listed in Table 3, which the genotype distributions of all polymorphisms in controls conformed to HWE. Therefore, our study population was a Mendelian population and had the representative.

### 3.2. The effect of AKT1 single polymorphism on CHD susceptibility

Single polymorphism was analyzed in Table 3, the results were that rs2494732 didn’t show any significant difference, either genotypes or allele ($P > .05$). In the contrary, rs1130214, rs3803300 were associated with CHD development. In rs1130214 polymorphism, T allele frequency in cases was obviously higher than that of the controls, compared with G allele (21.15% vs 14.14%, $P = .03$) and it was 1.63 times risk for the onset of CHD (OR = 1.63, 95%CI = 1.05–2.54). Its mutant genotype GT/TT was also associated with the increased susceptibility to CHD (OR = 1.71, 95%CI = 1.02–2.86).

Referring to rs3803300, people carrying the minor allele G had 1.48 times risk suffering from CHD, compared the common A allele (OR = 1.48, 95%CI = 1.04–2.11). What’s more, the GG genotype enlarge the risk to 2.48 times based on AA allele (OR = 2.48, 95%CI = 1.08–5.69).

### 3.3. The interaction analysis of AKT1 polymorphisms and environment in CHD

The combined analysis results of AKT1 polymorphisms and environmental factors were displayed in Table 4. In terms of birth weight and premature birth, we didn’t detect any significant correlation to 3 polymorphisms ($P > .05$). Furthermore, rs2494732 had no interaction with either birth weight, premature birth or family history, smoking ($P > .05$). Differently, People with a family history of CHD were tendency to carry GT/TT mutant genotype of rs1130214 and AG/GG genotype of rs3803300 ($P > .05$). Smoking was also considered as the interaction with rs1130214 and rs3803300 ($P > .05$). Therefore, AKT1 polymorphisms exerted role in the onset of CHD with environmental factors synergistically.
4. Discussion

Heart is the first organ to form in the process of embryogenesis and its development is a extremely complicated work, referring to the expression of multiple different genes in specific period and the proliferation, differentiation, migration of cells. [19] Accurate gene regulation is a safeguard in heart formation, furthermore, most of CHD patients occur in embryonic period, which may be caused by the structure and function of genes regulating the differentiation and morphogenesis of myocardial cells. [20, 21] The occurrence of CHD results in fetal abortion and newborns death, meanwhile, it puts a heavy burden on the economy and life of family. Therefore, it is very important to study the genetic mechanism of CHD at the molecular level.

With the development of modern medicine and medical technology, SNP as a novel tool is paid attention to explore the ethology of disease. After years of efforts, researchers achieve some relative information. Li et al summarize multiple populations of others researchers to analyze the association of the common polymorphism C667T in methylenetetrahydrofolate reductase gene with CHD risk, the results indicate that methylenetetrahydrofolate reductase C667T polymorphism is relevant to CHD with obvious heterogeneity in a meta-analysis. [22] In the study of Dinesh et al based on Indian population about the effect of NK2 homeobox 5 SNPs on CHD, NK2 homeobox 5 c.608A > G (p.E203G) and c.852G > A (p. N226D) polymorphisms were counted to be associated with CHD. [23] Endothelial NO synthase gene genetic variant is also detected to contribute to the risk of sporadic CHD. [24] In addition, TBXs, ACE, GATA4 genes polymorphisms are also the study targets in the onset of CHD.

Nowadays, genes regulating the heart development include 4 types: signal, cell adhesion, ion channels molecules and transcription factor. AKT participates in cellular survival and protein synthesis pathways as a key signaling protein. Phosphoinositide 3kinase/protein kinase B pathway is a key regulator in which mesoblastema, original cells of heart development in embryo stage, specially express transcription factors in heart. [25] AKT is key target molecule in the downstream of phosphoinositide 3 kinase and activated AKT is involved in a series of biological progresses, such as cell survival, growth and proliferation. [26]

In this article, AKT1 rs1130214, rs2494732, rs8303300 polymorphisms were analyzed the association with the onset risk of CHD. Firstly, the clinical information and family of CHD patients and relative controls were investigated, the results showed that birth weight, premature birth, family history of CHD and mother’s smoking had the significantly independent correlation to CHD, but not the other environmental factors in our study. However, in other studies, childbearing history and even mother’s pregnant age also participate in CHD occurrence. [27] Maybe different populations and small sample size result in this variant conclusion. In the study of AKT1 single polymorphism, rs2494732 wasn’t detected the association with CHD, either genotypes or alleles. Differently, both of rs1130214, rs8303300 polymorphisms were independent association with CHD susceptibility. The mutant genotype of rs1130214 had 1.71 times risk to suffer from CHD, compared with the common genotype, and even the mutant allele was also 1.63 times risk. In views of rs8303300, both of the genotypes and alleles showed significant differences between the case and control groups and it was also an independent susceptibility factor. However, any genes and polymorphisms don’t play roles in the development of disease independently. So the interaction of gene polymorphism with environmental factor was analyzed. In this study, people with a family history of CHD and GT/TT genotype of rs1130214 or AG/GG genotype of rs3803300 were easily attacked by CHD. In smoking population, we gained the similar conclusion.

In conclusion, environmental factors can affect the onset of CHD and AKT1 rs1130214, rs3803300 polymorphisms involve in the generation and development of CHD. What’s more, the combined effects of gene polymorphisms and environment on CHD are found in our study. Finally, due to the single population and small sample size, the results need to be confirmed in the future with well-design and large samples.

Author contributions

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