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ORIGINAL ARTICLE

# Associations of *NKX2-5* Genetic Polymorphisms with the Risk of Congenital Heart Disease: A Meta-analysis

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**Abstract** The *NKX2-5* gene is a vital regulator of cardiac formation and development. Recently, the roles of *NKX2-5* 63A>G polymorphism and 606G>C polymorphism in congenital heart disease (CHD) have been extensively studied, with conflicting results. The aim of the present study was to better elucidate the associations between *NKX2-5* genetic polymorphisms and CHD risk through a meta-analysis. Eligible articles were searched in PubMed, MEDLINE, EMBASE, Google Scholar and CNKI up to December 2015. Odds ratios (ORs) and 95 % confidence intervals were used to detect any potential associations between *NKX2-5* genetic polymorphisms and CHD risk. Heterogeneity between studies was assessed with Q test and $I^2$ statistic. Subgroup analysis and sensitivity analysis were performed to test the reliability and stability of the results, and funnel plots were applied to estimate publication bias. A total of 13 case–control studies including 2245 CHD patients and 1953 healthy controls were analyzed. The overall meta-analysis results showed that *NKX2-5* 63A>G polymorphism and 606G>C polymorphism were not significantly associated with CHD risk. Subgroup analysis was further performed for *NKX2-5* 63A>G polymorphism based on types of CHD and ethnicity of study population, and similar negative results were found in all subgroups. Our findings suggested that *NKX2-5* 63A>G polymorphism and 606G>C polymorphism may not be implicated in the pathogenesis of CHD.

**Keywords** *NKX2-5* · Congenital heart disease (CHD) · Genetic polymorphisms · Meta-analysis

## Introduction

Congenital heart disease (CHD), characterized by structural and/or functional anomaly of heart or intrathoracic great vessels due to abnormal cardiac development, is the most prevalent type of birth defect, with an estimated incidence of 4–50 in every 1000 live births, and is the leading non-infectious cause of infant death worldwide [1]. Despite rapid improvement in surgical treatment and interventional therapy over the past few decades, the morbidity and mortality of CHD patients remained significantly higher than that of the general population, and its associated complications such as heart failure, arrhythmia or sudden cardiac death may occur even after effective correction of cardiac abnormalities [2, 3].

To date, the exact cause of CHD is still unclear; however, there is growing evidence to support that genetic factors play a crucial part in its pathogenesis. Firstly, family clustering of CHD with variable phenotypes has been observed extensively, and a higher risk of developing cardiac malformations exists in offspring of CHD patients [4, 5]. Secondly, it was reported that 8–13 % of CHD patients have chromosome abnormalities [6], and mutations of several genes have been found to be associated with CHD [7–11]. Nevertheless, the etiology of CHD is highly complex and the genetic determinants underlying CHD are not fully elucidated.

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The *NKX2-5* gene, a highly conserved cardiac-specific transcription factor encoder located on chromosome 5q34, is a vital regulator of cardiac formation and development [12]. Previous studies have shown that knockout of *NKX2-5* gene in mice could lead to embryonic or premature death, with arrested heart development and various kinds of cardiac defects [13, 14]. Besides, over 40 missense and nonsense mutations of *NKX2-5* gene have been identified in CHD patients [15, 16], and abnormal expression levels of *NKX2-5* were also found to be associated with multiple cardiac malformations [17, 18]. Therefore, *NKX2-5* is thought to be an ideal candidate gene for CHD susceptibility.

Recently, numerous studies have been done to investigate the roles of *NKX2-5* genetic polymorphisms, particularly the exon 1 63A>G polymorphism (rs2277923) and the exon 2 606G>C (rs3729753) polymorphism in the pathogenesis of CHD. But results were controversial, and statistical power of individual studies was insufficient [19–31]. Therefore, we conducted the present meta-analysis to better assess the associations between *NKX2-5* genetic polymorphisms and the risk of CHD.

## Materials and Methods

### Literature Search Strategy

Electronic databases of PubMed, MEDLINE, EMBASE, Google Scholar and China National Knowledge Infrastructure (CNKI) were searched without time limit using the following keywords: "congenital heart disease," "congenital heart defect," "congenital cardiovascular malformation," "NKX2-5," "NKX2.5," "NK2 homeobox 5," "NK2 transcription factor-related, locus 5," "cardiac-specific homeobox," "CSX," "polymorphism," "variant," "genotype" and "allele." The initial search was conducted in May 2015, and the latest update was performed in December 2015. Besides, the reference lists of all relevant articles were reviewed manually for further identification of potentially eligible articles.

### Inclusion Criteria

The inclusion criteria for the present study were set prior to the literature search. Eligible studies met the following conditions: (1) article already published electronically; (2) case–control study of unrelated CHD patients and healthy controls; (3) evaluation of the association between *NKX2-5* 63A>G polymorphism (rs2277923) and/or 606G>C polymorphism (rs3729753) and the risk of CHD; (4) presentation of sufficient data to calculate the odds ratios (ORs) and corresponding 95 % confidence intervals (CIs); and (5) full text in English or Chinese available. If the report was duplicated or identical patients were enrolled in two studies, only the most recent and complete article was included. Pedigree studies, case reports, case series, reviews, editorials, expert opinions and studies with CHD being considered as a component of any known genetic disorders were intentionally excluded.

### Data Extraction and Quality Assessment

From each included studies, the following data were extracted: first author, year of publication, country of origin, ethnicity of study population, the number of cases and controls, types of CHD, allelic and genotypic frequency of *NKX2-5* 63A>G polymorphism and/or 606G>C polymorphism in CHD patients and control subjects, and whether the distributions of *NKX2-5* genetic polymorphisms in the control group were in accordance with Hardy–Weinberg equilibrium (HWE). The Newcastle–Ottawa quality assessment scale (NOS), a classical rating tool which evaluates the quality of non-randomized studies from three perspectives: selection, comparability and exposure, was used to assess the validity of all case–control studies included [32]. This rating system has a score range of 0–9, and studies with a score of more than 7 were assumed to be of high quality. Two reviewers (XCX and XHS) performed the data extraction and quality assessment independently. When necessary, the reviewers wrote to the authors for extra information or raw data. Any discrepancies between two reviewers were resolved by discussion until reaching a consensus. The final results were reviewed by a senior reviewer (LR).

### Statistical Analysis

All data analyses were performed with Review Manager version 5.3.3 (The Cochrane Collaboration, Software Update, Oxford, United Kingdom). HWE was explored by using the Chi-square test. ORs and corresponding 95 % CIs were used to evaluate associations of *NKX2-5* 63A>G polymorphism and 606G>C polymorphism with CHD risk. In addition, Q test and $I^2$ statistic were employed to evaluate heterogeneity between studies. If probability value ($P$ value) was less than 0.1 or $I^2$ was greater than 50 %, between-study heterogeneity was considered to be significant, and the random-effect model (REM) would be adopted for analyses. Otherwise, if studies were considered to be homogenous, the fixed-effect model (FEM) would be applied for analyses. Subgroup analysis was performed based on types of CHD and ethnicity of study population if a subgroup contained at least four individual studies. Sensitivity analysis was carried out through removing one individual study each time. Publication bias was further

assessed with funnel plots. And a *P* value of 0.05 or less was regarded as statistically significant in comparison between CHD patients and healthy controls.

## Results

### Included Studies

The literature search generated 243 results. After exclusion of irrelevant or duplicate articles by reading titles and abstracts, 74 articles were selected for further evaluation. Among these, a total of 13 case–control studies including 2245 CHD patients and 1953 healthy controls met our inclusion criteria [19–31] (see Fig. 1), 13/13 were about the *NKX2-5* 63A>G polymorphism, and 6/13 were about the *NKX2-5* 606G>C polymorphism. All included studies were published between 2005 and 2015. Of these, there were 11 studies of Asian ethnicity and 2 studies of Caucasian ethnicity. HWE test for the control group of each included studies revealed that 10 studies were in agreement with HWE, whether the remaining 3 studies by Ouyang et al. [25], Peng et al. [27] and Xiong et al. [29] met HWE were unavailable since only allelic frequencies were provided. Eight articles were published in English, and the other five were published in Chinese. Characteristics of included studies are summarized in Table 1, and distributions of *NKX2-5* 63A>G polymorphism and 606G>C polymorphism are summarized in Table 2 and Table 3.

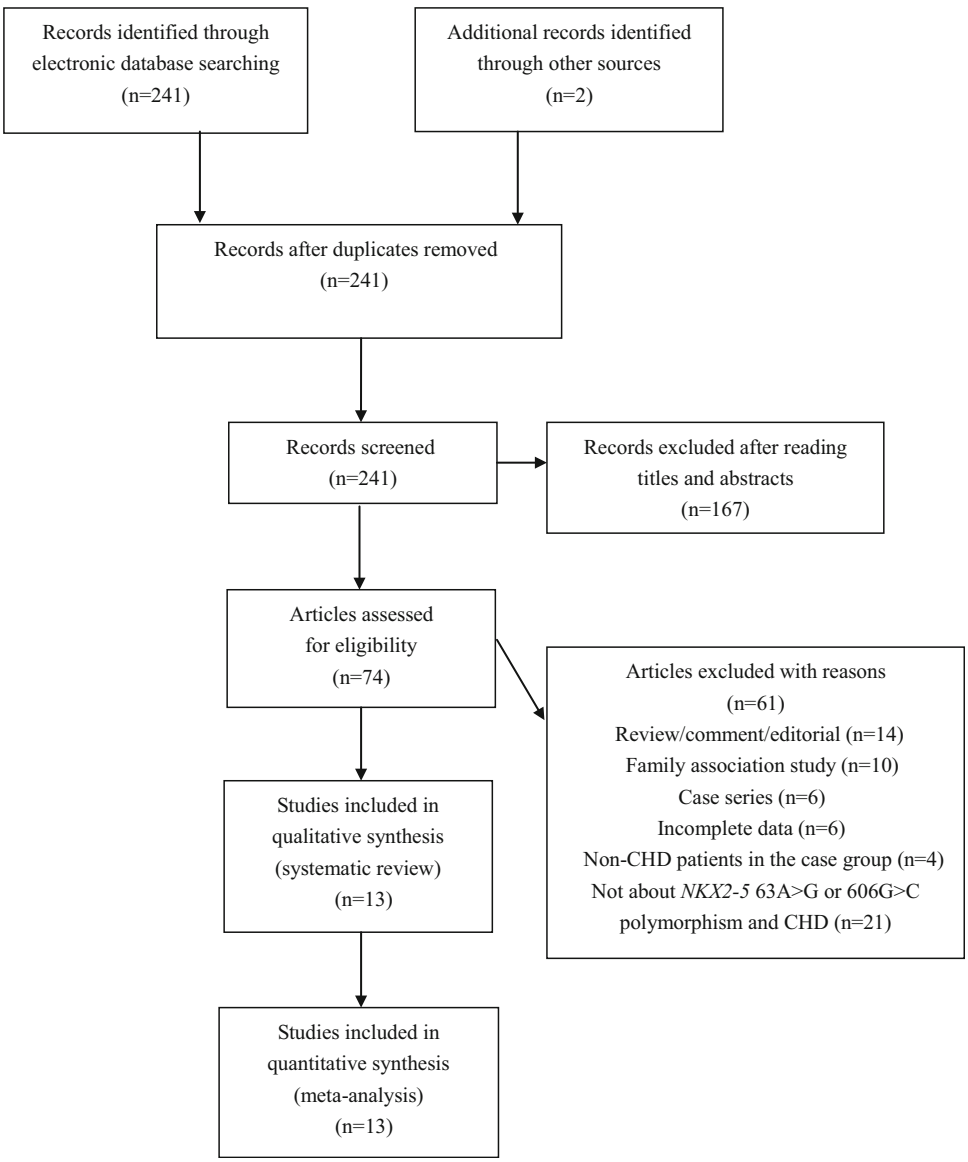


**Fig. 1** Flowchart of study selection for the present study

**Table 1** Characteristics of included studies

| First author | Year | Country | Ethnicity | Case/control | Polymorphisms | Types of CHD | HWE | NOS score |
|---|---|---|---|---|---|---|---|---|
| Cao | 2015 | China | Asian | ASD: 107/487<br>VSD: 391/487 | 63A>G<br>606G>C | ASD + VSD | Y | 7 |
| Dinesh | 2010 | India | Caucasian | 150/70 | 63A>G | Multiple | Y | 7 |
| Han | 2011 | China | Asian | 81/52 | 63A>G | Multiple | Y | 7 |
| Ketharnathan | 2015 | India | Caucasian | 50/50 | 63A>G | Multiple | Y | 8 |
| Liu (1)[a] | 2009 | China | Asian | 180/200 | 63A>G | ASD | Y | 7 |
| Liu (2)[a] | 2009 | China | Asian | 160/200 | 63A>G<br>606G>C | VSD | Y | 7 |
| Ouyang | 2011 | China | Asian | 125/105 | 63A>G | Multiple | NA | 7 |
| Pang | 2012 | China | Asian | 213/194 | 63A>G | VSD | Y | 8 |
| Peng | 2010 | China | Asian | 63A>G: 126/114<br>606G>C: 134/109 | 63A>G<br>606G>C | Multiple | NA | 7 |
| Shi | 2005 | China | Asian | 110/110 | 63A>G | Multiple | Y | 8 |
| Xiong | 2012 | China | Asian | 224/121 | 63A>G 606G>C | Multiple | NA | 7 |
| Zhang | 2009 | China | Asian | 230/200 | 63A>G 606G>C | Multiple | Y | 8 |
| Zhao | 2014 | China | Asian | ASD: 40/50<br>VSD: 50/50 | 63A>G<br>606G>C | ASD + VSD | Y | 7 |

*CHD* congenital heart disease, *HWE* Hardy–Weinberg equilibrium, *NOS* Newcastle–Ottawa quality assessment scale, *ASD* atrial septal defect, *VSD* ventricular septal defect, *NA* not available

[a] These two studies shared identical control subjects

**Table 2** Distribution of the *NKX2-5* 63A>G polymorphism for CHD cases and controls

| First author, year | CHD case | | | Control | | |
|---|---|---|---|---|---|---|
| | *n* | Genotypes GG/GA/AA | Alleles G/A (%) | *n* | Genotypes GG/GA/AA | Alleles G/A (%) |
| Cao, 2015 | 491 | 158/218/115 | 54.4/45.6 | 474 | 155/233/86 | 57.3/42.7 |
| Dinesh, 2010 | 150 | 24/49/77 | 32.3/67.7 | 70 | 11/25/34 | 33.6/66.4 |
| Han, 2011 | 81 | 30/43/8 | 63.6/36.4 | 52 | 23/25/4 | 68.3/31.7 |
| Ketharnathan, 2015 | 50 | 11/19/20 | 41.0/59.0 | 50 | 0/7/43 | 7.0/93.0 |
| Liu (1)[a], 2009 | 180 | 63/85/32 | 58.6/41.4 | 200 | 58/94/48 | 52.5/47.5 |
| Liu (2)[a], 2009 | 160 | 60/70/30 | 59.4/40.6 | 200 | 58/94/48 | 52.5/47.5 |
| Ouyang, 2011 | 125 | NA | 54.0/46.0 | 105 | NA | 63.3/36.7 |
| Pang, 2012 | 213 | 86/100/27 | 63.8/36.2 | 194 | 85/80/29 | 64.4/35.6 |
| Peng, 2010 | 126 | NA | 77.8/22.2 | 114 | NA | 77.2/22.8 |
| Shi, 2005 | 110 | 76/27/7 | 81.0/19.0 | 110 | 70/33/7 | 62.3/37.7 |
| Xiong, 2012 | 224 | NA | 79.2/20.8 | 121 | NA | 82.3/17.7 |
| Zhang, 2009 | 230 | 93/107/30 | 63.7/36.3 | 200 | 76/98/26 | 62.5/37.5 |
| Zhao, 2014 | 90 | 28/42/20 | 54.5/45.5 | 50 | 22/24/4 | 68.0/32.0 |

*NA* not available

[a] These two studies shared identical control subjects

## Risk of Bias in Included Studies

As shown in Table 1, the average NOS score of included studies was 7.31 (range from 7 to 8), which implied that all eligible articles were of relatively high quality. The improper selection of controls and mismatching age of CHD cases and control subjects were the major sources of biases.

**Table 3** Distribution of the *NKX2-5* 606G>C polymorphism for CHD cases and controls

| First author, year | CHD case | | | Control | | |
|---|---|---|---|---|---|---|
| | *n* | Genotypes GG/GC/CC | Alleles G/C (%) | *n* | Genotypes GG/GC/CC | Alleles G/C (%) |
| Cao, 2015 | 492 | 468/24/0 | 97.6/2.4 | 487 | 465/22/0 | 97.7/2.3 |
| Liu, 2009 | 160 | 145/15/0 | 95.3/4.7 | 200 | 191/9/0 | 97.7/2.3 |
| Peng, 2010 | 134 | NA | 98.1/1.9 | 109 | NA | 98.2/1.8 |
| Xiong, 2012 | 224 | NA | 97.8/2.2 | 121 | NA | 97.9/2.1 |
| Zhang, 2009 | 230 | 219/11/0 | 97.6/2.4 | 200 | 188/12/0 | 97.0/3.0 |
| Zhao, 2014 | 90 | 84/6/0 | 96.7/3.3 | 50 | 45/5/0 | 95.0/5.0 |

*NA* not available

### *NKX2-5* 63A>G Polymorphism and CHD Risk

A total of 13 studies with 2230 CHD cases and 1940 control subjects were enrolled for *NKX2-5* 63A>G polymorphism [19–31]. Since Ouyang et al. [25], Peng et al. [27] and Xiong et al. [29] failed to report genotypic frequency of *NKX2-5* 63A>G polymorphism in CHD patients and healthy controls, we accessed the association between *NKX2-5* 63A>G polymorphism and CHD risk by comparing distribution of alleles in CHD cases and control subjects. REM was employed for analysis on account of striking between-study heterogeneity ($I^2 = 81$ %, $P$ for the heterogeneity <0.00001), and no significant association with CHD risk was found for *NKX2-5* 63A>G polymorphism (OR 1.10, 95 % CI 0.88–1.38, $P = 0.39$) (see Fig. 2).

### *NKX2-5* 606G>C Polymorphism and CHD Risk

For *NKX2-5* 606G>C polymorphism, a total of 6 studies including 1330 CHD patients and 1167 healthy controls were investigated [19, 24, 27, 29–31]. In order to explore the association between *NKX2-5* 606G>C polymorphism and CHD risk, we compared allelic frequency in CHD cases and control subjects because Peng et al. [27] and Xiong et al. [29] did not present distribution of genotypes in CHD patients and healthy controls. Heterogeneity between studies was found to be trivial ($I^2 = 0$ %, $P$ for the heterogeneity = 0.59), and thus, analysis was performed with FEM. No significant association was detected between *NKX2-5* 606G>C polymorphism and CHD risk (OR 1.11, 95 % CI 0.78–1.58, $P = 0.57$) (see Fig. 3).

### Subgroup Analysis

For *NKX2-5* 63A>G polymorphism and CHD risk, subgroup analysis was performed by stratifying available data based on types of CHD and ethnicity of study population. In the ventricular septal defect (VSD) and atrial septal defect (ASD) subgroups, we failed to detect any significant association for *NKX2-5* 63A>G polymorphism (VSD, OR 1.12, 95 % CI 0.83–1.51, $P = 0.47$, $I^2 = 77$ %, $P$ for the

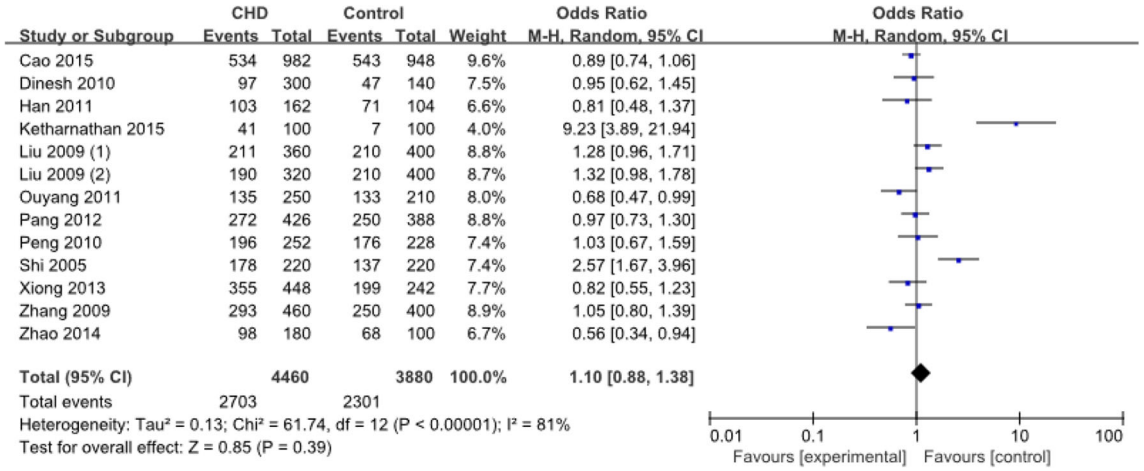


**Fig. 2** Forest plot on association between *NKX2-5* 63A>G polymorphism and CHD risk (G allele vs. A allele). Random-effect pooled OR 1.10, 95 % CI 0.88–1.38, $P = 0.39$; $I^2 = 81$ %, $P$ for the heterogeneity <0.00001

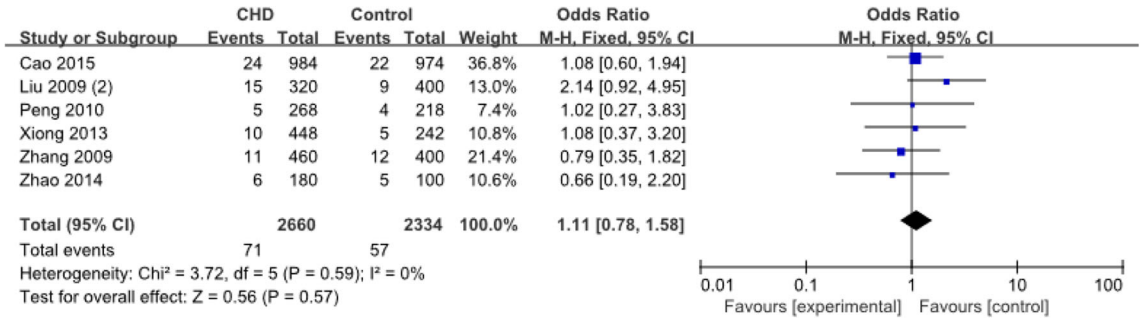


**Fig. 3** Forest plot on association between *NKX2-5* 606G>C polymorphism and CHD risk (C allele vs. G allele). Fixed-effect pooled OR 1.11, 95 % CI 0.78–1.58, $P = 0.57$; $I^2 = 0$ %, $P$ for the heterogeneity = 0.59

heterogeneity = 0.0002; ASD, OR 0.79, 95 % CI 0.53–1.19, $P = 0.26$, $I^2 = 74$ %, $P$ for the heterogeneity = 0.002) (see Table 4). In addition, no significant association was found between *NKX2-5* 63A>G polymorphism and the risk of CHD in the Asian subgroup (OR 1.02, 95 % CI 0.84–1.23, $P = 0.87$, $I^2 = 73$ %, $P$ for the heterogeneity <0.0001) (See Table 4). Subgroup analysis for *NKX2-5* 606G>C polymorphism and CHD risk could not be carried out due to lack of relevant data.

### Sensitivity Analysis

Sensitivity analysis was performed by removing one individual study each time. For *NKX2-5* 63A>G polymorphism and 606G>C polymorphism, removing any study did not impact the overall results. However, we found that between-study heterogeneity for *NKX2-5* 63A>G polymorphism was drastically reduced ($I^2 = 44$ %, $P$ for the heterogeneity = 0.06) after eliminating studies by Shi et al. [28] and Ketharnathan et al. [22], which suggested that these two studies were the main source of heterogeneity.

### Publication Bias

Potential publication bias was evaluated with funnel plots. Visual inspection of funnel plots revealed no apparent asymmetry for *NKX2-5* 63A>G polymorphism and 606G>C polymorphism. And these results suggested that significant publication bias was unlikely.

## Discussion

CHD contains a variety of structural cardiovascular malformations that are actually or potentially of functional significance [33]. Historically, few CHD patients reached adulthood, but owing to the tremendous advances in interventional therapies and surgical treatment, the average life expectancy of CHD patients has been significantly prolonged in the last two decades [34]. However, despite substantially improved prognosis, CHD remains to be the leading cause of mortality for infants worldwide.

To date, the etiology of CHD is still largely unknown in spite of extensive research. However, it has become evident recently that several genes are implicated in the pathogenesis of CHD [5]. Of these, the *NKX2-5* gene, which encodes a homeodomain and NK2-specific domain containing cardiac transcription factor of 324 amino acids, is a crucial regulator of cardiac formation and development [12, 26]. It is the earliest expressed transcription factor encoder during embryonic cardiac development and plays a vital role in regulating expression of genes essential for heart formation. Previous animal studies have demonstrated that disruption of *NKX2-5* gene would impact specification of cardiac progenitor cells, morphogenesis of

**Table 4** Subgroup analysis for *NKX2-5* 63A>G polymorphism and CHD risk

| Variables | No.[a] | Case/control | OR (95 % CI) | $I^2$ (%) | $P$ for the heterogeneity |
|---|---|---|---|---|---|
| Types of CHD | | | | | |
| VSD | 7 | 962/1150 | 1.12 (0.83–1.51) | 77 | 0.0002 |
| ASD | 6 | 385/956 | 0.79 (0.53–1.19) | 74 | 0.002 |
| Ethnicity | | | | | |
| Asian | 11 | 2030/1820 | 1.02 (0.84–1.23) | 73 | 0.0001 |

*OR* odds ratio, *CI* confidence interval

[a] The number of articles

heart, differentiation of cardiomyocytes as well as formation and development of cardiac conduction system [13, 35–37]. In addition, more than 40 mutations of *NKX2-5* gene have been found in both familiar and sporadic CHD cases, and altered transcription activity of *NKX2-5* was also shown to cause cardiac abnormalities [15, 16].

Recently, multiple studies have tried to explore the associations between certain *NKX2-5* genetic polymorphisms and the risk of CHD. Among these, the 63A>G polymorphism and 606G>C polymorphism were the two most intensively investigated sites. These two single-nucleotide polymorphisms are both synonymous variants. Although synonymous polymorphisms do not change the amino acid sequence of encoded proteins, accumulating evidence suggested that they may affect mRNA structure and stability, translation rate and fidelity as well as protein folding [38]. The 63A>G polymorphism (Glu21Glu) is located at exon 1 of *NKX2-5* gene. It was firstly reported by Benson et al. [39] in 1999. Afterward, several studies found that this polymorphism was significantly associated with CHD risk in different populations [22, 25, 28]. Moreover, luciferase reporter assays by Ouyang et al. [25] revealed that the A to G substitution of 63A>G polymorphism significantly reduced transcriptional activity of *NKX2-5* gene by 20 %, which may account for its association with the occurrence and development of CHD. The 606G>C polymorphism (Leu202Leu), which located at exon 2 of *NKX2-5* gene, was also thought to be implicated in the pathogenesis of CHD. However, no functional analysis has been reported about this polymorphism yet.

Despite potential theoretical mechanisms, results regarding associations of *NKX2-5* genetic polymorphisms with CHD risk were still contradicted. Hence, we conducted the present meta-analysis to solve the conflict and obtain a more conclusive result. And our overall results suggested that *NKX2-5* 63A>G polymorphism and 606G>C polymorphism may not be associated with CHD risk. Significant between-study heterogeneity for *NKX2-5* 63A>G polymorphism was found, and thus, we carried out stratified analyses by categorizing included studies into different subgroups on the basis of types of CHD and ethnicity of study population. Heterogeneity between studies remained notable in each subgroup, and similar to the result of overall meta-analysis, no significant association with CHD risk was detected for *NKX2-5* 63A>G polymorphism in any subgroups. Sensitivity analysis was further performed to test the stability of results and trace the source of between-study heterogeneity; for two *NKX2-5* genetic polymorphisms, removing any study did not change the overall results. However, we found that when studies by Shi et al. and Ketharnathan et al. were omitted, between-study heterogeneity for *NKX2-5* 63A>G polymorphism was drastically reduced, suggesting that these two studies were the major sources of heterogeneity.

It is worth noting that Wang et al. [40] performed a meta-analysis for associations between *NKX2-5* genetic polymorphisms and CHD risk of Chinese population in 2013. Compared with the present study, similar results were found for *NKX2-5* 606G>C polymorphism in the previous study, but results on *NKX2-5* 63A>G polymorphism were conflicting. Considering that our findings on *NKX2-5* 63A>G polymorphism were based on more eligible studies of relatively high quality and the sample size of the present analysis was significantly larger than that of the previous study (2230 cases and 1940 controls vs. 1243 cases and 1139 controls), maybe the results of the current study were more convincing. Overall, results from previous and current meta-analyses support that *NKX2-5* 606G>C polymorphism may not be involved in the pathogenesis of CHD; however, for *NKX2-5* 63A>G polymorphism and the risk of CHD, further studies are warranted to draw a more credible conclusion.

This study is certainly not without limitations. Firstly, the number of studies investigating the relationship between *NKX2-5* polymorphisms and CHD risk is still limited, and sample size of several included studies was obviously not sufficient, making our results not convincing enough. Secondly, although funnel plots revealed no apparent publication bias, we cannot eliminate the possibility of publication bias since only published studies were included. Thirdly, all included studies were published in English or Chinese; therefore, maybe some qualified articles in other languages were missed. Fourthly, genetic associations of certain *NKX2-5* polymorphisms with CHD may also be influenced by gene–gene and gene–environmental interactions. It is possible that one certain polymorphism may be associated with CHD risk, but due to interactions with multiple genes and environmental factors, the association would no longer be observed. Taken these limitations into consideration, the results presented by the current study should be interpreted with caution.

## Conclusions

In conclusion, the current meta-analysis suggested that *NKX2-5* 63A>G polymorphism and 606G>C polymorphism may not be implicated in the pathogenesis of CHD. However, considering that the present results were based on limited number of case–control studies, further multicenter studies with larger sample size from different populations are warranted to confirm our results. Besides, given that cardiac transcription factors play a pivotal role in heart formation, future investigations are needed to explore the potential roles of other polymorphisms of cardiac transcriptional factor genes in the occurrence and development of CHD.

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**Authors' contribution** X.C.X. and L.R. conceived of the study and participated in its design. X.C.X. and X.H.S. conducted the systematic literature review. X.S.X. performed data analysis. X.C.X. and L.R. drafted the manuscript. All authors have read and approved the final manuscript.

**Compliance with Ethical Standards**

**Conflict of interest** The authors report no conflicts of interest regarding the content herein.

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