

GENOTYPING OF GATA4 GENE VARIANT (G296S) IN MALAYSIAN CONGENITAL HEART DISEASE SUBJECTS BY REAL-TIME PCR HIGH RESOLUTION MELTING ANALYSIS

GENOTIPIZACIJA GENSKE VARIJANTE (G296S) GATA4 KOD MALEŽANSKIH SUBJEKATA SA UROĐENOM SRČANOM MANOM PUTEM METODE REAL-TIME PCR HRM

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Summary

Background: Congenital heart disease (CHD) is the most common birth defect; however, the underlying etiology is unrecognized in the majority of cases. GATA-binding protein 4 (GATA4), a cardiac transcription factor gene, has a crucial role in the cardiogenesis process; hence, a number of heterozygote sequence variations were identified as a cause of CHD. G296S heterozygote variant is the most frequently reported GATA4 gene sequence alteration. This study aims to investigate the role of G296S variant of the GATA4 gene in Malaysian CHD subjects.

Methods: We have investigated 86 Malaysian CHD subjects with cardiac septation defects for the presence of the GATA4 gene heterozygote variant (G296S) by the new technology of high resolution melting (HRM) analysis.

Results: Genotyping of G296S (c.886G>A) by HRM analysis shows that all the sample genotypes were of the wild GG

Kratak sadržaj

Uvod: Urođena srčana mana najčešći je defekt koji se javlja na rođenju. U većini slučajeva, međutim, njena etiologija ostaje nepoznata. GATA-vezujući protein 4 (GATA4), gen sa srčanim transkripcionim faktorom, ima ključnu ulogu u procesu kardiogeneze. Niz heterozigotnih varijacija u sekvencama identifikovane su kao uzroci urođenih srčanih mana. Utvrđeno je da je heterozigotna varijanta G296S alteracija u sekvenci gena GATA4 koja se najčešće javlja. Ova studija ima za cilj da istraži ulogu varijante G296S gena GATA4 kod maležanskih subjekata sa urođenom srčanom manom.

Metode: Kod 86 maležanskih subjekata sa urođenom srčanom manom, odnosno sa srčanim septalnim defektom, određivano je prisustvo heterozigotne varijante (G296S) gena GATA4 pomoću nove tehnologije HRM (*high resolution melting*).

Rezultati: Genotipizacija G296S (c.886G>A) pomoću analize HRM pokazuje da svi ispitivani uzorci genotipa pripadaju

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type genotype and the heterozygote mutant GA genotype was totally absent from this study cohort.

Conclusions: The results of our study showed that the G296S variant of the *GATA4* gene was not associated with the development of CHD in Malaysian subjects. The use of HRM analysis proved a cost-effective, high-throughput, specific and sensitive genotyping technique which eliminates the need for unnecessary sequencing.

Keywords: congenital heart disease, heterozygote, variant, cardiac transcription factor, real-time high resolution melting analysis

Introduction

Congenital heart disease (CHD) is defined as a defect in the structure of the heart or the great vessels that occurs during embryogenesis and is found at birth. It is the most common congenital anomaly, responsible for about one third of the major human congenital malformations (1). CHD includes a wide variety of defects such as atrial septation defect (ASD), ventricular septation defect (VSD), atrioventricular cushion defect, great blood vessels anomalies and many others. Despite the improvement in the diagnosis and management of this birth defect, it is still considered as one of the leading causes of neonatal mortality and the commonest cause of birth defect-related death in the infancy period, accounting for up to 24% of all of the birth defect-related mortality (2). The underlying etiology of non-syndromic CHD is not determined in the majority of the cases. However, a number of single gene defects have been identified as the cause of non-syndromic CHD. *GATA*-binding protein 4 (*GATA4*) was identified as a risk factor for CHD in many populations.

GATA4 gene [*600576] is one of the cardiac transcription factor genes, located at chromosome 8p23.1-p22 (3). The gene consists of 7 exons. It is expressed in the heart during embryogenesis and plays a major role in the process of heart development (4, 5). *GATA4* cardiac transcription factor regulates the expression of the cardiac alpha-myosin heavy chain (*MYH6*) gene (6). *GATA4* gene interacts with the *NKX2-5* cardiac transcription factor gene, acting together in activating the natriuretic peptide precursor (*NPPA*) which has an important role in cardiomyocytes differentiation and cardiac chambers formation (7). *GATA4* gene interacts also with the *TBX5* cardiac transcription factor gene (8) which in turn interacts and acts synergistically with the *NKX2-5* gene in regulating the differentiation of cardiomyocytes by binding to the *NPPA* gene promoter (9). As it is noticed, the *GATA4* gene forms an interaction network with important genes during the embryonic period which is fundamental to the cardiogenesis process.

GATA4 gene has been investigated for sequence variation in CHD subjects in different populations (Table I). In the Table I, it is clearly indicated that a

»divljem« GG tipu genotipa, dok je heterozigotni mutirani genotip GA bio potpuno odsutan u ispitivanoj populaciji.

Zaključak: Rezultati naše studije pokazali su da varijanta G296S gena *GATA4* nije povezana s razvojem urođenih srčanih mana kod maležanskih subjekata. Upotrebljena analiza HRM pokazala se kao isplativa, specifična i osetljiva tehnika genotipizacije sa visokom propusnom moći koja je otklonila potrebu za nepotrebnim sekvenciranjem.

Ključne reči: urođena srčana mana, heterozigot, varijanta, srčani transkripcioni faktor, *real-time high resolution melting analysis*

number of studies have not detected any sequence alteration in the gene sequence. On the other hand, *GATA4* gene sequence alterations were identified to cause CHD, such as the variants S52F (10), E359del and G296S (8). The variant G296S has been reported and analyzed in a number of studies. This variant

Table I Studies on *GATA4* gene sequence variants in CHD in different populations

Polymorphisms/Variants	Country	Reference
G296S, E359del	USA	(8)
S358RfsX45	Japan	(16)
S52F and E359Xfs	Japan	(10)
G296S	Italy	(15)
E216D	Lebanon	(26)
No variant detected	Denmark	(23)
V267M, V380M	China	(27)
A346V, P163S, 296C, L403M	USA	(28)
Gly93Ala, Gln316Glu, Ala411Val, Asp425Asn	USA	(29)
No variant detected	India	(25)
No variant detected	USA	(30)
46delS, 118-119insA, 125-126insAA, A6V, P163S, E359K, P407Q, S429T and A442V	China	(18)
P407Q, D425N	China	(19)
No variant detected	Indonesia	(24)
His28Tyr, His436Tyr	China	(17)
G303E and G296S	USA	(11)
Pro407Gln (reported)	China	(31)
No variant detected	Australia	(32)
Pro163Ser, Pro283Gln	China	(22)
M310V	China	(33)
T280M	China	(34)
D425N	Australia	(35)
No variant detected	Italy	(36)
No variant detected	Colombia	(37)

was first reported by Garg et al. (8) in familial cases of CHD in association with the development of ASD. It is located in exon 3 of the *GATA4* gene and is caused by G→A change at nucleotide 886 (c.886G>A) which predicts glycine to serine amino acid change at codon 296. Biochemical analysis of this variant demonstrates a reduction in the binding affinity of *GATA4* gene to the DNA and decrease in its transcriptional activity. Therefore, haplo-insufficiency of *GATA4* can cause cardiac septal defects, revealing the crucial function of the *GATA4* gene in cardiac septation and the separation of the four cardiac chambers. Moreover, the *GATA4* gene variant (G296S) disturbs its interaction with the *TBX5* gene (8). This variant will cause impairment of *GATA4* interaction with the *SMAD4* gene, which is another transcription factor essential for (BMP/TGF-β) signaling (canonical bone morphogenetic protein/transforming growth factor-β), a signaling pathway required for the formation of atrioventricular valves and the adjacent atrial and ventricular septae from the atrioventricular endocardial cushions (11).

So far, a large number of methods have been used for the genotyping of sequence variants. The use of the new genotyping technology of high resolution melting analysis in real-time (HRM real-time PCR) has advantages over the previously used genotyping technologies in being a high-throughput, simple, rapid, probe-free method that does not require the use of conventional post-PCR gel electrophoresis (12, 13).

Methods

The study cohort

Ethical Approval for this study was obtained from the Faculty of Medicine and Health Sciences (FMHS), University Putra Malaysia (Reference number: UPM/FPSK/PADS/T7-MJKEtikaPer/F01 (JSB_APR (10)05)). A written informed consent was obtained from all of the subjects recruited in this study.

A total of 86 CHD subjects treated at the National Heart Institute (Institut Jantung Negara – IJN) were recruited to take part in this study. All the patients visited the pediatric department of IJN from June 2010 till April 2011. The diagnosis of non-syndromic CHD and the classification of the type of cardiac defect had been done by an experienced consultant pediatric cardiologist based on the clinical and echocardiography findings +/- the diagnostic cardiac catheterization findings and surgical notes. The cardiac defects included in this study were septation defect (Ventricular Septal Defect (VSD) and Atrial septal defect (ASD)). Of the 86 CHD subjects, 52 had VSD and 16 had ASD. Eighty-six normal healthy subjects were included as a control group.

Molecular analysis

Genomic DNA extraction from the blood samples was done using the DNA Extraction Kit from Norgen Biotek Corp., Ontario, Canada. Genotyping of the *GATA4* gene variant G296S was done by the HRM analysis in the Rotor-Gene 6000 (Corbett Research-QIAGEN, Hilden, Germany) real-time rotary analyzer. Primers were designed to amplify the parts of the genome that harbor the G296S variant by using Primer 3 software (<http://frodo.wi.mit.edu/primer3/>). The primers: 5'-GAGTGGCCTCTCCTGTG-3' and for the reverse primer: 5'-GAGAGATGGGCATCA-GAAGG-3' were used for HRM analysis. Eva green dye was used as the double strand saturating DNA dye for the HRM analysis. The primers and the DNA concentrations and reaction conditions were optimized to have minimal noise in the machine and the best CT value. The PCR amplification was done in a 25 μL reaction mixture consisting of 5 μL of 2× SensiMix™ (Bioline Headquarters, London, UK) master mix, 0.5 mmol/L of a forward primer, 0.5 μmol/L of a reverse primer and 100 ng of genomic DNA, 1 μL of Eva green dye and finally distilled water was added to obtain the final volume of 25 μL. DNA amplification was done with an initial denaturation of 95 °C (10 minutes), followed by 40 cycles of 95 °C denaturation (45 seconds), 59 °C annealing (30 seconds) and 72 °C extension (45 seconds), the cycling followed by a final extension step at 72 °C (10 minute). The HRM analysis was done by adding a melting step ranging from 82 °C–92 °C with a rise of 0.1 °C per step and a hold of 2 seconds at each step.

HRM data analysis

Rotor-Gene 6000 software (software version 1.7, built 87) was used for the HRM analysis by generating a normalized melting curve, a difference graph and a derivative plot. In each run of HRM analysis, a sample with a known sequence of the wild GG genotype of the variant G296S (886A>G) was added to function as a negative control and help in the automated genotyping results generated from the machine software. Identification of the heterozygote variants can be done by using the difference and the normalized graphs, as it changes the shape of the melting curves. Besides that, in the derivative plot it will cause a low and/or broad melting.

DNA sequencing

Confirmation of the genotyping results was done by DNA sequencing. Sequencing was performed by an Applied Biosystems 3730×I DNA Sequencer. The sequencing results were aligned to the respective reference sequence from the NCBI-GenBank sequences using the sequence alignment MEGA4 software (14).

Results

HRM analysis genotyping results

In this study, eighty-six DNA samples from VSD and ASD subjects and eighty-six DNA samples from normal healthy individuals were assessed for the presence of the heterozygote GA genotype of the variant G296S (c.886G>A) by the real-time HRM analysis. Calling for the genotypes in the HRM analysis was based on observing the three generated curves from the software of the machine; the normalized melting curve, the difference graph and the derivative plot. Besides that, the machine semiautomated calling of the genotypes was also used.

Figure 1 shows the normalized melting curves generated from the rotor gene machine. As it is shown in Figure 1, all the samples had the same melting temperature, which can also be confirmed in the derivative plot (Figure 3) with no shifting in the melting curves. More importantly, this study is looking for the heterozygote genotype (GA) of this sequence vari-

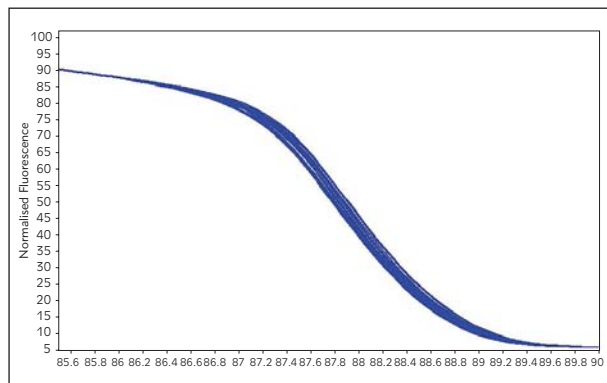


Figure 1 Normalised melting graph of HRM analysis for the genotyping of the G296S (c.886G>A) variant of GATA4 gene. All the samples had the same melting temperature with no shifting in the melting curves and no changes in the shape of the curve.

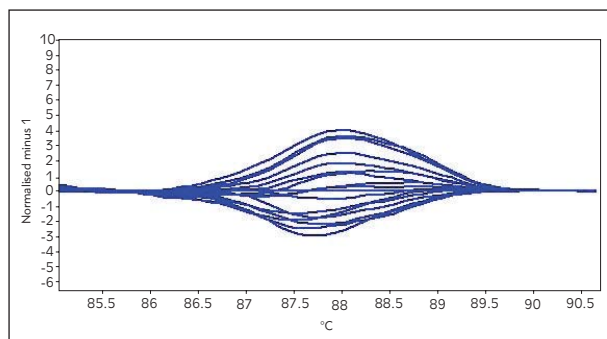


Figure 2 The difference graph of HRM analysis for the genotyping of the G296S (c.886G>A) variant of GATA4 gene. All the samples formed a bundle around the baseline indicating the wild GG genotype from both cases and control subjects.

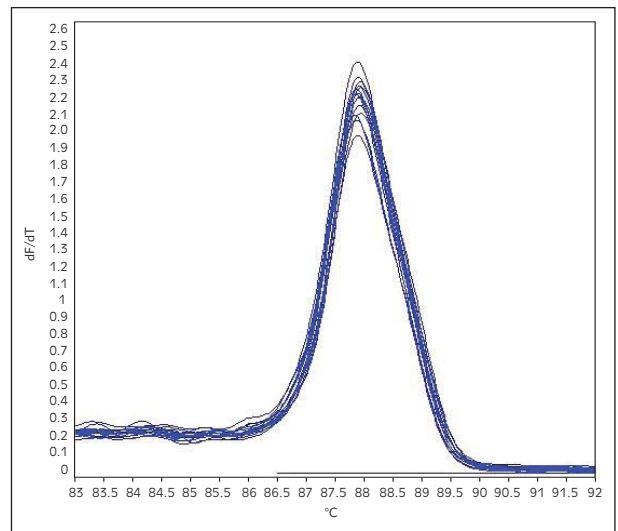


Figure 3 The derivative plot of HRM analysis for the genotyping of the G296S (c.886G>A) variant of GATA4 gene. All the samples have the same melting temperature with absence of the slow transition of the melting temperature, a characteristic of the heterozygote genotype in the HRM analysis.

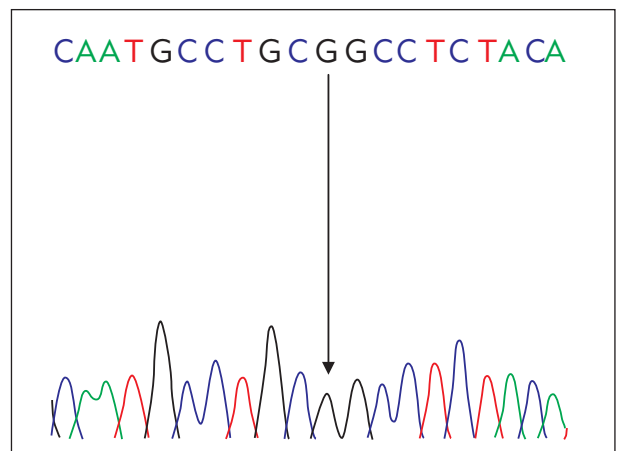


Figure 4 DNA sequencing chromatograph results. The arrow points to the site of expected G296S (886G>A) variant which shows the G allele of the wild GG genotype.

ation (G296S) that should cause a change in the shape of the melting curve. All the genotyped samples show no alteration of the generated melting curves. Also noted was the absence of slow transition of the melting temperature in the derivative plot which is a characteristic of the heterozygote genotype in the HRM analysis. On the other hand, in the difference graph all the samples formed a bundle around the baseline (Figure 2) which indicates that all the samples are of the wild GG genotype, as well as the total absence of G296S variant from both the case and control samples. In this study we utilized the Rotor gene machine software autocalling function of the genotypes after setting its specificity level to 90%.

DNA sequencing results

The DNA sequencing was done for 10 samples that were genotyped to confirm the results of the HRM analysis. *Figure 4* shows an example of DNA sequencing results for the amplicon that harbor the G296S sequence alteration. The samples were chosen on a random basis and the results of sequencing were 100% identical to those obtained from the HRM analysis.

Discussion

GATA4 gene is expressed in the heart during embryogenesis and plays a major role in the process of cardiogenesis (4, 5). Our study revealed the absence of the G296S missense variant of the GATA4 gene. However, the results of this study show total absence of the mutant allele A that causes the G296S (886A>G) sequence alteration of the GATA4 gene from the CHD and the control subjects in this study cohort. So far, the G296S variant of the GATA4 gene has been reported mainly in familial cases of CHD with the septation defect (8) (11, 15). Nevertheless, several studies (Japan (16), China (17–22), Denmark (23) and Indonesia (24)) failed to detect the G296S variant of the GATA4 gene in CHD patients despite including familial cases of CHD as well as cases with septation defects. Their reports were in agreement with the findings of our study, which included 52 cases of VSD and 16 case of ASD. One possible explanation for this is the tendency of this variant to cause familial cases of CHD rather than sporadic ones. However, our study cohort had included one family with a history of CHD over three generations; five members of this family had ASD or VSD. Despite that, we could not identify this variant in their genome. In a study conducted in India, sequencing was used in 21 CHD subjects to detect the G296S variant of the GATA4 gene but failed to detect the variant (25). Even in our study, we randomly selected 10 samples for DNA sequencing to detect the G296S

variant of GATA4 and failed to detect the variant, which is concordant with previous reports.

Conclusions

The results of our study show that the heterozygote genotype of the G296S variant of the GATA4 gene was totally absent from our study cohort and thus G296S is not associated with the development of CHD in Malaysian subjects. Other sequence alterations of the GATA4 gene as well as other transcription factor genes were identified in familial and sporadic cases of CHD, and therefore exploration of different variants of this gene in Malaysian CHD is warranted. The real-time HRM analysis used in this study as a genotyping method was a high-throughput technique, specific, sensitive and cost-effective, as it eliminated the need for unnecessary sequencing.

Authors' contributions. NFK carried out the molecular genetic studies and drafted the manuscript. RV participated in the design of the study and the manuscript drafts. PI conceived the study, participated in its design and coordination and helped to draft the manuscript. MA and AFA participated in designing the study. HAA, NM and AE helped in collection of the blood samples. All authors read and approved the final manuscript.

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Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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