Cellular and Molecular Biology

Al-Azzouny *et al. Cell. Mol. Biol.*2016, 62 (3): 10-14
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.3.3

Original Research

Detection and putative effect of GATA4 gene variants in patients with congenital cardiac septal defects

M. A. Al-Azzouny[1], M. O. El Ruby[2], H. A. Issa[1], E. G. Behiry[1], N. R. Elsayed[1], A. G. Fayez[3*]

[1] Faculty of Medicine, Banha University, Banha, Egypt
[2] Clinical Genetics Department, Human Genetics & Genome Research Division, National Research Centre, Dokki, Egypt
[3] Molecular Genetics and Enzymology Department, Human Genetics & Genome Research Division, National Research Centre, Dokki, Egypt

Abstract: The zinc finger transcription factor GATA4, located on chromosome 8p23.1–p22, has been implicated as a critical regulator of cardiac development during embryogenesis. Mutations of GATA4 appear to be responsible for some cardiac septal defects. The aim of this work was to screen for mutations in the GATA4 gene in sample of Egyptian patients affected by isolated and non-isolated cardiac septal defects. We examined 20 patients with atrial septal defect (ASD), ventricle septal defect (VSD), atrioventricular septal defects (AVSD) and A-V canal disturbance defect and compared with examined 10 unaffected individuals as normal control. The patients were referred from Congenital Heart Disease Clinic of the Clinical Genetics department at the National Research Centre. All patients were subjected to clinical evaluation, echocardiography and karyotyping. Genomic DNA was extracted from all cases and subjected to PCR followed by direct sequencing. The predicted effect of variants was done by a variety of proper prediction tools. We detected six variants in GATA4 gene, two of them are novel variants. Predicted functional analysis of the relevant variants was performed by *In silico* analysis. Further confirmatory studies on familial segregation and in vitro / in vivo functional analysis are recommended to support our results.

Key words: Heart, congenital, genetics, septation defect, GATA4, *In silico* analysis.

Introduction

Congenital heart diseases (CHDs) are the most common of all birth defects and accounts for nearly 30% of all major congenital anomalies leading to mortality in the first year of life (1, 2).Bassili, et al (2000) revealed that the prevalence of congenital heart diseases (CHD) among school children in Alexandria, Egypt accounted for 1.01/1000. Van der Linde et al (2011) reported that total CHD birth prevalence increased substantially over time, from 0.6 per 1,000 live births in 1930 till 1934 to 9.1 per 1,000 live births after 1995. Over the last 15 years (till 2010), stabilization occurred, corresponding to 1.35 million newborns with CHD every year.

Cardiac septation defects are among the most common birth defects in humans. The frequency of these defects might reflect the complexity of cardiogenesis. Major advances in the understanding of the underlying genetic etiologies of cardiac septation defects have provided insight into the genetic pathways involved. These genetic factors are most often transcription factors involved in the early stages of cardiogenesis as Tbx5, Nkx2-5, and GATA4 (5).

Many mutations in GATA4 gene has been reported in cases with ASD, VSD and tetralogy of Fallot (TOF) patients (6-11). 3'-UTR of mRNA contains regulatory elements that are essential for the appropriate expression of many genes. Indeed, 3'-UTR mutations have been associated with disease, but frequently this region is not analyzed. Seven hot spot sequence alterations in 3'-UTR of GATA4 gene has been detected to causing effect (12).

Materials and Methods

Subjects

The study included 20 unrelated affected children with isolated and non-isolated congenital cardiac septal defects referred from Congenital Heart Disease Clinic of the Clinical Genetics department at the National Research Centre (NRC) during the period from August 2014 to October 2015 (prospective case-control study design), They were 10 females and 10 males with female to male ratio is 1:1 of patients with age ranged from 5 months to 6 years. Ten normal age and sex matched individuals with no family history of cardiac diseases were included as normal control group. A full explanation of the study was discussed and clarified to the patients and/or their guardians. Official written consent was obtained. An ethical approval for the plan of work was provided from the Scientific Ethical Committee of the National research Centre (NRC).

Clinical evaluation

For each case, the clinical genetics team carried out three-generation pedigree constructions, Complete history for patients and their parents, Detailed general clinical examination. Special emphasis on cardiac and dysmorphic features examination and other manifesta-

Received December 15, 2015; **Accepted** March 15, 2016; **Published** March 20, 2016

*** Corresponding author:** A. G. Fayez, National Research Centre, 33 El Bohouth Street (Former El Tahrir Street), P.O. Box 12622, Dokki, Giza, Egypt. E-mail: afayez_nrc@yahoo.com

tions were done carefully to exclude known dysmorphic syndromes. Examination of different body system, Echo heart, electrocardiogram (ECG) and Plain chest X-rays were carried out for all cases and other investigations were performed whenever needed.

Cytogentics studies

Conventional cytogenetic analysis, using GTG banding technique, was performed for all patients. Fluorescence In Situ Hybridization (FISH) was also done on peripheral blood lymphocytes for all studied patients to exclude chromosomal aberration syndromes.

Samples and DNA Extraction

Three ml. of the peripheral blood samples was collected from the patients using potassium ethylenediaminetetra-acetic acid (K_2EDTA) as anticoagulant inside vacutainer sterile tubes. Genomic DNA was isolated from peripheral blood leukocytes by QIAamp DNAMini Kit (50 preps), catalog number 51304, Germany (13).

Mutation Analysis

Amplification of coding regions (exon 3-7) was carried out using specific PCR primers as previously reported by Okubo et al. (2004), PCR was cycled 35 times at 95˚C for 30 secs, at 56–61˚C for 30 secs, and at 72˚C for 1 min in 30 ul mixture containing 1ug of genomic DNA, 0.5U recombinant Taq DNA polymerase (2U/ul) (Thermo Fisher Scientific Inc., USA) with buffer, 0.2 mmol/l each dNTP and 0.8 pmol of each primer. Amplified fragments were verified by agarose gel (2%) electrophoresis containing ethidium bromide. The verified amplicons by UV transilluminator were purified from the gel by GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., USA) and directly sequenced in both directions using the Big Dye Termination kit (Applied Biosystems, Foster City, CA) and analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions.

In Silico Analysis

The sequencing chromatograms were checked by manual inspection of characteristic double peaks and analyzed by DNA Baser Sequence Assembler v4.7.0 (2013), Heracle BioSoft (15). Prediction of the disease causing mutations and putative effect of the mutations were identified using the MutationTaster tool (16) and Variant Effect Predictor (VEP), Ensemble genome browser v83.38, assembly GRCh38.p5 (Genome Reference Consortium Human Build 38) (17). Effect of variants on mRNA folding was performed by GeneQuest program (Lasergene 6.0) (18). miRanda-mirSVR v3.3a (19) was used in this study as online tool that combines two approaches (miRanda is used to identify candidate target sites and mirSVR is used to score them) to predict miRNA targets on 3'-UTR of GATA4 gene.

Results

Clinical evaluation

Twenty cases with congenital cardiac septal defects were diagnosed as isolated (isolated cardiac septal defects) and non isolated (not diagnosed as known syndromes). Their diagnosis performed by cardiac examination, Echo heart, Plain chest X-rays and electrocardiogram (ECG). Karyotyping and FISH were performed to exclude known dysmorphic features and chromosomal aberations cases. The clinical data are listed in table (1).

Table 1. Phenotype and genotype characteristics for studied cases.

Case No	Phenotype	Genotype
1	Isolated VSD	Normal
2	Isolated TOF	Normal
3	Isolated VSD	rs804280/ CA** rs867858*** rs1062219***
4	Isolated AVSD	rs804280/ AA**
5	ASD, dysmorphic features	Normal
6	DORV, VSD, dysmorphic features	Normal
7	ASD, hypoplastic aortic arch, dysmorphic features	S377G* rs804280/ CA**
8	TOF; VSD: 0.7cm, ASD: 0.3 cm, PS, overridding aorta, RV hypertrophy, polydactly, clenched hands	rs804280/ AA** rs867858***
9	VSD, overriding aorta, dysmorphic features, low birth weight, microcephaly	Normal
10	AVSD, dysmorphic features	Normal
11	ASD, PS, dysmorphic features	rs804280/ CA**
12	Hypoplastic LV, overriding aorta, VSD: 0.7, undescended testis dysmorphic	rs804280/ CA**
13	Complete A-V canal e' RVOTO, mental subnormality, dysmorphic features.	rs804280/ AA** g.81534*** leu403=***
14	ASD, VSD, PS, Digitalized thumb.	Normal
15	VSD 0.4 / O.6, PG: 40 mmHg LV dilatation, dysmorphic features, Delayed milestones	Normal
16	ASD, Reduction defects.	rs804280/ AA**
17	TOF, dysmorphic features	rs804280/ AA **
18	ASD, single ventricle, aortic coarc., PHT	Normal
19	TOF, MCA	rs804280/ AA**
20	VSD, PS, fingers defects	rs804280/ AA**

*** Predicted as pathogenic; ** Predicted as polymorphism; *** Predicted as risk factor**

MCA; Multiple **C**ongenital **A**nomalies**, TOF; T**etralogy **o**f **F**allot**, PHT; P**ulmonary **H**ypertension**, PS; P**ulmonary **S**tenosis**, VSD; V**entricle **S**eptal **D**efect, **ASD; A**trial **S**eptal **D**efect, **DORV; D**ouble **O**utlet **R**ight **V**entricle, **RVOTO; R**ight **V**entricular **O**utflow **T**ract **O**bstruction.

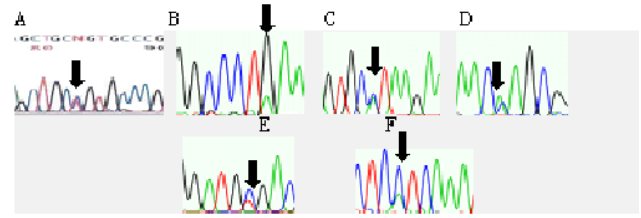

Figure 1. Partial sequence chromatogram displaying the detected variants in GATA4 gene for affected cases. The arrowed nucleotide indicates the position of S377G (A), p.L403L (B), rs804280 (C), rs867858 (D), rs1062219 (E) and g.81534C>A(F).

Mutation analysis

GATA4 is located on chromosome 8p23.1-p22, consists of seven exons and codes for a 442 amino-acid protein. We investigated the genomic DNA for sequence variations in the entire coding region from exon 3 to 7 and the first part of 3 prime untranslated regions (3'-UTR) of this gene for 20 patients with cardiac septal defects and 10 unaffected individual. We were detected six variants that are one missense mutation (S377G) reported previously in dbSNP database under *rs3729856*, one reported intronic variant in dbSNP database under *rs804280,* two reported variants located on 3'-UTR that are g.59622A>C (*rs867858*), and g.59694C>T (*rs1062219*), and two novel variants; one novel silent mutation (L403L) and another novel 3'-UTR variant (g.81534C>A) as shown in table (1) and figure (1). The ten control cases were negative for the detected variants except for rs804280 which was present in 5 out of the 10 control group. We detected two novel variants which are not reported in publicly available SNP database of National Center for Biotechnology Information, Ensembl, Human Gene Mutation Database (public HGMD), 1000 Genomes Project (TGB), ClinVar, Exome Aggregation Consortium (ExAC) browser, and NHLBI Exome Sequencing Project (ESP) server databases.

Molecular results interpretation

We applied three algorithms to predict the putative functional effect of S377G variant. In the first algorithm, we used Grantham Matrix to measure difference score in physico-chemical characteristics between serine and glycine with 56, so it is generally considered "conservative". In the second algorithm, we used phastCons to measure the evolutionary conservation of serine residue to score 0.995, so the serine residue considered highly conserved. In the last algorithm was used MutationTaster to predict any change in splicing sites to score a degree of 0.98 as donor increasing in the mutant type compared to 0.26 in the wild one, so S377G might lead to produce non-functional transcript, which would then lead to loss of phosphorylation legand in downstream aa 406.

3'-UTR g.81534C>A variant, it had conservation phastCons score 0.863 and might lead to change splice site to produce non-functional transcript that is based on donor increasing score 0.96 for the mutant type compared to 0.76 for the wild one.

Using the program GeneQuest (Lasergene 6.0), we determined the effect of the five sequence alterations (all the detected variants except rs804280)on RNA folding. GeneQuest uses the Vienna RNA folding procedure, taken from Zuker's optimal RNA folding algorithm, to predict variant dependent mRNA folding. when compared to the reference sequence, we reported 4 out of the 5 of these would result in faulty RNA folding, but 3'-UTR g.81534C>A would not likely lead to faulty RNA folding. The our results indicated the existence of differences in mRNA secondary structure between the reference (wild type) and the p.Leu403, p.Ser377Gly, also differences in structure and stability (minimum free energy) for rs867858, and compound heterozygote rs867858+ rs1062219 which suggests that change in mRNA stability and folding might contribute to the functional impact as shown in (Figs. 2 & 3).

Using miRanda-mirSVR tool to verify whether the detected 3'-UTR variants including in one of miRNA targets or not, we found that none of the conserved miRNA reported targets so far have been.

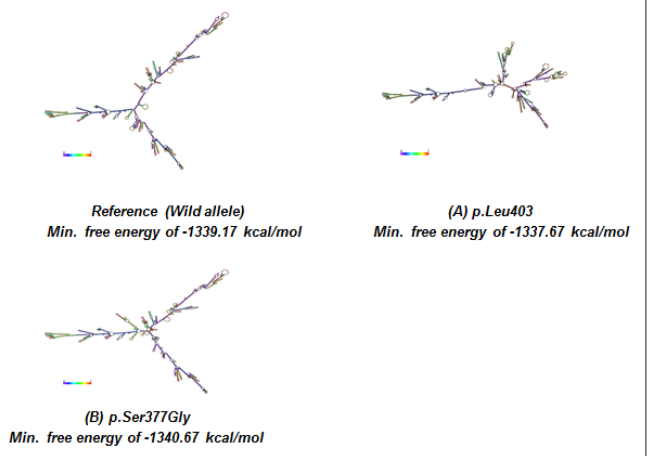

Figure 2. Prediction of mRNA secondary structure, as determined by the Vienna RNA folding procedure, showing differences in folding structure between reference (wild type) and p.Leu403 (A) and p.Ser377Gly (B), transcript variant 1, mRNA_ NCBI Reference Sequence: NM_001308093.1 was used for the prediction.

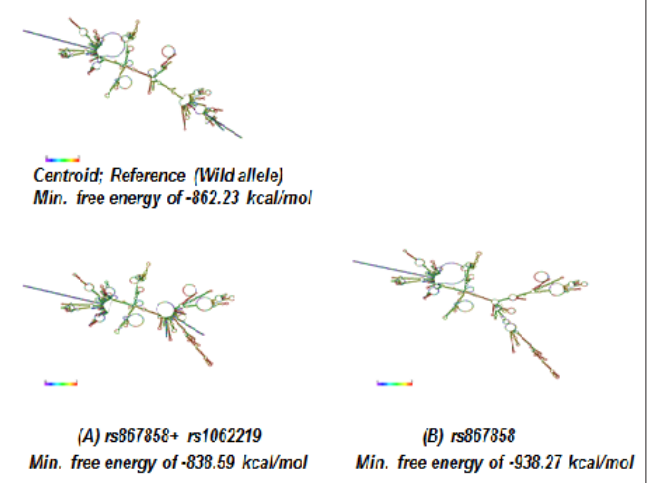

Figure 3. Prediction of GATA4 mRNA folding in centroid state: Prediction of mRNA secondary structure, as determined by the the Vienna RNA folding procedure, showing differences in folding and minimum free energy between reference (wild type) and combined rs867858 + rs1062219 (A), and rs 867858 only (B), transcript variant 1, mRNA_ NCBI Reference Sequence: NM_001308093.1 was used for the prediction.

Discussion

There are key genes known to be essential for cardiac development. For example, GATA4, NKX2-5, and TBX5 may function in a complex manner to regulate a subset of genes required for cardiac septal formation (20).

In our study, we used Sanger sequencing technique to detect possible genetic variants in GATA4 gene. We detected S377G (rs3729856), in one patient with TOF and absent in all 10 unaffected individuals, adjacent to the nuclear localization signal (NLS). GATA4 is a nucleic transcription factor that has been reported to localize entirely in the nucleus (Garg et al, 2003), Wang et al, (2011) revealed that S377G mutation led to effect on transcription factor GATA-4 protein trafficking by impeding normal nuclear localization and partial protein distribution in the cytoplasm beside the nucleus. That then prevent GATA4 from properly functioning. Alrasheed et al (2010) pointed to a significant causative association for the rs3729856 (p.S377G) with CHD and hypertensive patients. Our results could elucidate the causative association between S377G and CHD that reported previously by Alrasheed et al, 2010 and Wang et al, 2011, where our identification of putative S377G function based on phastCons and MutationTaster algorithms was pointed to high conservation score for serine residue and prediction to add extra the splice donor site, which would then lead to producing non-functional transcript with loss of phosphoserine legand in downstream aa 406, consequently could lead to partial loss of protein activation

We also detected two 3'-UTR reported variants (rs867858 and rs1062219) which predicted to change splice sites either by new donor gain or donor increased, and intronic rs804280 variant which predicted to be polymorphism.

3'-UTR of mRNA contains regulatory elements that are essential for the appropriate expression of many genes. These regulatory elements are involved in the control of nuclear transport, polyadenylation status, subcellular targetting as well as rates of translation and degradation of mRNA. Indeed, 3'-UTR mutations have been associated with disease, but frequently this region is not analyzed (12). The 3'-UTR of GATA4 is relatively long and likely contains regulatory elements essential for the regulation and transport of the mRNA transcript (24). Here we found two reported variants (rs867858 and rs1062219) and novel one (g.81534C>A) in 3'-UTR of GATA4 gene. Mattapally, et al (2015) found that rs867858 G / T genotype can significantly increase the risk for the VSD [odds ratio (OR) = 1.879,95% Confidence interval (95% CI) :1.173-3 .010, p = 0.008] and rs804280 is strongly associated with VSD, these reported results agree with our results, where we found rs867858 (G/T) in two patients with TOF and AVSD. Concerning of rs804280, we found rs804280 in 11 patients (11 out of 20 [55%]) with VSD & TOF, but it has also found in 5 out of the 10 healthy individuals [50%]. Through determining the expected mRNA folding by Vienna RNA folding procedure, we found that rs867858 and rs1062219 would affect RNA folding either in separate or co-existence state, and this change might have been function defect impact, and this agree with what concluded by Reamon-Buettner et al (2007) that rs1062219 led to faulty RNA folding. Beside that our results might be interpret what concluded by Mattapally, et al (2015) that rs867858 (G / T) can significantly increase the risk for the VSD, while this risk might be due to that rs867858 led to formation faulty mRNA folding as we resulted. Although that 3'-UTR of GATA4 gene having about nine miRNA targets, based on miRanda-mirSVR prediction results, but the detected 3'-UTR variants in our study are not included in these targets as we resulted.

Although we identified two coding variants and four non-coding ones of GATA4 gene, but furtherconfirmation study for familial segregation detection was recommended. Our putative functional results may provide a new insight to further promising functional studies aiming additional molecular rationale for CHD especially cardiac septal defects.

Acknowledgments

The authors are very grateful to patients and their family for their participation and cooperation during this study.

References

1. Bernier, P.L., Stefanescu, A., Samoukovic, G. and Tchervenkov, C.I., The challenge of congenital heart disease worldwide: epidemiologic and demographic facts. Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu. 2010, 13: 26-34.

2. Dolk, H., Loane, M., and Garne, E., European Surveillance of Congenital Anomalies (EUROCAT) Working Group. Congenital heart defects in Europe: prevalence and perinatal mortality, 2000 to 2005. Circulation. 2011, 123: 841-849.

3. Bassili, A., Mokhtar, S., and Dabous, N., Risk factors for congenital heart diseases in Alexandria, Egypt. Eur J Epidemiol. 2000, 16: 805–814.

4. van der Linde, D., Konings, E., Slager, M., Witsenburg, M., Helbing, W., Takkenberg, J., and Roos-Hesselink, J., Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. J Am Coll Cardiol. 2011, 58: 2241-2247.

5. McDermott, D.A., Basson, C.T., and Hatcher, C.J., Genetics of cardiac septation defects and their pre-implantation diagnosis. Methods Mol Med. 2006, 126:19-42.

6. Zhang, W., Li, X., Shen, A., Jiao, W., Guan, X., and Li, Z., GATA4 mutations in 486 Chinese patients with congenital heart disease. Europ. J. Med. Genet. 2008, 51: 527-535.

7. Peng, T., Wang, L., Zhou, S.-F., and Li, X., Mutations of the GATA4 and NKX2.5 genes in Chinese pediatric patients with non-familial congenital heart disease. Genetica. 2010, 138: 1231-1240.

8. Hussein, I. R., El-Ruby, M. O., Fahmi, A. A., El-Desouky, M.A., and Fayez, A.G., Detection of mutations in GATA4 and Nkx2.5 genes in patients with Fallot's tetralogy. Mid East J of Med Genet. 2012, 1:49–52.

9. Yang, Y.Q., Li, L., Wang, J., Liu, X.-Y., Chen, X.-Z., Zhang, W., Wang, X.-Z., Jiang, J.-Q., Liu, X., and Fang, W.-Y., A novel GATA4 loss-of-function mutation associated with congenital ventricular septal defect. Pediat. Cardiol. 2012, 33: 539-546.

10. Mattapally, S., Nizamuddin, S., Murthy, K., Thangaraj, K., and Banerjee, S., c.620C>T mutation in GATA4 is associated with congenital heart disease in South India. BMC Med Genet. 2015, 16:7-10.

11. Ding, J.D., Tong, T., Li, K., Fang, X., and Li, H., The novel mutations of GATA4 gene in Chinese patients with sporadic congenital heart defects. J Integr Cardiol. 2015, 2: 134-137.

12. Reamon-Buettner, s., Cho, S., and Borlak, J., Mutations in the 3'-untranslated region of GATA4 as molecular hotspots for congenital heart disease (CHD). BMC Medical Genetics. 2007, 10: 1186-1471.
13. http://snp-nexus.org/.
14. Okubo, A., Miyoshi, O., Baba, K., Takagi, M., Tsukamoto, K., and Kinoshita, A., A novel GATA4 mutation completely segregated with atrial septal defect in a large Japanese family. J Med Genet. 2004, 41:e97.
15. DNA Baser Sequence Assembler v4.7.0. (2013), Heracle BioSoft, http://www.DnaBaser.com
16. Schwarz, J.M., Cooper, D.N., Schuelke, M., and Seelow, D., MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods. 2014; 11:361-362. http://www.mutationtaster.org
17. Fiona, Cunningham, M. Ridwan Amode, Daniel Barrell, Kathryn Beal, Konstantinos Billis, Simon Brent, Denise Carvalho-Silva, Peter Clapham, Guy Coates, Stephen Fitzgerald, Laurent Gil, Carlos Garcín Girón, Leo Gordon, Thibaut Hourlier, Sarah E. Hunt, Sophie H. Janacek, Nathan Johnson, Thomas Juettemann, Andreas K. Kähäri, Stephen Keenan, Fergal J. Martin, Thomas Maurel, William McLaren, Daniel N. Murphy, Rishi Nag, Bert Overduin, Anne Parker, Mateus Patricio, Emily Perry, Miguel Pignatelli, Harpreet Singh Riat, Daniel Sheppard, Kieron Taylor, Anja Thormann, Alessandro Vullo, Steven P. Wilder, Amonida Zadissa, Bronwen L. Aken, Ewan Birney, Jennifer Harrow, Rhoda Kinsella, Matthieu Muffato, Magali Ruffier, Stephen M.J. Searle, Giulietta Spudich, Stephen J. Trevanion, Andy Yates, Daniel R. Zerbino and Paul Flicek. Ensembl 2015. Nucl. Acids Res. (28 January 2015) 43 (D1): D662-D669.
18. Zuker's RNA folding algorithm http://www.bioinfo.rpi.edu/~zukerm/seqanal/
19. Betel, D., Koppal, A., Agius, P., Sander, C., and Leslie, C., Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol. 2010, 11:R90 10.1186/gb-2010-11-8-r90.
20. Ware, S.M., and Jefferies, J.L., New Genetic Insights into Congenital Heart Disease. J Clin Exp Cardiolog. 2012, S8. pii: 003. doi:10.4172/2155-9880.S8-003
21. Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C., and Srivastava, D., GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature. 2003, 424: 443–447.
22. Wang, J., Fang, M., Liu, X.Y., Xin, Y.F., Liu, Z.M., Chen, X.Z., Wang, X.Z., Fang, W.Y., Liu, X., and Yang, Y.Q., A novel GATA4 mutation responsible for congenital ventricular septal defects. Int. J. Molec. Med. 2011, 28: 557-564.
23. Alrasheed, M., Muiya, P., Gueco, D., Andres· E., Alshahid, M. and Dzimiri, N., The Ser377Gly of the GATA4 gene is a susceptibility variant for hypertension. FASEB, 2010.
24. Hesketh, J., 3'-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. Biochem Soc Trans. 2004, 32: 990-993.