**HIRA Gene is Lower Expressed in the Myocardium of Patients with Tetralogy of Fallot**

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**Abstract**

**Background:** The most typical cardiac abnormality is conotruncal defects (CTDs) in patients with 22q11 deletion syndrome (22q11DS). *HIRA* (histone cell cycle regulator) gene, as one of the candidate genes located at the critical region of 22q11DS, was reported as possibly relevant to CTD in animal models. This study aimed to analyze the level of expression of the *HIRA* gene in tetralogy of Fallot (TOF) patients and the potential DNA sequence variations in the promoter region.

**Methods:** The messenger RNA (mRNA) expression was examined with quantitative real-time polymerase chain reaction in 39 myocardial tissues of the right ventricular outflow tract (RVOT) from TOF patients and 4 myocardial tissues of RVOT from noncardiac death children. The protein expression was detected using immunohistochemistry in 12 TOF patients and 4 controls. A total of 100 TOF cases and 200 healthy controls were recruited for DNA sequencing.

**Results:** The mRNA and protein expressions of the *HIRA* gene in the myocardium of the TOF patients were both significantly lower as compared to the controls (*P* < 0.05). Five single nucleotide polymorphisms (SNPs), including g.4111A>G (rs1128399), g.4265C>A (rs4585115), g.4369T>G (rs2277837), g.4371C>A (rs148516780), and g.4543T>C (rs111802956), were found in the promoter region of the *HIRA* gene. There were no significant differences of frequencies in these SNPs between the TOF patients and the controls (*P* > 0.05).

**Conclusion:** The abnormal lower expression of the *HIRA* gene in the myocardium may participate in the pathogenesis of TOF.

**Key words:** Congenital Heart Defects; Gene Expression; *HIRA*; Single Nucleotide Polymorphism; Tetralogy of Fallot

**Introduction**

Congenital heart defects (CHDs) are one of the most common congenital malformations and account for nearly one-third of all major birth defects, affecting 9.1 per 1000 live births worldwide.[¹] Conotruncal defect (CTD) is a spectrum of cyanotic CHD, which commonly causes hypoxemia and irreversible acidosis during the neonatal period, thus leading to early death. Tetralogy of Fallot (TOF), with a prevalence of 0.34 per 1000 live births,[¹] is the most common type of CTD and it is characterized by obstruction of the right ventricular outflow tract (RVOT), ventricular septal defect, overriding aortic root, and right ventricular hypertrophy.[²]

The 22q11 deletion syndrome (22q11DS) is a general term of some syndromes including velocardiofacial syndrome, DiGeorge syndrome, and conotruncal anomaly face syndrome.[³] Reports have shown that the most typical cardiac abnormality is CTD in patients with 22q11DS,[⁴,⁵] whereas some patients with CTD have a 22q11 deletion. The prevalence of 22q11 deletion is estimated at 0.014%–0.017% in general population, but 6.13%–14.8% in CTD patients. Thus, 22q11DS is considered to have a close relationship with the pathogenesis of CTD.

The *HIRA* (histone cell cycle regulator) gene, located at the critical region associated with 22q11DS, was present in the neural crest and the neural crest-derived tissues during embryonic development in animal models.[¹²,¹³] Weakening

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Received: 06-06-2016 Edited by: Li-Min Chen
How to cite this article: Ju ZR, Wang HJ, Ma XJ, Ma D, Huang GY. *HIRA* Gene is Lower Expressed in the Myocardium of Patients with Tetralogy of Fallot. Chin Med J 2016;129:2403-8.
the function of cHIRA in the chick cardiac neural crest leads to a high incidence of persistent truncus arteriosus (PTA), which is one kind of CTD. Therefore, we speculate that the HIRA gene may be an important candidate gene that is relevant to the cause of CTD.

In our previous studies, we sequenced five exons of HIRA gene from nonsyndromic CTD patients and found that one single nucleotide polymorphism (SNP) was associated with the susceptibility of TOF and PTA (data not published). One report discovered that the SNP (rs: 117447448) of 3’UTR region of the HIRA gene was related with TOF. However, the studies about the HIRA gene in patients with CTD were very seldom. To illustrate the association of the HIRA gene and CTD in human beings, our current study aimed to analyze the expression level of the HIRA gene in patients with TOF and the potential DNA sequence variations in the HIRA promoter region.

**Methods**

**Patients and controls**

Myocardial tissue samples were collected from 39 TOF patients undergoing cardiac surgery at the Children’s Hospital of Fudan University, Shanghai, China. Four normal myocardial tissues were obtained from noncardiac death children, who were provided by the Forensic Medicine Department of Fudan University, Shanghai, China. Blood samples were obtained from 100 TOF patients and 200 healthy children, who were enrolled at the same hospital.

These TOF patients were diagnosed by echocardiogram and then confirmed by surgery. Patients with any other abnormalities or known syndromes including Holt–Oram, Marfan, Noonan, Alagille, DiGeorge, and Char syndromes were excluded from the study. Family histories of CHD were not present in any of these cases. The healthy children did not have any sign of genetic diseases or birth defects.

**Ethical statement**

The study was approved by the Institutional Research Ethics Committee of Children’s Hospital of Fudan University. Informed consent was obtained from parents or guardians prior to recruitment.

**Quantitative real-time polymerase chain reaction**

The total RNA was extracted from the myocardium tissues of the RVOT using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s guidelines. A total of 500 ng RNA was reversely converted to complementary DNA (cDNA) in a 10 µl reaction mixture using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). The primers of the HIRA gene and the reference gene of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for quantitative real-time polymerase chain reaction (QRT-PCR) were designed by online Primer3 (version 0.4.0) [Table 1] and their specificity was tested by Basic Local Alignment Search Tool. Each pair of primers spanned one intron. The QRT-PCR was performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq (TaKaRa) with a reaction volume of 10 µl, including 5 µl of ×2 SYBR Premix Ex Taq, 0.25 µl of each primer, 0.25 µl of ×50 ROX Reference Dye, 1 µl cDNA, and 3.25 µl of double-distilled water. The amplification parameters were 30 s at 95°C, followed by 5 s at 95°C, and 34 s at 60°C for 40 cycles. Amplification specificity was monitored using melting curve analysis. The relative messenger RNA (mRNA) expression levels were transformed by the 2[−∆∆CT] method with GAPDH gene as control.

**Immunohistochemistry**

The collected myocardial tissues of RVOT were fixed in 10% neutral-buffered formalin followed by paraffin embedding. Tissues embedded in paraffin were cut into 4 µm slides and placed in 56°C oven and dried overnight. After deparaffination, hydration, and antigen retrieval with citric acid (0.01 mol/L, pH 6.0), the slides were blocked in 3% peroxide by ablating endogenous peroxidase. The sections were incubated with the rabbit antihuman polyclonal antibody to HIRA (dilution 1:400, Abcam, Cambridge, UK) for 1.5 h at 37°C and then 4°C overnight. Then, incubation with secondary antibody (Invitrogen, Carlsbad, CA, USA) was performed for 30 min at 37°C. Finally, the sections were stained with 3, 3’-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, and mounted for microscopic examination.

The staining was evaluated using the immunoreactive score (IRS) system. Staining intensity was classified as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong. The percentage of positivity was scored by providing values of 0 (focal or <10%), 1 (10%–30%), 2 (30%–50%), and 3 (>50%). The IRS was calculated by adding staining intensity and the percentage of positivity, which could range from 0 to 6.

**Sequencing analysis**

Genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). We selected 1000-bp DNA sequences of the upstream region from the transcription start site of HIRA gene (the standard sequence from NCBI GeneBank, NG_009231). The primers were designed by online Primer3 and their specificity was tested by BLAST [Table 2]. PCR was accomplished in a 10 µl reaction mixture, which contained

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**Table 1: Sequencing primers of QRT-PCR**

| Gene     | Primers (5’-3’)         | Product size (bp) |
|----------|-------------------------|-------------------|
| HIRA-F   | ACCGAGGCGCATCTGCTGTC    | 138               |
| HIRA-R   | CCCACACTGCTACCTCAT      |                   |
| GAPDH-F  | CACCCACCTTCACCCCTTTG    | 108               |
| GAPDH-R  | ACCACCGTGCTGCTGACCC    |                   |

QRT-PCR: Quantitative real-time polymerase chain reaction; F: Forward; R: Reverse; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.
1 μl of genomic DNA (10 ng/μl), 0.8 μl mixture of forward and reverse primers, 1.6 μl of dNTP (2.5 mmol/L each), 5 μl of ×2 GC Buffer I/II (Mg²⁺ plus), 0.1 μl of Hot Start DNA Taq polymerase (TaKaRa), and 1.5 μl of double-distilled water. The PCR mixture was preheated for 5 min at 95°C and then incubated for 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, followed by 72°C for 5 min. The PCR products were purified and sequenced by a commercial sequencing company (Jie Li Biology, Shanghai, China). We analyzed the sequencing outcome, comparing to the standard sequence from NCBI, with Mutation Surveyor Demo V3.25 software (version 3.25; SoftGenetics, LLC., PA, USA).

**Statistical analysis**

All QRT-PCR and IRS results were presented as median (interquartile range). Data were analyzed by GraphPad Prism (version 5.0; GraphPad Software Inc., San Diego, CA, USA) and SPSS software (version 17.0; SPSS Inc., Chicago, IL., USA). Mann–Whitney U-test was performed to compare the expression levels between the TOF patients and the controls. The allelic and genotypic frequencies were analyzed by Chi-square test. P < 0.05 was considered to be statistically significant.

**Results**

**Messenger RNA expression of the HIRA gene**

We used QRT-PCR to examine the HIRA mRNA expression in 39 TOF patients and four control children. The mRNA expression of HIRA in the RVOT myocardium was significantly lower in the TOF patients compared with the controls [0.29 (0.17–0.37) vs. 0.70 (0.48–0.89); U = 15.00, P = 0.009, as shown in Figure 1].

**Protein expression of the HIRA gene**

The HIRA gene was lower expressed in the transcriptional level in the TOF patients. To know its expression in the translational level, we further detected the HIRA protein expression in the myocardial tissues of RVOT from 12 TOF patients and four controls using immunohistochemistry. The HIRA protein was distributed in both nucleus and cytoplasm [Figure 2]. The cardiomyocytes from the TOF patients [Figure 2a] were hypertrophic, disorganized, and unevenly dyed. By contrast, the staining intensity of cardiomyocytes from the TOF cases [Figure 2a] was clearly weaker than that in the controls [Figure 2b]. By the statistical analysis, it was observed that HIRA protein expression was lower in the TOF patients as compared to the controls [2.25 (2.00–3.00) vs. 4.25 (3.88–4.50); U = 0.00, P = 0.0035, Figure 3].

**Discussion**

The most typical cardiac malformation is CTD in individuals with 22q11DS. The HIRA gene, one of the candidate genes located at the critical region of 22q11DS, was contributing to the phenotypes of these syndromes. Farrell et al.[14] have found that weakening the function of cHIRA in the chick cardiac neural crest will lead to a high incidence of PTA, which is one type of CTD. There were similar findings in mammal studies. The murine embryos carrying targeted mutant HIRA gene showed the failure of embryonic turning and heart looping.[15] One recent research has found that HIRA deficiency in murine cardiomyocyte will result in...
cardiomyocyte hypertrophy and alteration of cardiac gene expression. Based on these findings, it can be concluded that the regression expression of the HIRA gene can cause abnormal embryonic cardiac development. In our study, the lower expression of the HIRA gene in the myocardial tissues of TOF patients is coincided with the above findings in animal models. Thereby, we could deduce that the HIRA gene is essential for the early embryonic heart development. Furthermore, its abnormal expression may be relative to the cause of TOF.

HIRA was first recognized in yeast as a negative regulator for the expression of histone gene. It was renamed as HIRA because the most significant peptides were similar to Hir1p and Hir2p which were two histone gene repressor proteins from the yeast Saccharomyces cerevisiae. The two co-repressors were presumed to be functional on chromatin structure to control the transcription of histone gene. Researchers have reported that HIRA involves in nucleosome assembly independent of DNA synthesis and plays an important role in maintaining nucleosome structure. Moreover, HIRA may interact with other histone chaperones, such as ASF-1 and CAF-1, to regulate histone gene transcription and to promote heterochromatic gene silencing. Hence, considering all these above mechanisms, the abnormal expression of the HIRA gene may compromise the early development of embryo through these ways, especially the growth of embryonic heart.

Besides, HIRA was found expressed in the neural crest and the neural crest-derived tissues during embryonic development. It is well known that neural crest cells (NCCs) are a major element in cardiovascular development. They participate in the formation of aorticopulmonary septum, the tunica media of the great arteries, the outflow tract septum, and the semilunar valves throughout the embryonic development. It is not difficult to deduct that HIRA has a great meaning for the development of embryonic heart. Thus, we suspect that the lower expression of HIRA may interfere with the normal functioning of NCCs. Consequently, it will impact the normal cardiac development.

Eukaryotic gene expression and regulation are very intricate processes. The promoter, which is combined with the RNA polymerase and the transcription factors, regulates the start time of transcription and the gene expressive degree. Any change of the promoter may decrease its affinity with polymerase and transcription factors. Thus, the transcription or even the whole gene expression processes will be influenced.

Therefore, we analyzed 1000 bp DNA sequences of the promoter region of the HIRA gene and found five SNPs that have been reported. By the prediction of TFSEARCH website, there are three transcription factors, namely, GATA-1, GATA-2, and GATA-3, binding to the SNP of g.4543T>C. Any change in DNA sequence of this site will affect its binding with the transcript factors and influence the expression of HIRA gene. However, by the statistical analysis, there are no significant differences in allelic and genotypic frequencies of these SNPs between the TOF patients and the controls. In the future research, we can amplify the volume of sample size to know whether these five SNPs are responsible for TOF. In addition, analysis of these transcript factors that bind to the HIRA gene may help us understand more about the abnormal expression of this gene.

Besides, epigenetics can also modify the activation or the function of certain gene without changing the gene sequence. DNA methylation is one of the specific epigenetic processes and it is associated with the suppression of gene expression. In the future, we can check the DNA methylation status of the promoter region of the HIRA gene in TOF patients. It may help us know whether the lower
expression of *HIRA* is caused by the DNA methylation. In addition to epigenetics, microRNAs (miRNAs) can also inhibit gene expression at the post-transcriptional level. Studies have found that miRNAs play an important role in heart development and function.[33] It provides a new direction for our future research.

In summary, our current study has shown that the *HIRA* gene is lower expressed at both transcriptional and translational levels in TOF patients. Its abnormal expression may participate in the pathogenesis of TOF. However, there is no noticeable difference in the promotor region of the *HIRA* gene between the TOF patients and the controls. Given the complicated mechanism of the pathogenesis of CHDs, efforts should be made in the points of genetics, epigenetics, and environmental factors as well as the interaction between them to identify the pathogenic mechanism of TOF.

**Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.**

**Financial support and sponsorship**

This work was supported by grants from the Natural Science Foundation of China (No. 81370198, No. 81570283) and National Key Research and Development Program (No. 2016YFC1000500).

**Conflicts of interest**

There are no conflicts of interest.

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**Supplementary Table 1: Allelic frequencies of five SNPs**

| SNPs        | Alleles | TOF (%) | Control (%) | $\chi^2$ | P    |
|-------------|---------|---------|-------------|----------|------|
| g. 4111A>G  | A       | 96      | 94.75       | 0.116    | 0.733|
|             | G       | 4       | 5.25        |          |      |
| g. 4265C>A  | C       | 97.5    | 97          | 0        | 0.990|
|             | A       | 2.5     | 3           |          |      |
| g. 4369T>G  | T       | 45      | 47.5        | 0.129    | 0.720|
|             | G       | 55      | 52.5        |          |      |
| g. 4371C>A  | C       | 95.5    | 94.25       | 0.107    | 0.744|
|             | A       | 4.5     | 5.75        |          |      |
| g. 4543T>C  | T       | 96      | 94.75       | 0.116    | 0.733|
|             | C       | 4       | 5.25        |          |      |

SNPs: single nucleotide polymorphisms; TOF: Tetralogy of Fallot.

**Supplementary Table 2: Genotypic frequencies of five SNPs**

| SNPs        | Genotypes | TOF (%) | Control (%) | $\chi^2$ | P    |
|-------------|-----------|---------|-------------|----------|------|
| g. 4111A>G  | AA        | 92      | 90          | 1.239    | 0.538|
|             | AG        | 8       | 9.5         |          |      |
|             | GG        | 0       | 0.5         |          |      |
| g. 4265C>A  | CC        | 96      | 94          | 2.021    | 0.364|
|             | CA        | 3       | 6           |          |      |
|             | AA        | 1       | 0           |          |      |
| g. 4369T>G  | TT        | 25      | 24.5        | 0.798    | 0.671|
|             | TG        | 40      | 46          |          |      |
|             | GG        | 35      | 29.5        |          |      |
| g. 4371C>A  | CC        | 91      | 88.5        | 0.446    | 0.504|
|             | CA        | 9       | 11.5        |          |      |
|             | AA        | 0       | 0           |          |      |
| g. 4543T>C  | TT        | 92      | 90          | 1.239    | 0.538|
|             | TC        | 8       | 9.5         |          |      |
|             | CC        | 0       | 0.5         |          |      |

SNPs: single nucleotide polymorphisms; TOF: Tetralogy of Fallot.