GATA4 molecular screening and assessment of environmental risk factors in a Moroccan cohort with tetralogy of Fallot

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Abstract

Background: Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart defect (CHD) with an incidence of 1/3600 live births. This disorder was associated with mutations in the transcription factors involved in cardiogenesis, like Nk2 homeobox5 (NKX2-5), GATA binding protein4 (GATA4) and T-BOX1 (TBX1). GATA4 contributes particularly to heart looping and differentiation of the second heart field.

Objectives: The aim of this study was to screen a Moroccan cohort with tetralogy of Fallot for GATA4 mutations, and to assess environmental risk factors that could be involved in the occurrence of this disorder.

Methods: Thirty-one non-syndromic TOF patients, enrolled between 5th April 2014 and 18th June 2015, were screened for GATA4 mutations using direct sequencing of GATA4 coding exons. Statistical assessment of different risk factors, which is a retrospective study, was carried out using Chi-square and Fisher's exact tests.

Results: We identified seven exonic variants in nine patients (two missense and five synonymous variants); in addition of eight intronic variants. Assessment of environmental risk factors shows significant association of maternal passive smoking with TOF in the Moroccan population.

Conclusion: The present study allowed, for the first time, the molecular and environmental characterisation of Moroccan TOF population. Our findings emphasise particularly the strong association of passive smoking with the emergence of tetralogy of Fallot.

Keywords: Tetralogy of Fallot, GATA4, molecular screening, risk factors.

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Introduction

Tetralogy of Fallot (TOF) is the most common type of cyanotic congenital heart defect, affecting approximately 1/3,600 live births[1]. This cyanotic defect is characterised by the combination of four cardiac anomalies: ventricular septal defect; over-riding aorta; right ventricular outflow obstruction and right ventricular hypertrophy. Approximately 20-25% of TOF occurs within the context of

syndromes, particularly with 22q11 microdeletion syndrome[2].

TOF is one of the multifactorial disorders that results from interaction of environmental and genetic factors. Environmental factors include maternal toxic exposure (smoking, alcohol, organic solvents), maternal illness with use of medication, and consanguinity among others[1]. Whereas, genetic factors consists mainly of molecular alteration of genes involved in cardiogenesis[1].

Several genes were proved to be involved in non-syndromic Tetralogy of Fallot. The most evident are those encoding the cardiac transcription factors, such as GATA binding protein 4 (GATA4), which is involved in the second heart field differentiation and heart looping during the cardiogenesis process[3,4].

GATA4 (GATA-binding protein 4), member of GATA transcription factor family, is a zinc-finger protein that specifically bind to (T/A)GATA(A/G) motif in target genes. GATA4 interact with Nk2 homeobox 5 (NKX2-$_5$) and T-BOX 5 (TBX5) within a complex regulatory network to regulate the down-stream genes involved in heart development process[3].

In Lebanese and Chinese populations, GATA4 mutations are estimated to be present in 3 - 8% of patients with Tetralogy of Fallot[5,6]. Whereas, in other TOF populations, such as the American and Japanese, the GATA4 mutation rate seems to be very low, about 0.5 - 0.8%[4,7].

Therefore, the aim of this study was to identify, for the first time, the spectrum of GATA4 variants and to assess the environmental risk factors that may contribute potentially in the emergence of tetralogy of Fallot in a non-syndromic Moroccan population.

Methods

Study subjects

In this retrospective study, thirty-six patients, with ages ranging from 2 months to 15 year-old, were enrolled between 5th April 2014 and 18th June 2015 from the cardio-pediatics department. Each patient was evaluated via electrocardiography (ECG) and colour Doppler echocardiography, and confirmed to have tetralogy of Fallot. All patients were clinically evaluated for putative syndromic dysmorphology, and tested for chromosomal rearrangements (using karyotype) and 22q11 microdeletion by the Medical Genetics Department. Five subjects with confirmed or suggestive syndromic traits were excluded from the study. The included patients were interviewed to assess the personal and familial disease history.

Molecular screening

After obtaining informed consent as well as the Approval of the local ethic committee, the peripheral blood was collected from the 31 non-syndromic tetralogy of Fallot patients, then the DNA specimens were extracted from blood leukocytes. For each sample, we performed Polymerase chain reaction (PCR) to amplify GATA4 coding exons and their intron boundaries. Primers used were derived from previous study[8].

PCR mix was prepared in a 25 μL volume comprising 10× PCR buffer (Invitrogen, California, CA, USA), 25 mM $MgCl_2$, 10 mM dNTP, 1 U of Taq (Invitrogen, California, CA, USA), 10 pmol of each primer and 40 ng of genomic DNA. The PCR cycling conditions performed in the Veriti 96-well Thermal Cycler 9902 (Applied Biosystems, Massachusetts, MA, USA) were 94 °C for 5 min; 35 cycles of: 94 °C for 45 s, 59 - 62 °C (according to the specific annealing temperature relative to each pair of primers) for 40 s, and 72 °C for 45 s; and 72°C for 7 min. The purified PCR products underwent direct sequencing using the BigDye Terminator V1.1 Cycle Sequencing Kit (ABI Prism, Applied Biosystems, Massachusetts, MA, USA) and the Applied Biosystems 3500Dx Genetic Analyser, then chromatograms were analysed by Sequencing Analysis SeqA v.5.4 (Applied Biosystems, Massachusetts, MA, USA). The sequences thus obtained were analysed with the Bioinformatics analysis tool "Nucleotide Blast alignment program", (http://blast.ncbi.nlm.nih.gov) (NCBI, Maryland, MD, USA).

Statistical analysis

To assess the significance of differences between the various rates through the study, we used chi-square tests for large cohort (>5) and Fisher's exact test for smaller cohort sizes. The statistical analysis was performed using the R software package version 3.2.

Results

Environmental features

A total of 31 patients with non-syndromic tetralogy of Fallot were recruited for GATA4 mutation screening. The cohort included 14 females and 17 males. The average age was 1 year-old at diagnosis, and 6 year-old at enrolment. All enrolled subjects were Moroccan with 80% of them from the Central North region of Morocco.

Patients born to consanguineous marriages represented 20.8% of the studied population. Averages of maternal and paternal ages were 28 and 38 years respectively. More than a third of patients (38%) had at least one incident of neonatal sibling death or spontaneous abortion in maternal obstetric history. Interestingly, we noticed a high frequency (47.6%) of second-hand smoking during the concerned pregnancies.

Molecular screening

In this study, we investigated DNA samples of 31 non-syndromic TOF patients for GATA4 variants. Mutation screening revealed seven variants: two missense variants, rs114868912 and rs3729856; four synonymous variants that were previously reported, rs192122549, rs55788387, rs3729855 and rs112435835; in addition to a novel synonymous variant. We also identified eight polymorphisms spread over the intronic regions. Figure 1 and tables 1 and 2 show further details.

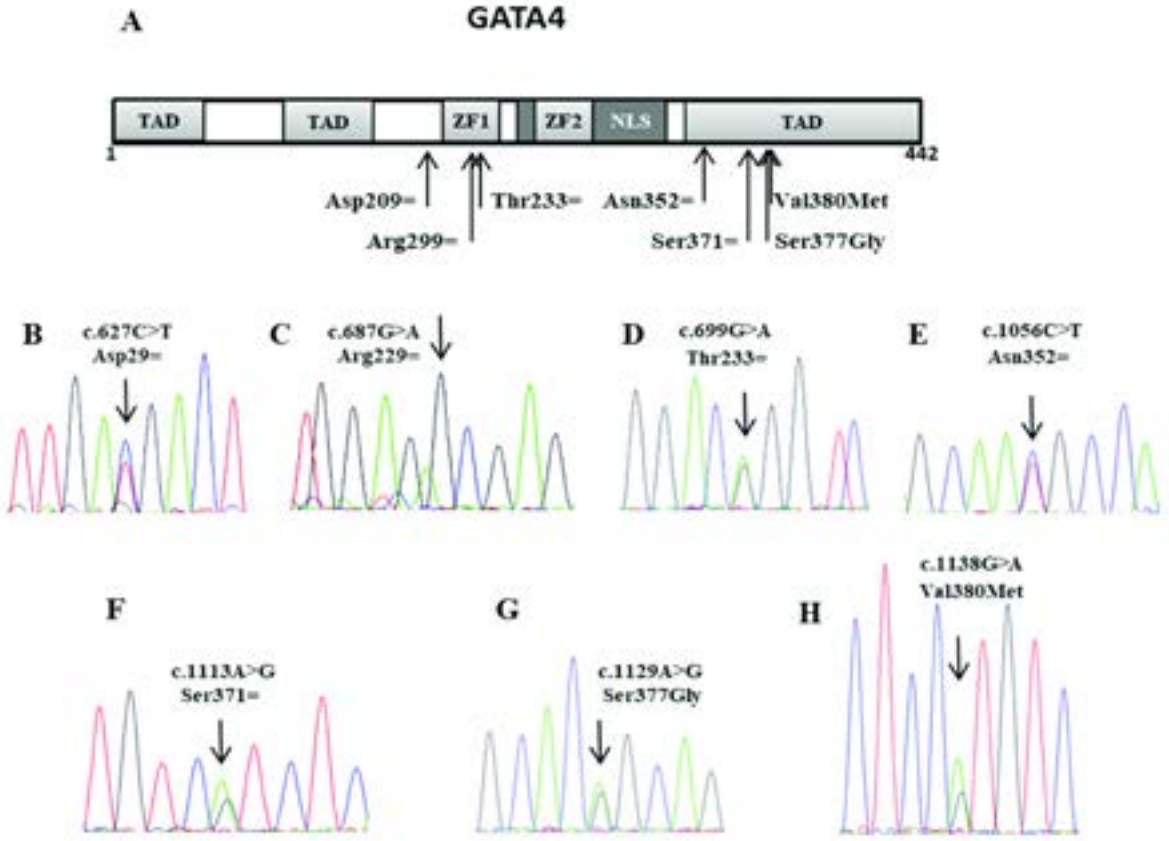

Figure 1: (a) Organisation of GATA4 domains with position of the identified exonic variants. (b-h) Sequencing chromatograms showing the exonic variants detected in *GATA4*. NLS: nuclear localization signal, TAD: Transcriptional activation domain, ZF: zinc finger.

Table 1: GATA4 exonic variants identified among 31 patients with non-syndromic TOF.

Variation ID	Chromosome localisation	Nucleotide substitution	Amino acid substitution	Exon	Mutation type	Patients
rs192122549	8:11606438	c.627C>T	Asp209Asp	2	Synonymous	P2
Novel	8:11606498	c.687G>A	Arg229Arg	2	Synonymous	P7
rs55788387	8:11606510	c.699G>A	Thr233Thr	2	Synonymous	P21
rs3729855	8:11614502	c.1056C>T	Asn352Asn	5	Synonymous	P6
rs112435835	8:11614559	c.1113A>G	Ser371Ser	5	Synonymous	P6
rs3729856	8:11614575	c.1129A>G	Ser377Gly	5	Missense	P2,P4, P8, P10, P11
rs114868912	8:11614584	c.1138G>A	Val380Met	5	Missense	P4

Table 2: GATA4 intronic variants identified among 31 patients with non-syndromic TOF.

Variation ID	Chromosome localisation	Nucleotide substitution	Exon/ intron	variant type	Patients	Frequency (%)
Novel	8:11606365	c.617-63C>A	Intron 1	Non-coding	P7	3.2
rs10503425	8:11606364	c.617-64G>C	Intron 1	Non-coding	P2, P4, P8, P10, P11	16
rs3735819	8:11606312	c.617-116T>C	Intron 1	Non-coding	P5, P7,	6.5
rs200555437	8:11606610	c.783+16G>A	Intron 2	Non-coding	P31	
rs804280	8:11612698	c.997+56C>A	Intron 4	Non-coding	P2, P4, P8, P9, P14, P15, P18, P20, P24, 26, P27, P29, P30	42
rs76808439	8:11612665	c.997+23A>T	Intron 4	Non-coding	P13, P14, P15, P20 P29,	16
rs3729854	8:11614329	c.998-115C>T	Intron 4	Non-coding	P1, P3,	6.5
rs114345849	8:11615782	c.1147-20G>A	Intron 4	Non-coding	P2	3.2

Discussion

GATA4 is a zinc-finger transcription factor that plays a major role during different stages of embryonic heart development. Association of GATA4 with congenital heart disease was emphasised, for the first time, in 1999 by Pehlivan et al. who were studying a cohort with 8p23 microdeletion, a region that carries GATA4[9]. Then, the subsequent studies confirmed the involvement of GATA4 mutations in different CHD types, with mutation rates varying across ethnic populations. Thus, the aim of the present study was to identify the spectrum and the rate of GATA4 mutations in a Moroccan population with non-syndromic TOF, and also to discuss the factors that may increase the risk of having tetralogy of Fallot.

Assessment of environmental risk factors

Sex-ratio in the studied TOF cohort was 1.2, reflecting a slightly higher proportion of males. An identical ratio has been reported by Sarikouch et al., 2011[10], Rauch et al., 2010[11] and Lindinger et al., 2010[12]. In our cohort, consanguinity rate accounts for 20.8%. This rate does not seem to be significantly different from the general consanguinity rate in Moroccan population, that is reported to be around 15.25% ($p = 0.4$)[13]. Indeed, the association between consanguinity and CHDs differs from a subset to another, and also across populations. Thus, our data is consistent with a previous study conducted in Saudi population[14], in which there was no significant relationship between consanguinity and tetralogy of Fallot sub-group, but different from Lebanese[15,16] and Indian[17] studies that reported a significant association. The non-association observed in our cohort suggests probably a different mode of inheritance or sporadic events.

According to previous studies, advanced parental age is said to have no particular association with tetralogy of Fallot alongside several CHD subsets[18,19]. We noticed in our cohort high rates of previous miscarriage in maternal obstetric histories and sibling's neonatal sudden death, respectively 28.5% and 9.5%. According to the National Survey on Population and Family Health, 2011 (ENPSF – 2011) conducted by Moroccan ministry of health in the general population, the rate of perinatal death is limited to 2.8%[20], which does not constitute a significant difference to our data ($p = 0.12$). Our conclusion confirms the studies conducted by Hassan et al.[21] and Roodpeyma et al.[22] respectively in a Pakistani and Iranian populations that attempted to assess the relationship between previous maternal abortion or perinatal sudden death among sibling and CHDs, but did not find a significant association. In contrast, other studies prove that previous abortions may be associated with the likelihood of having a child with CHD[23,24].

Considering the sociocultural and religious characteristics of the Moroccans, maternal smoking or alcohol exposure is very unusual, unlike the paternal's. Thus, to assess the relationship between this well-known cardiovascular risk factor and TOF, we rather checked the maternal passive smoking during the concerned pregnancy. Interestingly, we noticed in our cohort that 47.6% of mothers were exposed to passive smoking during the concerned pregnancy. This rate is significantly higher compared to that described in a Moroccan ministry of health report based on the Global Youth Tobacco Survey (GITS), in which the passive smoking rate in families of Moroccan general population was about 19.7% ($p = 0.003$)[25]. These findings lead us to conclude that exposure to passive smoking during pregnancy could be a potential risk factor for tetralogy of Fallot. This conclusion is consistent with the Heart Disease and Stroke Statistics -2016 Update[26].

GATA4 variants

GATA4 mutation screening of 31 non-syndromic TOF patients allowed us to identify two heterozygous missense variants. The first variant c.1138G>A (rs114868912), found in P4, leads to the Valine-to-Methionine amino acid change at the position 380. While the second missense variant c.1129A>G (rs3729856) found in five patients (16%) results in Serine-to-Glycine amino acid substitution at the position 377 (Table 1, Figure 1).

These two variants are located at the carboxy-terminal segment that plays an essential role in regulating the GATA4 transcription activity[8]. Indeed, Gallagher et al. proved that CDK4, a crucial protein required to activate theCyclinD2-GATA4 transcriptional synergy in cardiogenesis interacts directly with GATA4 via its C-terminal region (362-440 aa) , which shows the potential role of GATA4 C-terminal through the heart development process[27].

The non-synonymous variant S377G is located near to the nuclear localization signal (NLS), a domain responsible of transporting the GATA4 protein to the nucleus. In order to determine whether this mutation affects the NLS function, Wang et al. carried out immunofluorescence staining for GFP-tagged GATA4 in human cardiomyocytes cells. The result shows that a small amount of S377G GATA4 mutants, among other NLS adjacent mutants, was detected in the cytoplasmic region. Such localisation pattern was considered a partially abnormal, compared to the wildtype pEGFP-GATA4 that was exclusively localised in the nucleus. This finding suggests that S377G may partially impair the GATA4 protein trafficking[28].

However, since several studies reported that the variant Ser377Gly was frequently observed in both affected and healthy groups, often with no significant difference[6,29,30], it could be considered as possibly benign.

On the other hand, the non-synonymous variant Val-

380Met was proven to have no impact in the protein secondary structure and was detected among healthy groups in several studies. Moreover, the codon Val380 seems to be weakly conserved through species[4,31-33]. These data lead us to conclude that this variant is likely benign.
Mutation screening allowed us to identify five additional variants (c.627C>T, c.687G>A, c.699G>A, c.1056C>T, c.1113A>G), one of them constitutes a non-previously-reported variant, c.687G>A. These variants were identified in one patient each, and found to be synonymous mutations (Asp209Asp, Arg229Arg, Thr233Thr, Asn352Asn, Ser371Ser) (table 1, Figure 1).

As these silent mutations have obviously no damaging effect on protein conformation, we checked their possible earlier effect on splicing function during the post-transcriptional process[34-36]. We tested hence whether or not those variants are located in a critical exonic splicing enhancer (ESE) or silencer (ESS) sites using Human Splicing Finder tool (HSF 3.0)[37].
Interestingly we found that except the variant c.1113A>G (Ser371Ser) that has no particular impact, the remaining four variants have an interesting effect on splicing function. Indeed, the novel synonymous variant c.687G>A (Arg229Arg) leads to a potential alteration of splicing function by altering an exonic splicing enhancer (ESE) site. The synonymous variant c.1056C>T (Asn352Asn) results in a creation of anexonic splicing silencer (ESS) site, while the synonymous variants c.627C>T (Asp209Asp) and c.699G>A (Thr233Thr) lead to a creation of an ESS site, simultaneously with an alteration of an ESE site, which constitutes a potential source of splicing impairment. Disruption of splicing function with such variants can result in skipping the involved exons and thus forming alternative isoforms that may be non-functional, or at least contribute in destabilising the GATA4 isoforms balance[7].

Given that GATA4 is a highly dosage-sensitive protein, a slight change in the level of protein expression by such isoform balance disruption, may lead to potential cardiac morphogenesis impairment, involving life-threatening embryonic complications[38].
This significant impact is related to the pivotal role that GATA4 plays in proliferation of cardiomyocytes, development of right ventricle and endocardial cushion, and in septation of the outflow tract[39,40].

Tomita et al. noticed in TOF subgroup a significantly higher rate of silent variants compared to the non-synonymous variants ($p = 0.03$), and thus suggested that silent variants may be a potential genetic cause involved specifically in TOF subset[7]. In our cohort, we noticed a high number of synonymous mutations, but the difference was not significant.
In addition to these exonic variants, we identified several non-coding polymorphisms that were clustered mainly in introns 1 and 4 (Table 2). We used the Human Splicing Finder (HSF 3.0)37 to check the impact of these non-coding variants on splicing machinery, but we did not found a particular altering effect.

It is worth to mention that 71% of the studied patients carry at least one variant that is, in 90% of cases, non-coding polymorphism. This data matches the conclusion made by Tomita and al. who noticed that amongst all CHD patients, those with conotruncal carry a substantial number of non-coding variants, unlike control groups that did not harbour such amounts. This may suggest a possible CHD-predisposing effect, though the molecular mechanism has yet to be elucidated[7].
Taken together, this data shows the absence of pathogenic or likely pathogenic mutations of GATA4 gene in the studied Moroccan population. This prompts further molecular investigations in the other genetic factors of cardiogenesis pathways, particularly, those involved in the second heart field differentiation and the heart looping process (e.g. NKX2.5, TBX1 …).

Conclusion

The present study emphasises particularly the association of GATA4 silent mutations with conotruncal defects, and supports the critical role of GATA4 with its highly sensitive dosage in the developmental process of the outflow-tract. On the other hand, this study confirms the strong involvement of maternal passive smoking in increasing the risk of TOF, and underlines notably the heterogeneity of the cardiac defects aetiology. Finally, to have a full overview about the aetiology of TOF in Morocco, it would be pertinent to conduct a case-control study in a larger cohort; to screen GATA4 gene in a control group as well, in order to compare variant frequency between healthy and affected cohorts; and also to consider more additional genetic and environmental factors.

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Conflict of interest
The authors declare no conflict of interest.

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