Mutational analysis of the PITX2 coding region revealed no common cause for transposition of the great arteries (dTGA)

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Abstract

Background: PITX2 is a bicoid-related homeodomain transcription factor that plays an important role in asymmetric cardiogenesis. Loss of function experiments in mice cause severe heart malformations, including transposition of the great arteries (TGA). TGA accounts for 5–7% of all congenital heart diseases affecting 0.2 per 1000 live births, thereby representing the most frequent cyanotic heart defect diagnosed in the neonatal period.

Methods: To address whether altered PITX2 function could also contribute to the formation of dTGA in humans, we screened 96 patients with dTGA by means of dHPLC and direct sequencing for mutations within the PITX2 gene.

Results: Several SNPs could be detected, but no stop or frame shift mutation. In particular, we found seven intronic and UTR variants, two silent mutations and two polymorphisms within the coding region.

Conclusion: As most sequence variants were also found in controls we conclude that mutations in PITX2 are not a common cause of dTGA.

Background

With a frequency of up to 1%, congenital heart disease represents one of the most common major congenital anomalies [1-3]. Transposition of the great arteries (TGA) accounts for 5% of all congenital heart defects [4]. TGA manifests during the early fifth week of development affecting the septation of the common outflow tract into aorta and pulmonary arteries, and has been suggested to represent a laterality defect of the heart [5]. The more common dTGA (dextro-looped TGA) represents a complete inversion of the great vessels (atrioventricular concordance and ventriculoarterial discordance). In the less
common dTGA (laevo-looped TGA), both atrioventricular and ventriculoarterial discordance is present. Despite the high prevalence and clinical importance of TGA, we are just beginning to unravel the etiology of this heterogeneous disease. Up to now, three genes have been suggested to be involved in the etiology of dTGA in humans: PROSIT240, a novel TRAP240-like gene, has been recently isolated and several mutations are suggested to be responsible for a subset of TGA patients [6]. Isolated mutations in ZIC3 [7] and CFC1 (human CRYPTIC gene) [8,9] have also been detected in patients with TGA. ZIC3 and CFC1 have been shown before to be involved in laterality defects in humans [8,10]. However the total number of mutations detected so far within these three genes is not sufficient to explain the high incidence of dTGA and point towards strong heterogeneity.

As cardiac neural crest cells contribute to the formation of the outflow septum that divides the common outflow tract, an association between neural crest disturbance and TGA has been suggested. Extirpation experiments in chick could show that neural crest cells contribute to normal aorticopulmonary septation. Deletion of those cells causes malformation of the aorticopulmonary septum resulting in common arterial outflow channels or transposition of the great arteries [11,12]. Pitx2, a bicoid-related homeodomain transcription factor involved in eye, heart and craniofacial development and establishment of left-right asymmetry, is expressed in several tissues of the developing mouse embryo including neural crest derived organs [13]. In humans, PITX2 haploinsufficiency causes Axenfeld-Rieger Syndrome (ARS), an autosomal dominant disorder involving ocular, dental and umbilical defects [14] and, in some patients with unknown mutations, also cardiac defects [15,16]. Most interestingly, Pitx2 loss of function experiments in mice cause severe cardiovascular defects including transposition of the great arteries [17-20]. Kioussi et al. reported that Pitx2−/− mice, that survive up to E15, invariantly exhibit major cardiac outflow tract abnormalities, amongst which 30% show incomplete septation of the great arteries, that may develop with double outlet right ventricle (DORV) or transposition of the great arteries [20]. Deletion of the Dvl2 gene [21], which is regulated by the same pathway as Pitx2, leads to the same severe outflow tract malformations, indicating a strong implication of this pathway in the outflow tract phenotype. These lines of evidence prompted us to investigate whether PITX2 mutations in humans can also contribute to the etiology of TGA.

**Methods**

**Human subjects and genomic DNA**

Peripheral-blood samples were taken from healthy individuals and patients with simple dTGA after informed consent had been obtained, after approval by the institutional review board of ethics of the Medical Department of the University of Heidelberg and the Newcastle and North Tyneside Health Authority Joint Ethics Committee. Genomic DNA was prepared using the Puregene DNA Isolation Kit (Gentra, Inc., USA).

**PCR and mutation screening**

Amplifications were performed using the High Fidelity System (Roche) according to the manufacturer’s protocol. Primers were designed according to the PITX2 sequence gene bank accession number AF238048 and respective sequences are given in table 1. Mutation screening was performed using denaturing high performance liquid chromatography (DHPLC). A WAVE DNA-Fragment Analysis System (Transgenomic Inc., Cheshire) was used.

**Table 1:** Primer pairs used for mutation analysis, covering the coding region of PITX2.

| exon | primer name          | sequence 5’>3’                                                                 | TA°C | reference     |
|------|----------------------|-------------------------------------------------------------------------------|------|---------------|
| 2    | PITX2-exon2for:      | tag tct cat cag acc cct gc                                                    | 60   | Ref: [25] this paper |
|      | PITX2-exon2rev:      | ggc att tgg ttc tga ttt cct                                                  |      | this paper    |
| 3    | PITX2-exon3for:      | ttg ctc ttt gtc ctt ctt tct cct                                              | 60   |
|      | PITX2-exon3brev:     | cgg aat gtc taa gtt caa gca gca                                               |      |
| 4a   | PITX2-exon4afor:     | cgg cct ctg gtt tta aga tg                                                    | 60   | this paper    |
|      | PITX2-exon4arev:     | gca aag acc ccc tct ttc tc                                                    |      |
| 4b   | PITX2-exon4bfor:     | ctt gac act tct ctc tca gg                                                    | 60/56/52* | Ref: [25] |
|      | PITX2-exon4breve:    | aag cgg gaa tgt ctg cag g                                                      |      |
| 5    | PITX2-exon5for:      | cag ctc ttc cac ggc tcc t ttc tct cct gtt cta ctt gg                          | 60   | Ref: [25]    |
|      | PITX2-exon5rev:      | ttc tct cct gtt cta ctt gg                                                    |      |
| 6    | PITX2-exon6for:      | gta act tgc act gtt gca tc                                                    | 60   | Ref: [25]    |
|      | PITX2-exon6rev:      | agt ctt tca agg ggc gag tt                                                    |      |

TA: Annealing temperature

* step down PCR was performed with three temperatures for 10/10/15 cycles.
Sequencing

Sequencing was performed on a MegaBACE sequencer (Amersham Bioscience, Piscataway) using the DYEnamic™ ET terminator Cycle Sequencing Kit following the manufacturer's protocol. Sequencing reactions were performed on both DNA strands. Sequences were analyzed using the Clustal program (German Cancer Research Center, Biocomputing Facility HUSAR, Heidelberg).

Results and Discussion

96 patients with dTGA were analyzed for mutations in PITX2 by DHPLC and direct sequencing. All coding exons of PITX2 (exon 2 to 6, including both alternatively spliced exons 4a and b) were amplified by intron-specific exon-flanking primers to screen exon-intron junctions (table 1, figure 1). Non-coding regions (exon 1 and the 3’part of exon 6), intronic regions beyond the intronic sequences covered by the amplification, and promoter elements were not examined. We identified seven intronic and UTR variants, two silent mutations and two polymorphisms within the coding region. Most of these variants were found also in similar frequencies in 100 control individuals and are therefore unlikely to be of functional relevance. The missense mutation detected in exon 4b (204C>A, P65T) most likely represents a polymorphic variant compared to the sequence in the database, as the heterozygous form was invariantly detectable in all tested patients and controls. This finding also excludes large deletions in the patients affecting the whole gene locus. Three intronic (IVS2+7A>G, IVS3+11G>T, IVS4a-62C>A) and one silent mutations (30G>C Ser10Ser) were not detectable in 100 controls. One variant in the 5’UTR (2–40T>C) and one missense mutation (30C>T S27F) were only found once in control individuals (table 2).

We report on the mutation screening of PITX2, as we considered it to be an interesting candidate gene for TGA due
to its role in regulating asymmetric cardiac morphogenesis [22] and interesting data from mouse studies. Impaired Pitx2 function in mice leads to severe cardiac malformations [17-20]. It has been suggested that altered PITX2 expression in the outflow tract could underlie either TGA or DORV [22].

PITX2 comprises three major isoforms, formed by differential splicing or alternative promotor usage: PITX2a, b, c, as well as one minor isoform PITX2d (Fig 1). We have included all coding exons in our screening as all forms exhibit a differential expression pattern [18,19]. Pitx2c is of special interest, as only this isoform is asymmetrically expressed within the lateral plate mesoderm and the heart and governs asymmetric organ morphogenesis in a dose-dependent manner [23,19]. Furthermore, the newly identified minor isoform, PITX2d, that in fact does not bind to DNA, was included in the study since it may influence expression levels of the other splice variants and also regulate the transcriptional activity of the major isoforms on protein level [24]. As only low amounts of PITX2 are required for normal cardiac development and as the different isoforms can possibly compensate for each other in some cell populations, it might require a combination of different sequence variants within different isoforms of the gene to dramatically reduce PITX2 function and therefore manifest a cardiac phenotype.

Conclusion
To address whether altered PITX2 function could also contribute to the formation of dTGA in humans, we screened the coding regions as well as exon-intron boundaries of the PITX2 gene for mutations in 96 patients with dTGA. The majority of detected variants, however, were also found in controls with comparable frequency. Three intronic and one silent mutation could not be detected in 100 controls. As they were only found once in the cohort of 96 patients and as none of the variants was found within the evolutionary conserved homeodomain, we consider them to be rare polymorphisms rather than functional mutations, although we cannot totally exclude the latter possibility. Further investigations will have to evaluate whether these sequence variants might change splicing processes. Due to the study design we can also not exclude mutations in the very 5' and 3' UTRs and within introns as well as the promoter regions of the gene. Nevertheless, we conclude that the detected mutations in PITX2 are not a common cause of dTGA.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
NM designed the study, BN participated in the mutation analysis and drafted the manuscript, RR and KS performed PCRs and sequencing reactions. HJR, JG and EG provided patient care and collected blood samples. GR was involved in study design and supervision and finalized the manuscript. All authors read and approved the final manuscript.

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Table 2: Summary of PITX2 sequence variations in the dTGA study cohort

| type of variation: | specific variation | variant frequency (%) | number of controls | frequency (%) |
|-------------------|--------------------|-----------------------|--------------------|--------------|
| intronic/UTR      |                    |                       |                    |              |
| variations:       | 2–40T>C (5'UTR exon 2) | 10 (10.4%)            | 100                | 12 (12%)     |
|                   | 2–18T>C (5'UTR exon 2) | 0 (0%)                | 100                | 1 (1%)       |
|                   | IVS2+7A>G (intron 2)  | 1 (1.04%)             | 100                | 0 (0%)       |
|                   | IVS2–106C>A (intron 2)| 17 (17.7%)            | 100                | 20 (20%)     |
|                   | IVS3+11G>T (intron 3) | 1 (1.04%)             | 100                | 0 (0%)       |
|                   | IVS4a+11G (intron 4a) | 30 (31.25%)           | 100                | 39 (39%)     |
|                   | IVS4a-62C>A (intron 4a)| 1 (1.04%)          | 100                | 0 (0%)       |
| silent mutations: | 30G>C (S10S) (exon 2) | 1 (1.04%)             | 100                | 0 (0%)       |
|                   | 63C>T (A21A) (exon 4b) | 1 (1.04%)             | 100                | 2 (2%)       |
| polymorphism within coding region: | 30C>T (S27F) (exon 3) | 0 (0%)                | 100                | 1 (1%)       |
|                   | 204C>A (P65T) (exon 4b) | 96 (100%)            | 100                | 100 (100%)   |

UTR: untranslated region
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