Mutations in *PTF1A* are not a common cause for human VATER/VACTERL association or neural tube defects mirroring *Danforth's short tail* mouse

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Received May 26, 2014; Accepted February 27, 2015

DOI: 10.3892/mmr.2015.3486

Abstract. *Danforth's short tail (Sd)* mutant mice exhibit defects of the neural tube and other abnormalities, which are similar to the human vertebral anomalies, anal atresia, cardiac defects, tracheosophageal fistula and/or esophageal atresia, renal and radial abnormalities, and limb defects (VATER/VACTERL) association, including defects of the hindgut. *Sd* has been shown to underlie ectopic gene expression of murine *Ptf1a*, which encodes pancreas-specific transcription factor 1A, due to the insertion of a retrotansposon in its 5' regulatory domain. In order to investigate the possible involvement of this gene in human VATER/VACTERL association and human neural tube defects (NTDs), a sequence analysis was performed. DNA samples from 103 patients with VATER/VACTERL and VATER/VACTERL-like association, all presenting with anorectal malformations, and 72 fetuses with NTDs, where termination of pregnancy had been performed, were included in the current study. The complete *PTF1A* coding region, splice sites and 1.5 kb of the 5' flanking promotor region was sequenced. However, no pathogenic alterations were detected. The results of the present study do not support the hypothesis that high penetrant mutations in these regions of *PTF1A* are involved in the development of human VATER/VACTERL association or NTDs, although rare mutations may be detectable in larger patient samples.

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Key words: anorectal malformation, *Danforth's short tail* mouse, neural tube defects, *PTF1A* gene, urogenital malformation, VATER/VACTERL association

Introduction

The *Danforth's short tail (Sd)* mouse, first reported in 1930, represents a semidominant spontaneous mutant, which is characterized by a broad range of anomalies, including neural tube and spinal defects, as well as anorectal, renal and urogenital malformations (1,2). More recently, the molecular basis of *Sd* was elucidated by three groups who reported the insertion of a retrotansposon in the 5' regulatory domain of the murine *Ptf1a* gene that encodes pancreas-specific transcription factor 1A (3-5). As a consequence, and contrary to observations in wildtype littermates, *Sd* mice exhibited ectopic *Ptf1a* expression in the notochord and hindgut at embryonic day (E) 8.5 to E9.5, which extended to the cloaca and mesonephros at E10.5, and to the pancreatic bud at E10.5 and E11.5 (4). The resultant phenotype of this *Sd* mutation mirrors the phenotype observed in the human vertebral anomalies, anal atresia, cardiac defects, tracheosophageal fistula and/or esophageal atresia, renal and radial abnormalities, and limb defects (VATER/VACTERL) association and in neural tube defects (NTDs).

The VATER/VACTERL association (MIM no. 192350), with a birth prevalence of 1 in 10,000 to 1 in 40,000 (6-8), refers to the non-random co-occurrence of the following component features (CFs): Vertebral defects (V); anorectal malformations (A); cardiac defects (C); tracheoesophageal fistula, with or without esophageal atresia (TE); renal malformations (R); and limb defects (L) (9). The condition is defined by the presence of at least three major CFs (7,9). However, patients often present with additional features, in particular, urogenital anomalies. Although in the majority of cases, genetic determinants have yet to be identified, the observation of familial occurrence, an increase in the prevalence of CFs in first-degree relatives of affected individuals, higher concordance rates among monozygous twins, chromosomal (micro-)aberrations and the presence of single gene mutations

in individuals with VATER/VACTERL association all point to the importance of genetic factors in the etiology of this non-random congenital multisystem defect (10).

NTDs (MIM no. 182940), affecting the precursor to the central nervous system, the brain and spinal cord, have a prevalence of 1 in 2,000 births (11). NTDs are hypothesized to arise from a complex combination of environmental and genetic factors, of which maternal folate intake is particularly important (12), as well as maternal pregestational obesity and diabetes (13,14). In addition to the genes involved in folate metabolism (15), an increased risk of NTDs is associated with variants in the *T*-locus and the *CCL2* gene, encoding chemokine monocyte chemoattractant protein 1 (16,17). In rare cases, heterozygous mutations in the genes encoding Vang-like proteins 1 and 2, the coat protein (COPII) vesicle component gene, *SEC24B* (encoding the transport protein Sec24B), and the protein fuzzy homolog *(FUZZY)* gene have been detected in familial and sporadic cases of NTD (18-21).

From the observations made in the *Sd* mouse, it was hypothesized that gain-of-function mutations in human *PTF1A* or promoter variants may exert a similar effect to that of the mouse retrotransposon insertion. Lee *et al* (22) have shown, that specific single point mutations in mouse steroidogenic transcription factor (SF) 1 are involved in SF-1 sumoylation, by interfering with normal endocrine tissue development, thereby inducing ectopic expression of SF-1 target genes, such as sonic hedgehog. Therefore, the present study investigated the *PTF1A* gene and 1.5 kb of its 5' flanking sequence in 103 patients exhibiting VATER/VACTERL features, with or without renal or urogenital defects, and in samples from 72 fetuses diagnosed with NTDs, in which termination of pregnancy had been performed.

Materials and methods

The present study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (Bonn, Germany), and written informed consent was obtained from all patients or parents prior to study entry.

Patients with VATER/VACTERL association. The sample contained 103 patients (33 females and 70 males) presenting with at least two CFs from the VATER/VACTERL spectrum (Table I). The age of the patients ranged from 3 to 54 years. Of these patients, 79 also exhibited renal and/or additional urogenital anomalies. As reported earlier, due to the recruitment procedure, all of these patients displayed anorectal malformations (23). All patients were of Central European origin and all reported an unremarkable family history. None of the patients investigated exhibited copy number variations in previous studies by this group (23-25) or mutations in *TRAP1* (26).

Fetuses with NTD. The present cohort of 72 fetuses following termination of pregnancy with NTD (37 females, 31 males and 4 of unknown sex) was sampled during this procedure, through the Department of Prenatal Medicine at the University of Bonn. Parents were of Central European origin in 60 of these cases. All except 4 reported an unremarkable family history. For 1 fetus, diagnosed with trisomy 18, NTD had previously also been diagnosed in a maternal and a paternal cousin. In the second case, NTD was observed in a second cousin. Two women reported a previous pregnancy which had been affected by NTD. Table II lists further associated malformations observed in all the fetuses. One fetus was diagnosed with NTD and VATER/VACTERL association, and one fetus presented with additional bladder exstrophy.

Table I. Number and phenotype of 103 patients investigated.

Phenotype	No. of patients
VACTERL-like (two cardinal features)	10
VACTERL-like including renal/urogenital anomaly	19
VACTERL (at least three cardinal features)	14
VACTERL including renal/urogenital anomaly	60

VACTERL, vertebral anomalies, anal atresia, cardiac defects, tracheosophageal fistula and/or esophageal atresia, renal and radial abnormalities, and limb defects.

DNA isolation. Blood or saliva samples were obtained from the patients and their parents, and for 97 cases both parents were available. Isolation of genomic DNA was conducted using a Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany) or, in the case of saliva samples, the Oragene DNA kit (DNA Genotek Inc., Kanata, ON, Canada).

Sequence analysis. Analysis of the two coding exons and 1,500 bp of the 5' untranslated promotor region (UTR) of the human *PTF1A* gene was performed using polymerase chain reaction (PCR). Primers for *PTF1A* exons with their adjacent splice sites were (1F) 5'-GGGAGGGAGGGGCTCGGAC-3', (1R) 5'-TGGCAGTCAGCTCCCCCAGC-3'; and (2F) 5'-GGGGACGGTGGGGACTTGAAG-3', (2R) 5'-CATAGG GTGTTTGGAAGGTGGCC-3'. The 5'-UTR was amplified with the following primers: (5'-1F) 5'-TCTCAGAAGAAA CGCGCCCCG-3', (5'-1R) 5'-TCTAAGGCCCGTCCGAG CCC-3', (5'-2F) 5'-GGATAAGGGGGTGGCGGAATG-3' and (5'-2R) 5'-AGTTCCTAGGGGTGCGCGCC-3'. All primers were obtained from Metabion (Planegg, Germany). Blockcycler (Biometra T3000; Biometra, Göttingen, Germany) PCR was initiated by a 5 min denaturation step at 94˚C. The PCR program consisted of 35 cycles with a 30 sec denaturation step at 94˚C, followed by a 30 sec annealing step at 63˚C (exon products) or at 68˚C (promotor products) and a 180 sec extension step (exon products) at 72˚C; for the promotor products, elongation time was 75 sec. A 7 min final extension step at 72˚C completed the PCR. PCR-amplified DNA products were subjected to direct automated sequencing (3130Xl Genetic Analyzer, Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. The two strands of each amplicon were sequenced and PCR primers also served as sequencing primers. *PTF1A* nucleotide and amino acid

Table II. Phenotype of the fetuses with NTD.

Affected system	No. of fetuses (%)
Myelomeningocele	
Lumbosacral	46 (63.9)
Thoracic	11 (15.3)
Sacral	8 (11.1)
Frontal/occipital/cervical	7 (9.7)
Associated anomalies[a]	
Arnold-Chiari malformation	53 (73.4)
Hydrocephalus/ventriculomegaly	46 (63.9)
Lower limb anomalies secondary to peripheral nerve defects	27 (37.5)
Additional malformations[a]	
Further cerebral defects	4 (5.6)
Cardiac	4 (5.6)
Limb	4 (5.6)
Renal	3 (4.2)
Vertebral	2 (2.8)
Genitourinary	1 (1.4)

[a]In addition to myelomeningocele, fetuses may have had >1 of the associated anomalies and additional malformations listed. NTD, neural tube defects.

positions were numbered according to Ensembl transcript ID ENST00000376504 with the A of the start-methionine as no. 1.

Results

Examination of all *PTF1A* exons and their adjacent splice sites did not reveal any likely causative sequence variant in the genes from the fetal or patient samples. A total of five heterozygous variants were detected, which were all unlikely to be causative for the occurrence of the VATER/VACTERL association or for NTDs. Three of these variants (non-synonymous coding in exon 2, rs7918487; non-coding in the 3'UTR, rs10828415 and rs149560393), deposited in dbSNP (Build 138), were frequently detected in our samples, and displayed similar frequencies as reported in the databases. A novel, heterozygous p.Gly90Ala substitution due to a c.269G>C transversion was found in one NTD fetal sample, where the patients declined to participate. It was therefore not possible to test the de novo occurrence of this variant. From validated protein sequences available, the amino acid position affected appears to be highly conserved among mammals (UniProtKB/Swiss-Prot entries G3UEX6, F7IDX3, G3T9V8, I3LMI3, Q9QX98 and Q64302, annotated for orangutan, marmoset, African elephant, pig, mouse and rat, respectively). However, a number of publically available prediction programs [Mutation Taster (http://www.mutationtaster.org/), MutPred (http://mutpred.mutdb.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html)] classified this amino acid change as benign.

A fifth, unreported heterozygous variant was detected in the 5'UTR of *PTF1A* in two cases with NTD, and one patient with VATER/VACTERL. This G-to-A transition at position -477 upstream of the start codon is located in a TFSEARCH-predicted (27) low-scored binding site for upstream stimulatory factor (USF), a ubiquitously expressed cellular transcription factor (28). However, comparison with the consensus binding sequence (NCACGTGN), revealed that this *PTF1A* variant only affects a variable position (gCACGcGg/a). Furthermore, the variant was transmitted from an unaffected mother in one NTD case and in the patient with VATER/VACTERL (in the second NTD case, the parents' samples were unavailable). Therefore, its involvement in the etiology of the respective phenotype remains unclear.

Discussion

The embryopathogenesis of VATER/VACTERL association and NTDs affects various different anatomical systems, namely the early central nervous system, and the skeletal, respiratory, cardiac, gastrointestinal, renal and urogenital systems. A number of the malformations appear early during human embryogenesis (16-30 days post conception), while others may arise up to 15 days later (29,30). In accordance with these assumptions, the most recent findings in the *Sd* mouse provide a preliminary model, indicating how these anomalies may co-occur in a nonrandom fashion (3-5). *Sd* mice exhibited embryonic perturbance of the spatiotemporal expression pattern of a single gene, *Ptf1a*, initially in the notochord and hindgut, then extending to the cloaca and mesonephros, and later to the pancreatic bud (4). Thus, the various interactions of transcription factor *Ptf1a* affect multiple organs, interfering with the time window for their correct formation. As a consequence, the phenotype of the semidominant *Sd* mutation comprises defects of the neural tube and spinal column, the skeleton, and the renal, urogenital

and anorectal system, mirroring the VATER/VACTERL association and NTDs.

Homozygous *Sd* mice are born alive, but die within 24 h of birth, whereas heterozygotes are less severely affected and are fertile, with some surviving into adulthood. Heterozygotes exhibit displaced kidneys, renal hypoplasia or renal unilateral agenesis, whereas homozygotes consistently present with spina bifida, bilateral renal agenesis, or severely malformed and dislocated kidneys. Bladder, urethra and external genitalia are absent in some homozygotes, while they are present in others. Heterozygotes frequently exhibit duplication of the ureteric buds (2,31,32). In addition, whereas heterozygote *Sd* mice have no reported anorectal anomaly, homozygotes uniformly present with an imperforate anus (2,31,32).

Previously in humans homozygous loss-of-function mutations in *PTF1A* have been identified in a total of four families with pancreatic and cerebellar agenesis (MIM: #609069) (33-35). Although no pancreatic involvement has been reported in the *Sd* mouse, *Ptf1a* null mice also lack a pancreas and die shortly after birth (36). In the present sequencing study, no causative *PTF1A* variant was observed in 103 patients with VATER/VACTERL association (Table I) or in the 72 fetuses with NTDs. The heterozygous p.Gly90Ala variant detected in one NTD sample affects an amino acid residue conserved in mammals. However, it was not predicted to be pathogenic *in silico* and, furthermore, it does not map to an important functional domain of the protein, meaning that it is unlikely to have a causative role.

As the complex temporospatial expression pattern of *Ptf1a* has previously been reported to be controlled by ≥3 regions, occupying ~30 kb in the 3' and 5' flanking regions of the gene (37), it may be that a more extended sequence analysis may have identified causative variants in the two patient cohorts in the present study. Furthermore, genes targeted by *PTF1A* may be considered candidates for the VATER/VACTERL association or NTDs, and further studies, including larger numbers of patients, may identify rare causative mutations contributing to the manifestation of these defects. In this respect, the Currarino syndrome (MIM: #176450), which consists of hemisacrum, anorectal malformations and presacral mass, may be caused by mutations affecting the transcription factor Motor neuron and pancreas homeobox protein 1 (MNX1/HLXB9), which is involved in pancreas development and function. As murine Mnx1 is a direct target of *Ptf1a* (38), a common pathway, yet to be elucidated, may be affected in each of these non-random congenital multisystem defects.

Acknowledgements

Markus Draaken, Heiko Reutter, and Michael Ludwig are members of the 'Network for the Systematic Investigation of the Molecular Causes, Clinical Implications and Psychosocial Outcome of Congenital Uro-Rectal Malformations (CURE-Net)' which is supported by a research grant from the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF; grant no. 01GM08107). Greta Große is supported by the BONFOR program of the University of Bonn (grant no. O-149.0104). Nirmala Gurung is supported by the Richard-Winter-Stiftung.

The authors would like to thank patients and their parents for their cooperation, and to acknowledge the German self-help organization for people with anorectal malformations (SoMA e.V.). The authors would also like to thank Pia Uerdingen for technical assistance.

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