Comprehensive genotype–phenotype analysis in 230 patients with tetralogy of Fallot

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ABSTRACT

Background Tetralogy of Fallot (ToF), the most frequent cyanotic congenital heart disease, is associated with a wide range of intra- and extracardiac phenotypes. In order to get further insight into genotype–phenotype correlation, a large cohort of 230 unselected patients with ToF was comprehensively investigated.

Methods and results 230 patients with ToF were studied by karyotyping, comprehensive 22q11.2 deletion testing and sequencing of *TBX1, NKX2.5* and *JAG1*, as well as molecular karyotyping in selected patients. Pathogenic genetic aberrations were found in 42 patients (18%), with 22q11.2 deletion as the most common diagnosis (7.4%), followed by trisomy 21 (5.2%) and other chromosomal aberrations or submicroscopic copy number changes (3%). Mutations in *JAG1* were detected in three patients with Alagille syndrome (1.3%), while *NKX2.5* mutations were seen in two patients with non-syndromic ToF (0.9%). One patient showed a recurrent polyalanine stretch elongation within *TBX1* which represents a true mutation resulting in loss of transcriptional activity due to cytoplasmatic protein aggregation.

Conclusion This study shows that 22q11.2 deletion represents the most common known cause of ToF, and that the associated cardiac phenotype is distinct for obstruction of the proximal pulmonary artery, hypoplastic central pulmonary arteries and subclavian artery anomalies. Atrioventricular septal defect associated with ToF is very suggestive of trisomy 21 and almost excludes 22q11.2 deletion. We report a further patient with a recurrent polyalanine stretch elongation within *TBX1* and for the first time link TBX1 cytoplasmatic protein aggregation to congenital heart defects.

INTRODUCTION

Tetralogy of Fallot (ToF) is estimated to occur in 3.3 per 10 000 live births and accounts for 6.8% of all congenital heart defects.[1] Trisomy 21 (OMIM 190685) and 22q11.2 deletion (OMIM 188400, 192430, 611867) are the most frequent associated genetic anomalies. The microdeletion within chromosomal band 22q11.2 is associated with a variety of syndromes,[2–4] including DiGeorge and Shprintzen (velocardiofacial) syndromes (DGS/VCFS; OMIM 188400 and 192430). The majority of patients harbour a deletion of either 3 or 1.5 Mb.[5] Of the more than 40 commonly deleted genes, *Tbx1* is the only gene that, after an extensive functional analysis in the mouse, has been found to be haploinsufficient with a convincingly similar phenotype to the human syndrome.[6] *TBX1* missense and truncating mutations were found in up to 30% of non-deleted patients with DGS/VCFS phenotype,[7–9] while mutational screening in smaller cohorts of patients with isolated ToF failed to reveal *TBX1* mutations,[10 11] or reported rare variants of unknown significance.[12] In contrast, mutations in the *NKX2.5* gene located in 5q35 were reported in 4% of non-syndromic ToF patients.[13 14] Mutations of *JAG1* (20p12) cause Alagille syndrome showing clinical overlap with 22q11.2 deletion including right heart abnormalities.[15] Isolated ToF was also reported to be caused by *JAG1* mutations in one large pedigree[16] and in two non-familial cases.[17] To assess the incidence and to evaluate genotype–phenotype correlation of these potentially frequent causes of ToF, we performed chromosomal analysis, comprehensive 22q11.2 microdeletion testing and sequencing of *TBX1, NKX2.5* and *JAG1* in 230 unselected patients with ToF.

PATIENTS AND METHODS

Patients were recruited prospectively in two tertiary centres from January 2003 to June 2007. All patients presenting in cardiology units of Erlangen and Tuebingen with ToF (excluding children with pulmonary atresia and ventricular septal defect (PAVSD) but including those with absent pulmonary valve (APV)) were approached. Of a total of 236 patients, 230 consented to participate in the study (103 female, 127 male, median age 9.9 years). The study protocol was approved by the ethical committees of both medical faculties. Written informed consent was obtained from the parents and all patients >11 years of age. A questionnaire was filled out at presentation, stating social situation and previous medical history (hearing and speech development, frequency of infections, seizures, non-cardiac surgeries, education, profession). Mental development was classified according to Goldmuntz *et al* depending on neuropaediatric evaluation or special care during pre-school or school service.[18] The documentation included the main clinical features including neurologic, otolaryngeal, urogenital, skeletal and craniofacial anomalies. Frequent upper respiratory tract infections were defined as >4 infections per year.[19] The cardiac phenotype was characterised on the basis of angiocardiographies in 218 and echocardiography in all patients. Differences in frequencies were evaluated by χ^2 test for categorical variables and by Student unpaired t test for continuous variables.

Genetic testing

Chromosomal analysis was performed after GTG banding at a 450–500 banding resolution (International System for Human Cytogenetic

Nomenclature (ISCN) 2005) from cultured lymphocytes following standard procedures. Fluorescence in situ hybridisation (FISH) analysis was performed on metaphase spreads with 10 DNA probes covering the common and atypical deletion intervals as described earlier[5]: 6E8 (D22S427), 51H3 (D22S1649), 70A2 (D22S1694), PAC 140D4 (TUPLE1/HIRA), co23 (UFD1L), D0832 (COMT), 48c12 (D22S264), cHKAD26 (D22S935), cosmid 109G12 (109G12-1), BAC 438P22 (D22S425). In 13 patients in whom parents agreed only to the collection of saliva or in whom the lymphocyte cultures failed, multiplex ligation dependent probe amplification (MLPA) with the 22q11.2 MRC-Holland kit P023 was performed on DNA extracted from peripheral blood or saliva with standard methods. In patients with mental retardation, normal conventional or no karyotype and normal molecular testing (total 19 patients), molecular karyotyping using an Affymetrix GeneChip 100 K SNP array was performed.[20] *TBX1, NKX2.5* and *JAG1* mutational analysis in patients with normal karyotype was performed by direct sequencing of polymerase chain reaction (PCR) amplificates using intronic primers for all coding exons (table 1).

Functional testing

Constructs

We used a TBX1 wild-type expression construct, containing the complete cDNA of human *TBX1* isoform C, and a 2xTtkGL2 reporter construct, containing two palindromic T-box binding sites inserted upstream of the tk promoter.[9] The P290S mutation was inserted using the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA). The dup30-plasmid was constructed by replacing the respective part of the *TBX1* gene in the expression vector by a fragment containing the dup30, that was amplified from the patient's DNA. The complete cDNA of *NKX2.5* was amplified from cDNA of fetal spleen (Clontech, Mountain View, California, USA) with reverse transcriptase PCR (RT-PCR) and cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, California, USA). Mutagenesis was performed using the Quick Change Site Directed Mutagenesis Kit (Stratagene). As a binding site for NKX2.5 the reported proximal promoter consisting of the adjacent 135 base pairs upstream of the atrial natriuretic factor gene[21] was amplified from DNA of a healthy individual and cloned into a pGL3 luciferase reporter vector (Promega, Madison, Wisconsin, USA).

Table 1 Primer sequences used to amplify and sequence all coding exons of the three TBX1 isoforms (A, B, C) and exon/intron boundaries

TBX1	Orientation	Sequence	Fragment size
Exon 1	F	GAGCAGATGTCTCAGCCCAG	344 bp
	R	CCACACTCCTCTTCACCTGC	
Exon 2	F	CTGTCTCCCCGAGCCAGT	263 bp
	R	CAAGAGCTGCCTCCACCTAC	
Exon 3	F	GCAGCTCTCGCATTTCTGC	622 bp
	R	GGCGGAGGATAGGTGTTAGGAG	
Exon 4	F	GCTAAGCCAGGAAAGATGGAG	394 bp
	R	CGGGTTGATCGGGCAGCATCGC	
Exon 5	F	CAGAGGGTTCAATCTCACAGG	382 bp
	R	GGATTCTACAGGCCTCTTAGG	
Exon 6	F	CATTGCCAACTCAGACCTCAG	361 bp
	R	CACAGCCTGCAGGTCTAAGC	
Exon 7 and 8	F	CTTGTCAGGGCAGCAGAAAGG	418 bp
	R	TAGAGCGCGCACAGGGCC	
Seq exon 8	R	GCGGAGAGAAGGCTCTTG	
Exon 9a	F	TCAGACACTGGACATTTGTGC	453 bp
	R	ACTGGGAGTGTGACTCTATGGA	
Exon 9b	F	AGGCCACAAACACTTTGACC	264 bp
	R	TTCCATCACAGCCTCTTCAC	
Exon 9c	F	GACTGGTCGGGGAACACC	692 bp
	R	AACGTATTCCTTGCTTGCCC	

Luciferase assays

As described previously, we used JEG3 cells which are derived from chorioncarcinoma for the transcriptional reporter assay, since the wildtype did not activate t-boxes in HEK293 cells, which apparently lack transcriptional co-factors necessary for proper TBX1 function.[9] For the NKX2.5 reporter assay HEK293 and COS7 cells were cultivated in RPMI medium containing 10% FCS and then, like for the TBX1 assay, were transiently co-transfected with 100 ng of the wild-type or mutant constructs, 1 μg of the reporter construct and a renilla luciferase vector used for normalisation. Cells were harvested 48 h after transfection and luminescence measurements were performed with a Dual Luciferase Kit (Promega) and a luminometer.

Real-time PCR

We performed a quantitative real-time PCR assay to analyse mRNA levels of *TBX1* wild-type and mutants in transfected JEG3 cells.[9] Cells were co-transfected in duplicate with the TBX1 wildtype or mutant vector and a renilla luciferase vector and were harvested after 48 h. Quantitative multiplex PCR was performed with a tagged TBX1-specific TaqMan probe and a control probe detecting renilla luciferase, to normalise for transfection efficiency. Data from quadruplicate measurements were analysed using the DDCt (delta delta Ct) method.

Immunohistochemistry was performed in JEG3 cells transfected with the empty cytomegalovirus (CMV) vector, the construct containing the wildtype and the vector containing the mutant HA tagged *TBX1* with a primary Anti-HA antibody (Sigma-Aldrich, Brooklyn, New York, USA) and a secondary, Cy3 labelled anti-rabbit antibody (Abcam, Santa Cruz, California, USA) 24 h after transfection. 100 Cy3 positive cells each were microscopically analysed for staining pattern. Immunostaining was performed as described.[22]

RESULTS

Results of genetic testing and associated phenotypes are summarised in tables 2–5. FISH analysis showed deletion of probes 51H3, 70A2, PAC 140D4, co23, D0832, 48c12 and cHKAD26 indicating the common 3 Mb deletion in 16 patients, and deletion of the probes 51H3, 70A2, PAC 140D4, co23 and D0832 indicating the proximal 1.5 Mb deletion in one patient. Details about the four submicroscopic imbalances detected by molecular karyotyping were reported elsewhere.[20]

We found two unknown variants in *TBX1* and two in *NKX2.5*, as well as the known *NKX2.5* missense mutation R25C in a further two patients. The *JAG1* missense mutation R937Q was reported before in a patient with Alagille syndrome[23] and excluded by us in 284 normal control individuals. The *NKX2.5* mutation R25C was supposed to be functionally relevant but with low penetrance.[13 24 25] The two unknown *NKX2.5* variants C270Y and V315L (table 3) were not detected in 280 control individuals and were evolutionary conserved for many but not all species. Although functional testing using a transcriptional reporter assay did not reveal a relevant reduction in transcriptional activity, we cannot exclude the possibility that they might cause alterations not detectable by this assay (figure 1). Likewise, the *TBX1* variant P290S was evolutionarily conserved for many but not all species and was not observed in 174 control individuals, but was found to be inherited from the healthy father and showed no alteration in the transcriptional reporter

Table 2 Summary of genetic findings in 42 out of 230 patients with tetralogy of Fallot

n	%	Gene/locus	Mutation/aberration	Phenotype
17	7.4	22q11.2	Common 3 Mb microdeletion (16x) Proximally nested 1.5 Mb deletion (1)	DiGeorge/VCFS spectrum
12	5.2	Trisomy 21	47,XY,t(4;21)(q12;q11.2),+21 46,XX,der (15;21)(p10;p10)+21 47,XX/XY,+21 (7x) 47,XY,+21 in female patient* 100 K SNP array enh(21) (1x)	Down syndrome; Down syndrome and Swyer syndrome*: AVSD, hypoplastic right ventricle, right aortic arch, external and internal genitalia (uterus) female, frequent infections
3	1.3	Chromosomal aberrations other than trisomy 21	der(19)(pter::p13.2-13.3::13.3-qter) de novo	Height/weight <P3, mental retardation, impaired hearing, frequent infections, craniofacial dysmorphy, restrictive VSD, LAA, aberrant subclavian artery
			del(21)(q22.3) de novo	Height/weight <P3, mental retardation, cleft palate, inguinal hernias, hip dysplasia, LSVC, perimembranous VSD, additional VSD, juxtaductal coarctation, LAA, aberrant subclavian artery
			47,XXX	height/weight >P97, scoliosis, frequent infections, long, spherical infundibulum, juxtaductal coarctation
4/19 tested	1.7	Microaberrations other than 22q11.2 deletion	del(1)(p32.2p31.1), 14 Mb, parents not available	Mental retardation, height/length <P3, agenesis of corpus callosum, hydrocephalus, seizures, renal dysplasia, jejunal atresia, clubfoot, coronary arteries abnormal, LCA from RCA, RAA, right vertebral arteria from aortic arch
			del(6)(q16.1q21), 12.80 Mb, de novo	Mental retardation, behavioral problems, thymic aplasia, frequent infections, DORV
			del(7)(p22.3p22.2), 1.50 Mb, de novo	Mental retardation, cleft palate, facial dysmorphism, scoliosis, DORV, RAA
			del(19)(p13.11p12), 4.5 Mb and dup(19)(q13.1), 2.70 Mb, both de novo	mental retardation, craniofacial anomalies, coronary artery anomalies (LAD from RCA)
3	1.3	JAG1	c.2810G→A (p.R937Q)	Alagille facies, mild mental retardation, RAA, singular origin of left subclavian artery, truncus bicaroticus
			c.439C→T (p.Q147X), parents not available	Alagille facies, severe mental retardation, hypernasality, dysplastic ears, flat nasal bridge, wide-set nipples, extrahepatic biliary atresia, peripheral pulmonary stenosis
			c.2508_2509insTG (p.D837WfsX870)	Alagille facies, premature birth, microcephaly, dystrophy, severe mental retardation, extrahepatic biliary atresia (liver transplantation at 2 years of age), juxtaductal coarctation, peripheral pulmonary stenosis, truncus bicaroticus
2	0.9	NKX2.5	R25C	Dextroposition of the aorta >50% from right ventricle, aberrant subclavian artery
			R25C and 46,XY,inv(5)(p14.2q13.1)	Normal facies, no mental retardation, elongated infundibulum, LAA
1	0.4	TBX1	c.1399–1428dup30, excluded in mother, father not available	Scoliosis, facial asymmetry, upslanting palpebral fissures, absent PV, isolated left pulmonary artery

* In a female one would expect an XX karyotype; to clarify that this patient in addition to trisomy 21 (Down syndrome) also has a disorder of sexual development, which is named 'Swyer syndrome'.
AVSD, atrioventricular septal defect; DORV, double outlet right ventricle; LAA, left aortic arch; LAD, left anterior descending coronary artery; LSVC, left superior vena cava; PV, pulmonary vein; RAA, right aortic arch; RCA, right coronary artery; ToF, tetralogy of Fallot; VCFS, velocardiofacial syndrome; VSD, ventricular septal defect.

assay (figure 2). The *TBX1* gross insertion of 30 bp within exon 9c (GCCGCGGCCGCCGCCGCCGCTGCCGCAGCT; c.1399-1428dup30) was determined by cloning the aberrant PCR fragment and sequencing of clones. It was excluded in 185 control individuals as well as in the patient's mother. The father, who was a smoker and obese, died at the age of 40 years following a myocardial infarction. Despite normal mRNA levels as revealed by RT-PCR, the transcriptional reporter assay showed severely reduced transcriptional activity of the mutant construct containing the c.1399-1428dup30 (figure 2). Since this insertion leads to the expansion of a polyalanine tract, we assumed that it might lead to aggregation of the protein. We therefore performed immunofluorescence studies which indeed demonstrated aggregation of the protein in the cytoplasma (figure 3).

Comparison of cardiovascular and extracardiac phenotype in patients with and without 22q11.2 deletion.

There was no significant difference in z-scores for weight (p=0.16, Mann–Whitney U test), but there was for height (p=0.045), between patients with 22q11.2 deletion and those without detectable genetic abnormality (table 4). Mild mental retardation was present in most of the deleted (82%) but only in a minority of genetically normal patients (15%, p<0.001). Velopharyngeal insufficiency (47% vs 2%, p<0.001) was predominantly observed in patients with 22q11.2 deletion, while upper respiratory tract infections, palatal, skeletal and genito-urinary abnormalities did not reach statistical significance. The cardiovascular phenotype differed with respect to the pulmonary artery (PA) anatomy and the aortic arch vessels (table 5): 8/34 patients (24%) with juxtaductal coarctation or hypoplastic PA had 22q11.2 deletion, while the incidence of this microdeletion among the remaining patients was 5% (9/196) (p<0.001). Similar findings were observed for subclavian artery anomalies (SAA): 9/31 patients (29%) with SAA had the deletion, but only 8/199 (4%) without SAA (p<0.001). 22q11.2 deletion was independent of the laterality of the aortic arch (p=0.14). The most frequent anomaly of the subclavian artery was aberrant origin from the descending aorta, followed by isolation, which was found exclusively in combination with right aortic arch (seven patients). Cervical origin of the right subclavian artery was detected in one patient with 22q11.2 deletion. The shape of the infundibulum did not differ between both groups and most of the patients had combined valvular and infundibular stenoses. Coronary artery anomalies, patent ductus arteriosus in the neonatal period, major aortopulmonary

Table 3 Overview of structural polymorphisms and rare single nucleotide variants identified in six out of 230 patients with tetralogy of Fallot

n	%	Gene/locus	Mutation/aberration	Phenotype
3	1.3	Structural polymorphisms	45,XX,der(13;14)(q10;q10)pat Uniparental disomies 13 and 14 excluded	Height/weight <P3, mental retardation, Dandy–Walker cyst, hydrocephalus, omphalocele, juxtaductal coarctation
			inv(2)(p11.2q13)	Typical ToF
			inv(9)(p12q13)	Typical ToF
2	0.9	NKX2.5	C270Y, absent in controls, parents not tested	Horseshoe kidney, mild pectus carinatus, MAPCA from the descending aorta, RAA without SAA
			V315L, absent in controls, parents not tested	Obesity, aberrant subclavian artery
1	0.4	TBX1	P290S inherited from healthy father	Absent PV, frequent infections

RAA, right aortic arch; SAA, subclavian artery anomaly; ToF, tetralogy of Fallot.

collateral arteries (MAPCAs), pronounced dextroposition of the aorta (>50% from right ventricle), or APV were not specifically associated with the 22q11.2 deletion. None of our patients with 22q11.2 deletion had associated atrioventricular septal defect (AVSD). Patients with 22q11.2 deletion showed facial features compatible with the DiGeorge/Shprintzen syndrome phenotype.

Phenotype of patients with TBX1, NKX2.5 or JAG1 mutations

Patients with functionally proven mutations in *TBX1, NKX2.5* or *JAG1* did not differ significantly from the non-mutated patients in height and weight (table 4). While patients with *TBX1* or *NKX2.5* mutations showed no specific facial dysmorphism and attended regular schools, all three patients with *JAG1* mutations had indicative facial gestalt and mild or severe mental retardation. The female patient with the *TBX1* c.1399-1428dup30 mutation was born after an uneventful pregnancy at 40 weeks of gestation to healthy unrelated parents of Turkish origin. Birth measurements were 3250 g (weight), 51 cm (length), and 34 cm (head circumference) (proportionate within normal ranges for Turkish percentiles) and APGAR scores were reported as 9/10/10.[26] ToF with APV and isolation of the left PA was corrected at the age of 3 years. Frequent infections were reported during early childhood, but all regular vaccinations were tolerated without problems. During puberty she developed scoliosis. Psychomotor development was normal and the girl attended secondary school. At the age of 20 years her height was 157 cm (3rd–10th centile), weight was 56 kg (25th–50th centile), and head circumference was 52 cm (3rd–10th centile). Her facial gestalt was not indicative of 22q11.2 deletion (figure 4). She had some broadening of the uvula and small teeth.

Among our group of six patients with bona fide mutations she was the only one with additional muscular ventricular septal defect (VSD). Two out of three patients with *JAG1* mutations had peripheral pulmonary stenoses typical for Alagille syndrome. APV in association with ToF was neither characteristic for the classical 22q11.2 deletion (one patient) nor for *NKX2.5* and *JAG1* mutations. The patient with *TBX1* mutation had APV with isolated left PA.

Phenotype of ToF patients with Down syndrome

Patients with Down syndrome were significantly lower in z-scores for height and weight ($p<0.0001$) than the remaining

Table 4 Extracardiac abnormalities in 230 patients with tetralogy of Fallot

	All	No genetic abnormality	Del 22q11.2	1 TBX1, 2 NKX2.5, 3 JAG1	Down	Further chromosomal abberations	Polymorphisms
N (%)	230	182 (79.1)	17 (7.4)	6 (2.6)	12 (5.2)	7 (3.0)	6 (2.6)
Mean age (years) at inclusion in our study (range)	10.9 (0/34.0)	10.9 (0/34.0)	7.4 (0/21.8)	8.5 (0.4/16.4)	14.7 (0.9/32.4)	7.9 (0.3/14.3)	18.2 (3.5/33.5)
Mean time (years) post surgery (range)	9.2 (0.2/28.8)	9.5 (0.2/28.8)	6.3 (0/21.0)	5.3 (0/12.9)	10.5 (2.7/23.8)	6.1 (0.2/10.8)	14.5 (3.0/14.5)
Weight z-scores (min/max)	−0.6 (−11/5)	−0.4 (−11.1/5.0)	−0.9 (−5.2/2.2)	−1.5 (−6.3/0.9)	−2.0 (−3.7/−0.5)	−1.1 (−5.0/2.2)	−0.1 (−2.8/1.8)
Height z-scores (min/max)	−0.1 (−5.8/9.6)	0.07 (−5.7/9.7)	−0.6 (−4.1/1.4)	−0.2 (−3.3/2.8)	−2.1 (−3.8/−0.2)	−0.8 (−5.7/3.1)	−0.4 (−2.4/0.8)
CNS imaging performed (%)	74 (32)	56 (31)	7 (41)	2 (33)	3 (25)	4 (57)	2 (33)
CNS imaging pathologic (%)	25 (11)	16 (9)	2 (12)	0	2 (17)	3 (43)	2 (33)
Mental retardation (%)							
No	163 (71)	151 (83)	3 (18)	3 (63)	1	0	5 (83)
Mild	55 (24)	28 (15)	14 (82)	2 (JAG1) (25)	9 (82)	1 (14)	1 (17)
Severe	12 (5)	3 (2)	0	1 (JAG1) (12)	2 (18)	6 (86)	0
Seizures	18 (8)	11 (6)	4 (24)	0	2 (17)	1 (14)	
Velopharyngeal insufficiency	12 (5)	3 (2)	8 (47)	1 (JAG1) (17%)	0	0	0
Cleft palate	8 (3)	4 (2)	1 (6)	0	0	2 (29)	0
Impaired hearing	10 (4)	7 (4)	0	0	2 (17)	1 (14)	0
Skeletal abnormality	57 (25)	36 (20)	4 (24)	2 (2 JAG1) (33)	8 (73)	6 (86)	1 (17)
Genitourinary abnormalities	29 (13)	19 (10)	3 (18)	0	3 (25)	2 (29)	2 (33)
Frequent infections ≥1/month	38 (17)	26 (14)	5 (29)	0	3 (25)	3 (43)	1 (17)

CNS, central nervous system.

Table 5 Cardiac phenotype in 230 patients with tetralogy of Fallot

	All	No genetic abnormality	del 22q11.2	1 TBX1, 2 NKX2.5, 3 JAG1	Down	Further chromosomal aberrations	Polymorphisms
N (%)	230	182 (79)	17 (7.4)	6 (2.6)	12 (5.2)	7 (3.0)	6 (2.6)
Situs solitus	230 (100)	182 (100)	17 (100)	6 (100)	12 (100)	7 (100)	6 (100)
LSVC	22 (10)	20 (11)	1 (6)	0	0	1 (14)	0
PAPVC	5 (2)	4 (22)	0	0	0	0	0
PFO/ASD II	185 (80)	149 (82)	13 (76)	5 (83)	8 (67)	6 (86)	4 (66)
ASD I	5 (2)	2 (1)	0	0	3 (25)	0	0
Sinus venosus ASD	1 (0.4)	1	0	0	0	0	0
Malalignment VSD	226(98)	182 (88)	17 (100)	6 (100)	9 (75)	7 (100)	5 (83)
AVSD	4 (2)	0	0	0	3 (25)	0	1 (17)
Additional VSDs	20 (9)	16 (9)	0	1 TBX1 (17)	1 (8)	2 (29)	0
Infundibulum							
Short	111 (48)	90 (49)	6 (35)	1 JAG1) (17)	4 (33)	4 (57)	6 (100)
Elongated	71 (31)	57 (31)	5 (29)	2 NKX2.5 (33)	5 (42)	2 (29)	0
ToF-type	32 (14)	27 (15)	2 (12)	3 (1 TBX1, 2 JAG1) (50)	0	0	0
Unknown	19 (8)	10 (5)	4 (24)	0	3 (25)	1 (14)	1 (17)
Pulmonary stenosis							
Predominantly valvular	21 (9)	16 (9)	2 (12)	1 JAG1 (17)	1 (8)	0	1 (17)
Predominantly infundibular	11 (5)	11 (6)	0	0	0	0	0
Combined	182 (79)	144 (79)	12 (71)	5 (83)	10 (83)	6 (86)	4 (67)
Unknown	10 (4)	7 (4)	2 (12)	0	0	1 (14)	0
Juxtaductal coarctation/PA hypoplasia	34 (15)	20 (11)	8 (47)	2 (JAG1) (33)	0	3 (43)	1 (17)
Peripheral PA stenosis	14 (6)	9 (5)	2 (12)	2 (2 JAG1) (33)	0	0	1 (17)
MAPCA							
Yes	12 (5)	9 (5)	2 (12)	0	0	0	1 (17)
No	216 (94)	173 (95)	15 (88)	6 (100)	12 (100)	7 (100)	5 (83)
Unknown	2 (1)	2 (1)	0	0	0	0	0
PDA							
Yes	47 (20)	36 (LAA17, RAA 18) (20)	3 (LAA 1, RAA 2) (18)	0	2 (LAA/RAA) (17)	5 (71) (4 LAA)	1 (17) (LAA)
No	21 (9)	15 (8)	2 (11)	1 (17)	2 (17)	0	1 (17)
Unknown	165 (72)	133 (58)	12 (71)	5 (83)	9 (75)	2 (29)	4 (67)
Additional cardiac defects							
Absent PV	10 (4)	7 (4)	1 (6)	1 (TBX1) (17)	0	0	1 (17)
Origin of the aorta >50% from right ventricle	14 (6)	9 (5)	2 (11)	1 (NKX2.5) (17)	0	2 (29)	0
Coronary arteries							
Normal	203 (88)	158 (87)	16 (94)	6 (100)	12 (100)	5 (71)	6 (199)
Single	5 (2)	5 (3)	0	0	0	0	0
Accessory	13 (6)	12 (7)	1 (6)	0	0	0	0
LAD from RCA	9 (4)	7 (4)	0	0	0	2 (29)	0
Unknown	3 (1)	0	0	0	0	0	0
Long asc. aorta	15 (7)	12 (7)	2 (12)	0	1 (8)	0	0
Aortic arch							
Left	159 (69)	126 (68)	9 (53)	5 (83)	10 (83)	5 (71)	5 (83)
Right	71 (31)	58 (32)	8 (47)	1 (JAG1) (17)	1 (8)	2 (29)	1 (17)

Continued

Table 5 Continued

	All	No genetic abnormality	del 22q11.2	1 TBX1, 2 NKX2.5, 3 JAG1	Down	Further chromosomal aberrations	Polymorphisms
Subclavian artery anomalies	31 (13)	17 (LAA 6, RAA 11) (9)	9 (LAA 3, RAA 6) (53)	1 (NKX2.5, LAA 1) (17)	1 (LAA) (8)	2 (29) (2 LAA)	1 (17) LAA
Aberrant	24 (10)	12 (7)	6 (35)	2 (33)	1 (8)	2 (17)	1 (17)
Isolation	7 (all RAA) (3)	5 (3)	2 (12)	0	0	0	0
Distal ductal origin	0	0	0	0	0	0	0
CORSA	1	0	1 (6)	0	0	0	0

ASD, atrial septal defect; AVSD, atrioventricular septal defect; CORSA, cervical origin of the right subclavian artery; DORV, double outlet right ventricle; LAA, left aortic arch; LAD, left anterior descending coronary artery; LSVC, left superior vena cava; MAPCA, major aortopulmonary collateral arteries; PA, pulmonary artery; PAPVC, partial anomalous pulmonary venous connection; PDA, patent ductus arteriosus; PFO, persistent foramen ovale; RAA, right aortic arch; RCA, right coronary artery; SAA, subclavian artery anomaly; VSD, ventricular septal defect.

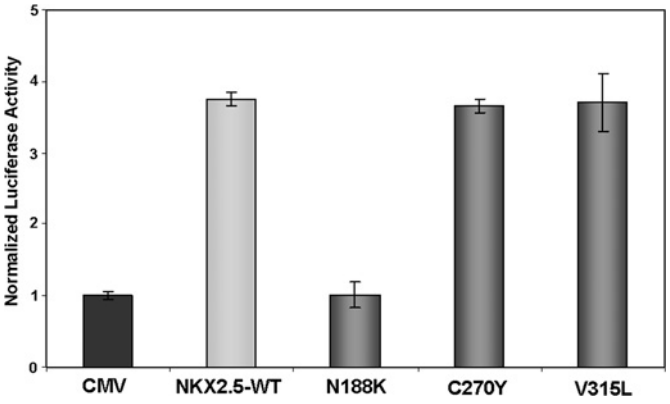

Figure 1 Transcriptional activation of a luciferase reporter construct by wild-type (WT) and mutant NKX2.5. HEK cells were co-transfected with the atrial natriuretic factor gene (ANF) promoter reporter construct, containing NKX2.5 binding sites, and either a cytomegalovirus (CMV) control vector, the NKX2.5 WT construct, or the mutant constructs. In comparison to the CMV vector, WT NKX2.5 results in a fourfold increased activation of the luciferase reporter. While the positive control N188K showed reduced transcriptional activity as reported before,[25 65] our variants C270Y and V315L showed no altered activity compared to the WT in this assay. Results are normalised for transfection efficiency to a cotransfected renilla luciferase vector and expressed as average values±SEM of two independent transfections. Results were confirmed in a second cell line (COS7, data not shown).

patients. Three of the 12 patients had AVSD (table 5). No stenoses of bifurcation or peripheral PA occurred in patients with Down syndrome, who showed neither MAPCAs nor abnormal coronaries. All but one had left aortic arch, and one with left aortic arch had an aberrant subclavian artery.

DISCUSSION

Genetic findings

In contrast to our findings of cytogenetically visible chromosomal abnormalities in 6.5% (15/230), previous population based studies from the 1980s and early 1990s have documented such abnormalities in as much as 12–13% of patients with ToF.[27 28] Since the common trisomies of chromosomes 13, 18 and 21 account for the vast majority of aberrations in the older studies, the significant difference can be explained by the lack of patients with trisomy 13 and 18 and the decrease of children with trisomy 21 in our cohort. Nowadays these trisomies are frequently detected and aborted prenatally when associated with complex heart defects. The Baltimore-Washington Infant study included 236 patients with ToF and reported chromosomal abnormalities in 11.9%, Mendelian syndromes in 2.5%, non-Mendelian associations in 4.6%, multiple anomalies in 2.1%, and organ defects in 11%.[29] With the introduction of FISH, and molecular karyotyping, it became evident that a significant number of patients with ToF had 22q11.2 deletion or other syndromes caused by submicroscopic chromosomal imbalances. The latter were found in 21% of our patients with ToF and mental retardation investigated by molecular karyotyping (4/19), which is in line with the reported finding of 17% of submicroscopic chromosomal imbalances in patients with congenital heart defects selected for a 'chromosomal phenotype'.[30]

The reported frequency of 22q11.2 deletion in ToF varies between 6–23%.[31–39] This variability can be explained at least partially by inclusion or exclusion of patients with PAVSD and

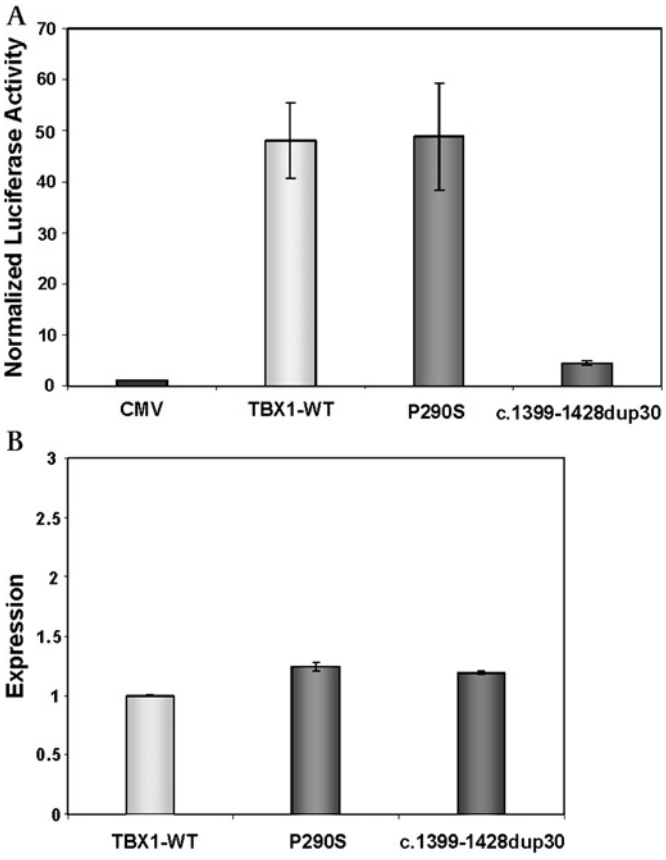

Figure 2 (A) Transcriptional activation of a luciferase reporter construct by wild-type (WT) and mutant TBX1C. JEG3 cells were co-transfected with the 2xTtkGL2 reporter construct, containing T-Box binding elements, and either a cytomegalovirus (CMV) control vector, the TBX1C WT construct, or the mutant constructs. In comparison to the CMV vector, TBX1C shows a 50-fold increased activation on the luciferase reporter. While the paternally inherited missense variant P290S does not show any alteration in transcriptional activity compared to the WT, the c.1399-1428dup30 mutant shows a lack of transcriptional activation, thus demonstrating loss of function. Results were normalised for transfection efficiency to a co-transfected renilla luciferase vector and expressed as average values±SEM of three independent transfections. Results were confirmed in a second experiment with triple transfections. (B) Expression of WT and mutant TBX1C in JEG3 cells. In order to investigate possible differences in TBX1 expression as the cause of the lack of activity in the 30 bp dup mutant, mRNA levels of TBX1C WT and mutants were analysed by quantitative RT-PCR using a probe specific to the tagged transcript in RNA extracted from the cultured cells. No altered expression was observed for the mutants in comparison to the wild type. Results were normalised for transfection efficiency against the co-transfected renilla vector and expressed as average value±SEM of quadruplicate measurements of two independent transfections, respectively.

MAPCAs, the extreme variant of the anatomic spectrum of ToF, since the incidence of 22q11.2 deletion is significantly higher in the latter group.[36 40] In our study of ToF patients (excluding those with PAVSD) the incidence of 22q11.2 deletion was 7.4%, representing the most frequent genetic abnormality. There is some bias, however, since we recruited patients of all age groups, therefore neglecting some patients who died younger. All but one patient had the classical 3 Mb deletion and none showed an atypical distal deletion, which apparently causes an overlapping but distinct phenotype.[5 41 42]

So far mutations of *TBX1* were reported in five independent families with variable features of the DiGeorge/Shprintzen/VCFS spectrum.[7–9] In our large cohort we found evidence for the first time that intragenic *TBX1* mutations may cause non-syndromic ToF. The insertion of 30 bp within exon 9c of *TBX1* (c.1399-1428dup30), detected in one patient with unsuspicious facial gestalt and scoliosis, was previously reported as an unclear variant in a patient with isolated interruption of the aortic arch.[12] We were able to show that this in-frame polyalanine stretch expansion results in almost complete loss of transcriptional activity due to cytoplasmatic aggregation of the mutant protein. This finding adds a novel pathogenetic mechanism of *TBX1* mutations to the formerly known haploinsufficiency, gain of function or loss of nuclear localisation signal.[20 43]

While expansions of polyglutamine repeats have been known as cause for a variety of neurodegenerative diseases, only recently mitotically stable expansions of alanine tracts particularly in transcription factor genes emerged as a disease mechanism in congenital malformations, skeletal dysplasia and nervous system anomalies.[44] A common mechanism seems to be enhanced degradation due to protein misfolding and/or aggregation resulting in cellular dysfunction. Like we found in *TBX1*, for most of the known transcription factors associated with polyalanine stretch expansion disorders, cytoplasmatic accumulation and cytoplasmatic and nuclear aggregation of the mutant protein could be observed.[45] Our novel finding of pathogenetic polyalanine stretches in *TBX1* links, for the first time, this mechanism to the aetiology of congenital heart defects. A genotype–phenotype correlation with polyalanine expansions causing a milder phenotype than other mutations was reported for ARX, RUNX2, FOXL2 and PHOX2B.[44] Since, in contrast to patients with *TBX1* point mutations, both currently known patients with the *TBX1* polyalanine expansion[12] do not show the classical 22q11.2 deletion phenotype, it is tempting to speculate about a similar genotype–phenotype correlation.

In contrast to earlier studies reporting *NKX2.5* mutations in 1.7–4% of patients with ToF,[13 14 46] we found only the low penetrant R25C variant in 0.9% of all patients and in 1% of patients without other diagnoses. We cannot exclude the possibility, however, that the rare variants C270Y and V315L, which were not detected in 280 control individuals and showed no functional impairment in our transcriptional assay, may cause alterations not detected by our testing system. Though a report on a *JAG1* mutation segregating with isolated ToF in a family[16] and two cryptic mutation reports indicated a possible role in isolated ToF,[17] our systematic study showed *JAG1* mutations only in patients with clinical diagnosis of Alagille syndrome.

Extracardiac and cardiac phenotype of 22q11.2 deletion

Ryan and colleagues reported that one third of deleted patients, irrespective of associated cardiac anomalies, were below the 3rd centile for either height or weight, suggesting a strong link between growth retardation and 22q11.2 deletion.[3] Our study excludes the bias of comparing patients with cardiac anomalies to those without and found only a tendency towards lower z-scores for weight and a weak significance for lower height in 22q11.2 deleted patients. Corrective surgery in patients with ToF results in significant catch up growth of weight and height within 2 years.[47] The majority of our patients were beyond this postoperative interval, explaining why no pronounced differences were found, even compared to normal values of patients without cardiac defect. Learning and behavioural difficulties were identified as significant discriminators between deleted and non-deleted adults with congenital heart disease (79% vs 44%),[48] which is in line with the finding of mild mental retardation or

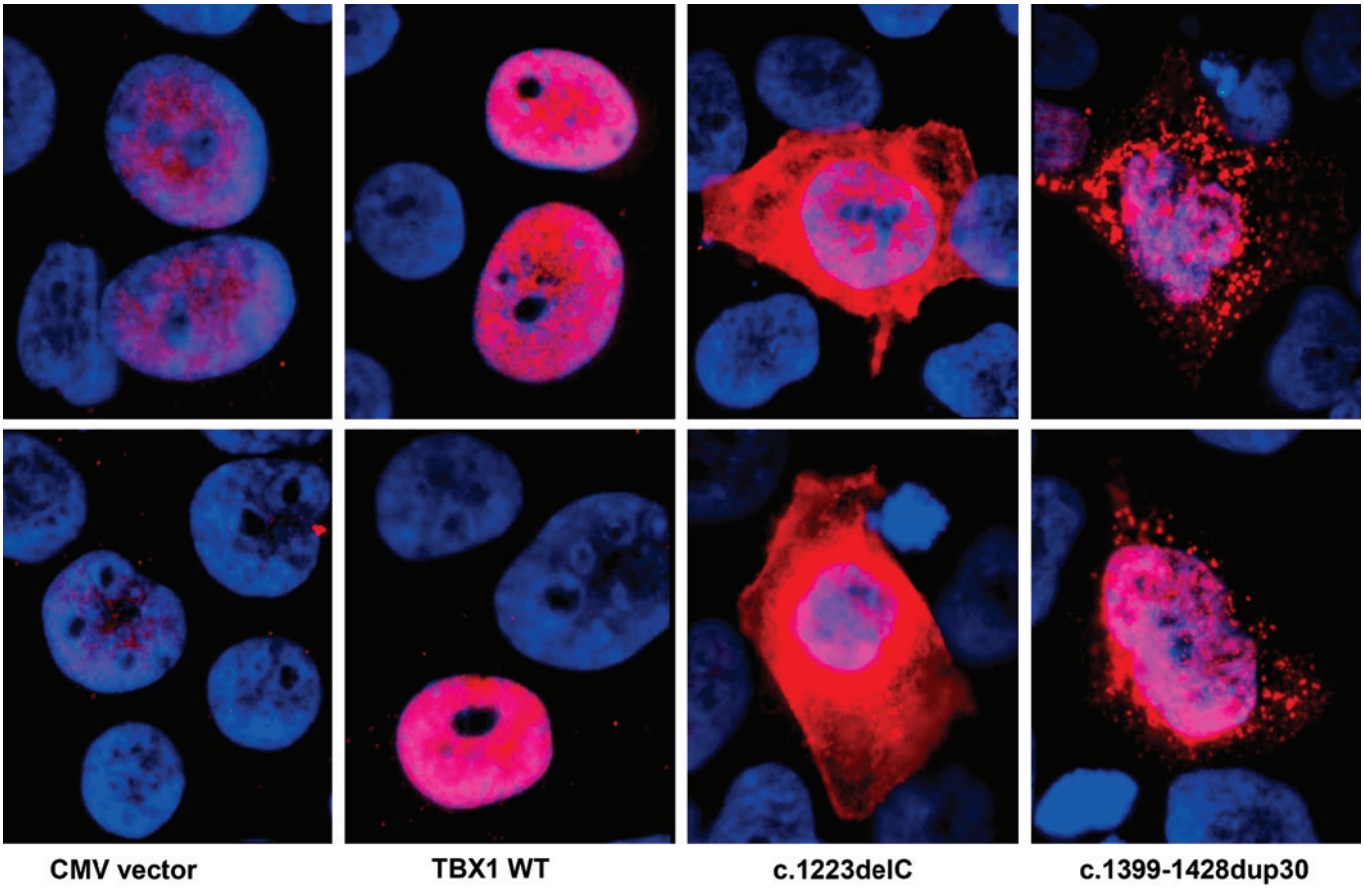

Figure 3 Representative results of immunhistochemistry of TBX1C wild-type and loss of function mutants. JEG3 cells were transfected with either an empty cytomegalovirus (CMV) vector, TBX1 wild type, a previously reported 1223delC mutant,[43] or the c.1399-1428dup30 mutant. While the empty CMV vector did not significantly stain transfected cells, cells transfected with wild-type TBX1 showed homogeneous nuclear localisation as previously described. As a positive control we analysed the c.1223delC mutation, which was previously characterised in detail and shown to result in cytoplasmatic localisation of TBX1 due to the loss of a c-terminal nuclear localisation signal.[43] Our results for the c.1223delC mutant confirm the published findings obtained with a different reporter system and prove the reliability of our assay. The 30 bp duplication in exon 9c (c.1399-1428dup30) resulted in protein aggregation in cytoplasm and nucleus, due to elongation of a polyalanine stretch.

need for special care during school documented in 82% of our patients with 22q11.2 deletion. As learning and behavioural difficulties were significantly less frequent in our patients without genetic aberration, and since previous studies included deleted patients without congenital heart disease,[3] mental retardation can be attributed to a large extent to the genetic disorder. While velopharyngeal insufficiency was present in 47% of patients with 22q11.2 deletion, there was no significant difference in the incidence of frequent infections, impaired hearing, cleft palate and genitourinary anomalies.

In our cohort SAA contralateral to the side of the aortic arch represented the undisputable cardiovascular feature of 22q11.2 deletion and discriminated significantly between patients with and without the deletion. The incidence of SAA among children with 22q11.2 deletion in our study (53%) was similar to the previously reported incidence among 22q11.2 deletion patients with PAVSD (57%) or truncus arteriosus (50%), but lower than in children with interrupted aortic arch (75)%.[35 38 49] According to the pioneering work of Kutsche and van Mierop, SAA contralateral to the aortic arch can be explained by unilateral impairment of development of the respective fourth aortic arch. Resulting SAA include aberrant origin from the descending aorta, isolation, distal ductal origin from the PA, and cervical origin.[50] The latter anomaly has been described exclusively in patients with 22q11.2 deletion,[49] an observation further supported by the present study. The association of SAA with 22q11.2 deletion is of clinical significance, since echocardiography achieves a high sensitivity in the detection of these anomalies, and allows in patients with cervical origin of the right subclavian artery to establish the clinical diagnosis of 22q11.2 deletion.[51]

Our study revealed anomalies of the PA in patients with ToF to be significantly associated with 22q11.2 deletion. Almost 50% of patients with 22q11.2 deletion had either juxtaductal PA coarctation resulting from abnormal insertion of the arterial duct, or hypoplastic PA, as compared to 11% of non-syndromic patients. This confirms previous observations of anomalous central PA in patients with 22q11.2 deletion both with normal intracardiac anatomy and PAVSD.[39 52] This is of significant clinical importance, as central PA hypoplasia has been found to be an independent predictor of mortality after surgical correction of ToF.[53 54] Contrarily, MAPCAs, associated with 22q11.2 deletion in patients with PAVSD,[55] were no indicator for this deletion in ToF patients with patent pulmonary valve. Therefore hypoplastic PA appears as a key predictor of 22q11.2 deletion both in ToF and in PAVSD.

There was no distinctive pattern of intracardiac anatomic features in 22q11.2 deletion. However, none of our four patients with ToF and AVSD and none of the 64 patients investigated by Vergara *et al* had 22q11.2 deletion, confirming that AVSD almost excludes 22q11.2 deletion.[56–58]

Figure 4 Clinical photographs of the patient with the TBX1 c.1399-1428dup30 mutation. The facial phenotype of the 20-year-old woman was not considered suggestive of 22q11.2 deletion or any other syndrome, but the palpebral fissures were mildly upslanting and the chin was large and asymmetric. The columella was somewhat pointed. In addition there was clinodactyly of fingers and toes, with the middle fingers being most affected, and mild syndactyly of toes 2 and 3.

APV, a distinct variant of ToF, is characterised by absent or rudimentary pulmonary valve cusps and massive dilation of the PA. Johnson *et al* found a specifically high incidence of 22q11.2 deletion (6/8 patients) among these children.[59] To the best of our knowledge our study represents the largest cohort of patients with APV with genetic screening. Only 1/10 had 22q11.2 deletion, while another patient had a mutation in *TBX1*. Since the latter had only few extracardiac anomalies, *TBX1* mutations might explain familial occurrence of non-syndromic ToF and APV in whom only 22q11.2 deletion was excluded.[60]

Comparison of cardiac phenotype between 22q11.2 deletion and *TBX1*

Our patient with *TBX1* mutation had an additional VSD and ToF with APV, neither of which where common features of 22q11.2 deletion in our cohort. In *Tbx1* deficient mouse models short and narrow outflow tracts were described, but not an isolated and hypoplastic left PA. To date, there are no reports of humans with mutations in *TBX1* and isolated PAS; however, detailed descriptions of the cardiac phenotypes are lacking in the small number of previously reported patients. Recent studies in mice lacking *Tbx1* have displayed severe defects in the development of the pharyngeal derivates leading to abnormal coronary artery patterning.[61] None of our patients with 22q11.2 deletion or the patient with *TBX1* mutation had coronary anomalies.

Cardiac phenotype in Down syndrome

Our study confirmed previous results that about 25% of patients with ToF and Down syndrome have AVSD, and in children with ToF and associated AVSD the presence of genetic syndromes, mostly Down syndrome, is very high (>85%).[56] In our study patients with Down syndrome did not present coarctation of the PA, MAPCAs or coronary artery abnormalities. Furthermore, our study confirmed that pronounced dextroposition of the aorta is very rare in patients with Down syndrome.[56] Our finding of a single patient with Down syndrome and SAA is in line with previous data showing SAA in one out of 43 patients.[56] Thus, ToF in Down syndrome seems to be associated with a relatively uniform cardiac phenotype. Interestingly, in one patient with Down syndrome, ToF and SAA, co-occurrence of 22q11.2 deletion and trisomy 21 mosaicism was reported.[62]

CONCLUSIONS

We found the common 22q11.2 deletion to represent the most frequent genetic diagnosis in patients with ToF, followed by Down syndrome. Atypical proximal deletions in 22q11.2 as well as mutations in *TBX1, NKX2.5* and *JAG1* are far less frequent. We were able to show that intragenic *TBX1* mutations may cause non-syndromic ToF and demonstrated a novel pathogenetic mechanism of *TBX1* mutations with loss of transcriptional activity due to aggregation of the mutant protein. Submicroscopic chromosomal imbalances detectable by molecular karyotyping seem to play a major role in patients with ToF and mental retardation, although total numbers were small and comparison between groups had low power. Results from cardiovascular phenotype–genotype correlation of our study may be used to perform focused testing in high risk cohorts defined by prenatal or postnatal echocardiography. AVSD associated with ToF is very suggestive of trisomy 21 and almost certainly excludes 22q11.2 deletion. Our postnatal study could not confirm previous prenatal observations—that SAA are frequently associated with trisomy 21.[63 64] SAA and obstruction of the proximal left or right PA at the insertion of the ductus arteriosus as well as hypoplastic central PAs are associated with an increased incidence of 22q11.2 deletion. While it is probably impossible to discriminate the latter patients by fetal echocardiography, there is increasing experience with the prenatal diagnosis of aortic arch anomalies and SAA,[63 64] which may be helpful to recognise those fetuses at risk for 22q11.2 deletion.

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Competing interests None.

Ethics approval This study was conducted with the approval of the ethical committees of the medical faculties of Erlangen and Tuebingen.

Patient consent Obtained.

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