Detection of chromosomal abnormalities and the 22q11 microdeletion in fetuses with congenital heart defects

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Abstract. Chromosomal abnormalities and the 22q11 microdeletion are implicated in congenital heart defects (CHDs). This study was designed to detect these abnormalities in fetuses and determine the effect of genetic factors on CHD etiology. Between January 2010 and December 2011, 113 fetuses with CHD treated at the Beijing Obstetrics and Gynecology Hospital were investigated, using chromosome karyotyping of either amniotic fluid cell or umbilical cord blood cell samples. Fetuses with a normal result were then investigated for the 22q11 microdeletion by fluorescence *in situ* hybridization. Of the 113 patients, 12 (10.6%) exhibited chromosomal abnormalities, while 6 (5.3%) of the remaining 101 cases presented with a 22q11 microdeletion. The incidence of chromosomal abnormalities was significantly higher in the group of fetuses presenting with extracardiac malformations in addition to CHD (P<0.001), although the detection of the 22q11 microdeletion was not significantly different between the two groups (P=0.583). In addition, all fetuses with the 22q11 microdeletion occurred *de novo*. In conclusion, genetic factors are important in the etiology of CHD. Where fetuses present with cardiac defects, additional chromosomal analysis is required to detect extracardiac abnormalities. Fetuses with heart defects should also be considered for 22q11 microdeletion detection to evaluate fetal prognosis, particularly prior to surgery.

Introduction

Congenital heart defects (CHDs) are among the most common malformations in live-born infants. Complicated structural and functional cardiovascular fetal malformations have high rates of mortality and morbidity, and may result in fetal, neonatal or adolescent fatality, stillbirth, preterm labor or

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disability. Studies in the USA and China have reported that the prevalence of CHD is 4-8 in every 1,000 live births (1-3). This may be as high as 19-74 in every 1,000 live births when particular minor malformations are included, such as bicuspid aortic valves (4,5). The number of reported cases of CHD in China is 1.98-13.8 per 1,000 live births (6) and in large areas of China, CHDs are the most common birth defects (7). However, these results have been obtained from epidemiological studies within small areas and this area of investigation lacks systematic nation-wide epidemiological research.

CHDs occur due to a number of factors; 90% of CHDs result from multifactorial disorders, 8% from single gene disorders and 2% from environmental teratogens (8). The current predominant method of correcting CHD is through cardiac surgery. The development of techniques and equipment has enabled doctors to execute more complicated heart operations and surgery is performed earlier; where as it used to be conducted in childhood, it is now carried out at the neonatal and even the prenatal stage. Early diagnosis allows surgeons to pay close attention to the classification and severity of the cardiac malformations and to plan treatment accordingly. Notably, 20-45% children with CHDs also present with extracardiac defects (9,10), which may be caused by genetic factors (11-13). CHDs are commonly observed in fetuses or neonates with chromosomal abnormalities, such as trisomy 21, trisomy 18, trisomy 13, chromosome 4P⁻ syndrome, Cri-du-chat syndrome and DiGeorge syndrome.

22q11 microdeletion syndrome may present as DiGeorge syndrome or velocardiofacial syndrome (also termed conotruncal anomaly face syndrome), and the prevalence is ~1 in 4,000-6,000 live births (14,15). The syndrome is caused by a microdeletion on chromosome 22 at the q11.2 band. The majority of affected individuals have an identical 3 megabase deletion, which encompasses a region containing 30-40 genes. 22q11 microdeletion is commonly accompanied by CHD (75-100% cases), immunodeficiency (~80% cases), neonatal hypocalcemia (49-60% cases), palate anomalies (81% cases, in particular, submucous cleft palate), renal and skeletal anomalies, feeding disorders, growth retardation, speech and language disabilities, and behavioral and psychiatric disorders (14,16-18).

As heart surgery may not improve the prognosis of CHD fetuses with additional problems, it is important to evaluate the requirement for cardiac surgery taking into account the results of genetic screening, and to explore other options, such as termination. Accurate and prompt genetic diagnosis of a fetus with CHDs may aid parents in making such a decision. In the majority

of countries, chromosome analysis and 22q11 microdeletion detection are commonly investigated in children or adolescents, but are not routinely used on fetuses with CHDs, therefore chromosomal abnormalities may not be detected. A study by Zyblewski et al (19) found that, compared with the severity of the heart defect, the influence of fetal chromosomal abnormality was more significant on the parental decision to terminate the pregnancy or provide special postnatal nursing (20). In China, >50,000 CHD surgeries are performed annually (21). Among these, ~50% of operations are conducted on infants aged 1-2 years. Cardiac surgery is currently following a trend towards operating on younger patients, thus the proportion of surgical procedures performed on newborns is increasing. Appropriate genetic tests are hypothesized to become an important method of diagnosis and vital in evaluating fetal prognosis.

The etiology of CHD may correspond to the area of residence and ethnicity of the patient due to the multiple factors involved in development of the disease. Beijing Obstetrics and Gynecology Hospital (Beijing, China) is the biggest specialist hospital in Obstetrics and Gynecology in the North of China and thus may provide important information regarding the detection of CHD in a large area. The aim of the present study was to analyze chromosomal abnormalities and 22q11 microdeletions in fetuses with CHD, in order to evaluate fetal prognosis, and to inform the parents and the cardiac surgeons of the result.

Materials and methods

Study group. The study was conducted between January 2010 and December 2011. Subsequent to obtaining written informed consent, all 113 pregnant females with fetuses with heart malformations detected by routine prenatal diagnostic ultrasound screening that consented to further investigation were enrolled in the study group in the Beijing Obstetrics and Gynecology Hospital (Beijing, China); there were no exclusions. Fetal samples were collected from either amniotic fluid or umbilical cord blood according to gestational age: Amniotic fluid cells were collected from patients with gestational age 16-23 weeks and cord blood cells were collected from patients with gestational age 24-35 weeks. G-banding chromosome karyotyping was performed. The 101 cases with apparently normal chromosomes were then further investigated for the 22q11 microdeletion following the obtention of written informed consent a second time. The parents of the 22q11 microdeletion-positive fetuses were then investigated themselves for the same microdeletion. The study was approved by the Beijing Obstetrics and Gynecology Hospital Medical Ethics Committee of Capital Medical University (Approval ID: Ky200912).

Echocardiographic methods. The cardiac defects of all 113 fetuses were diagnosed by certified ultrasound specialists in the prenatal diagnostic center using ultrasound equipment (Philips iU22, GE V730 and Voluson E8; Philips, Amsterdam, The Netherlands). The cardiovascular malformations were screened by a two-dimensional color Doppler appliance (Philips iU22, Amsterdam, The Netherlands) and then confirmed by fetal echocardiography (GE V730, GE, Fairfield, CT, USA). The types of CHD detected were grouped according to 'Fetal heart screening guidelines' (22). Real-time examination included four-chamber, left- and right- heart, long-axis,

short-axis, aortic arch and arterial duct views. The different types of cardiac abnormalities were recorded.

Cytogenetic methods. Following genetic consultation and informed consent, amniotic fluid cells were collected from the 48 cases with a gestational age of 16-23 weeks. In addition, cord blood cells were collected from the remaining 65 cases with a gestational age of 24-35 weeks. Chromosomal analysis was conducted using standard methods, including cell culturing, harvesting and histology (23). G-banded metaphase chromosomes were screened at 500-550-band level. The chromosomes were analyzed and karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN 1995; Fig. 1) and the results were recorded.

Fluorescence in situ hybridization (FISH) methods. FISH analysis of samples collected from the 101 pregnant females whose fetus had normal chromosomes and the 12 parents whose fetus exhibited the 22q11 microdeletion was performed. The analysis was performed on metaphase chromosome spreads using a domestically manufactured probe (Beijing Jinpujia Medical Technology Co., Ltd., Beijing, China), which maps to the TUPLE1 region (22q11.2, spectrum red), combined with the ARSA region (22q11.3, spectrum green) as a control probe (24,25).

FISH analysis was conducted as follows: Following extraction, 5-10 ml amniotic fluid sample was centrifuged at 900 x g for 8 min, and the supernatant was removed and discarded. The pelleted cells were incubated in 5 ml hypotonic 0.075 mol/l KCl solution at 37°C for 12 min and then 2 ml Carnoy's fixative (methanol:acetic acid=3:1) was added. The cells were then pelleted by centrifugation at 250 x g for 10 minutes, and fixed twice for 8 min with Carnoy's fixative at room temperature. Fresh Carnoy's fixative was added to adjust the final volume to provide an optimal cell concentration for cell spreading and mixing when the cell suspension was dripped onto a microscope slide. For hybridization, the prepared slides were rinsed twice with 2X saline-sodium citrate (SSC; pH 7.0) for 5 min at room temperature, treated with 0.1 mol/l HCl for 5 min, then incubated with pepsin in 0.01 mol/l HCl at 37°C for 12 min. The slides were rinsed again with 2X SSC for 5 min at room temperature, then dehydrated with a series of ethanol dilutions at 70, 85 and 100% in sequence, air-dried and heated to 56°C prior to hybridization. The probe mixture (containing 2 ml probe, 7 ml hybridizing buffer and 1 ml deionized water), was denatured at 76°C for 5 min. The slides were denatured separately in 70% formamide/2X SSC at 76°C for 5 min and then dehydrated with -20°C precooled ethanol at 70, 85 and 100% in sequence and air-dried. The denatured probe mixture was dropped onto the cell smear on each prepared slide, covered with a cover slip and sealed with sealing glue. The hybridization was performed in a wet chamber at 42°C overnight. On the second day, with the cover slip removed, subsequent to washing three times with 50% formamide/2X SSC at 46°C for 10 min, 2X SSC for 10 min and 2X SSC/0.1% NP-40 for 5 min, the air-dried slides were restained with 15 μl 4',6-diamidino-2-phenylindole dihydrochloride for 10-20 min prior to analysis.

The slides were observed with a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan); the filters used were MBE44720, MBE45600 and MBE41300. For each specimen,
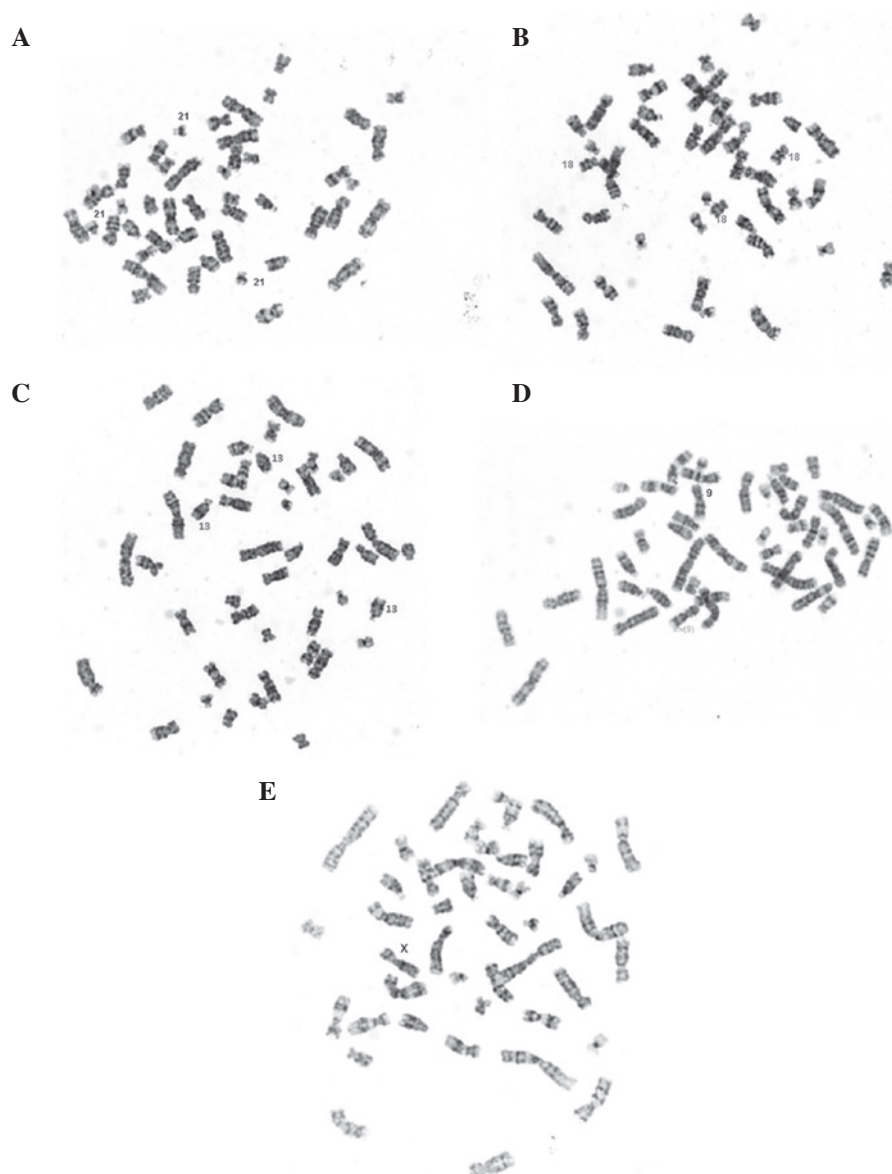
Figure 1. Examples of karyotyping. (A) Trisomy 21 karyotyping (original magnification, x1,000). (B) Trisomy 18 karyotyping (original magnification, x1,000). (C) Trisomy 13 karyotyping (original magnification, x1,000). (D) Inversion 9 karyotyping (original magnification, x1,000). (E) 45, X karyotyping (original magnification, x1,000).
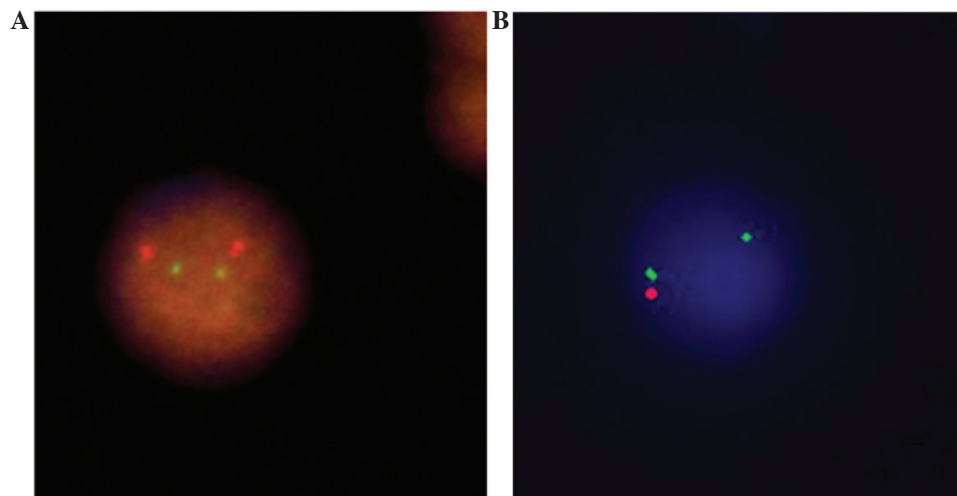

Figure 2. Fluorescence *in situ* hybidisation using a TUPLE1 probe to the 22q11.2 DNA fragment. (A) Two red and two green signals indicate a normal cell (original magnification, x1,000). (B) One red and two green signals signify a microdeletion of the TUPLE1 gene (original magnification, x1,000).

Table I. Clinical data for 113 pregnant females carrying fetuses with CHD.

Factor	Parameter
Age, mean±SD (range)	30.73±4.59 (20-47)
Gestational weeks, mean±SD (range)	25.12±4.37 (16-35)
Parity, n (%)	
Primipara	98 (86.7)
Pluripara	15 (13.3)
Gender of fetus, n (%)	
Male	67 (59.3)
Female	42 (37.2)
Unknown	4 (3.5)

CHD, congenital heart defect; SD, standard deviation.

Table II. Classification of CHDs in 113 fetuses.

Types of CHD	n	%
Non-cyanotic		
Ventricular septal defect	47	41.6
Atrial septal defect	11	9.7
Pulmonary stenosis	7	6.2
Pulmonary valve stenosis	4	3.5
Aortic stenosis	2	1.8
Aortic valve stenosis	2	1.8
Tricuspid valve dysplasia	3	2.7
Aortic widened	2	1.8
Major blood vessel abnormality	2	1.8
Acleistocardia	1	0.9
Cyanotic		
Tetralogy of Fallot	17	15.0
Endocardial cushion defect	10	8.0
Transposition of the great arteries	5	4.4
Uniatrial heart	4	3.5
Univentricular heart	4	3.5
Hypoplastic left heart syndrome	4	3.5
Truncus arteriosus	3	2.7
Double outlet right ventricle	2	1.8
Hypoplastic right heart syndrome	2	1.8
Total number of fetuses affected	113	

Fetuses with more than one type of cardiac defect were counted separately. The % data was determined by the total number of fetuses (113), not by the number of defects (132). CHD, congenital heart defect.

at least 50 nuclei were evaluated. If 90% cells detected were normal, the specimen was classified as normal (Fig. 2A). If 60% cells were abnormal, the specimen was diagnosed as affected

Table III. Chromosome analysis and 22q11 microdeletion results of each cardiac defect.

Type of CHD	Chromosomal abnormality		22q11 microdeletion (n)
	Type	n	
VSD	Trisomy 21	2	2
	Trisomy 18	4	
	Trisomy 13	1	
	Inv (9)	1	
	45, X	1	
PS	Trisomy 21	1	
AW	Trisomy 21	1	1
TOF	Trisomy 18	1	2
ECD	Trisomy 21	1	1
DORV			1
TGA			1
UA			2
UV			2
Total number of fetuses affected	12 (10.6%)		6 (5.3%)

Fetuses with more than one type of cardiac defect were counted separately. The data were determined by the total number of fetuses (113), not the number of defects. CHD, congenital heart defect; VSD, ventricular septal defect; PS, pulmonary stenosis; AW, aortic widened; TOF, tetralogy of Fallot; ECD, endocardial cushion defect; DORV, double outlet right ventricle; TGA, transposition of the great arteries; UA, uniatrial heart; UV, univentricular heart.

(Fig. 2B). In cases where there was any doubt, the number of cells evaluated increased to 100 cells and results were reported as uninformative if the above criteria were not met.

Statistical analysis. The data are presented as the mean ± standard deviation for continuous variables. Differences in categorical variables were measured using the χ^2 test. Analyses were performed with SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the study group. A total of 113 fetuses with major structural CHD were identified at 16 to 35 (25.12±4.37) weeks gestation. The age of the pregnant females ranged between 20 and 47 years (mean, 30.73±4.59). A total of 98 of the pregnant females (86.7%) were primipara and the remaining 15 pregnant females were pluripara (13.3%). A total of 67 fetuses were identified as male and 42 female, while the genders of the remaining 4 were unknown, since terminations of the pregnancies occurred at an early gestational stage. The basic clinical data are listed in Table I.

Table IV. Genetic testing of fetuses with CHD for extracardiac abnormalities.

Extracardiac abnormalities	Cases (n) (% of group)	Chromosomal abnormalities (n) (% of group)	22q11 microdeletion (n) (% of group)
With	15 (13.2)	8 (53.3)	1 (6.7)
Without	98 (86.8)	4 (4.1)	5 (5.1)
Total	113 (100)	12 (10.6)	6 (5.3)
P value		<0.001	0.583

CHD, congenital heart defect.

Ultrasound characteristics of study group. The classification of CHDs is determined by the International Paediatric and Congenital Cardiac Code. This divides all types of CHD into 10 categories and 23 subcategories according to a multi-dimensional approach encompassing anatomic, diagnostic and therapeutic criteria (26). Of the fetuses with non-cyanotic cardiac defects, 47 cases (41.6% of the whole group) of ventricular septal defects (VSD) were identified, followed by 11 cases (9.7%) with atrial septal defects (ASD), seven cases (6.2%) with pulmonary stenosis, four cases (3.5%) with pulmonary valve stenosis, two cases with aortic stenosis, two cases with aortic valve stenosis, three cases with tricuspid valve dysplasia, two cases with aortic widening, two cases with major blood vessel abnormality and one case with acleistocardia.

Of the fetuses with cyanotic cardiac defects, 17 cases (15.0%) with tetralogy of Fallot (TOF) were detected, followed by 10 cases (8%) with endocardial cushion defects, five cases (4.4%) with transposition of the great arteries, four cases (3.5%) with a uniatrial heart, four cases (3.5%) with a univentricular heart, four cases (3.5%) with hypoplastic left heart syndrome, three cases with truncus arteriosus, two cases with a double outlet right ventricle and two cases with hypoplastic right heart syndrome. The types and numbers of CHDs are listed in Table II.

CHD type and chromosomal abnormalities in the study group. The associations among CHD type, the incidence of chromosomal abnormalities and the 22q11 microdeletion are shown in Table III. Twelve patients (10.6%) had chromosomal abnormalities and 6 patients (5.3%) exhibited the classic 22q11 microdeletion.

Table IV reveals the number of the CHD cases that presented with extracardiac abnormalities. The incidence of chromosomal abnormalities was significantly higher in the CHD with extracardiac abnormalities group, than in the CHD without extracardiac abnormalities group (P<0.001); however, the difference in the incidence of the 22q11 microdeletion was not significant between these two groups (P=0.583). The parents of the six fetuses with the 22q11 microdeletion did not present with the same microdeletion.

Discussion

The present study was conducted in order to investigate chromosomal abnormalities and the 22q11 microdeletion in CHD fetuses in a Chinese population. The genetic results revealed 12 patients (10.6%) had chromosomal abnormalities and 6 patients (5.3%) exhibited the classic 22q11 microdeletion. Altogether, ~1 in 6 (18/113) of the CHD cases may be explained by genetic factors. Therefore, genetic factors are likely to be important in CHD etiology.

In total, of the 15 CHD cases exhibiting extracardiac anomalies, eight (53.3%) had chromosomal abnormalities, while among the remaining 98 CHD cases without extracardiac defects, only four (4.1%) exhibited chromosomal abnormalities (P<0.001), suggesting that CHD fetuses with extracardiac defects are more likely to have chromosomal abnormalities than those fetuses with cardiac defects only. Of the 113 CHD cases, six (5.3%) were identified to have the 22q11 microdeletion. Among the 15 CHD cases with extracardiac defects, only one (6.7%) was found to have the 22q11 microdeletion, while in the remaining 98 cases without extracardiac defects, five (5.1%) exhibited the 22q11 microdeletion; the difference between the two groups was not significant (P=0.583). This finding is consistent with the results of studies conducted by Hartman *et al* (27) and Lammer *et al* (28), but contradicts those of studies by Fokstuen *et al* (29) and Borgmann *et al* (30), who observed that all cases of CHD caused by the 22q11 microdeletion presented with extracardiac defects. However, these authors analyzed unwell infants with CHD, whose extracardiac anomalies were comparatively easy to diagnose; the present study focused on fetuses in the second and third trimesters. Considering the difficulty in ultrasound diagnosis of typical fetal structural malformations, including palate anomalies (particularly submucous cleft palates), renal anomalies and skeletal anomalies, it is difficult to detect all abnormalities during routine ultrasound screening. Song *et al* (31) demonstrated that as many as 46.4% of fetal abnormalities are not identified prior to birth. The present study is also not in accordance with the study by Bellucco *et al* (32), who failed to detect any 22q11.2 deletions. This may be associated with the smaller sample size in that study and ethnic differences in the study populations.

Isolated heart defects, such as ASD, VSD and TOF, are rectified quite well by surgery following birth, with good prognosis. However, an infirm infant with genetic abnormalities commonly presents with a complicated clinical syndrome, with intelligence defects, immunodeficiency and endocrinology abnormalities, as well as behavioral and psychiatric disorders, and the prognosis may not be markedly improved by

cardiac surgery alone. In the present study, among 47 fetuses with VSDs, 11 (23.4%) exhibited genetic abnormalities, while in 17 fetuses with TOF, three (17.6%) had genetic abnormalities. Thus, CHDs accompanied by extracardiac defects should become an indicator of the requirement for chromosome analysis, while fetuses with cardiac defects alone should be an indicator for investigating for the presence of the 22q11 microdeletion during pregnancy. With this extra information, parents are fully informed and consulted as to whether the fetuses will undergo surgery, particularly intrauterine operations; and can make an informed decision regarding termination.

In the present study, the 12 parents of the six fetuses (5.3%) with the 22q11 microdeletion exhibited negative results for the same microdeletion, suggesting that these six cases occurred *de novo*; this is concurrent with previous reports demonstrating that ~90% microdeletion cases occurred *de novo*, with no known family history (14). Therefore, routine screening of the parents is not required to detect fetuses that may have this microdeleiton. In the future, the use of novel technology (e.g. chromosomal microarray) may allow more sensitive detection of abnormalities and thus may increase the identified contribution of chromosomal abnormalities further.

The sample size in the present study was relatively small, thus, further studies are required to determine the exact frequency of chromosomal abnormalities and microdeletions, since the etiology of cardiac defects may vary in different ethnic groups.

In conclusion, CHD is currently the most common birth defect in numerous areas of the world, including China, and genetic factors are important in the etiology. As such abnormalities are not rectified without surgery, it is essential to identify the genetic factors involved, including chromosomal abnormalities and the 22q11 microdeletion, a process essential in determining fetal prognosis. These tests provide information allowing appropriate prenatal consultation and assessment of the recurrent risk.

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