Analysis of gene copy number variations in patients with congenital heart disease using multiplex ligation-dependent probe amplification

Objective: At the molecular and cellular levels, heart development entails the precise orchestration of genetic events such as the interplay of master transcriptional regulators, signaling pathways, and chromatin remodeling. Recent studies among patients with congenital heart disease (CHD) have shown the importance of recurrent copy number variations (CNVs) in a significant number of patients. Recurrent CNVs that span several genes may affect other important organs, besides the heart. Because CHD may be the first presenting symptom in such patients, the analysis of recurrent CNVs in the genomic regions containing genes associated with CHD in patients referring to cardiology clinics may lead to an early diagnosis and the treatment of extracardiac symptoms in these patients. In this study, we aimed to screen CNVs in the genomic regions including GATA4, NKX2-5, TBX5, BMP4, and CRELD1 genes and to analyse the 22q11.2 chromosomal region in apparently nonsyndromic patients with cardiac septal defects.

Methods: Genomic regions including GATA4, NKX2-5, TBX5, BMP4, and CRELD1 genes and the 22q11.2 chromosomal region were analyzed in apparently nonsyndromic 45 patients with cardiac septal defects using the MLPA P-311 A2 Congenital Heart Disease kit. Multiplex ligation-dependent probe amplification (MLPA) is an established technique for the detection of known CNVs. MLPA is substantially less expensive than array CGH and is relatively simple to use for clinicians without specific expertise in genomic technology; thus, MLPA could be used as a first-tier screening assay.

Results: We screened 45 patients with cardiac septal defects for CNVs using the MLPA P-311 A2 kit. We identified three CNVs (n=3/45, 6.66%) and three 22q11 deletions. The CNVs were confirmed using fluorescence in situ hybridization.

Conclusion: Our study confirmed that the analysis of recurrent CNVs using the MLPA assay within pediatric cardiology clinics can lead to an early syndrome diagnosis in nonsyndromic patients with CHD. (Anatol J Cardiol 2018; 20: 9-15)

Keywords: congenital heart disease, cardiac septal defect, multiplex ligation-dependent probe amplification, copy number variant, 22q11 deletion syndrome

Introduction

Congenital heart disease (CHD) refers to structural or functional heart defects that arise before birth (1, 2). It affects 19-75 of 1000 live births (3). CHD is the leading non-infectious cause of mortality in newborns. Despite its clinical importance, little is known regarding the etiopathogenesis of CHD. The major cause of CHD is thought to be mutations in the regulators that play roles in heart development during embryogenesis (4) however epidemiologic data have also suggested a role of environmental factors (5). Recent studies among patients with CHD have shown the importance of recurrent copy number variations (CNVs) in a significant number of patients. Recurrent CNVs that span several genes may affect other important organs, besides the heart. Because CHD may be the first presenting symptom in these patients, the analysis of recurrent CNVs in the genomic regions containing genes associated with CHD in patients referring to cardiology clinics may lead to an early diagnosis and the treatment of extracardiac symptoms in these patients (6). MLPA is an established technique for the detection of known CNVs. The advantage of the MLPA assay over array CGH is that it is relatively simple to use for clinicians without specific expertise in genomic technology and its cost is substantially less than that of array CGH. In this study, we aimed to screen CNVs in 45 apparently nonsyndromic patients with cardiac septal defects using the MLPA assay.
Methods

Forty-five apparently nonsyndromic patients with CHD diagnosed with atrial septal defect (ASD), ventricular septal defect (VSD), and atrioventricular septal defect (AVSD) at the Pediatric Cardiology Department of the Hacettepe University were included. Patients with CHD with extracardiac malformations were included, whereas those with a confirmed diagnosis of a known syndrome were excluded. Clinical phenotypes and age of patients at blood sampling were recorded (Table 1). Informed consent was obtained from the patients and their parents, and the study was approved by the Non-Invasive Clinical Researches Ethics Committee of Hacettepe University Faculty of Medicine.

After the clinical evaluation in the Pediatric Genetics Department, the MLPA assay of DNA extracted from blood samples collected from patients was performed according to the standard protocol supplied by the manufacturer (MLPA®DNADetection/Quantification Protocol, MRC-Holland, Amsterdam, The Netherlands) in Hacettepe University İhsan Doğramacı Childrens Hospital Genetics Diagnoses Laboratory. In this study, the SALSA MLPA P 311-A2 Congenital Heart Disease kit was used. Capillary electrophoresis was performed using the ABIPRISM®3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using Coffalyser MLPA software (www.mlpa.com).

The SALSA P 311-A2 MLPA Congenital Heart Disease kit contains 34 probes for GATA4, NKX2-5, TBX5, BMP4, and CRELD1
and for the chromosome region 22q11 (DiGeorge). In addition, 10 reference probes are included in this probemix, which detect several different autosomal chromosomal locations. \textit{GATA4} (seven exons) spans approximately 55.8 kb of genomic DNA and is located on chromosome 8p23; probes for each of the seven exons and two probes upstream and downstream of the gene are included in the P311-A2 probemix. \textit{NKX2-5} (two exons) spans approximately 3.2 kb of genomic DNA and is located on chromosome 5q35; two probes for each exon are included. \textit{TBX5} (10 exons) spans approximately 52.2 kb of genomic DNA and is located on chromosome 12q24; probes for eight of the ten exons are included, with two probes for exons 9 and 10. \textit{BMP4} (five exons) spans approximately 4.8 kb of genomic DNA and is located on chromosome 14q22; probes for four of the five exons are included. \textit{CRELD1} (11 exons) spans approximately 11.6 kb of genomic DNA and is located on chromosome 3p25; probes for exons 3 and 10 are included. Furthermore, this probemix contains three probes for the chromosome region 22q11 (DiGeorge).

CNVs detected in the MLPA assay were confirmed using the FISH QBiogene PDEL 5141 DiGeorge DGSCR2 (CLTD) and Vysis 32-190012 DiGeorge Region N25 probes.

**Results**

The MLPA assay detected three CNVs in three (6.6%) of 45 patients with CHD, thereby leading to early syndrome diagnoses in the three patients. Patients CHD21, CHD29 and CHD50 had

| Patient | Gender | Type of CHD | Other clinical symptoms | Age (month) |
|---------|--------|-------------|-------------------------|------------|
| 27-CHD29 | M | VSD | Growth retardation, overriding foot fingers, low-set and antevert ears, prominent nasal root and nasal bridge, bulbous nasal tip, asymmetric crying facies | 67 |
| 28-CHD30 | F | VSD+ASD | Growth retardation | 45 |
| 29-CHD32 | F | ASD | Kidney cyst | 177 |
| 30-CHD34 | F | ASD | Hypopigmentation at foot | 56 |
| 31-CHD36 | F | VSD | Growth retardation and developmental delay | 170 |
| 32-CHD37 | F | VSD+ASD | Intracranial cyst, hypothyroidism, growth retardation | 5 |
| 33-CHD38 | F | VSD+ASD | Esophage atresia | 27 |
| 34-CHD39 | M | ASD | Stuttering, developmental delay | 34 |
| 35-CHD40 | M | VSD | | 25 |
| 36-CHD41 | F | VSD | Growth retardation and developmental delay | 15 |
| 37-CHD42 | M | ASD | | 6 |
| 38-CHD43 | M | ASD | | 83 |
| 39-CHD44 | F | ASD | | 4 |
| 40-CHD45 | F | ASD | | 78 |
| 41-CHD46 | F | VSD | Micrognathia, coanal atresia | 105 |
| 42-CHD47 | F | ASD | Cleft lip, cleft palate, antevert ear | 9 |
| 43-CHD48 | M | VSD+ASD | | 77 |
| 44-CHD49 | F | VSD | Growth retardation, scoliosis, Prominent nasal root and nasal bridge | 3 |
| 45-CHD50 | F | VSD+ASD | | 82 |

<sup>1</sup>F - female; M - male  
<sup>2</sup>Age at blood sampling
For these three patients, the results of MLPA assay were confirmed using fluorescence in situ hybridization (FISH). In patients CHD21 and CHD29, 22q11.2 deletions were confirmed using the QBiogene PDEL 5141 DiGeorge DGSCR2 (CLTD) probe; however, in patient CHD50, 22q11.2 deletion was not identified using the Vysis 32-190012 DiGeorge Region N25 probe. The MLPA assay also detected three deletions and two duplications of exon 9 of CTSB. In 8p23.1 chromosomal region, patients CHD42, CHD43, and CHD50 had a deletion of exon 9 of CTSB downstream of GATA4, and patients CHD21 and CHD29 had a duplication. Deletions and duplications of exon 9 of CTSB were considered polymorphisms (Table 2).

### Table 2. Copy number variations and copy number polymorphisms identified by MLPA analysis in 45 patients with cardiac septal defects

| Patient number | Imbalance | Chromosome band | Status | Phenotype | Age |
|----------------|-----------|-----------------|--------|-----------|-----|
| CHD42          | Deletion  | 8p23.1          | CNP    | ASD       | 6 month |
| CHD43          | Deletion  | 8p23.1          | CNP    | ASD       | 7 years |
| CHD21          | Deletion  | 22q11.2         | Causative CNV | ASD | 15 years |
|                 | Duplication | 8p23.1       | CNP    | VSD       | 6 years |
| CHD29          | Deletion  | 22q11.2         | Causative CNV | VSD+ASD | 7 years |
|                 | Duplication | 8p23.1       | CNP    | VSD       | 6 years |
| CHD50          | Deletion  | 22q11.2         | Causative CNV | VSD | 7 years |
|                 | Deletion  | 8p23.1          | CNP    | VSD       | 7 years |

1Age at blood sampling.
ASD - atrial septal defect; VSD - ventricular septal defect; CNV - copy number variant; CNP - copy number polymorphism

### Patient CHD21, 22q11.2 deletion

This patient was a girl. An ASD was identified using echocardiography. At her latest examination at the age of 15 years, she had weight and height retardation by −3 standard deviations (SD). She held her head up at 1 year of age and walked at 3 years of age. Our examination revealed growth retardation and developmental delay (Table 1, Fig. 1). She had a short neck, antevert ears, separate teeth, and scoliosis. She was the second child of her non-consanguineous healthy parents. She was aged 15 years and had a brother and a sister who were alive and healthy. Speech and cognitive developments were delayed; therefore, we evaluated whether she was mentally retarded. Analysis using the P311-A2 kit (MRC-Holland) showed the presence of a deletion in 22q11.2 chromosomal region with decreased ratio (0.25-0.35) of three probes targeting the CDC45-1, GP1BB-2, and DGCR8-14 exons, respectively.

### Patient CHD29, 22q11.2 deletion

This patient was a boy, born via the C/S section after 38 gestational weeks with a birth weight of 2700 g. He was his mother’s (age, 30 years) second and twin gestation. At his 5 years of age, cardiac murmur was noticed and a VSD was identified by echocardiographic examination. He had operated at the year of 2010. At his latest examination at the age of 6 years he had weight retardation by −3 SD and length retardation by −10 SD (Table 1, Fig. 1). He had prominent nasal bridge, low-set and antevert ears, overriding foot fingers. His sister and twin brother were alive and healthy. He was the second child of non-consanguineous healthy parents. He had an aunt who had been operated for cleft lip and palate and died at 8 months. MLPA analysis of patient CHD29 showed decreased ratio (0.34–0.37) of three probes targeting the CDC45-1, GP1BB-2, and DGCR8-14 exons in 22q11.2 chromosomal region.

### Patient CHD50, 22q11.2 deletion

This patient is a girl, born at term by spontaneous vaginal delivery (NSVD) with a birth weight of 3200 g. At 4 months of age ASD+VSD was identified by echocardiographic examination and she was operated. At her latest examination at the age of 7 years she had weight and length retardation by −3SD (Table 1, Fig. 1). She had prominent nasal bridge and scoliosis. She had an inguinal hernia operation at the age of 5 years. Her brother and sister were alive and healthy. She was the second child of her consanguineous parents. The fathers of the grandfathers of her parents were brothers. Her parents, brother, and sister were alive and healthy. The MLPA assay showed decreased ratio (0.65–0.67) of two probes targeting the CDC45-1, and GP1BB-2 exons, respectively, located in 22q11.2 chromosomal region.

**Figure 1.** Facial characteristics of patients CHD 21, CHD 29 and CHD 50
Patients CHD42 and CHD43 were brothers. Their mother had six gestations but had three alive children. She had an intrauterine loss (at 4 months of gestation), a loss of postnatal 16 days, and a loss of postnatal 4.5 months from dilated cardiomyopathy. Her sister and brother also died from dilated cardiomyopathy. Patients CHD42 and CHD43 were clinically examined, which did not reveal developmental delay, growth retardation, and extracardiac abnormalities (Table 1).

Deletions and duplications of exon 9 of *CTSB*, located in 8p23.1 chromosomal region were considered polymorphism according to the reports of the Database of Genomic Variants and UCSC Genome Browser that reported healthy individuals with deletions or duplications of exon 9 of *CTSB*. CTSB-9 probe is located downstream of GATA4 in the P-311 A2 kit to give an idea regarding the length of the deletion in case a deletion is found in GATA4; therefore, we considered deletions and duplications of exon 9 of *CTSB* to be not pathological but polymorphisms. Consequently, we identified 22q11.2 deletions in three of apparently nonsyndromic 45 patients with congenital cardiac septal defects (n=3/45, 6.66%) using the MLPA P311 A2 Congenital Heart Disease kit (Fig. 1 and 2).

**Discussion**

CHDs are often among the first presenting symptoms in syndromes involving CHD as a part of the clinical spectrum. Recent studies among patients with CHD have shown the importance of CNVs in a significant number of patients (6, 7). In the latter report, the MLPA screening of patients with CHD led to an early syndrome diagnosis for three patients with 22q11 deletions. 22q11 deletion syndrome (22q11DS) can affect several organs and systems; therefore, it has a very wide phenotypic spectrum. Although the patients CHD21, CHD29, and CHD50 share several clinical features reported in patients with 22q11 deletions, in the absence of classical findings, such as hypocalcemia and immune deficiency, a correct diagnosis had not been made prior to the MLPA assay. 22q11DS is the most common microdeletion syndrome in humans, although it is likely to be more prevalent and under recognized because of its inherent clinical variability and heterogeneity (8). Most studies have reported a prevalence of one in every 4,000 newborns; however, population-based estimates of the incidence and prevalence of 22q11DS differ (9). It is believed that this number is low because of its underdiagnosis. Consistent with this familial occurrence is the most frequent cause of diagnosis in adults at genetic centers (10).

22q11DS presents with a very wide phenotypic spectrum, including facial dysmorphisms, congenital cardiac defects, velopharyngeal insufficiency with or without cleft palate, thymic hypoplasia, developmental delay, learning disabilities, psychiatric disorders, renal, ocular and skeletal malformations, hearing loss, and laryngeal abnormalities. In the latter report, the patient CHD21 was a 15-year-old girl. An ASD was identified using echocardiography. At her latest examination, she had weight and height retardation by −3 SD. The patient CHD29 was a 6-year-old boy. A VSD was identified using echocardiography. He had weight retardation by −3 SD and height retardation by −10 SD. The patient CHD50 was a 7-year-old girl. ASD+VSD was identified using echocardiography. She had weight and height retardation by −3 SD. CHDs are present in approximately 80% of patients with 22q11DS, and 22q11 deletion is responsible for the...
etopathogenesis in approximately 5% of CHD cases. Although conotruncal and aortic arch defects are the most typical cardiac malformations associated with 22q11DS, nonconotruncal defects such as VSDs, ASDs, and AVSDs have also been reported (11-13). 22q11DS patients usually have mild facial features, and careful physical examinations using anthropometric measures are very important (14, 15).

The fascial characteristics of individuals with 22q11DS are a long face; malar flattening; hypertelorism; short palpebral features; a wide and prominent nasal root and nasal bridge; a bulbous nasal tip; micrognathia; a small mouth; asymmetric facial movements; and malformed, small, low-set ears (16). The patient CHD21 had antevert ears, separate teeth, and a long palpebral fissure. The patient CHD29 had low-set and antevert ears, prominent nasal root and nasal bridge, bulbous nasal tip, and asymmetric crying facies. The patient CHD50 had a prominent nasal root and nasal bridge consistent with 22q11DS.

Congenital cardiac defects associated with neonatal hypercalcemia are the most frequent features that lead to diagnosis in the first 2 years of age. In patients aged >2 years, the most common symptoms leading to diagnosis are neuropsychological manifestations, otorhinolaryngologic manifestations, and typical facial findings (8, 10, 14, 17, 18). The neurocognitive profile of 22q11DS is highly variable. Borderline intellectual function (IQ of approximately 70–75) is the most common intellectual disability in these patients. Attention difficulties, visual spatial abnormalities, and impaired executive function are also common. Most children achieve higher scores in verbal tasks than in non-verbal tasks. Learning difficulties are very common in preschool- and primary school-going children (14, 17, 18). Psychiatric problems, including attention deficit/hyperactivity disorders, anxiety disorders, depression, and autism spectrum disorders, have also been described in children and adolescents (19). In the latter report, the patient CHD21 had held her head at the age of 1 year and walked at the age of 3 years. Our examination revealed developmental delay. Speech and cognitive development was also delayed; therefore, we evaluated whether she was mentally retarded. She had attention deficit disorder and had difficulties passing her examinations in school. The patients CHD29 and CHD50 yet cope with school with no sign of failure.

22q11DS patients have an increased risk of spinal deformities, such as cervical spinal abnormalities and scoliosis (8, 20). Consistent with 22q11DS, the patients CHD21 and CHD50 had scoliosis. Renal anomalies, such as renal agenesis, dysplastic kidneys, obstructive abnormalities, and vesicoureteric reflux, are frequent in 22q11DS patients (8, 17). The patient CHD50 had renal pelvis dilatation.

Multiplex ligation-dependent probe amplification (MLPA) is an established technique for the detection of known CNVs. The cost of MLPA is less than that of array CGH, and it is simple to use with no specific expertise in genomic technology. Some recent studies investigated the etiopathogenesis of CHD using array CGH and MLPA. A study of Lu et al reported a 21.8% detection rate among newborns with apparently isolated and syndromic CHDs using array CGH (21). Breckpot et al. (22) reported a 19% detection rate among patients with apparently syndromic CHDs and a 3.6% detection rate among patients with isolated CHDs using array CGH. They reported that using array CGH among apparently syndromic CHD patients was more favorable (22). Erdogan et al. (7) reported a similar detection rate for isolated and syndromic CHDs (17%) using array CGH and proposed that array CGH led to early syndrome diagnosis. Sørensen et al. (6) screened 402 patients with CHD for CNVs using the MLPA assay and identified 14 rare CNVs in 13 patients and a 3.2% detection rate. In the latter report, the MLPA assay led to an early syndrome diagnosis of three patients among 45 apparently isolated (non-syndromic) patients with cardiac septal defects. We reported a 6.6% detection rate using the P311-A2 MLPA kit and proposed that the MLPA assay could be used in pediatric cardiology clinics as a first-tier screening to detect clinically relevant CNVs and identify syndromic patients. The MLPA analysis was confirmed using FISH. FISH is the current method of choice for detecting 22q11.2 microdeletions. It is a highly accurate and reliable test; however, it is limited to a single target sequence and some atypical and the smaller deletions can be missed. As in our study, 22q11.2 deletions were confirmed for patients CHD21 and CHD29 but not for the patient CHD50 using FISH. MLPA is a cost-effective method and also can detect the smaller deletions missed by FISH.

The identification of genomic imbalances in 6.6% of the patients confirms that recurrent CNVs are associated with nonsyndromic CHD (6, 7). The early diagnosis of 22q11DS provides the best opportunity for modifying the course of illness and optimizing the patient outcomes and also for the genetic counseling of family and patients when they reach the reproductive age.

In the latter study, we did not identify CNVs in genomic regions containing GATA4, NKK2-5, TBX5, BMP4, and CRELD1. Searching for point mutations in the transcription factor genes GATA4, NKK2-5, and TBX5 among the study group can be planned for another study.

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References

1. Hoffman JI. Incidence of congenital heart disease: II. Prenatal incidence. Pediatr Cardiol 1995; 16: 155-65.
2. Hoffman JI. Incidence of congenital heart disease: I. Postnatal incidence. Pediatr Cardiol 1995; 16: 103-13.
3. Hoffman JI, Kaplan S. The incidence of congenital heart disease. J Am Coll Cardiol 2002; 39: 1890-900.
4. Pierpont ME, Basson CT, Benson DW Jr, Gelb BD, Giglia TM, Goldmuntz E, et al.; American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation 2007; 115: 3015-38.
5. Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, et al.; American Heart Association Council on Cardiovascular Disease in the Young. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation 2007; 115: 2995-3014.
6. Sørensen KM, El-Segaier M, Fernlund E, Errami A, Bouvagnet P, Nehme N, et al. Presenting symptoms in adults with the 22q11.2 deletion syndrome. Eur J Med Genet 2011; 54: 251-6.
7. Erdogan F, Larsen LA, Zhang L, Tümer Z, Tommerup N, Chen W, et al. Prevalence of 22q11 deletion in fetuses with conotruncal cardiac defects: A 6-year prospective study. J Pediatr 2001; 138: 520-4.
8. Rauch A, Hofbeck M, Leipold G, Klinge J, Trautmann U, Kirsch M, et al. Incidence and significance of 22q11.2 hemizygosity in patients with interrupted aortic arch. Am J Med Genet 1998; 78: 322-31.
9. Borgmann S, Luhmer I, Arslan-Kirchner M, Kallfelz HC, Schmidtke J. A search for chromosome 22q11.2 deletions in a series of 176 consecutively catheterized patients with congenital heart disease: no evidence for deletions in non-syndromic patients. Eur J Pediatr 1999; 158: 958-63.
10. Botto LD, May K, Fernhoff PM, Correa A, Coleman K, Rasmussen SA, et al. Defining new guidelines for screening the 22q11.2 deletion based on a clinical and dysmorphic evaluation of 194 individuals and review of the literature. Eur J Pediatr 2013; 172: 927-45.
11. Sørensen KM, El-Segaier M, Fernlund E, Errami A, Bouvagnet P, Nehme N, et al. Screening of congenital heart disease patients using multiplex ligation-dependent probe amplification: early diagnosis of syndromic patients. Am J Med Genet A 2012; 158A: 720-5.
12. Cheung EN, George SR, Andrade DM, Chow EW, Silversides CK, Bassett AS. Neonatal hypocalcemia, neonatal seizures, and intellectual disability in 22q11.2 deletion syndrome. Genet Med 2014; 16: 40-44.
13. Schneider M, Debbané M, Bassett AS, McDonald-McGinn DM, Devriendt K, Digilio MC, Goldenberg R, Habel A, et al.; International 22q11.2 Deletion Syndrome Consortium. Practical guidelines for managing patients with 22q11.2 deletion syndrome. J Pediatr 2011; 159: 332-9.
14. Monteiro FP, Vieira TP, Sgardiolli IC, Malck MC, Damiano AP, Souza J, et al. Defining new guidelines for screening the 22q11.2 deletion syndrome. Acta Paediatr 2008; 97: 1113-7.
15. Kobrynski LJ, Sullivan KE. Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes. Lancet 2007; 370: 1443-52.
16. Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scherer SW, Scher...