

CLINICAL REPORT

De novo 22q11.2 deletions and auricular findings in two Chinese patients with microtia

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Funding information

CAMS Innovation Fund for Medical Sciences, Grant/Award Number: 2021-I2M-1-052; the National Natural Science Foundation of China, Grant/Award Number: 31801046; the Key Laboratory of congenital craniofacial malformation of Chinese Academy of Medical Sciences, Grant/Award Number: 2018PT31051; the Fund of CAMS Plastic Surgery Hospital, Grant/Award Number: YS202003 and YS202036

Abstract

Background: Congenital microtia is a common craniofacial malformation resulting from both environmental and genetic factors. Recurrent chromosomal imbalances were observed in patients with microtia. The 22q11.2 deletion is one of the most common microdeletions in human beings. The cell division cycle 45 gene (*CDC45*) embedded in the proximal 22q11.2 deleted region is involved in craniofacial development. However, only a few studies have focused on the 22q11.2 deletion as genetic etiology in microtia patients and studied its associated external ear deformity characteristics in detail.

Methods: In this research, a total of 65 patients from north China with sporadic microtia were studied. Copy number variations of *CDC45* were screened using AccuCopy assay. The 22q11.2 deletion harboring *CDC45* was identified by whole-genome sequencing and targeted next-generation sequencing. A parental test was carried out to determine the origin of the deletion.

Results: *CDC45* copy number loss was identified in two patients with microtia. A set of qPCR assays demonstrated two patients carried a typical proximal 22q11.2 deletion between the low-copy repeats on chromosome 22q11.2 (LCR22A and LCR22D), encompassing *CDC45*. The 22q11.2 deletions were de novo in each patient. In-depth auricular phenotype assessment showed these two patients have a distinct concha-type ear malformation while other microtia patients have lobule-type microtia among the 65 microtia patient cohort in this study.

Conclusion: Here we present two additional Chinese microtia patients with de novo 22q11.2 proximal deletion harboring *CDC45* and further report these patients' distinct ear malformation.

KEYWORDS

22q11.2 deletion, auricular phenotype, *CDC45* gene, copy number variations, microtia

Nuo Si and Zeya Zhang contributed equally to this work.

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1 | INTRODUCTION

Microtia (MIM #600674) is a congenital malformation involving the middle and external ear. The reported incidence of microtia ranges from 0.83 to 17.4 per 10,000 live birth worldwide (Luquetti et al., 2011) and is estimated 3.06 per 10,000 in China (Deng et al., 2016). Microtia is due to the abnormal development of the first and second pharyngeal arch and the first pharyngeal cleft during the embryonic period. Clinical heterogeneous and various expressivity were observed in microtia patients. The majority of cases occur as isolated, while some cases are associated with other congenital malformation or syndrome. Therefore, different terms were used to describe microtia or related conditions, such as hemifacial macrosomia (HFM), oculoauriculovertebral spectrum (OAVS), Goldenhar syndrome (GS), etc.

The underlying etiology of microtia remains mostly unknown. Both environmental and genetic factors have been associated with microtia. Recurrent chromosomal imbalances have been implicated in OAVS patients, including those on chromosome 4 and 22 (Beleza-Meireles et al., 2014; Bragagnolo et al., 2018). On chromosome 4, the region associated with microtia is 4p16, and one of the responsible genes for ear development is *HMX1*. Copy number variations involving long-range enhancers of *HMX1/Hmx1* lead to isolated ear malformation cross-species (Si et al., 2020). However, less is known about the responsible gene for ear phenotype on chromosome 22.

Chromosome 22q11.2 deletions are among the most frequently reported chromosomal rearrangements, and it occurs in up to 1:4,000 to 6,000 live births (Botto et al., 2003). The diseases caused by chromosome 22q11.2 deletions are collectively called 22q11.2 deletion syndrome (22q11.2DS). Various dysmorphic facial features were frequently found in 22q11.2DS, including external ear abnormalities. Campbell et al. reported that 63% of patients with 22q11.2 deletions have at least one abnormal ear helix, and other subtle or remarkable ear malformations are found in 22q11.2DS patients with a percentage of 0.7% to 26%, such as protuberant, posteriorly rotated, low-set ear, ear tag, and ear pit (Campbell et al., 2018; McDonald-McGinn, 2018).

Haploinsufficiency of different genes within 22q11.2 has been thought to account for the phenotypes in various organs and systems in 22q11.2DS. However, the responsible gene for ear phenotype is not well understood. The loss of cell division cycle 45 gene (*CDC45*, MIM #603465) embedded in 22q11.2 has been reported to be related to craniofacial malformations, as in Meier-Gorlin syndrome and craniosynostosis (Miller et al., 2017; Parker et al., 2018). As one of the components in replicative helicase Cdc45-MCM2-7-GINS (CMG), *CDC45* functions in

DNA replication which is crucial in many developmental processes (Schmit & Bielinsky, 2021; Tognetti et al., 2015). We therefore speculate that the loss of *CDC45* on 22q11.2 is an important causal mutation for ear malformation. Here, we recruited 65 patients with congenital microtia from a single auricular reconstruction center in north China. The 22q11.2 deletion was screened through CNVs involving the *CDC45* gene, which is encompassed in most of the 22q11.2 deletions. The aim of this study is to detect whether *CDC45* is responsible for ear malformation in these patients, and to report the corresponding ear phenotypes.

2 | METHOD

2.1 | Patient recruitment

A total of 65 patients diagnosed with congenital microtia in the plastic surgery hospital of Chinese academy of medical sciences from 2016 to 2018 were recruited. Demographic data and clinical features of ear malformation were collected. After informed consent, 4 ml peripheral blood of each patient was collected. Parents of two patients with 22q11.2 deletion were also recruited, and their blood samples were collected for parental confirmation. Genomic DNA was extracted using the QIAamp DNA blood mini kit (Qiagen, Venlo, Netherlands).

2.2 | Copy number variation analysis of *CDC45*

Two target genomic segments within the *CDC45* gene were detected by AccuCopy assay. The method is based on a multiplex fluorescence competitive PCR and is performed as described in (Du et al., 2012). A custom-by-design Multiplex AccuCopy™ Kit (Genesky Biotechnologies Inc., Shanghai, China) was used following the manufacturer's manual. Primer sequences for *CDC45* copy number variation are available upon request. The products were sequenced on an ABI3730XL sequencer, and raw data were analyzed by GeneMapper 4.0. For each segment, sample/competitive (S/C) peak ratio was calculated and normalized to reference fragment, the median value in all samples for the segment, and then averaged. For patient 1, the *CDC45* copy number loss was confirmed by real-time qPCR assay. The primer sequences were the same as those used in the Accucopy assay. The reaction mix is prepared with SYBR Premix Ex Tag GC (Takara Bio., Dalian, PR China) following the manufacture's instruction. Reactions were performed on a Roter-Gene 6000 instrument (Qiagen, Hilden, Germany) following the procedure

as 95°C for 10 min and 40 cycles of 95 °C 10 s, 60 °C 15 s, 72 °C 20 s. The relative copy number of each segment was calculated with the delta-delta CT method.

2.3 | Next-Generation sequencing to detect the structure variation involving *CDC45* deletion

Structure variations (SVs) in the whole genome of patient 1 were detected by whole-genome sequencing (WGS). The sequencing library was prepared with the NEBNext Ultra II kit and was sequenced on an Illumina HiSeq2500 platform (Illumina, USA) in a pair-end 150 bp configuration. Generated reads were aligned to the GRCh37/hg19 human reference sequence using the Burrows-Wheeler Aligner (BWA, v.0.7.8-r455). SVs were detected using the Clipping Reveals Structure (CREST v.V0.0.1) algorithm. All NGS procedures and bioinformatics analysis were performed at the Novogene Corporation (Beijing, China). Genomic sequences on 22q11.2 of patient 2 and his parents were captured respectively using an Agilent custom-designed chip and sequenced at the CapitalBio Corporation (Beijing, China). CNVnator was used for detecting and genotyping of CNVs at the targeted 22q11.2 region with a sliding window size of 100bp. Integrative Genomics Viewer (IGV) was used for visualizing the detected CNV.

3 | RESULTS

3.1 | Screening of *CDC45* copy number variations in a microtia cohort

We screened *CDC45* copy number changes by Accucopy assay in a total of 65 patients with microtia. Among them, 42 are male, 23 are female. The ages are between one year old and 54 years old. Eight of them are bilaterally affected, while 57 of them are unilaterally affected. For unilaterally affected ears, 41 are right, and 16 are left. The analysis of the average copy number of *CDC45* showed two patients have copy number loss (Figure 1a). These two patients showed one copy in *CDC45*. The average copy number is 1.07 and 1.14, respectively, while the other 63 patients showed approximately two copies.

3.2 | Clinical features of two patients with *CDC45* copy number changes

Patient 1 is a 6-year-old boy who has a malformed, protruding and simple right ear (Figure 2a). His right ear is anteverted and has underdeveloped superior and inferior

crus of antihelix. No typical structure of triangular fossa or scaphoid fossa is observed. Abnormalities of tragus, antitragus, and earlobe are noticed as well. In addition to malformed pinna, he also has stenosis of the external auditory canal in the right ear. Facial asymmetry is noticeable with downturned corners of the mouth and anteverted nares. Thoracic CT scan, cardiac ultrasound, and abdominal ultrasound showed no apparent abnormalities. No other physical problems and family history were reported. Patient 2 is a 4-year-old boy. He had bilateral malformed outer ears, and abnormality of the pinna is consistent with patient 1. Patient 2 had postaxial polydactyly in both feet (Figure 2c–f). Doppler echocardiography showed heart defects, including atrial septal defects, patent *foramen ovale*, and patent ductus arteriosus. Parents of both patients were all phenotypically normal.

3.3 | 22q11.2 deletions embedded the *CDC45* in two patients

CDC45 deletion was confirmed in patient 1 by two qPCR assays designed within *CDC45* coding regions. Relative copy number of *CDC45* showed ~0.5 in patient 1 and ~1.0 in both parents (Figure 1b). This result indicated the hemizygous status of the *CDC45* gene and the deletion is de novo in patient 1. Further, whole-genome sequencing in Patient 1 showed the *CDC45* deletion was embedded in a 2.55-Mb gross deletion on 22q11.2. The precise proximal and distal breakpoints detected by WGS were chr22:18910000 and chr22:21459999 (hg19), respectively. The breakpoints were in the low-copy repeats (LCR) on chromosome 22q11.2, LCR22s A and D (Figure 3). Since patient 2 has a typical manifestation as 22q11DS, we use the 22q11.2-targeted high-throughput sequencing to examine the identified deletion involving *CDC45*. A ~3Mb deletion between LCR22s A and D was called in patient 2 with a 1000-bp window size (Figure 1c). In contrast, his unaffected parents do not carry the deletion (Figure 1d,e). The mean sequencing depth in patient 2 (65×) is significantly lower within the detected region than his parents' (80×). The deletion is consistent with the typical recurrent proximal 22q11.2 microdeletions in DG/VCFs between LCR22s A and D.

4 | DISCUSSION

The 22q11.2 DS has a variable phenotype and affects multiple systems. These phenotypes varied even across population groups. *Kruszka* et al found congenital heart disease and learning problems were common in most participants (greater than 60%) (*Kruszka* et al., 2017). Interestingly,

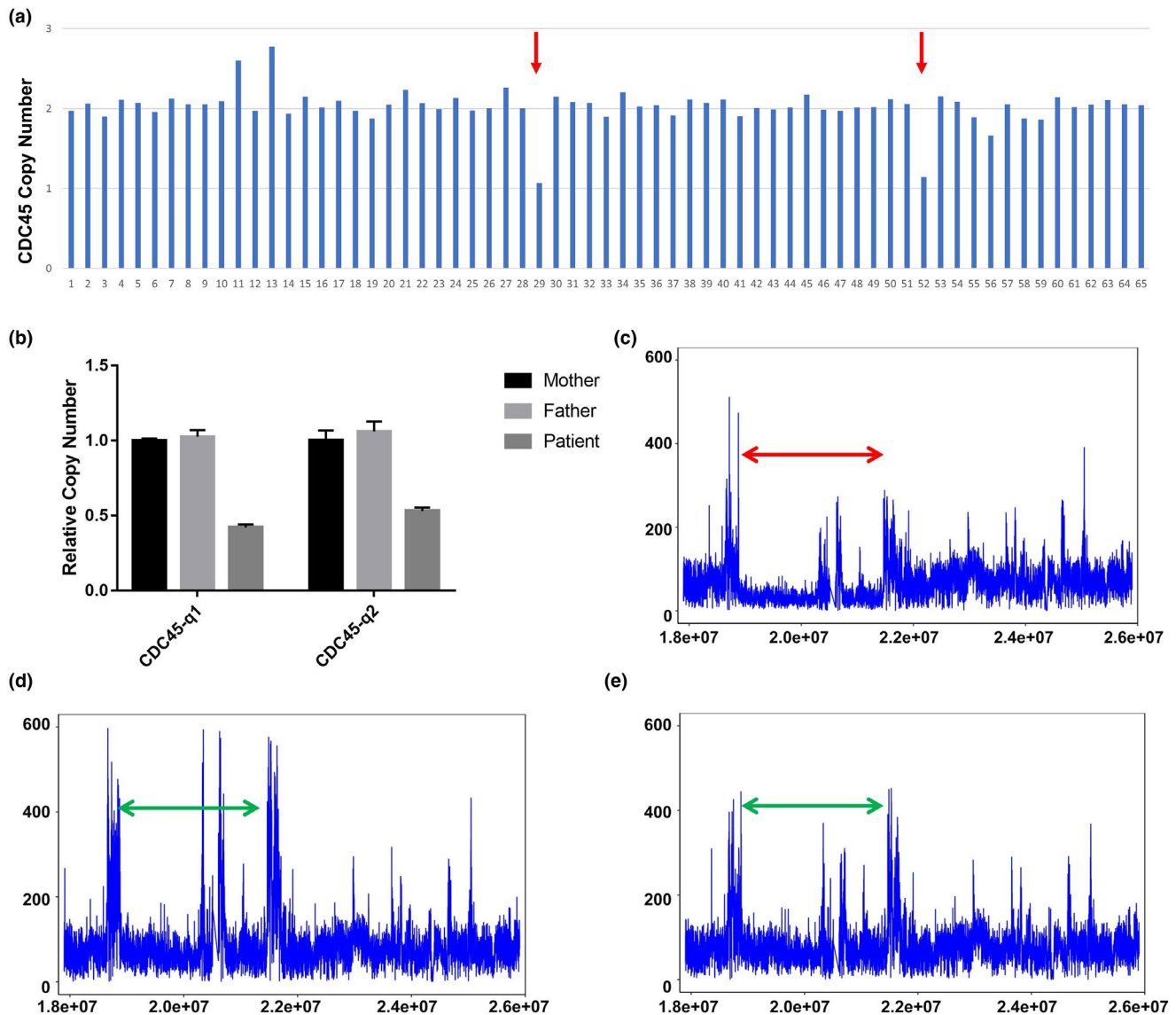


FIGURE 1 De novo 22q11.2 deletions harbor the *CDC45* gene in two patients with microtia. (a) *CDC45* copy number variation screening in 65 patients detected by Accucopy Assay. Red arrows indicate two patients have one copy of *CDC45*. (b) *CDC45* copy number loss in patient 1 confirmed by two qPCR assays. (c–e) Targeted high-throughput sequencing showed typical 22q11.21 deletion in patient 2 (c) but not in his unaffected father (d) or mother (e)

among all these clinical features, learning problems and ear anomalies were independent of the population groups. For the two reported patients in this study, we found different systemic clinical features except ear anomalies. We found heart defects in the patient 2 but not in the patient 1. Learning problems were not reported according to the patient's complaint. For patient 1, facial features (facial asymmetry, downturned corners of the mouth, and anteverted nares) were also observed, although not obvious. Besides, polydactyly was obviously noticed only in patient 2. We did not find other reported clinical features (such as narrow palpebral fissure, hooded eyelids, long face), which may be because these clinical features are not obvious or lack of standards. Other specific clinical features

such as immune deficiency and hypocalcemia are not the focus of our plastic surgery specialists.

In OAVS patients, recurrent genomic alterations in chromosome 22q were reported. Glaeser et al. reviewed the clinical characterization in 22 patients with OAVS in chromosome 22 abnormalities and reported the detailed auricular alterations in these patients (Glaeser et al., 2020). Kruszka also reported 59% (53/90) ear anomalies in 22q11.2 DS patients (Kruszka et al., 2017). These auricular alterations include preauricular tags, hearing loss, and agenesis/atresia of the external auditory canal. However, a clinical feature of pinna has not been described in detail. In both cases in the present study, distinct malformed pinna was noticed, including enlarged auricular cranial

FIGURE 2 Clinical findings in two microtia patients with 22q11.2 deletions. (a,b) Facial and auricular findings in Patient 1. (c,d) Bilateral ear malformation in patient 2. (e,f) Postaxial polydactyly of both feet in patient 2

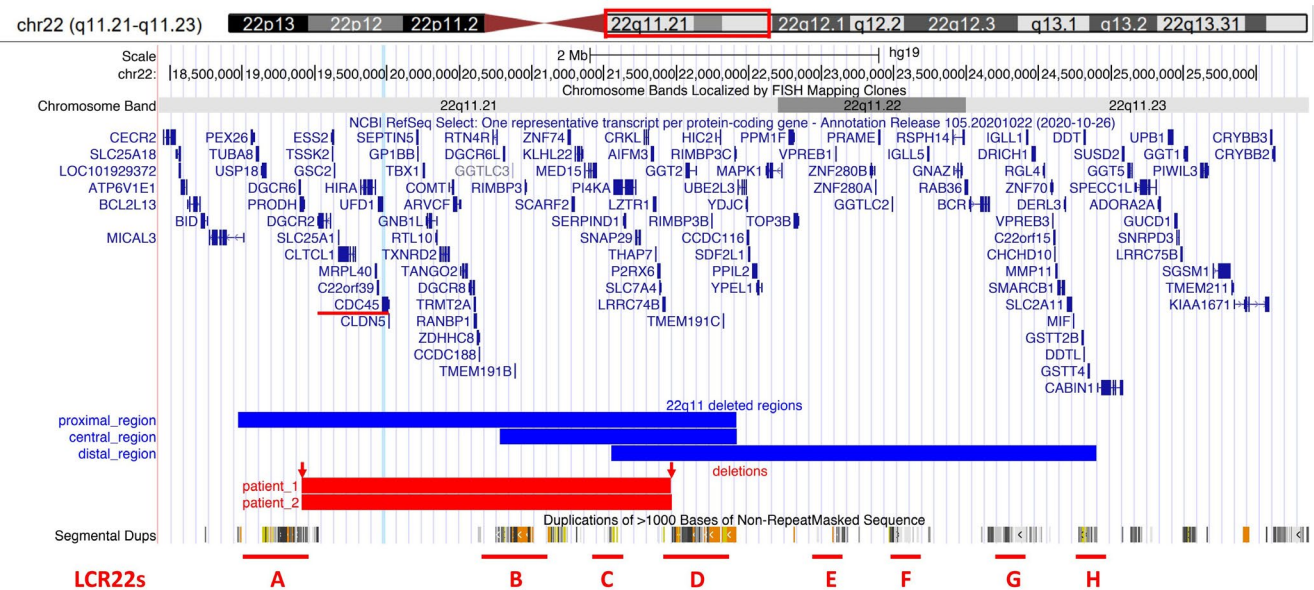


FIGURE 3 Schematic illustration of Genomic Structure of 22q11.2. Showing position of the CDC45 gene, distribution of LCR22s A-H, extend of proximal, central, and distal deletion, and detected deletion in the present study. The red arrows indicate the breakpoints in each patient

angle, lacking antihelix, triangular fossa, scapha, antitragus, and malformed tragus and ear lobe. Hemifacial macroglossia (HMF) is also suggested as one of the minimum diagnostic criteria for OAVS patients with 22q11 abnormalities (Glaeser et al., 2020). Accordingly, we observed slight facial asymmetry in patient 1 and downturned corners of the mouth and anteverted nares, although these facial features are not as apparent as the malformed pinna. Thus, chromosome 22q alteration should be examined when distinct malformed pinna in microtia patients with marked variable phenotypes are noticed.

On chromosome 22q11.2, there are eight low-copy repetitive sequences (LCR) that could mediate non-allelic homologous recombination, named LCR22A to LCR22H. Therefore, deletions, duplications, and complicated structure variations frequently occur in 22q11.2 (Shaikh et al., 2000, 2007). The phenotypes associated with 22q11.2DS are highly variable, although the underlying chromosomal deletions that cause the syndrome are almost the same in size among different patients. The most common condition of 22q11.2DS is described as DiGeorge syndrome (DGS, MIM#188400), with phenotypes of parathyroid/thymic hypoplasia and outflow tract defect of the heart. Meanwhile, physicians working on specific areas of expertise also reported different conditions due to 22q11.2 deletions, such as velocardiofacial (VCFS), conotruncal anomaly face syndrome (CTAF), Opitz G/BBB syndrome, and Cayler Cardiofacial syndrome (McDonald-McGinn, 2018). These conditions have overlapping phenotypic features, but each focuses on different manifestations in a specific area.

Constant effort in seeking responsible genes for each specific phenotype of 22q11.2DS has been made. To date, no responsible gene for auricular alteration was identified. According to the classification of 22q11.2 alteration, as proximal, central, and distal type, different candidate genes was proposed. Six OAVS patients with distal deletion at 22q11.2 between LCR22D-E present a minimal overlapping genomic region. The region encompasses three candidate genes involved in pharyngeal arches development, including *HIC2*, *YPEL1*, and *MAPK1*. However, the typical auricular phenotype in these patients is preauricular tags but not microtia (Schmit & Bielinsky, 2021). One OAVS patient with central 22q11.2 deletion between LCR22B-D was reported. For auricular phenotype, he presents severe microtia, but the central deleted region does not encompass any gene involved in pharyngeal arch development; thus, disruption of the interaction between a regulatory element and corresponding effect gene is more likely underlying the pathogenesis. The two patients in the present study have typical proximal 22q11.2 deletion. Although proximal type deletion in 22q11DS is the most common, OAVS patients with proximal deletion are rarely reported. Besides, those cases with proximal deletion were detected

by FISH using a limited probe, thus making the extent of the deletion hardly known. Previous studies mainly focused on two candidate genes within the 22q11.2 proximal region, the *TBX1* and *GSC2* (Glaeser et al., 2020). *TBX1* is closely related to retinoic acid (RA) signaling, while *GSC2* is implicated in the development of neural crest-derived structures (Glaeser et al., 2020; Guris et al., 2006). Nonetheless, no causal relationship has been established between these two genes and ear malformation due to heterogeneous phenotypes and limited cases with mutations only affecting the candidate gene.

We focused on the *CDC45* gene in the proximal region, considering its biological function and correlation with craniofacial malformation. The *CDC45* protein functions as a cofactor in the eukaryotic replicative helicase called the CMG complex (Makarova et al., 2012). It functions in the elongation of the replication process in the S-phase (Köhler et al., 2016). Disruption of the *CDC45* function causes impaired cell division during periods of rapid proliferation (Pollok et al., 2007). Causative mutations in *CDC45* were found in Meier–Gorlin syndrome (MGS), an osteodysplastic syndrome with growth retardation, microtia, and aplastic/hypoplastic patella as the core clinical findings (de Munnik et al., 2015; Fenwick et al., 2016). Biallelic *CDC45* mutations conferred varying degrees of protein dysfunction and depletion in MGS patients (Ting et al., 2020). Patients with *CDC45* mutations and 22q11.2 deletion in each allele present craniosynostosis in addition to MGS phenotype (Unolt et al., 2020). All these findings implicate *CDC45* is dosage sensitive. In the present study, we did not find copy number changes affecting *CDC45* alone in microtia patients. Thus, the haploinsufficiency of *CDC45* may not be sufficient to cause ear malformation.

Most patients with microtia cannot be attributed to a definitive cause, while tremendous variations in clinical features in 22q11.2DS makes a long diagnostic odyssey. Here, we report two additional Chinese microtia patients with 22q11.2 deletion. Although we do not have direct evidence indicating *CDC45* loss is responsible for the ear malformation in these patients, concha-type microtia was observed in two patients with 22q11.2 deletion, which distinguished them from other lobule-type microtia patients. We therefore suggest 22q11.2 deletion should be considered in the patient with microtia who presents the described ear malformation. Our genetic findings and observed phenotypes shed light on new information concerning ear abnormalities in microtia and 22q11.2DS.

ACKNOWLEDGMENTS

The authors thank all the participants in this study. This work was supported by the CAMS Innovation Fund for Medical Sciences (2021-I2 M-1-052), the Key Laboratory of congenital craniofacial malformation of Chinese Academy

of Medical Sciences (2018PT31051), the Fund of CAMS Plastic Surgery Hospital (YS202036,YS202003), and the National Natural Science Foundation of China (31801046).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the institutional research committee's ethical standards and with the 1964 Helsinki declaration.

AUTHOR CONTRIBUTIONS

NS, BP, and HJ contributed to conception and design of the study. BP, ZZ, XH, CW, and PG collected patients and performed phenotypic assessment. NS and ZZ performed the statistical analysis and genetic analysis. NS wrote the first draft of the manuscript. ZZ wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

ETHICAL COMPLIANCE

The study was approved by the institutional ethical committee of the plastic surgery hospital of Chinese Academy of Medical Sciences and performed following the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Si, N., Zhang, Z., Huang, X., Wang, C., Guo, P., Pan, B., & Jiang, H. (2022). De novo 22q11.2 deletions and auricular findings in two Chinese patients with microtia. *Molecular Genetics & Genomic Medicine*, 10, e1862. <https://doi.org/10.1002/mgg3.1862>