is the most challenging. Current treatment options include gene therapy and epidermal stem cell therapy. But the most cautious treatment strategy is mild wound treatment and limits the further development of the condition.[17] It is worth noting that patient reported in this paper had 2 redundant tissues in her oral cavity. This special structure has not yet been reported. And the authors intend to remove these 2 tissues by surgical resection at palatoplasty.

CONCLUSION

The authors report 1 extremely rare patient with AEC syndrome. Because AEC syndrome is an autosomal genetic disease that is characterized by a wide range of clinical manifestations, the appearance and development of the patients will be severely affected. Therefore, the authors must conduct a thorough examination of the patients and consult relevant departments to avoid missed diagnosis and misdiagnosis. And make an effective treatment plan, and carry on the comprehensive sequence treatment.

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OPEN

A Targeted, Next-Generation Genetic Sequencing Study on Tetralogy of Fallot, Combined With Cleft Lip and Palate

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Background: Congenital heart disease (CHD), plus cleft lip and palate (CLP) are currently the most common types of structural malformation in infants. Many genes have been investigated for their involvement in CHD with CLP. Targeted next-generation sequencing can analyze large amounts of genetic information rapidly, and thus address this question.

Methods: The authors designed a targeted, next-generation sequencing gene panel for 455 genes previously implicated in CHD or CLP. A single-subject patient served as a genetic source. Variants that affect protein-coding regions were classified into silico and filtered through databases, such as the Single-Nucleotide Polymorphism Database, Yan Huang, the Exome Sequencing Project, and the 1000 Genomes Project. The authors then predicted the function of gene mutations by PolyPhen-2, SIFT, and Mutation Taster. To confirm the related disease genes, the authors surveyed relevant literature on PubMed. Finally, the variant was verified by Sanger sequencing.

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Results: A total of 1520 mutations were successfully found in a patient using combined tetralogy of Fallot and CLP by the targeted next-generation sequencing. However, there were 6 gene mutations (*ZNF528*, *PVRL2*, methylenetetrahydrofolate reductase [*MTHFR*], *EVC2*, *DAND5*, *CCDC39*) that were not found on Single-Nucleotide Polymorphism Database, Yan Huang, Exome Sequencing Project, and 1000 Genomes Project. Four genes (*ZNF528*, *PVRL2*, *EVC2*, *CCDC39*) were all predicted to be "tolerated," "benign," or "polymorphic" by SIFT, PolyPhen-2, and Mutation Taster. The *DAND5* gene was predicted to be "possibly damaging" and "disease causing" respectively by PolyPhen-2 and Mutation Taster, but the SIFT program predicted this mutation to be "tolerated." Likewise, the *MTHFR* gene mutation was predicted to be "damaging," "possibly damaging," and "disease causing" respectively by SIFT, PolyPhen-2, and Mutation Taster. There is no relevant report about *MTHFR* gene mutation (c.G586A, p.G196S) on PubMed.

Conclusion: Using targeted, next-generation sequencing technology, the authors identified for the first time a mutation (c.G586A, p.G196S) in the *MTHFR* gene as a possible cause of TOF and CLP in a patient.

Key Words: Cleft lip and palate, targeted next-generation sequencing, tetralogy of Fallot, the *MTHFR* gene

Congenital heart diseases (CHD), cleft lip and cleft palate (CLP) comprise the highest incidence of birth defects in the world. The results of epidemiological investigation show a rising incidence of CHD and CLP year by year.[1,2] Congenital heart diseases include ventricular septal defect, atrial septal defect, tetralogy of Fallot (TOF), and so on. Tetralogy of Fallot consist of pulmonary artery stenosis, ventricular septal communication, rightward deviation of the aorta's origin, and hypertrophy of the right ventricle. However, due to the complex and long cycle of treatment procedures for CHD and CLP, these diseases can bring a heavy burden to family and society and seriously affect neonatal health. The high heritability of CHD, estimated to be between 0.6 and 0.7, suggests a strong genetic component and numerous genes, which have been linked to syndromic and nonsyndromic forms of CHD.[3] Together, the above factors drive the need to identify the disease genes responsible for CHD with CLP.

Many genes have been implicated in the development of congenital heart disease or cleft lip and palate. With the rapid development of technology, many methods also had been used to find these disease genes, such as Array-SNP, CNVs, targeted, next-generation sequencing, and whole exome sequencing. Targeted, next-generation sequencing rapidly analyzes large amounts of genetic information. In this paper, we wanted to find disease genes through targeted next-generation sequencing.

METHODS

Patient

The study protocol was approved by the Review Board at Second Xiangya Hospital of Central South University (China), and the related study subject gave informed consent. All experiments were performed in accordance with relevant guidelines and regulations. We enrolled a patient in whom we observed cardiac structure, leading to diagnosis of TOF and CLP by transthoracic echocardiogram.

DNA Extraction

Genomic DNA was extracted from peripheral blood lymphocytes of the patient. Genomic DNA was prepared for testing by DNeasy Blood and TissueKit (Qiagen, Valencia, CA) on the QIAcube automated DNA-extraction robot (Qiagen, Hilden, Germany), as previously described.[4] The quality and quantity of the DNA sample were measured by the use of the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA), from which 3 μg of DNA of each sample was used for analysis.

Targeted Next-Generation Sequencing

Targeted, next-generation sequencing (NGS), including library construction, capture, and sequencing, was carried out at Oxford Gene Technologies (Oxford, UK). Enrichment of target regions and library preparation were performed by the use of a SureSelectXT2 Custom kit (1–499 kb, 16) in accordance with SureSelect protocol (version 1.2; Agilent Technologies, Changsha, China). Library concentrations were determined using Agilent's QPCR NGS Library Quantification Kit (G4880A), with each sample at a final concentration of 10 nmol/L. A HiSeq2000 sequencer ordered the samples using TruSeq chemistry and protocols (version 3, Illumina Inc, San Diego, CA). The TOF with CLP patient was analyzed separately using all CHD or CLP patients grouped into 1 enrichment kit and sequencing run.[3]

Data Analysis and Filtering

The preliminary data analyses, including read alignment, variant calling, and annotation, were carried out by Oxford Gene Technologies. All variants that affect protein-coding regions for each sample were categorized into "novel" and "known" categories according to their presence in Single-Nucleotide Polymorphism Database (dbSNP) 137. Minor allele frequencies of all known variants were reported according to Exome Sequencing Project (ESP) or Yan Huang (YH), 1000 Genomes Project (TGP) if not present in dbSNP137. All variants were subjected to in silico analysis, which included prediction programs, such as SIFT, PolyPhen-2, and Mutation Taster.

Variant Validation

Variants warranting further investigation included novel variants, which were predicted to be "probably damaging," "disease causing," or "damaging," according to PolyPhen-2, mutation tasting, and SIFT predictions, or these variants were known to be "probably damaging" and possessed minor allele frequencies $<0.1\%$. Variants were verified by Sanger sequencing. To confirm the related disease genes, we surveyed the relevant literature on PubMed (https://www.ncbi.nlm.nih.gov/pubmed).

Polymerase Chain Reaction

Entire exons and exon–intron junctions of *MTHFR* (Refseq:NM_005957) were amplified by polymerase chain reaction (PCR, primer sequences will be provided upon request). Sequences of the PCR products were determined using an ABI 3100Genetic Analyzer. The primer sequences are as follows:

Forward primer: 5′ ATAGGTGACCAGTGGGAAGA 3′,
Reverse primer: 5′ CTGATCACTGTGTCCTGAACC 3′.

RESULTS

In our study, there were 1390 SNPs and 130 indels mutations (a total of 1520 mutations) that successfully passed the filtering criteria as identified by targeted, next-generation sequencing. In addition, there were 6 gene mutations (*ZNF528*, *PVRL2*, methylenetetrahydrofolate reductase [*MTHFR*], *EVC2*, *DAND5*, consensus coding

TABLE 1. The Results of Targeted Sequencing

Category	
The total number of mutations	1520
The number of SNP	1390
The number of missing mutations	82
The number of insertion mutations	48
Database of dbSNP	515
Database of TGP	173
Database of YH	46
Database of ESP	6

dbSNP, Single Nucleotide Polymorphism Database; ESP, Exome Sequencing Project; TGP, 1000 Genomes Project; YH, Yan Huang.

sequence [*CCDC39*]) not found in the databases used, such as dbSNP, YH, ESP, and TGP (Table 1).

Four genes (*ZNF528, PVRL2, EVC2, CCDC39*) were all predicted to be "tolerated," "benign," and/or "polymorphic" by SIFT, PolyPhen-2, and Mutation Taster. The *DAND5* gene was predicted to be "possibly damaging" and "disease causing" respectively by PolyPhen-2 and Mutation Taster, but the SIFT program predicted this mutation to be "tolerated." The *MTHFR* gene mutation was predicted to be "damaging," "possibly damaging," and "disease causing" respectively by SIFT, PolyPhen-2, and Mutation Taster (Table 2). At last, we verified the variant by Sanger sequencing (Fig. 1). There is no relevant report about *MTHFR* gene mutation (c.G586A, p.G196S) on PubMed.

DISCUSSION

With the rapid development of technology, many methods had been used to find the disease-causing genes, such as Array-SNP, CNVs, targeted, next-generation sequencing, and whole exome sequencing. Targeted, next-generation sequencing offers opportunities for genetic testing and can analyze large amounts of genetic information rapidly. We consider targeted, next-generation sequencing to be more clinically useful than whole exome sequencing, due to speeder turnaround time (reduced sequencing volume and associated data analysis), higher and more reliable coverage, plus the ability to avoid incidental findings.[3] In the last few years, many researchers have published reports on gene mutations, discovered by targeted sequencing, for many genetic diseases, including CHD and CL/P.

In this study, a total of 1520 mutations were successfully found in the patient via the targeted, next-generation sequencing. However, there were 6 genes (*ZNF528, PVRL2, MTHFR, EVC2, DAND5, CCDC39*) with mutations not found in existing databases, such as dbSNP, YH, ESP, and TGP. We predicted character of the named gene mutations through PolyPhen-2, SIFT, and Mutation Taster programs. The *ZNF528, PVRL2, EVC2, CCDC39* genes were all predicted to be "tolerated," "benign," and "polymorphic" respectively by SIFT, PolyPhen-2, and Mutation Taster. Thus, we can eliminate those genes mutations. The *DAND5* gene was predicted to be "possibly damaging" and "disease causing," respectively by PolyPhen-2 and Mutation Taster, while the SIFT program predicted *DAND5* to be "tolerated." However, there is a close relationship between the said gene and spiradenoma according to our survey of published reports. The *MTHFR* gene mutation (c.G586A, p.G196S) was predicted to be "damaging," "possibly damaging," and "disease causing" in turn, by SIFT, PolyPhen-2, and Mutation Taster. These predictions support the notion that the *MTHFR* gene variant may contribute to TOF and CLP pathogenesis. We also studied the subject's parents using Sanger sequencing, but the results were meaningless. The result should be further verified through a model organism, such as zebra fish or mouse.

The human *MTHFR* gene, consisting of 11 exons, catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for the remethylation of homocysteine to methionine, which plays a key role in neural tube and vascular defects during embryogenesis.[5] According to the Human Gene Mutation Database, 74 mutations on the *MTHFR* gene have been reported in several congenital heart diseases. So far, more than 8 genes (*JAG1, NKX2–5, GATA4, MTHFR, ZFPM2, GDF1, TBX1, GATA6*) have been implicated in TOF.[6–11] Moreover, more than 12 genes (*MSX1, MTHFR, IRF6, PVRL1, SUMO1, BMP4* in addition to the previous list) have been reported in CLP.[12–15] Among these genes, *MTHFR* is a disease gene with high-penetrance mutations.

Over the last few years, impaired *MTHFR* has been widely investigated to establish its potential role as a risk factor or marker of cardiovascular disease, neural tube defect, maxillofacial malformation, cognitive disorders, and cancer.[16–21] Abnormal folate metabolism has been previously described as a possible risk factor for TOF.[6] It has been hypothesized that genetic polymorphisms in folate-metabolizing enzymes affect global DNA methylation as well as changes the synthesis and repair of DNA. Furthermore, animal experiments have shown that disruption of the *MTHFR* gene results in decreased methylation capacity.[22] There are, however, no reports about mutations on the *MTHFR* gene in TOF with CLP.

By contrast, this study found a novel *MTHFR* mutation (c.G586A, p.G196S) located in the functional region that may affect the enzyme activities. For the first time, levels of homocysteine in a patient with TOF and CLP were shown to be affected by the mutation using targeted, next-generation sequencing, which mutation had not been described in any previous reports or databases. The physiological function of *MTHFR* gene is changed by the mutation, a defect that leads to methionine deficiency and overaccumulation of homocysteine, in turn which leads to lowered *MTHFR* enzymatic activity or lowered folate levels. As the result of reduced enzymatic activity and elevated plasma homocysteine

TABLE 2. The Results of Prediction

Genes	Chromosome	SIFT	PolyPhen 2	Mutation Taster	CCDS	Amino Acid
ZNF528	Chr19	Tolerated	Benign	Polymorphism	A598G	S200G
PVRL2	Chr19	Tolerated	Benign	Polymorphism	C214G	R72G
MTHFR	Chr1	Damaging	Probably damaging	Disease causing	G586A	G196S
EVC2	Chr4	Tolerated	Benign	Polymorphism	G2623A	V875I
DAND5	Chr19	Tolerated	Possibly damaging	Disease causing	G515A	R172Q
CCDC39	Chr3	Tolerated	Benign	Polymorphism	G1035T	R345S

CCDS, the consensus coding sequence; *MTHFR*, methylenetetrahydrofolate reductase.

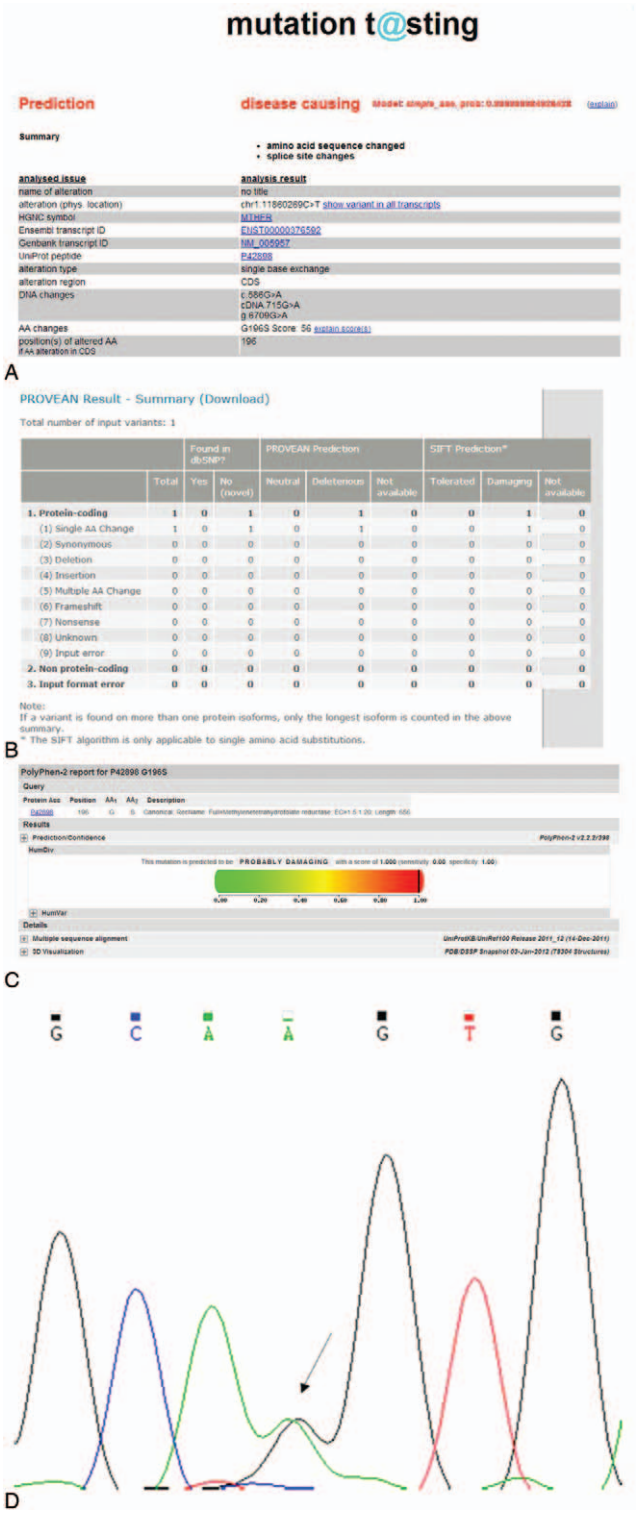

FIGURE 1. The results of prediction programs. (A) Mutation Taster prediction. (B) SIFT prediction. (C) Polyphen-2 prediction. (D) The results of Sanger sequencing.

levels, the patient may exhibit congenital malformations (tetralogy of Fallot, plus cleft lip, and palate). Our study broadens the mutation spectrum of the *MTHFR* gene and suggests that this approach will facilitate etiological elucidation of this congenital malformation, which causes TOF and CLP, by effective identification of the causative genetic mutations.

In spite of this study has found a new mutation by the new research method, but there are still some shortcomings in this study. On the one hand, the sample size is not enough. On the other hand, this research has not carried out further functional studies on the new mutation found such as zebra fish or mouse.

CONCLUSIONS

Using targeted, next-generation sequencing technology, we identified for the first time a mutation (c.G586A, p.G196S) in the *MTHFR* gene as a possible cause of TOF and CLP in a patient. Due to the complex and long cycle of treatment procedures for CHD and CLP, these diseases can bring a heavy burden to family and society and seriously affect neonatal health. It becomes urgent to reduce the birth rate of children with CHD and CLP by screening of the pathogenic genes and avoid surgical treatment by gene therapy in Chinese rural areas.

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Stereophotogrammetric Evaluation of Labial Symmetry After Surgical Treatment of a Lymphatic Malformation

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Abstract: Lymphatic malformations (LMs) are rare, nonmalignant masses, frequently involving the head and neck, potentially causing impairment to the surrounding anatomical structures. Major LMs frequently cause facial disfigurement with obvious consequences on self-esteem and social functioning. The attempt to restore symmetry is thus one of the main goals of treatment. In this study, the authors present a not-invasive method to objectively quantify the symmetry of the labial area before and after surgical treatment of a LM, affecting a 16-year-old woman. This was done with sequential three-dimensional stereophotogrammetric imaging and morphometric measurements. The method showed a high reproducibility and supplied quantitative indicators of the local degree of symmetry, helping clinicians in its objective assessment, and facilitating treatment planning and evaluation. A quantitative appraisal of the results can additionally improve patient adherence to a usually multistage therapy.

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Lymphatic malformations (LMs) are rare and nonmalignant masses, made of lymph containing vessels and chambers, which frequently involve the head and neck. Depending on their localization, they can compress or obstruct surrounding structures, thus causing different problems that can compromise facial appearance and aesthetics.[1] Treatment for macrocystic LMs is generally based on sclerotherapy, while microcystic LMs require a surgical approach based on gross debulking and/or camouflage procedures.[2,3]

Diagnosis and follow-up evaluations of craniofacial diseases can take advantage of computed tomography (CT) and magnetic resonance imaging. Unfortunately, these techniques present some disadvantages such as high costs, radiation exposure (CT), and quite long acquisition time, which make them not suitable for daily use and for repeated follow-up examinations.[4,5] While the visualization of inner hard tissues can be obtained only using these volumetric techniques, the advancement of noninvasive surface technologies has permitted new solutions for the morphological analysis of the external soft tissues only.[6]

In particular, optical systems, like stereophotogrammetry, can obtain three-dimensional (3D) reconstructions of the facial soft tissues in a safe and rapid way, thus allowing repeated assessments, with high levels of accuracy and reproducibility.[6,7]

In this study, we present a series of follow-up stereophotogrammetric evaluations of the labial symmetry of a Caucasoid 16-year-old woman affected by a microcystic LM, who was surgically treated. The evaluations were performed to objectively monitor the treatment progression and final outcome.

The treatment of microcystic LM has always been quite challenging. Indeed, the risk of recurrence is high because complete removal of the malformation is impossible or not advisable, being microcystic LMs generally multifocal.[8,9] Due to the difficulties in the surgical treatment and the recurrence of the disease, patients can lose motivation to adhere to a multistage (more frequently than not) treatment. The method described here allows for easy and fast assessment of the on-going achieved results, providing objective and easy-to-understand indicators, both for the surgeon and, especially, for the patients, who can be encouraged to carry on the treatment phases.

METHODS

The patient involved in this study came at our observation when she was 16 years old. She had a microcystic LM of her right hemiface that had been treated with several partial removals in another hospital since she was 12 years old. During one of these surgical sessions, the facial nerve was injured. The long-standing unilateral facial paralysis was treated with a free gracilis muscle transfer, innervated by homolateral masseteric nerve.[10] The residual deformity involved the right facial soft tissues in the labial area, parasymphysis, and mandibular body.

During the first surgical phase, by careful inspection of the muscle and vermilion, a new commissure was made by removing 2 myomucosal wedges at the angle of the mouth, to symmetrize the