

Prenatal Whole Exome Sequencing Reveals a Novel CDH1 Mutation Associated With Blepharo-Cheilo-Dontic Syndrome

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Case Report

Keywords: Cleft lip with palate, CDH1, whole-exome sequencing, noninvasive prenatal testing, Blepharo-Cheilo-Dontic Syndrome

Posted Date: December 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-135776/v1>

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Abstract

Background: The cleft lip with or without palate (CL/P) is the most prevalent congenital craniofacial abnormality. This study aims to provide molecular diagnosis for patients with CL/P in a Chinese family, and then offer suggestions for future pregnancy for this family.

Methods: Karyotyping, single nucleotide polymorphism array analysis, whole-exome sequencing (WES), and Sanger sequencing were applied to identify the underlying genetic cause for the clinical phenotypes. The functional effect of the identified mutation was evaluated through immunofluorescent analysis of the expression of wild or mutant protein that was transiently expressed. Non-invasive prenatal testing (NIPT) and ultrasound testing were conducted for a new pregnancy of this family.

Results: A novel missense mutation (c.1418T>A/p.(Val473Asp)) in *CDH1* was identified in the family members affected with CL/P. Functional analysis showed that this mutation changed the subcellular localization of the protein. The NIPT and ultrasound testing respectively revealed the new pregnancy carried no *CDH1* mutation and facial dysmorphic features, and a healthy baby was delivered.

Conclusion: The affected family members were diagnosed with a syndromic CL/P called Blepharo-Cheilo-Dontic(BCD) Syndrome. This study identified a novel *CDH1* mutation for CL/P, expanded the mutation spectrum, and contributed to the genetic diagnosis and counseling of this disorder. This study also provided an example of the application of NIPT for BCD Syndrome.

1. Introduction

The cleft lip with or without palate (CL/P) is the most prevalent congenital craniofacial abnormality. The newborn incidence of CL/P is approximately 1/700 to 1/1000 globally and about 1.66/1000 in China[1]. Clinically, CL/P has a unilateral or bilateral gap between the lateral side of the upper lip with philtrum, extending from the upper mouth and lower jaw to the nostrils with cleft palate. CL/P can be classified as syndromic and non-syndromic, depending on whether other congenital anomalies accompany it or not. Syndromic CL/P includes more than 500 different conditions, such as Blepharocheilodontic syndrome[2], making it challenging for the precise diagnosis of CL/P, especially for prenatal diagnosis of CL/P.

The etiology of CL/P is complex and multifactorial, including genetic and environmental factors[2, 3]. Various chromosomal disorders or monogenic diseases are resulting in CL/P syndromic forms. Moreover, the variation in different regions of some genes, such as *IRF6*, *CHD1*, have been reported implicated in both syndromic and non-syndromic CL/P patients, possibly because of incomplete penetrance and/variable expression among individuals affected[4–6]. Unfortunately, the genes mutation involved in CL/P has only been identified just in a small portion of patients. Thus, identifying novel genes or novel mutations related to CL/P is crucially important for the genetic diagnosis and counseling for this disorder.

Traditional karyotyping and single nucleotide polymorphism (SNP) array analysis are widely used tools for genetic diagnosis. However, the limitation of their low resolution makes them unsuitable for detecting

the causes of monogenic diseases. Whole exome sequencing (WES) is characterized with high resolution at a single base-pair level, and is being used to identify genetic causes, especially when karyotyping and microarray are not diagnostic[7–9]. WES has been widely used to identify genetic causes in patients of CL/P[10, 11]. Non-invasive prenatal testing (NIPT) is a method of determining whether the fetus carries specific genetic abnormalities or not through analyzing cell-free DNA (cfDNA) from the blood of the pregnant woman. NIPT was initially used to screen for chromosomal aneuploidy, such as trisomy 21, trisomy 18, trisomy 13[12, 13]. With the increased sequencing depth, NIPT is now being used for screening genomic disorders caused by microdeletion or duplication, Mendelian single-gene disorders[14–16]. Currently, there was no report on the application of NIPT for CL/P.

In this study, we reported a China mother and several fetuses displayed cleft lip with palate or other facial dysmorphic features. Molecular testing identified a novel *CDH1* mutation as the potential genetic lesion for the patients. The effect of the pathogenic mutation on protein function was analyzed. Moreover, NIPT has firstly been utilized to guides parents for eugenics.

2. Materials And Methods

2.1 Clinical information

In 2016, the woman (II2, 25 years old) referred to our clinical genetic diagnosis center due to the finding that her 4th fetus (the proband) was affected with cleft lip and cleft palate by prenatal ultrasound test (Fig. 1A). According to the family information, the 1st fetus was infected with cytomegalovirus and then aborted. The 2nd and the 3rd fetuses were affected with cleft lip diagnosed by prenatal ultrasound testing, and the pregnancies were terminated upon the request of the parents(Data not show). Physical examination showed that the woman had dysmorphic facial features, including bilateral CL/P, lower eyelid ectropion, upper eyelid distichiasis, lagophthalmos, and conical teeth (Fig. 1C). She underwent orthopedic surgery at three years old. Based on the clinical information and the results of molecular analysis in the present study, the affected member of this family was diagnosed as a syndromic CL/P called Blepharo-Cheilo-Dontic(BCD) Syndrome.

In 2017, The woman had pregnancy again and a prenatal ultrasound examination at 13 weeks gestation revealed that the 5th fetus had cleft lip (Fig. 1B). The 5th fetus-mother-father were detected by whole-exome sequencing. Except for the first, second, third fetuses diagnosed as CL/P by prenatal ultrasounds in a local hospital, the other family members were detected by Sanger sequencing in our clinical genetic diagnosis center to identify the underlying genetic causes of the disorder.

In 2019, with prenatal ultrasound examination and NIPT, the women had the healthy 6th fetus did not have CL/P(Fig. 3).

2.2 Whole-exome sequencing

Novaseq6000 platform (Illumina, San Diego, USA) with 150 bp pair-end reads was applied for sequencing. The general procedure for WES was as follows: genomic DNA was sheared to a fragment size of around 150 bp and blunt-ended followed by addition with deoxyadenosine at the 3'ends of the fragments. The genomic DNA library was created by ligating adaptors to the ends of double DNA strands that enable sequencing. The library was amplified by PCR and then hybridized to a pool of biotinylated oligo probes specific for exons. Streptavidin magnetic beads were used to capture DNA-probes hybrids. Raw image files were processed through CASAVA v1.82 for base calling and generating raw data with adequate CCDS coverages (93.93–94.96%, for depth ≥ 20). Burrows-Wheeler Aligner tool was used to map the sequencing reads to the human reference genome (hg19/GRCh37), and PCR duplicates were removed by Picard v1.57 (<http://picard.sourceforge.net/>). Variation annotation was conducted according to the American College of Medical Genetics and Genomics (ACMG) guidelines[17] and the Enliven® Variants Annotation Interpretation System authorized by Berry Genomics. Co-segregation analysis of the variant in other family members was performed by Sanger sequencing.

2.3 Cell culture, plasmids, transfection and antibodies

Human embryonic kidney HEK293 cells were obtained from ATCC and maintained in DMEM high glucose medium containing 10% fetal bovine serum (Gibco). Human CDH1 expression plasmid with Flag tag at the COOH terminal (HG10204-CF) was purchased from Sino Biological (Beijing, China). The mutant human CDH1 (c.1418T > A, p.Val473Asp) plasmid was constructed by a QuikChange site-directed Mutagenesis Kit. Transfection of *CDH1* or *CDH1* mutation plasmids to HEK293 cells were performed by Lipofectamine 2000 reagents (Invitrogen, Fisher Scientific, Illkirch, France). The primary antibody against FLAG (14793, rabbit) was obtained from Cell Signaling Technology. The secondary antibody was from Thermo Fisher Scientific.

2.4 Immunofluorescence analysis

Immunofluorescence staining was performed as previously described[18]. Briefly, cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde. After permeabilized with 0.1% Triton X-100/PBS, cells were blockaded with 5% bovine serum albumin. Then cells were incubated with 1:100 anti-FLAG and Alexa-conjugated secondary antibody. The nucleus was labeled with DAPI. Samples were imaged using a confocal microscope (Leica, TCS SP5).

2.5 Non-invasive prenatal diagnosis by cfBEST

For assessing whether the new pregnancy (the sixth fetus) contains the mutation identified in the family members affected with CL/P, 10 ml of peripheral blood was drawn from the mother at 31 weeks of gestational age for NIPT. The next-generation sequencing-based cell-free DNA Barcode-Enabled Single-Molecule Test (cfBEST) was performed as previously described[15]. Briefly, cfDNA was extracted from the peripheral blood of the pregnant woman using the QIAamp Circulating Nucleic Acid Kit (QIAGEN). The concentration of purified cfDNA was measured with the Qubit3.0 dsDNA HS Assay Kit (Thermo Fisher Scientific). Four specific primers were designed for the mutation site identified according to the principle of cfBEST, and the primers were synthesized by Sangon Biotech (Shanghai, China). The DNA libraries for

next-generation sequencing were constructed according to cfBEST's protocol[15]. Briefly, the ends of the cfDNA are repaired and A-tailed at the 3' end. T-tailed DNA was ligated to cfBEST Tag adaptors and then subjected to PCR for ten cycles. The library was then split into two equal portions (referred to as "F" and "R") and separately subjected to two rounds of nested PCR for ten cycles with 2 × KAPA HiFi HotStart Ready Mix (Kapa Biosystems). The first round of PCR was performed using a universal primer (U1) complementary to the universal adapter and a specific primer (CDH1-F1 and CDH1-R1 for the F and R parts, respectively). After 1.0X Ampure XP bead cleanup, the second PCR was executed using U1 and another specific primer (CDH1-F2 and CDH1-R2 for F and R, respectively). Primer CDH1-F2 and CDH1-R2 contained a particular part and a universal tail similar to another universal primer (U2). After Ampure XP bead cleanup, the two parts of the PCR products were pooled together to serve as the templates of the third PCR with U1 and U2. Then, the product of the third PCR was used for the following paired-end sequencing procedure. Sequencing libraries were subjected to massively parallel sequencing on the NextSeq CN500 (Illumina) to generate 15 million paired-end reads (2 × 75 bp). The cfBEST bioinformatics analysis was performed through a five-step workflow that includes preprocessing, mapping and filtering, consensus sequence calling, allele counting, and genotyping[15].

3. Result

3.1 Molecular analysis results

Karyotyping analysis by G-banding showed a normal chromosomal karyotype (46, XX), and SNP array analysis detected no common pathogenic variants for the 4th fetus(Data not show).Variant annotation and filtration detected a novel heterozygous mutation in *CDH1*(NM_004360.3 c.T1418 > A p.(Val473Asp)) by WES. Sanger sequencing results showed that the mutation was only present in the woman and his 4th and 5th fetus but not in other family members (Fig. 2A). This mutation was not present in all population frequency reference databases, including gnomAD_exome, ExAC,1000 Genomes.

3.2 Functional analysis results

The protein product of *CDH1* is E-cadherin, a multiple-domains protein that comprises an extracellular domain, a transmembrane domain, and an intracytoplasmic domain (Fig. 2C). The extracellular domain consists of five protein modules of immunoglobulin-like fold called extracellular cadherin(EC)domain (EC1 to EC5). The protein sequence alignment showed that the Val473 amino acid residue of E-cadherin is highly conserved among different species and located at the extracellular cadherin-EC domain 3 (Fig. 2D). Analysis with the silicon modeling algorithms POLYPHEN predicted that the mutation is probably damaging on protein function with a score of 0.999 (the maximal score is 1).

To further investigate the functional effect of the CDH1 mutation, expression plasmids of wildtype E-cadherin or mutant E-cadherin comprising a Val473Asp substitution were constructed and transfected into HEK293 cells and detected by immunofluorescence analysis. As shown in Fig. 2E, E-cadherin protein was mainly located at the plasma membrane in wild-type groups. Simultaneously, the Val473Asp protein

was decreased expression in the cytoplasmic membrane and accumulation in intracytoplasmic perinuclear. Therefore, these findings suggest that the *CDH1* variant affects E-cadherin subcellular localization.

3.3 NIPT results

To determine whether the 6th fetus carried *CDH1* mutations, the NIPT assay was performed after the prenatal ultrasound examination was normal (Fig. 3a-3c). The design and sequence of the primers were shown in Figs. 3d and 3e. The NIPT results showed that the fetal fraction of cfDNA in maternal plasma was 21.8%. The allele depths for c.1418 of *CDH1* were 2364, including 1464 for mutant allele T and 900 for wild-type allele A, and thus the relative ratio for T was 38.1% (Fig. 3f). The maternal genotype is heterozygous. According to the principles of relative mutation ratio (RMD), the theoretic RMD in the sixth fetus can be calculated as $(1 - \text{fetal fraction of cfDNA}) \times 1/2 + \text{fetal fraction of cfDNA} \times 1$ (fetal genotype is homozygous for T allele), $\times 1/2$ (fetal genotype is heterozygous for T allele), or $\times 0$ (fetal genotype is homozygous for A allele). The relative ratio for the T allele from sequencing analysis (38.1%) was close to the theoretic RMD under the condition when the fetal genotype is homozygous for the wild-type allele (39.1%). As such, the fetus does not contain the mutant allele T. The result of NIPT was further confirmed by Sanger sequencing (Fig. 3g). A healthy baby was eventually delivered (Fig. 3c).

4. Discussion

In the present study, we reported a Chinese family in which the woman and her five fetuses were affected with CL/P. The woman also displayed lower eyelid ectropion, upper eyelid distichiasis, and conical teeth. Molecular analysis demonstrated that the affected individuals carried a novel heterozygous missense mutation in *CDH1* (c.1418T > A p.(Val473Asp)). The variant was not found in the public population database such as 1000 g, Exac, and gnomAD. The affected residue (Val473) at the EC3 of E-cadherin is highly conserved, and In-silico prediction tools predicted this variant as pathogenic. According to the ACMG guidelines, the mutation (c.1418T > A p. (Val473Asp)) in *CDH1* is classified as likely pathogenic (PM2 + PS2 + PP3 + PP1).

The *CDH1* exists on chromosome 16q22.1 and encodes calcium-dependent cell adhesion proteins Cadherin-1 (CDH1). Cadherin-1 has an important role in developing lip, palate, eyelid, craniofacial, tooth, and hair in human and mouse embryos [19]. In 2016, Nishi et al. first reported a CL/P patient with *CDH1* heterozygous mutation (Asp676Glu). The patient characteristics with cleft lip and palate, meningoencephalocele, tetralogy of Fallot, and developmental delay [20]. In another study, Ghomid et al. identified five heterozygous missense mutations in *CDH1* in patients with CL/P (BCD syndrome), including two missense mutations (Asp254Tyr, Asp257Val) that were predicted to affect two highly conserved residues at the “linker” between EC1 and EC2, two splicing mutations (c.1320G > T, c.1320 + 1G > C) that removed a major part of the EC3 domain, and a deletion mutation (p.Val454del) that removed a conserved hydrophobic residue at the C-terminal end of the EC3 area (Fig. 2C) [10]. They further demonstrated that these mutations reduced the cytoplasmic membrane staining and the stability of E-cadherin. Moreover, Kievit et al. identified novel heterozygous mutations, including Asp254Asn,

Asn256Lys, Asp288His and Pro373Arg, and five de novo splicing mutations that resulted in the removal of the major portion of EC3 domain (Fig. 2C)[11]. They provided further evidence that these mutant proteins caused dominant-negative effects on wild-type E-cadherin, which impaired the trafficking of E-cadherin to the cell membrane[11]. These findings, together with ours in the present study, demonstrated that the *CDH1* mutations were related to CL/P with impaired E-cadherin functions.

Besides BCD syndrome, *CDH1* mutations have also been linked to non-syndromic forms of CL/P, BCD with gastric or breast cancer, or cancer only[5, 19]. In our research, the mother didn't have any cancers.

The development of next-generation sequencing will facilitate the clinical utilization of NIPT in the screening for genetic disorders. Recent clinical studies have demonstrated that NIPT can provide valuable molecular information to detect a wide spectrum of dominant monogenic diseases[15]. However, the clinical validity of NIPT for the detection of CL/P remains unknown. In this study, by using the next-generation sequencing-based NIPT strategy called cfBEST, we found that the new pregnancy (the sixth fetus) in the family did not carry the *CDH1* mutation (c.1418T > A)(Fig. 3f), which was further confirmed by Sanger sequencing(Fig. 3e). Prenatal ultrasound testing revealed no structural anomalies(Figs. 3a and 3b). A healthy boy was delivered. These findings suggested that NIPT can provide valuable molecular information for a screening of CL/P. For NIPT, the fetal fraction of cfDNA in maternal plasma is a crucial factor determining the reliability of a cfDNA screening result. Yang et al. proved that the genotypes called by cfBEST were 100% accuracy when the “fetal” DNA fraction was 5% or higher, while accuracy dropped to 40–80% when the fraction comes to 3%. NIPT detected genotype correctly in 99.78% of fetuses with the monogenic disorder β -thalassemia, yielding a sensitivity of 99.19% and a specificity of 99.92%[15]. In the present study, the concentration of “fetal” DNA fraction is 21.8%, reaching the requirement for high accuracy of the NIPT method according to the literature. What's more, this is the first report of the application of NIPT in the diagnose of CL/P. The use of NIPT gives pregnant women at high risk of amniocentesis a better choice for the diagnosis of single-gene genetic diseases and avoids the risk of fetal flow. In the future, we will continue to conduct a large sample of positive tests for single-gene genetic disorders in a non-invasive condition, which also contributes to promoting these single-gene genetic diseases, like trisomy 21, became the routine testing programs during pregnancy.

In summary, we identified a novel heterozygous missense mutation in *CDH1*(c.1418T > A p.(Val473Asp)) that was associated with hereditary BCD syndrome in a Chinese family, and further demonstrated that NIPT in combination with prenatal ultrasound testing represented an efficient way in screening for this syndrome. These findings broaden the mutation spectrum of *CDH1*, which contributes to the understanding of molecular mechanisms of BCD syndrome and the genetic diagnosis and counseling for patients of this syndrome.

Declarations

Ethical Compliance

The study was approved by the ethics committee of Hunan Provincial Maternal and Child Health Care Hospital. The research was conducted according to the Declaration of Helsinki and with written consent to use clinical data and samples.

Consent for publication

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

Availability of data and material

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

Competing interests

All authors declare that they have no commercial or other conflicting interests.

Funding information

Major Scientific and Technological Projects for Collaborative Prevention and Control of Birth Defects in Hunan Province, Grant/Award Number: 2019SK1013; National Natural Science Foundation of China, Grant/Award Number: 81870474, 31601035; Natural Science Foundation of Hunan Province, Grant/Award Number: 2018JJ3274, 2019JJ40415.

Author contribution

Y Peng and H Wang designed the study. Y Peng and C Tang prepared the manuscript. Y Peng and S Yang collected the clinical information of patients. L Shu and H Xi interpreted the data of WES. X Wang interpreted the data of NIPT. C Tang performed immunofluorescence staining. J Pang performed the SNP array analysis. Y Luo and C Liang performed the prenatal ultrasound testing.

Acknowledgment

The authors wish to thank all the patients, family members and collaborators that participated in this study.

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Figures

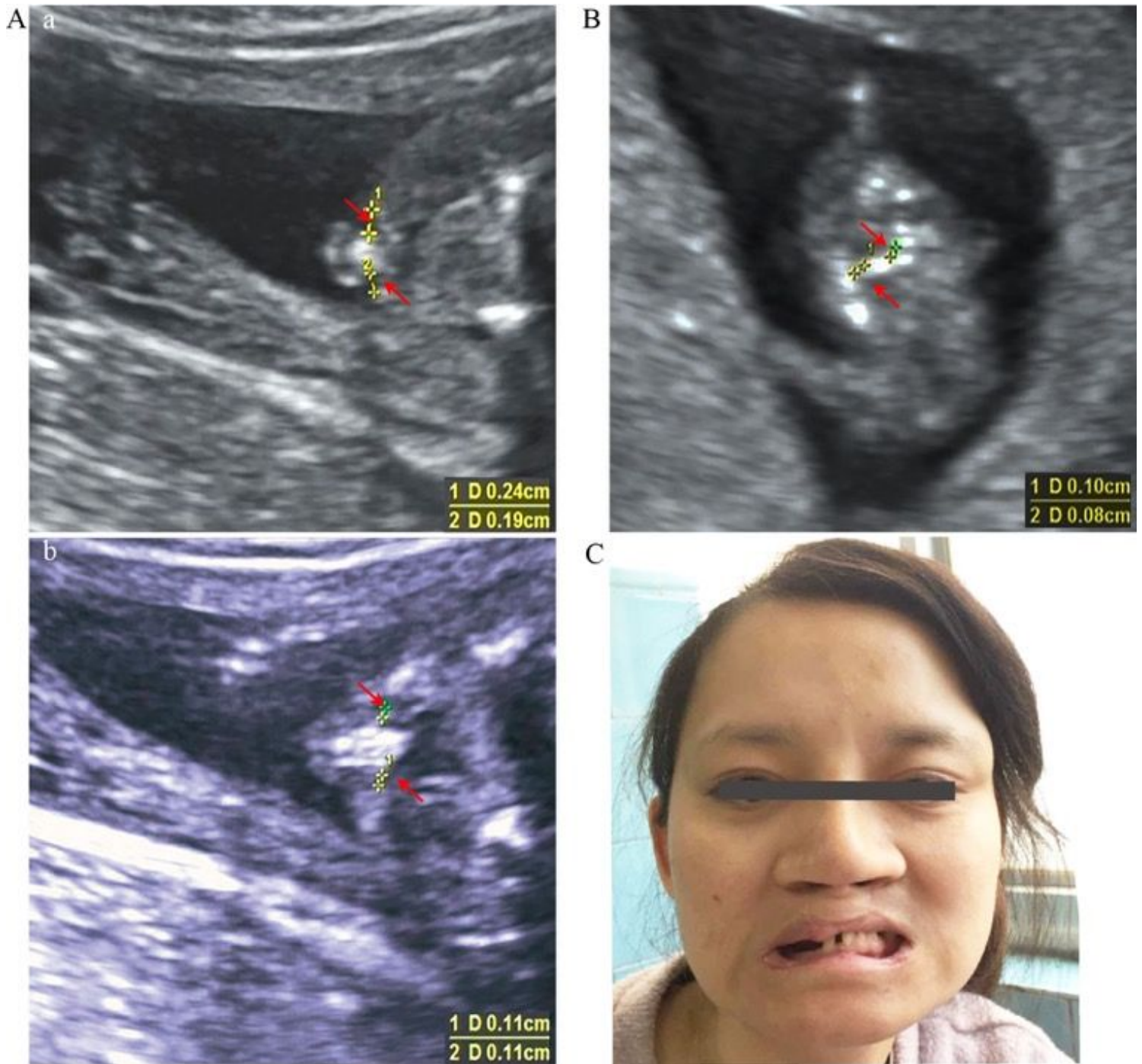


Figure 1

Clinical features of affected individuals. (A) Images of ultrasound examination of the 4th fetus at 18 weeks gestation revealed 0.19cm and 0.24cm width of echo discontinuous in the upper lip (a), and two 0.11cm widths of echo discontinuous in the alveolar process (b). (B) Images of ultrasound examination of the 5th fetus at 13 weeks gestation showed 0.1cm and 0.08cm width of echo discontinuous in the alveolar process. (C) Facial features of the mother including bilateral CLP and lower eyelid ectropion, upper eyelid distichiasis, lagophthalmos and conical teeth.

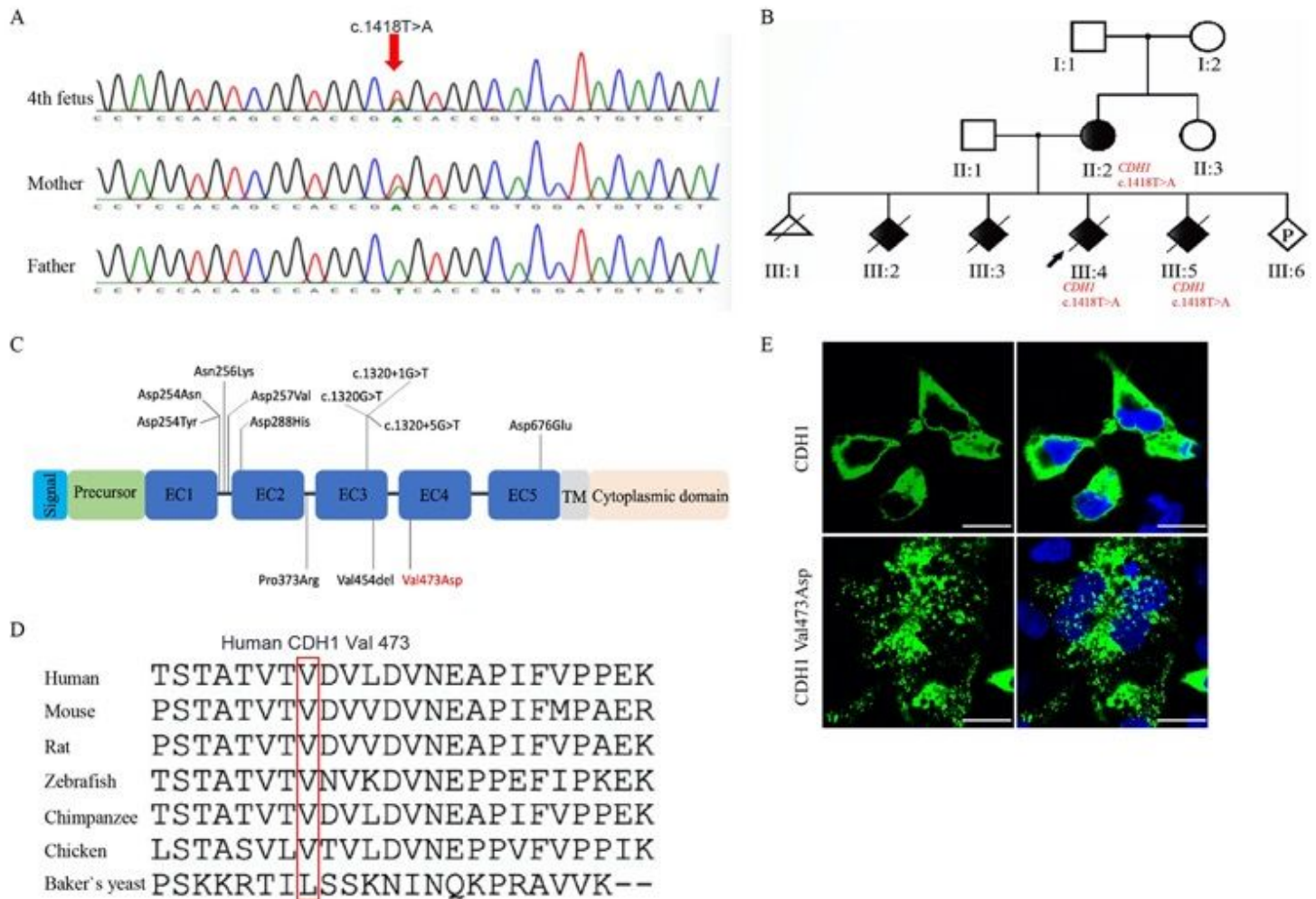


Figure 2

Analysis of the CDH1 mutation in the family (A) Sanger sequencing results of the 4th fetus, the women contained a heterozygous mutation CDH1 (c.1418T>A p. Val473Asp). (B) Family pedigrees of the family. (C) Structure of CDH1 and the localization of BCD syndrome-related germline mutations. CDH1 comprises protein signal peptide, precursor sequence, extracellular domain (EC), transmembrane domain (TM), and a cytoplasmic domain. (D) The residue Val473 is highly conserved. (E) Confocal microscopy images of HEK239T cells expressing wild-type CDH1 and CDH1 Val473Asp. Wild-type CDH1 is mainly located at the plasma membrane, whereas E-cadherin Val473Asp induce loss of cytoplasmic membrane staining and show intracytoplasmic perinuclear accumulation.

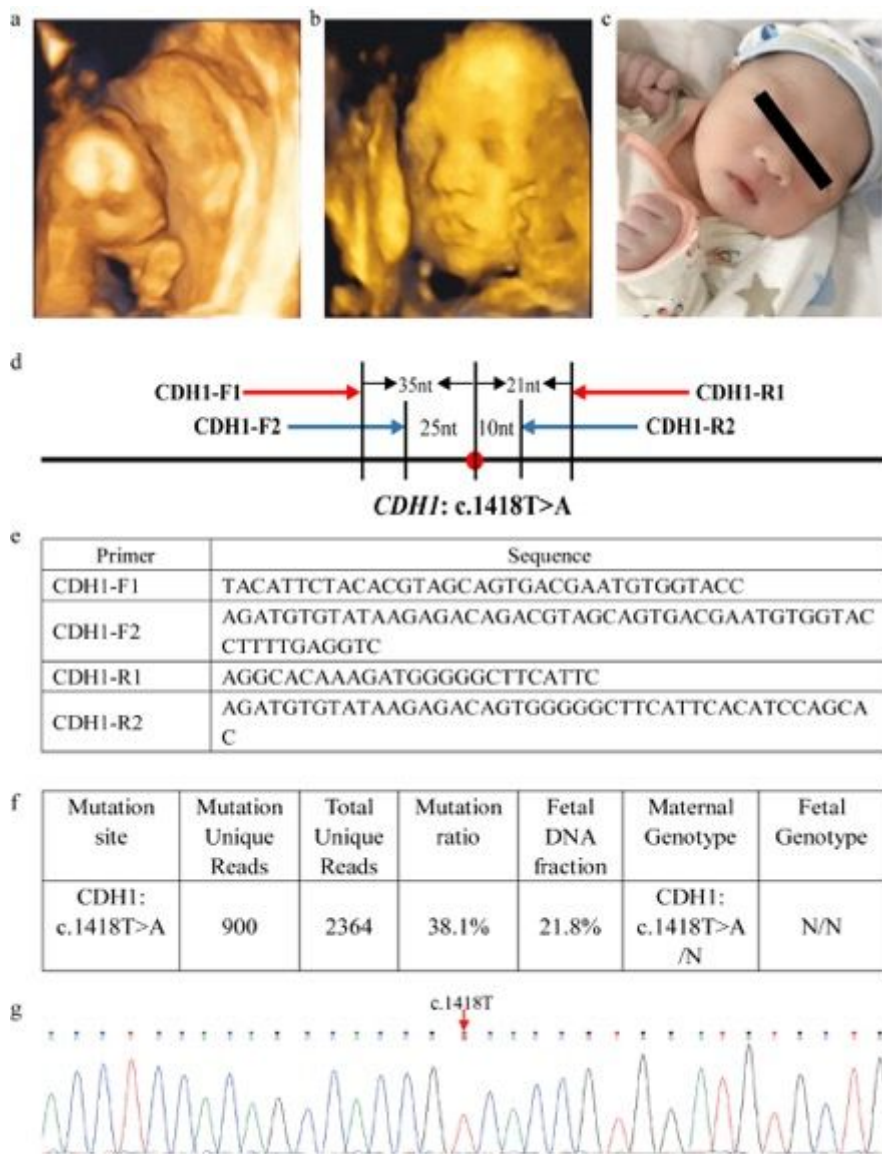


Figure 3

NIPT for the 6th fetus. (a,b) Ultrasound images of the frontal view of the face of fetus 6 at 16 weeks gestation(a) and 30 weeks gestation(b). (c) The picture of fetus 6 after birth. (d) The schematic representation of the primers used for NIPT. The red dot indicates the position of CDH1:c.1418T>A. (e) The primer sequences. (f) Results of NIPT analysis. (g) Sanger sequencing results for fetus 6.