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Genetic analysis of CHARGE syndrome identifies overlapping molecular biology

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Abstract

Purpose—CHARGE syndrome is an autosomal dominant, multiple congenital anomaly condition characterized by vision and hearing loss, congenital heart disease, and malformations of craniofacial and other structures. Pathogenic variants in *CHD7*, encoding ATP-dependent Chromodomain Helicase DNA binding protein 7, are present in the majority of affected individuals. However, no causal variant can be found in 5-30% (depending on the cohort) of individuals with a clinical diagnosis of CHARGE syndrome.

Methods—We performed whole exome sequencing (WES) on 28 families from which at least one individual presented with features highly suggestive of CHARGE syndrome.

Results—Pathogenic variants in *CHD7* were present in 15 of 28 individuals (53.6%), whereas four(14.3%) individuals had pathogenic variants in other genes (*RERE*, *KMT2D*, *EP300*, or *PUF60*). A variant of uncertain clinical significance in *KDM6A* was identified in one (3.5%) individual. The remaining eight (28.6%) individuals were not found to have pathogenic variants by WES.

Conclusion—These results demonstrate that the phenotypic features of CHARGE syndrome overlap with multiple other rare single-gene syndromes. Additionally, they implicate a shared molecular pathology that disrupts epigenetic regulation of multiple-organ development.

Keywords

CHARGE; Genetics; Exome; Oligogenicity; Chromatin

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Introduction

Epigenomic regulation is essential for human development. *De novo* pathogenic variants in genes that encode chromatin effectors are important for developmental transcriptional plasticity and make important genetic contributions to developmental disorders. CHARGE syndrome (MIM: 214800) is an autosomal dominant multiple congenital anomaly condition. Individuals with CHARGE syndrome present with a combination of distinct findings such as coloboma of the eye, choanal atresia, cleft palate, vestibular abnormalities, cranial nerve and other brain anomalies, and characteristic dysmorphism particularly of the ear and face¹. It occurs in about 1 in 10,000 births worldwide². CHARGE syndrome can be caused by heterozygous pathogenic variants in *CHD7*, encoding Chromodomain Helicase DNA binding protein 7, an ATP-dependent chromatin remodeler³. However, a pathogenic variant in *CHD7* cannot be found in up to 30% of individuals with clinical features of CHARGE syndrome, suggesting other genes or pathologies may be involved in a subset of cases.

CHARGE syndrome is one of several multi-organ developmental disorders attributed to pathogenic variants in genes encoding chromatin remodelers of the CHD family. Despite similarities in protein domain structure between CHD family members, individual CHD genes are associated with clinically distinct syndromes. Pathogenic variants in *CHD4* are implicated in Sifram-Hitz-Weiss syndrome (MIM: 617159), and *CHD8* is an important genetic risk factor for a subtype of autism spectrum disorders (MIM: 615032). Pathogenic variants in CHD genes are characterized by variable expressivity and reduced penetrance. The same pathogenic *CHD7* variant within a single family can present with disparate features, ranging from multiple organ system involvement to mild developmental delay or isolated clefting and hearing loss⁴. While there is minimal clinical overlap between individual CHD related disorders, a number of syndromes that mimic CHARGE have been described, including 22q11 Deletion syndrome (MIM: 188400), Kabuki syndrome (MIM: 147920, 300867), Renal-coloboma syndrome (MIM: 610536), and Mandibulofacial dysostosis (MIM: 610536)⁵⁻⁸. This clinical variability highlights the importance of genetic testing in diagnosis of developmental syndromes.

Within this clinical diversity of developmental disorders, a major unanswered question is whether pathogenic variants in genes other than *CHD7* might explain the genetic etiology in individuals not previously diagnosed with *CHD7* pathogenic variants. We performed WES for a cohort of 28 individuals with clinical features of CHARGE who had no previous history of genetic testing or tested negative for pathogenic variants, deletions or duplications in *CHD7*. WES revealed pathogenic variants in genes previously shown to cause other developmental disorders, including Rubinstein-Taybi syndrome (*EP300*), Kabuki syndrome (*KMT2D*), Verheji syndrome (*PUF60*, MIM: 615583), and *RERE*-associated developmental disorder (*RERE*, MIM: 616975). These results illustrate a higher degree of oligogenicity than previously recognized for individuals with clinical CHARGE features, suggesting common underlying molecular biological mechanisms that contribute to development of affected organs and tissues.

Materials and Methods

Subject recruitment and sample collection

The study protocol was approved by the Institutional Review Board for the Protection of Human Subjects at The University of Michigan Medical School. Individuals with clinical features of CHARGE and their family members were recruited using standard practices at the 12th International CHARGE Syndrome Foundation conference (Chicago, Illinois, 2015), the Pediatrics Genetics clinic at The University of Michigan C.S. Mott Children's Hospital, and external healthcare facilities. We included individuals with features of CHARGE syndrome for which a genetic etiology had not been identified and excluded individuals with features of CHARGE syndrome with a previously identified *CHD7* pathogenic variant. Study participation involved informed written consent, a clinical history questionnaire, and either a saliva or peripheral blood sample. DNA was extracted per the manufacturer's instructions from saliva samples using DNA Genotek Prep-IT L2P (catalog no. PT-L2P) or blood samples using Qiagen DNeasy Blood & Tissue Kit (catalog no. 69504). DNA quantification was employed using Life Technologies Quant-iT PicoGreen dsDNA Assay Kit (catalog no. P7589) with a microplate reader to obtain 3 µg of DNA for WES.

Exome sequencing

DNA samples were submitted to the University of Washington Center for Mendelian Genomics (UW-CMG) where library construction, WES and analysis were performed. Briefly, sequencing libraries were generated for each DNA sample in an automated, 96-well plate format (Perkin-Elmer Janus II). Sample libraries were constructed from 1 µg of genomic DNA which underwent a series of shotgun library construction steps including: acoustic sonication (Covaris), end-repair, A-tailing ligation of unique sequencing adaptors, and PCR amplification. Sample libraries were hybridized to the Nimblegen SeqCap EZ v2.0 target (~36.5 Mb) in multiplex for a period of 72 hrs. Captured DNA was then purified, PCR amplified, and normalized for sequencing.

Captured DNA was sequenced on Illumina HiSeq machines using paired-end sequencing. Raw sequence data in FASTQ format were aligned to the human genome reference hg19 using the BWA algorithm for the generation of BAM files⁹. The quality of each sample was assessed for coverage (80% of sequenced target with 20× coverage and 90% of target with 8× coverage) and Ts/Tv ratios. Additionally, samples were quality controlled to confirm sex using PLINK (v1.90b2m) software, and estimations of kinship were corroborated with pedigrees using KINGv1.4.0 software.

Variant detection, interpretation and validation

Variants and indels were detected and genotyped using HaplotypeCaller from GATK with hard filtering parameters. A multi-sample VCF was generated for all samples following GATK best practices¹⁰. The multi-sample VCF was annotated using Variant Effect Predictor (VEP) tool v83 within GEMINI (<http://uswest.ensembl.org/info/docs/tools/vep/index.html>). Copy number variants were detected from exome sequence data using the program CoNIFER¹¹.

GEMINI v0.19.1 was used to filter variants and indels detected in affected individuals¹². Variants were excluded if allele frequency in reference populations (ExAC, NHLBI Exome Sequencing Project (ESP) 6500, or 1000 Genomes) exceeded 0.005 or exceeded 0.05 in the UW-CMG internal database. Variant impact determined by VEP was also utilized to exclude variants and indels with an impact severity of “low” (ex. intergenic, intronic, synonymous, 5’UTR, and 3’ UTR variants). Variant prioritization was based on the following parameters: variant impact, presence in a disease-associated gene, conservation of impacted nucleotide by GERP, or predicted deleteriousness (CADD > 15)^{13; 14}. Interpretation of detected CNVs was based on size, frequency in the UW-CMG internal database, and overlap with known disease-associated regions. In addition, CNVs were filtered based on phenotypic overlap according to the ACMG guidelines for postnatal CNV calling¹⁵.

Sanger sequencing was used to validate rare, protein-altering variants. Variant segregation was also used to confirm inheritance patterns when family member samples were available.

Results

Cohort

We assembled a cohort of 28 affected individuals and their parents (Table 1). Probands ranged in age from 18 months to 40 years old, with 14 probands being <18 years of age. The majority of probands (26/28) presented with two or more major clinical CHARGE criteria, achieving a diagnosis of CHARGE as defined by *Hale et al.*¹⁶. Probands also exhibited minor clinical features according to the diagnostic criteria (Table 1 and Supplemental Table 1). Nineteen of the families in the cohort were trios, one a duo and the remaining eight were proband only. A previous history of negative *CHD7* genetic testing was reported for 12 probands with the remaining 16 probands reporting no prior history of clinical genetic testing at the level of *CHD7* or WES.

CHD7

WES and analysis were conducted at the UW-CMG. Fifteen probands, one of whom had a previous history of negative clinical *CHD7* genetic testing, were found to have pathogenic variants in *CHD7* [GenBank: NM_017780] by WES (Table 2). Of these 15 *CHD7* variants, 11 were designated as *de novo* because the variant was absent in parental samples and 1 variant was inherited from a clinically affected mother. For three probands, parental DNA was unavailable for segregation analysis. The majority of *CHD7* variants (13) were classified as either a stop-gained or frameshift, consistent with the previously described allelic spectrum of *CHD7* variants that cause CHARGE syndrome¹⁷. One *CHD7* splicing variant and one missense variant were also detected. Seven of the fifteen *CHD7* variants were described in 1000 Genomes with an association with CHARGE syndrome by The Human Gene Mutation Database or ClinVar. Otherwise, all *CHD7* variants were absent in ExAC, 1000 Genomes, and NHLBI ESP Exome Variant Server. CNV analysis of WES data did not reveal pathogenic changes (Supp. Table 3).

RERE

Proband 517, a female at the age of 8 years old at the time of study recruitment, presented with a medical history consistent with CHARGE syndrome. Notably, she exhibited bilateral choanal atresia, right inferior iris coloboma, abnormal external ears, progressive sensorineural hearing loss and bilateral cochlear dysplasia identified by temporal bone CT. Additionally, proband 517 was also noted to exhibit growth retardation, short stature, microcephaly, developmental delay, and truncus arteriosus repaired surgically at one-month of age. Consistent with the results of her previous CMA and *CHD7* genetic testing, proband 517 was not found to harbour a pathogenic variant in *CHD7* or CNV by WES (Supplemental Table 2). Rather, a *de novo* in-frame duplication (c.4313_4318dupTCCACC [p.Leu1438_His1439dup] [GenBank: NM_001042681]; hg19 chr1: 8,418,276) in the RE (Arginine-glutamic acid) dipeptide repeats gene *RERE* was identified. This in-frame duplication occurs in a histidine rich region of *RERE* (UniProt: QP2R6), and is absent in publically available databases ExAC, 1000 Genomes, and NHLBI ESP Exome Variant Server. The *RERE* variant detected for proband 517 is the same duplication identified for male subject S2 in *Fregeau et al.* who exhibits similar features to proband 517 including choanal atresia, coloboma, growth retardation, short stature, developmental delay, and microcephaly. Such phenotypic overlap suggests that this variant contributes to the clinical findings of proband 517¹⁸.

KMT2D and KDM6A

Variants in *KMT2D* and *KDM6A*, genes that underlie Kabuki syndrome, were identified in our cohort. Proband 518, a 16-year-old female, had clinical features of CHARGE syndrome (Table 2). Specifically, her medical history was notable for external ear abnormalities, mild hearing loss in the right ear, moderate to severe hearing loss in the left ear, long palpebral fissures, everted lashes, and persistent fingertip pads. Multiple ophthalmologic abnormalities were also noted, including bilateral chorioretinal colobomas, strabismus, and nystagmus. In addition, proband 518 exhibits cardiac and renal abnormalities, small stature, precocious puberty, developmental delay, and skeletal related problems including kyphosis and scoliosis. However, previous genetic testing did not identify a pathogenic variant in *CHD7*. WES reconfirmed previous negative *CHD7* genetic testing results and identified a single nucleotide duplication in *KMT2D* (c.9602dupT [p.Ser3202Glufs*13] [GenBank:NM_003482]; hg19 chr12: 49,431,536) (Supplemental Table 2). This duplication is predicted to result in a frameshift, generation of a premature stop codon in the central portion of *KMT2D* (UniProt: O14686), and nonsense mediated decay. The c.9602dupT variant in *KMT2D* is absent from the ExAC, NHLBI ESP Exome Variant Server and 1000 Genomes databases, and resemble previously described *KMT2D* variants ascribed to Kabuki syndrome¹⁹.

Proband 519, a male just under 18 months of age at the time of recruitment, had clinical findings consistent with CHARGE syndrome including bilateral choanal atresia, bilateral iris coloboma and asymmetric microphthalmia, low external ear placement on the left side with normal hearing, but no *CHD7* pathogenic variant. A family history of unilateral choanal atresia in a maternal great-grandfather was also reported. WES confirmed absence of a pathogenic *CHD7* variant (Supplemental Table 2). A hemizygous missense variant in

KDM6A (c.250A>G [p.Ile84Val] [GenBank: NM_021140]; hg19 chrX: 44,820,553) was identified just N-terminal to the first tetratricopeptide motif (UniProt: O15550). A GERP conservation score of 5.660 and a CADD score of 17.16, along with the variant's absence from publically available databases ExAC, 1000 Genomes and NHLBI ESP Exome Variant Server, suggest deleteriousness. The inheritance of the *KDM6A* variant from a self-reported unaffected mother is unexpected as carrier mothers are reported to exhibit mild features of Kabuki syndrome, but this may reflect favorable skewing of X-inactivation¹⁹. Consequently, we classify the p.Ile84Val variant observed in proband 519 as a variant of uncertain clinical significance. Further experimental studies including X-inactivation profiling, and a clinical evaluation of the mother of proband 519 would be required to change the variant classification.

EP300

Proband 520 was an 11-year-old male with a clinical diagnosis of CHARGE syndrome with a normal chromosomal microarray and *CHD7* sequencing. Significant findings included right-sided choanal atresia, sensorineural hearing loss, external ear abnormalities, dysphagia, developmental delay, growth deficiency, a ventricular septal defect and patent ductus arteriosus with spontaneous closure. He was born prematurely with polyhydramnios. Facial features included level palpebral fissures, a short nose with anteverted nares, an underbite with a symmetric facial grimace, and left-sided esotropia. He was found to have a *de novo* splice acceptor variant in *EP300* 3' to exon 18 (c.3262-2A>G [Genbank: NM_001429]; hg19 chr22: 41,553,171). Pathogenic variants in *EP300* are a minor cause of Rubinstein-Taybi syndrome (RTS) (OMIM: 180849)²⁰. The c.3262-2A nucleotide encompasses the AG splice acceptor site, which is well-conserved as described by a GERP score of 5.700. The transition to G is predicted to impact splicing (UniProt: Q09472). The c.3262-2A>G *EP300* splice acceptor variant was not observed in ExAC, 1000 Genomes, and NHLBI ESP Exome Variant Server databases. Moreover, the pathogenic role of the c.3262-2A>G variant is supported by the similarity between proband 520 and descriptions of RTS, and that RTS *EP300* variants are located throughout the gene.

PUF60

Proband 521 was a male recruited into our CHARGE cohort at 22 years of age with a clinical history of global developmental delay, irido-retinal colobomas, mild hearing loss, small stature, genital abnormalities, and a ventricular septal defect corrected surgically. Surgical history was also notable for multiple back surgeries. Consistent with his previous history of normal *CHD7* genetic testing, WES did not identify a pathogenic variant in *CHD7* or a CNV (Supplemental Table 2). Instead, a *de novo* missense variant in the RNA recognition motif of *poly-U-binding splicing factor 60* (*PUF60*) (c.389G>A [p.Arg130His] [GenBank: NM_078480]; hg19 chr8: 144,900,664) was identified. *PUF60* encodes a protein involved in pre-mRNA splicing²¹. Heterozygous loss of *PUF60* is predicted to contribute to the clinical features of recurrent microdeletions of 8q24.3, also called Verheij syndrome (OMIM: 615583)²², characterized by a constellation of features including microcephaly, craniofacial dysmorphisms, ocular colobomata, developmental delay, short stature, and skeletal, cardiac and renal abnormalities. Conservation of the *PUF60* c.389G>A variant based on GERP score, pathogenicity predicted by CADD, and absence of the missense

variant in the online databases ExAC, 1000 Genomes, and NHLBI ESP Exome Variant Server all provide evidence to support the pathogenicity of this variant (Table 2).

FLNA

Proband 522 was a 10-year-old male with clinical features suggestive of CHARGE syndrome including bilateral retinal colobomas, bilateral cleft lip and palate, bilateral hypoplastic vestibular apparatus and semicircular canals, and abnormal external ears. He also has a history of hearing loss, resolved patent ductus arteriosus, developmental delay, dysphagia, left hydronephrosis, hiatal hernia, genital anomalies, and normal brain imaging. Consistent with his previous genetic workup, proband 522 was not found to have variants impacting *CHD7* nor CNVs by WES. Instead, he was found to have a missense variant (c.2309A>G [p.Asn770Ser] [GenBank: NM_001456]; hg19 chrX: 153,591,124) in the rod-domain repeat 6 (UniProt: P21333-2) of filamin A (*FLNA*) that was inherited from his unaffected mother. Variants in *FLNA* often cluster in the calponin homology domain 2 and the rod-domain repeat 10, and are associated with several X-linked disorders together termed otopalatodigital spectrum disorders including Otopalatodigital syndrome types I and II (OPD, MIM: 311300 and 304120), Melnick-Needles syndrome (MIM: 309350), and Frontometaphyseal dysplasia type I (MIM: 305620)²³. Some clinical features of proband 522 overlap with features of *FLNA*-associated conditions, specifically OPD1, including hearing loss and bilateral cleft lip/palate. However, he had only mild syndactyly of the left second and third toes, which is less severe than the skeletal or limb abnormalities typically observed in *FLNA*-related disorders. Important in our analysis was additional genetic testing of an unaffected male sibling, who was found to be positive for the c.2309A>G *FLNA* variant. This information provided support for a variant classification of likely benign according to ACMG guidelines.

Discussion

We report results of WES of a cohort of 28 individuals with clinical features of CHARGE syndrome. Pathogenic variants in *CHD7* were discovered in 15/28 (53.6%) individuals and 4/28 (14.3%) individuals had pathogenic variants in four genes other than *CHD7*, including *RERE*, *KMT2D*, *EP300*, and *PUF60*. Our results suggest that careful phenotype-genotype analysis in the setting of comprehensive sequencing studies can uncover new relationships between genes and phenotypes, and sheds light on the underlying molecular pathology contributing to CHARGE syndrome and related disorders.

Our findings broaden the spectrum of clinical features associated with pathogenic variants in genes such as *EP300*. RTS is characterized by intellectual disability, growth delay, and distinctive craniofacial and digital dysmorphisms²⁴. CHARGE can typically be differentiated from RTS by the presence of semicircular canal dysgenesis, choanal atresia, facial asymmetry, gonadal hypoplasia, and syndrome-specific dysmorphology. While hearing loss has been reported in individuals with *EP300*-associated RTS, the choanal atresia remains unique to proband 520²⁵. It is possible that choanal atresia is a low penetrance phenotype of *EP300*-associated RTS, but remains seldom detected due to the limited number of individuals with *EP300* variants to date. This illustrates how the unbiased use of WES can

broaden the phenotypic spectrum associated with known disease genes to uncover novel phenotypic overlap between developmental disorders. Alternatively, dual molecular diagnoses may account for his unique RTS presentation. Although WES did not uncover any additional variants suggestive of this alternative, the possibility remains that proband 520 possesses an undiscovered genetic or non-genetic etiology.

The substantial clinical overlap between *RERE*-associated developmental disorder and CHARGE syndrome raises the possibility of classifying *RERE* as a genetic cause of CHARGE syndrome. Heterozygous pathogenic variants in *RERE* have been reported in 10 individuals with complex and variable phenotypes that overlap with those observed in CHARGE syndrome, including structural brain malformations, intellectual disability, seizures, coloboma, sensorineural hearing loss, choanal atresia, cardiac malformations, and gastroesophageal reflux disease¹⁸. Clinical evaluation of individuals with *RERE* pathogenic variants shows that two of the ten individuals meet clinical criteria for CHARGE syndrome^{17; 18}. Inner ear dysplasia and hypogonadotropic hypogonadism are not reported in *RERE*-associated developmental disorder and the craniofacial features differ from those commonly observed in CHARGE syndrome (square shaped facies, auricular anomalies, facial asymmetry and seventh nerve palsies). These differences suggest that pathogenic variants in *RERE* and *CHD7* are the genetic etiologies of distinct disorders.

Our results show that complex phenotypes presenting as CHARGE syndrome may be caused by pathogenic variants in *CHD7* or other genes associated with other syndromes. The high degree of phenotypic overlap among these disorders raises the question of whether CHARGE syndrome should be considered as a single gene disorder associated only with pathogenic variants in *CHD7* or an oligogenic disorder²⁶. Isolated features of CHARGE, such as congenital heart disease and congenital hypogonadotropic hypogonadism, can occur due to pathogenic variants in multiple individual genes, and are therefore considered to be oligogenic²⁷. Careful attention to both clinical diagnosis and molecular results is sure to continue shedding light on this important question.

Phenotypic overlap between CHARGE and Kabuki syndromes is well described, and may reflect direct interactions between *CHD7*, *KMT2D* and *KDM6A* or shared effects on DNA methylation^{28; 29}. Furthermore, overlapping features with Rubinstein-Taybi, Verheij and *RERE*-associated syndromes may also indicate shared molecular pathology between proteins encoded by *CHD7*, *KMT2D*, *KDM6A*, *EP300*, *PUF60* and *RERE*, the genetic findings in this study^{21; 30-34}. Such functional synergy is supported by experimental observations from primary cells derived from individuals with CHARGE syndrome and animal models. *RERE* is a co-regulator of retinoic acid (RA) signaling and forms a complex with NR2F2 (nuclear receptor subfamily 2, group F, member 2), P300 and retinoic acid receptors (RARs/RXRs), which is recruited to the retinoic acid response element (RARE) of retinoic acid transcriptional targets, such as *FGF8* (Figure 1)³². Activation of RAREs by RA promotes an exchange of repressive histone modification (H3K27Me3) in favor of activating ones (H3K27Ac) and translocation of nucleosomes to expose DNA to initiate transcription, functions attributed to *KDM6A*, P300, *CHD7* and *PUF60* respectively. A shared molecular biology is also supported by evidence of direct interaction between *CHD7* and P300-incorporating complexes³⁴.

This functional synergy may be pertinent to understand the epistatic relationship between *CHD7* and *FGF8* discovered in the *Chd7* knockout mouse³⁵. While mice lacking one working copy of *Chd7* or *Fgf8* exhibit normal cerebellar vermis development, those lacking one functional copy each of *Chd7* and *Fgf8* exhibit cerebellar vermis hypoplasia. Importantly, *CHD7* and *FGF8* do not directly interact and the molecular underpinning of this epistatic effect have not been described. *Fgf8* is a retinoic acid (RA) responsive gene. In the absence of RA, RAR–RXR heterodimers bind to RARE sequences, accompanied by histone deacetylases, H3K27 methyltransferase polycomb repressive complex 2 (PRC2) and histone H3K4 mono- and di- methyltransferase KMT2D to repress transcription of RA responsive genes³¹. In response to RA the RERE/NR2F2/P300 complex binds retinoic acid receptors at RAREs³². Activation of the RAREs promotes nucleosome translocation, an important function of *CHD7* that allows for KDM6A occupancy concomitant with H3K27 demethylation and activating H3K27 acetylation by P300, setting the stage for coordinated transcriptional regulation of *Fgf8*^{30; 36}. These findings also provide insights into the ability of RA to rescue structural inner ear defects in the conditional *Chd7* knockout mouse models³⁷. Future studies will be required to determine if chromatin based co-regulators of RA responsive transcription may cooperate to mediate the *Chd7-Fgf8* epistatic effect, and underlie a shared molecular pathology between these developmental disorders.

Nuclear receptor NR2F2 is a ligand-dependent transcription factor that regulates expression of genes critical for a variety of biological processes, including development, growth, and differentiation³³. The observation that interaction between RERE, P300 and NR2F2 is required for body patterning and somite formation early in embryogenesis implicates *NR2F2* in the etiology of developmental disorders (Fig 1). While *NR2F2* variants were not detected in our study, they have been identified in individuals with congenital heart defects (MIM 615779) which are also prominent in CHARGE syndrome³⁸.

Classifying variants is an inherent challenge of WES interpretation, as demonstrated by the *FLNA* variant identified in proband 522. Such an example illustrates the value of testing for segregation of the allele with the disorder among family members, especially for genes like *FLNA* that are associated with diverse malformations and exhibit complicated patterns of variation and X-inactivation²³. In total, 8/28 (28.6%) probands in our cohort had no pathogenic variant detected by WES. Further genome-wide analysis may be instrumental in identifying an underlying genetic etiology for these individuals. It is possible that pathogenic variants in non-coding regions may be identified to cause CHARGE syndrome. This potential etiology is particularly relevant for CHARGE, since human *CHD7* is a large gene (188 kb) and *CHD7* is known to bind preferentially to tens of thousands of H3K4me-enriched regions of the genome, including promoters, enhancers, and super-enhancers^{39; 40}. Other potential causes of CHARGE syndrome that could escape detection by standard chromosomal microarray and single gene, gene panel or whole exome sequencing include (a) differences in DNA methylation, (b) cryptic chromosomal rearrangements, and (c) somatic mosaicism. A recent study showed, for example, that DNA methylation signatures differ greatly between individuals with CHARGE and Kabuki syndromes²⁸, despite extensive overlap in clinical presentation. Potential environmental causes of features of CHARGE syndrome may include retinoic acid exposure or vitamin D deficiency, which also present with craniofacial malformations, ocular and auricular anomalies, and heart defects.

Further studies using larger cohorts of individuals with CHARGE and those with related clinical features, accompanied by methylation and genome sequencing studies will help explore and distinguish among these possibilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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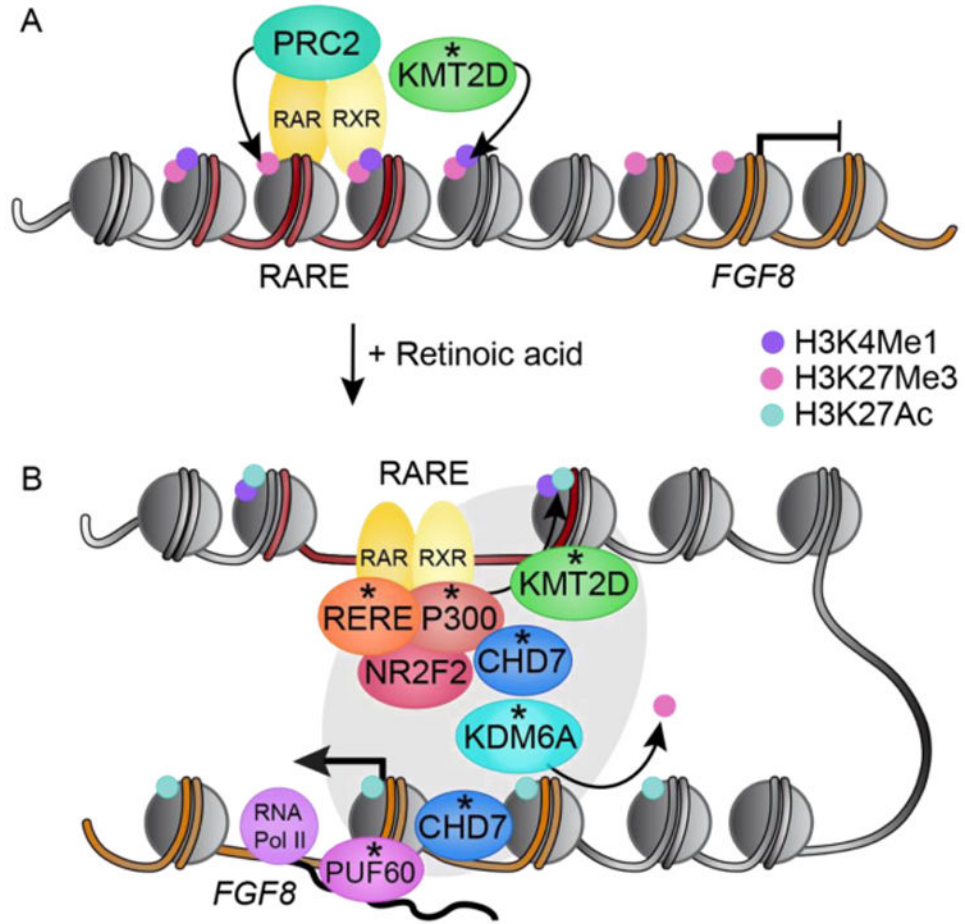


Figure 1. Model of molecular synergy implicated by genetic determinants (asterisks) that share clinical features of CHARGE syndrome. Cis-regulatory retinoic acid response elements (red DNA; RAREs) control cell-specific transcription of retinoic acid (RA) responsive genes, like *FGF8* (orange DNA). (A) In the absence of RA, Polycomb repressive complex 2 (PRC2) and KMT2D are recruited to RAREs, where they catalyze H3K27Me3 and H3K4Me1 respectively, promoting *FGF8* transcriptional repression. (B) In response to RA, the RERE/ NR2F2/P300 complex forms, binding nuclear heterodimers of retinoic acid receptors (RAR/ RXR) at RAREs. Histone acetyltransferase P300 and KMT2D cooperate to activate enhancer and enhancer-promoter looping that requires nucleosome translocation along the DNA by CHD7. KDM6A demethylates the repressive H3K27Me3 histone modifications. Enhancer-promoter looping is stabilized by the mediator complex (grey oval). CHD7 translocates nucleosomes to permit transcription of *FGF8* by RNA pol II. PUF60 helps form the transcription bubble required for RNA Pol II transcription and pre-mRNA splicing.

Table 1
Cohort Demographics and Clinical Features

Cohort	
Total individuals	67
Number of probands	28
Trios (proband and parents)	19
Duo (proband and mother)	1
Singletons	8
Proband Demographics	
Age	18m – 40yrs
Males	13 (46.4%)
Females	15 (53.6%)
Proband History of <i>CHD7</i> Genetic Testing	
Negative	12 (42.9%)
Not previously tested	16 (57.1%)
Proband Major Clinical Features	
Coloboma	26/28 (92.9%)
Choanal Atresia or Cleft Palate	19/28 (67.9%)
Abnormal External, Middle, or Inner Ears	19/28 (67.9%)
Proband Minor Clinical Features	
Cranial Nerve Dysfunction (including hearing loss)	20/28 (71.4%)
Dysphagia/feeding difficulties	8/28 (28.6%)
Structural brain abnormalities	3/28 (10.7%)
Developmental delay, intellectual disability or autism spectrum disorder	22/28 (78.6%)
Hypothalamo-hypophyseal dysfunction and genital anomalies	13/28 (46.4%)
Heart or esophageal malformations	20/28 (71.4%)
Renal anomalies or skeletal/limb anomalies	12/28 (42.9%)

Table 2

Probands, CHARGE major diagnostic features, gene variants, and pathogenicity evidence

Proband	CHARGE Major Criteria			Gene	Genomic Position	cDNA variant	Protein alteration	CADD Score	GERP Score	Inheritance	Status
	Coloboma	Choanal atresia or cleft palate	Abnormal external, middle or inner ears								
502	+	+	+	<i>CHD7</i>	chr8: 61,761,664	c.5355G>A	p.Trp1785*	48	5.87	de novo	known
503	+	+	-	<i>CHD7</i>	chr8: 61,655,332	c.1342_1343delAG	p.Arg448Glufs*126	N/A	5.539	de novo	novel
504	+	+	+	<i>CHD7</i>	chr8: 61,754,595	c.4835delA	p.Asn1612Ilefs*28	N/A	3.85	unknown	novel
505	+	+	+	<i>CHD7</i>	chr8: 61,773,655	c.7802dupA	p.Tyr2601*	N/A	5.73	de novo	novel ^a
506	+	-	+	<i>CHD7</i>	chr8: 61,736,406	c.3209delT	p.Val1070Glyfs*2	N/A	5.69	de novo	novel ^a
507	+	+	-	<i>CHD7</i>	chr8: 61,742,962	c.3606_3616dupAGAAACTAATTA	p.Ile1206Lysfs*9	N/A	5.47	de novo	novel ^a
508	+	+	+	<i>CHD7</i>	chr8: 61,757,970	c.5210+2T>C		22.6	5.48	de novo	novel ^b
509	+	-	-	<i>CHD7</i>	chr8: 61,714,150	c.2440C>T	p.Gln814*	44	6.17	inherited	known
510	+	+	+	<i>CHD7</i>	chr8: 61,750,761	c.4480C>T	p.Arg1494*	48	4.42	unknown	known
511	+	-	-	<i>CHD7</i>	chr8: 61,763,056	c.5409T>G	p.Tyr1803*	49	-1.18	de novo	novel
512	+	-	+	<i>CHD7</i>	chr8: 61,654,916	c.925C>T	p.Gln309*	40	5.56	unknown	known
513	+	+	+	<i>CHD7</i>	chr8: 61,765,241	c.6079C>T	p.Arg2027*	49	3.64	de novo	known
514	+	-	+	<i>CHD7</i>	chr8: 61,734,662	c.2915A>G	p.Gln972Arg	24.7	5.53	de novo	novel
515	+	+	+	<i>CHD7</i>	chr8: 61,736,402	c.3205C>T	p.Arg1069*	45	5.69	de novo	known
516	+	+	+	<i>CHD7</i>	chr8: 61,735,210	c.3106C>T	p.Arg1036*	45	5.53	de novo	known
517	+	+	+	<i>RERE</i>	chr1: 8,418,276	c.4313_4318dupTCCACC	p.Leu1438_His1439dup	N/A	5.61	de novo	known
518	+	-	+	<i>KMT2D</i>	chr12: 49,431,536	c.9602dupT	p.Ser3202Glufs*13	N/A	-0.809	unknown	novel
519	+	+	+	<i>KDM6A</i>	chrX: 44,820,553	c.250A>G	p.Ile84Val	17.16	5.66	X-linked recessive	novel
520	-	+	+	<i>EP300</i>	chr22: 41,553,171	c.3262-2A>G		24.4	5.7	de novo	novel
521	+	-	-	<i>PUF60</i>	chr8: 144,900,664	c.389G>A	p.Arg130His	25.9	5.18	de novo	novel

Known or novel status of *CHD7* variants were defined according to presence in the *CHD7* database at molgenis51.gcc.rug.nl on June 29, 2017. Known *CHD7* variants were also observed in 1000 Genomes with an association to CHARGE syndrome by the Human Gene Mutation Database or ClinVar. Abbreviations are as follows: known, identified in database and defined as pathogenic;

^a novel, known frameshifts observed at this protein position and defined as pathogenic;

^p novel, a splice site mutation is observed one nucleotide away at c.5210+3A>G and defined as pathogenic; N/A, not applicable.

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