

Original Paper

A Functional Mutation in HDAC8 Gene as Novel Diagnostic Marker for Cornelia De Lange Syndrome

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Key Words

Cornelia de Lange Syndrome • HDAC8 • Gene mutation • Enzymatic activity • X-chromosome inactivation

Abstract

Background/Aims: Cornelia de Lange Syndrome (CdLS) is a rare genetic disorder classically characterized by distinctive facies, growth retardation, intellectual disability, feeding difficulties, and multiple organ system anomalies. Previously, the diagnosis of CdLS was based mainly on identifying the typical phenotype in patients. However, with the advances in clinical molecular genetic diagnostic techniques, more patients, especially patients with milder phenotypes, are being diagnosed from detecting pathogenic mutation. **Methods:** Pathogenic mutation in a female patient with a milder phenotype was detected using whole-exome sequencing (WES), and was further characterized using bioinformatic analysis and *in vitro* functional experiments, including X-chromosome inactivation analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and enzyme activity assay. **Results:** This patient was found to harbor a novel missense mutation (c.806T>G, p.I269R) in the coding region of the *HDAC8* gene, which was predicted to be pathogenic. Compared with other CdLS patients with *HDAC8* mutation, the patient lacked typical facies, including synophrys and arched eyebrows. *In vitro* functional experiments showed the presence of skewed X-chromosome inactivation. Furthermore, the novel mutation decreased the dissolubility and enzymatic activity of HDAC8 protein. **Conclusions:** The present study identified a novel missense mutation (c.806T>G, p.I269R) in the *HDAC8* gene leading to CdLS, which not only provided strong evidence for diagnosis in this present patient, but also expanded the spectrum of pathogenic mutations for CdLS.

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Introduction

Cornelia de Lange Syndrome (CdLS) is a rare genetic disorder classically characterized by distinctive facies, growth retardation, intellectual disability, feeding difficulties, and multiple organ system anomalies. The worldwide incidence of CdLS is reported to range from 1 to 3 per 30,000 births [1]. However, in view of the wide clinical variability and limited clinical knowledge about the disease, the actual global incidence of CdLS may be higher. At present, the diagnosis of CdLS is based primarily on distinctive facies and other typical phenotypic features [2]. Thus, it is difficult to make an accurate diagnosis in patients with milder phenotypes based on phenotypic features alone. With the development of clinical molecular genetic diagnostic techniques however, such patients can now be diagnosed by detecting mutations in CdLS-related genes, mainly including *NIPBL*, *SMC1A*, *SMC3*, *RAD21*, and *HDAC8* [1].

Dysregulation of CdLS-related genes plays an important role in the occurrence of CdLS [3]. The proteins encoded by these genes are involved the composition and function of the cohesin ring, which contributes to regulating chromosome separation, DNA repair, and gene expression [4]. Among clinically diagnosed CdLS patients, more than 60% of patients have *NIPBL* mutation, about 5% patients have *SMC1A* or *HDAC8* mutation, and less than 1% patients have *SMC3* or *RAD21* mutation [5]; in the remaining 30% of patients the genetic causes are unknown [5]. Of these CdLS-related genes, *HDAC8* contains 11 exons and spans approximately 243 kilobases on human chromosome Xq13.1. It encodes a 42kDa zinc-dependent metalloproteinase, which regulates deacetylation of the SMC3 protein. Pathogenic variation of the *HDAC8* gene results in CdLS 5 (OMIM#300882) which has an X-linked dominant inheritance and was first reported in 2012 [6]. In 2014, Kaiser et al. summarized the phenotypes of 35 CdLS patients with *HDAC8* mutation, and found that most patients had phenotypes similar to the typical CdLS phenotype including synophrys, intellectual disability, and feeding difficulties [7]. However, upper limb defect, growth retardation, and microcephaly were rarely reported in these patients. The absence of these typical phenotypic features in female patients may be due to skewed X-chromosome inactivation (SXI). Recently, we identified a novel missense mutation [NM_018486.2:c.806T>G (p.I269R)] in *HDAC8*, in a 1.5-year-old female patient who had the following phenotypic features: small for gestational age, feeding difficulties in infancy, microcephaly, and growth retardation, but no distinctive facies. According to the clinical diagnostic criteria for CdLS, this patient was not a typical case of CdLS [7]. In addition, it was unclear whether the novel *HDAC8* mutation is a pathogenic mutation. In order to make an accurate diagnosis in this patient who had a milder phenotype, we carried out a literature search, summarized the phenotypic features of CdLS patients with *HDAC8* mutation, and then compared their phenotypes with those of our patient. Eventually, the pathogenicity of the novel *HDAC8* mutation was predicted by using bioinformatics tools, and was verified with SXI analysis and HDAC8 enzymatic activity assay, which provided strong evidence for accurate genetic diagnosis of our patient.

Materials and Methods

Ethical statement

This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was certified by the Ethics Committee of Xinhua Hospital affiliated with the Shanghai Jiaotong University School of Medicine. Written informed consent was obtained from the parents of the patient.

Patient

The patient was a 1.5-year-old female patient with growth retardation. Her height was 67 cm, weight 7 kg, and head circumference 41 cm. She had no distinctive facies or other phenotypic anomalies, except for delayed motor and speech development (Fig. 1). In infancy, she had no sucking reflex and was

usually fed using a dropper. However, the patient did not meet the diagnostic criteria for CdLS [2]. Previous evaluation showed that our patient had elevated 3-hydroxydecanoylcarnitine (C18-OH) concentration on plasma acylcarnitine profile analysis, normal levels of plasma very long chain fatty acids, negative *ABCD1* gene testing, widened extracerebral space in the frontoparietal region bilaterally, developmental quotient score of 63, normal karyotype, and normal thyroid function and other biochemical indices. Karyotype analysis and chromosomal microarray were also normal. Thus, we performed whole-exome sequencing (WES) to make a definitive diagnosis.

Identification of the putative pathogenic mutation

Genomic DNA was isolated from peripheral blood leukocytes using the Lab-Aid Nucleic Acid (DNA) Isolation Kit (Xiamen Zeesan Biotech Co., Ltd, China), captured using the TruSeq DNA Sample Preparation Kit (Agilent Technologies, Ltd., Santa Clara, CA), and sequenced on an Illumina Genome Analyzer IIx sequencing system (Illumina, Inc., San Diego, CA), generating 2×100 bp paired-end reads. Exome data processing, variant calling, and variant annotation were performed as previously described [8]. Data analysis was performed independently by two investigators. Any disagreement was resolved by discussion with a third expert. Regarding the pattern of inheritance, the putative pathogenic mutation was validated via Sanger sequencing and DNA-based paternity testing. Sequencing products were analyzed using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). For DNA-based paternity testing, multiplex polymerase chain reaction (PCR) amplification was performed with the Identifiler™ system, and PCR products were genotyped with capillary electrophoresis in an ABI 3730xL DNA Analyzer (Applied Biosystems).

Analysis of phenotypic features of CdLS patients with HDAC8 mutation

To determine the phenotypic features of CdLS patients with *HDAC8* mutation, we searched for and identified all related articles in the PubMed database (<https://www.ncbi.nlm.nih.gov/pubmed/>). Patients with *HDAC8* mutation were first reported in 2012, and so the publication year search was limited to 2012–2016. Phenotypic features of all patients were sorted according to the phenotypic items of CdLS 5 in OMIM. For patients with height records but without a corresponding height standard deviation score (Ht SDS), WHO growth charts (≤ 2 years of age) or Centers for Disease Control and Prevention growth charts (>2 years of age) were used to calculate the corresponding Ht SDS (<https://www.cdc.gov/growthcharts/>); Ht SDS <-2 was defined as short stature.

SXCI analysis

In line with a previous design, three pairs of PCR primers were synthesized and used to amplify exon 1 of the human androgen receptor (*AR*) gene [9]. All forward primers were labelled with the fluorescent dye 6-carboxyfluorescein. Primer sequences are listed in Table 1. Of these, the HAR primer pair was used to detect the presence of short tandem repeat (STR) polymorphisms in *AR* exon 1, which could help identify the source of the X chromosome. Genomic DNA was treated with bisulfite for methylation analysis using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's instructions. *AR-M* and *AR-U* primer pairs used bisulfite-treated DNA as a template to amplify DNA from the inactive and active X chromosomes, respectively. SXCI was determined by calculating the ratio of peak areas of the methylated short fragments versus long fragments as previously reported [9]. Ratios of peak area $>80/20$ or $<20/80$ were considered indicative of SXCI.



Fig. 1. Photographs of the female patient with HDAC8 c.806T>G mutation (A: 1.5 years old; B: 3 years old).

Table 1. Primer sequences used for skewed X-chromosomal inactivation analysis

Primer name	Primer sequences (5'-3')
HAR-F	TCCAGAATCTGTTCCAGAGCGTGC
HAR-R	CTCTACGATGGGCTTGGGAGAAC
AR-M-F	GCGAGCGTAGTATTTTTCGGC
AR-M-R	AACCAATAACCTATAAACCTCTACG
AR-U-F	GTTGTGAGTGTAGTATTTTTCGGT
AR-U-R	CAAATAACCTATAAACCTCTACA

Function prediction for the putative pathogenic mutation

In the process of WES data annotation, all mutations had been predicted by using MutationTaster (<http://www.mutationtaster.org/>), SIFT (<http://sift.jcvi.org>), and Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>) software [10-12]. We further assessed evolutionary conservation of the putative pathogenic mutation locus using HomoloGene (<https://www.ncbi.nlm.nih.gov/homologene>) and Clustal Omega (<http://www.clustal.org/omega/>) tools. The effect of the putative pathogenic mutation on protein structure was also predicted using Swiss-PdbViewer 4.1 software (<http://spdbv.vital-it.ch/>) [13].

Biosynthesis and acquisition of HDAC8 protein

Normal human HDAC8 cDNA was synthesized and inserted between the EcoRI and XhoI sites of a pGEX-6p-1 plasmid by using recombinant DNA technology. The new recombinant plasmid, pGEX-WT, was subjected to site-directed mutagenesis via PCR. Primer sequences for site-directed mutagenesis (c.806T>G, p.I269R) are presented in Table 2. PCR products were linked by T4 polynucleotide kinase and T4 DNA ligase, and named pGEX-I269R. The pGEX-WT and pGEX-I269R plasmids were separately transformed into *E. coli* BL21(DE3), and then induced using isopropyl β -D-thiogalactopyranoside (IPTG) (Amresco Inc., Solon, OH). After 14 h of induction, the bacterial cells were lysed by sonication. Samples were then centrifuged and the supernatant containing recombinant HDAC8 protein was collected. An aliquot of the supernatant was used to determine the dissolubility of recombinant HDAC8 protein using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining supernatant was subjected to GST-tag affinity chromatography to separate recombinant HDAC8 proteins. Finally, the recombinant proteins were digested using human rhinovirus 3C protease to obtain HDAC8 wild-type and variant proteins, which were purified using the AKTA™ Start protein purification system (GE Healthcare Life Sciences, Chicago, IL).

HDAC8 activity assay

The enzymatic activity of HDAC8 wild-type and variant proteins was measured by using an HDAC activity assay kit (Active Motif, Carlsbad, CA). Briefly, Boc-Lys (acetyl)-AMC substrate was deacetylated using co-incubation with HDAC8, and then hydrolyzed with HDAC developer solution to produce AMC fluorophores. HDAC8 activity was then assessed by calculating the fluorescence intensity.

Statistical analysis

Data analysis was carried out using SPSS statistical software package version 13.0. Continuous and categorical variables are presented as the mean $\pm$ standard deviation (SD) and number (%), respectively. The activity of HDAC8 wild-type and variant proteins was compared using the independent samples t-test. $P < 0.05$ was considered statistically significant.

Results*Identification of the putative pathogenic mutation*

A novel missense mutation (c.806 T>G, p.I269R) in the *HDAC8* gene was identified as a pathogenic mutation based on WES data annotation (Fig. 2A). To validate the putative pathogenic mutation, Sanger sequencing and DNA-based paternity testing were carried out for the patient and her parents. Results showed that the novel missense mutation was a *de novo* mutation (Fig. 2B).

Table 2. Primer sequences for HDAC8 gene (c.806T>G, p.I269R) site-directed mutagenesis

Primer name	Primer sequences (5'-3')
I269R-F	cgtGCTGGGGATCCCATGTGCTC
I269R-R	TGTGTGTCAGTCCAGCTGTAAG

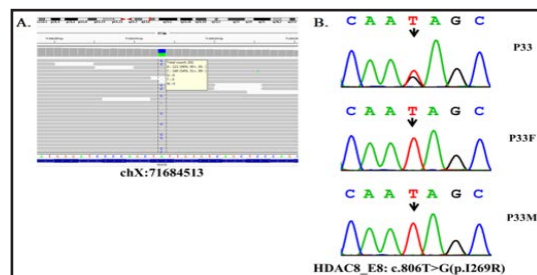


Fig. 2. The novel HDAC8 gene mutation identified using WES (A) and Sanger sequencing (B) [P33: our patient; P33F (father) and P33M (mother) confirmed by paternity testing].

Table 3. Phenotypic features of CdLS patients with HDAC8 mutation. Note: Some phenotypes were not included due to limited number of cases. ^a The patient in this report was aged 1.5 years. ± suspicious phenotype but was considered negative

System/organ	Phenotype	Our patient ^a	Harakalova et al. ^[14]	Kaiser et al. ^[7]	Feng et al. ^[15]	Fieremans et al. ^[16]	Parenti et al. ^[17]	Total
		Total = 1	Total = 10	Total = 35	Total = 1	Total = 1	Total = 11	59
Sex (M/F) No.		F	7M3F	8M27F	F	F	3M8F	18M41F
Height	Short stature	+	5/10 (50.0%)	18/29	NA	+	11/11	69.2%
	SGA	+	NA	13/27	-	NA	NA	48.3%
Weight	Truncal obesity	-	8/10 (80.0%)	2/29 (6.9%)	NA	NA	NA	25.0%
Head	Brachycephaly	-	7/9 (77.8%)	24/34	NA	NA	10/11	74.5%
	Microcephaly	+	NA	13/29	-	NA	4/11 (36.4%)	42.9%
Face	Arched eyebrows	-	NA	30/34	NA	±	10/11	85.1%
	Synophrys	-	NA	30/33	+	+	10/11	89.4%
	Long eyelashes	-	NA	15/32	+	NA	NA	47.1%
	Wide eye distance	+	NA	16/34	NA	NA	10/11	58.7%
	Myopia	NA	NA	11/26	+	NA	7/11 (63.6%)	50.0%
	Ptoisis	-	NA	6/32 (18.8%)	NA	NA	NA	18.2%
	Depressed nasal bridge	±	NA	15/33	+	NA	5/11 (45.5%)	45.7%
	Anteverted nostrils	-	NA	26/34	+	NA	6/11 (54.5%)	70.2%
	Wide/bulbous nose	±	NA	22/33	+	NA	9/11 (81.8%)	69.6%
	Downturned corners of the mouth	+	NA	19/33	NA	NA	6/11 (54.5%)	57.8%
	High arched palate	-	NA	8/22 (36.4%)	NA	NA	NA	34.8%
	Cleft palate	-	NA	5/27 (18.5%)	NA	NA	NA	17.9%
	Long philtrum	-	NA	19/33	NA	NA	4/11 (36.4%)	51.1%
	Micrognathia/retrognathia	±	NA	20/33	NA	+	10/11	67.4%
Teeth	Widely spaced teeth	NA	NA	16/26	+	NA	NA	63.0%
Eyes	Hearing loss	-	NA	18/29	NA	NA	7/11 (63.6%)	61.0%
Neck	Short neck	±	NA	12/25	NA	NA	NA	46.2%
Gastrointestinal	Feeding difficulties (in infancy)	+	NA	25/29	NA	NA	5/11 (45.5%)	75.6%
	Gastroesophageal reflux	NA	NA	19/28	NA	+	4/11 (36.4%)	60.0%
Hands	Small hands	±	5/7 (71.4%)	28/29	+	NA	8/11 (72.7%)	85.7%
Feet	Small feet	±	NA	27/30	+	NA	4/8 (50.0%)	80.0%
	Toe syndactyly	-	NA	13/28	NA	NA	1/11 (9.1%)	35.0%
Hair	Low anterior hairline	-	NA	23/34	NA	NA	NA	65.7%
	Hirsutism	NA	NA	21/32	NA	NA	NA	65.6%
Central Nervous	Intellectual disability/ delayed	+	8/10 (80.0%)	33/33	+	+	11/11	96.5%

Phenotypic features of CdLS patients with HDAC8 mutation

Phenotypic features of CdLS patients with *HDAC8* mutation were collated from a PubMed search. Phenotypic records were available for a total of 58 patients and are summarized in Table 3. Height and Ht SDS records were available for 37 patients, including 24 female patients and 13 male patients, [median Ht SDS: -2.1 [-6.6~0.9]]. Short stature was observed in 12/24 female patients [median Ht SDS: -2.0 [-4.5~0.4]] and 9/13 male patients [median Ht SDS: -2.3 [-6.6~0.9]]. The height defect was milder in females compared with male patients. Of all female patients, the height defect of our patient was the most severe (Ht SDS: -4.8). Thus, short stature was found to be a common feature of CdLS patients with *HDAC8* mutation. Besides short stature, intellectual disability was also observed in most CdLS patients with *HDAC8* mutation, but the degree of intellectual disability differed among patients. CdLS patients with *HDAC8* mutation also had some common facies, mainly wide nose with a broad nasal bridge, arched eyebrows with synophrys, anteverted nostrils, and micro-/retrognathia. Our patient, however, did not have typical facies such as arched eyebrows and synophrys. Other phenotypic features, including small hands and feet and feeding difficulties in infancy, were also observed in CdLS patients with *HDAC8* mutation.

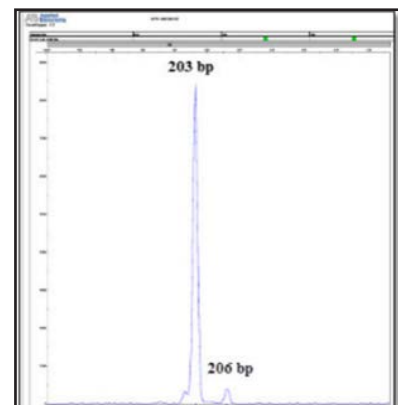
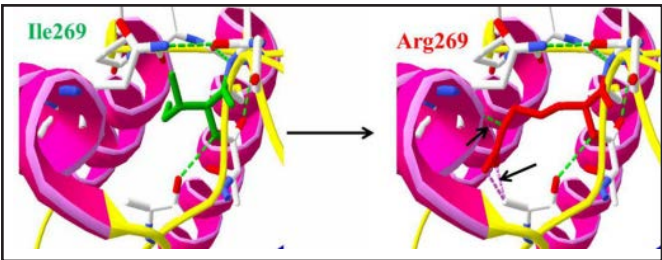


Fig. 3. Skewed X-chromosome inactivation of the patient (using AR-M primers).

Fig. 4. The evolutionary conservation of the novel HDAC8 gene mutation locus (Homo sapiens, Pan troglodytes, Macaca mulatta, Canis lupus, Bos taurus, Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio and Xenopus tropicalis).



Fig. 5. The three-dimensional models of HDAC8 wild-type (Ile269) and variant (Arg269) proteins (The green and pink dotted line indicated by the arrow represents hydrogen bond and steric hinerance, respectively).



SXCI analysis

Results of SXCI analysis showed that our patient had a polymorphic STR locus in the amplification region of the *AR* gene, which produced two fragments of different length (203 bp and 206 bp), thus distinguishing the source of the X chromosome. The ratio of peak areas of the methylated short and long fragments was 88.9/11.1, confirming SXCI (Fig. 3).

Prediction of function of the putative pathogenic mutation

The novel *HDAC8* gene mutation (c.806 T>G, p.I269R) in our patient had been predicted to be pathogenic based on snpEFF, Polyphen2-HVAR, SIFT, and MutationTaster. Therefore, we proceeded to predict the evolutionary conservation of the locus of this mutation, and the effect of the novel missense mutation (p.I269R) on HDAC8 protein structure. The I269 locus was found to be relatively highly conserved (Fig. 4). The variant amino acid (R), located in a β turn of HDAC8 protein, not only had a larger molecular weight compared with the wild-type amino acid (I), but also carried a positive electrical charge, which might affect the proper folding of the HDAC8 protein (Fig. 5).

In vitro expression of recombinant HDAC8 proteins

SDS-PAGE showed that *in vitro* expression of the recombinant HDAC8 proteins was successfully induced by IPTG. Assay of the supernatant obtained also indicated a lower dissolubility of recombinant HDAC8 variant protein versus that of recombinant HDAC8 wild-type protein (Fig. 6).

Enzymatic activity of HDAC8 wild-type and variant proteins

Enzymatic activity assay showed that the enzymatic activity of HDAC8 wild-type and variant proteins was 356.7 ± 27.5 and 41.8 ± 1.6 nmol-product/ μ mol-enzyme/min, respectively ($P < 0.01$), indicating that the novel missense mutation significantly decreased the activity of HDAC8 protein (Table 4).

Table 4. Enzymatic activity of HDAC8 wild-type and variant proteins

Activity (nmol-product/ μ mol-enzyme/min) mean $\pm$ standard deviation (n = 3)	
WT	356.7 ± 27.5
I269R	41.8 ± 1.6

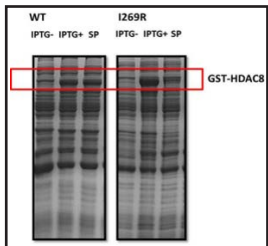


Fig. 6. In vitro expression of recombinant HDAC8 proteins (IPTG-: non-induced bacteria, IPTG+: induced bacteria lysed by sonication, SP: supernatant liquid after bacterial lysis).

Discussion

With the advent of high-throughput sequencing technologies, an increasing number of genetic causes of disease are being identified. However, because of a lack of clinical appreciation of diseases and atypical phenotypes of patients, correctly interpreting high-throughput sequencing data remains a challenge. The current study has described a 1.5-year-old female patient, initially diagnosed with growth retardation and who was further evaluated using WES. We found that the patient harbored a novel missense mutation (c.806T>G, p.I269R) in the *HDAC8* gene, which was predicted to be pathogenic. In 2012, Deardorff et al. found that pathogenic mutations of the *HDAC8* gene could cause CdLS 5 [6]. Thus, in order to make an accurate diagnosis for our patient with *HDAC8* c.806T>G mutation, we collated the phenotypic features of CdLS patients with *HDAC8* mutation from a literature search, and found that most patients had the following facial features: arched eyebrows with synophrys, wide bulbous nose, anteverted nostrils, and micro-/retrognathia. However, there was a report of another female patient without arched eyebrows and synophrys, similar to the present patient. In addition, Kline et al. found that the facies of CdLS patients could change with age [18]. Therefore, it is likely that the facies of these two female patients without synophrys and arched eyebrows may become more typical with advancing age. Although our patient had some phenotypic features that differed from those of typical CdLS patients, she also had intellectual disability, a common feature of CdLS patients with *HDAC8* mutation. Previous studies indicated that atypical phenotypes of female CdLS patients with *HDAC8* mutation might be due to SXCI [6, 7, 16]. In view of this, SXCI analysis of the present patient was also carried out and confirmed the presence of SXCI, which could explain the atypical phenotype of the patient. To quantify the degree of phenotypic variance, we then applied the scoring system proposed by Kline et al. and the phenotypic classification described by Guillis et al. Quantitative analysis showed that the patient had a milder phenotype, and thus a milder form of CdLS [2, 19].

Although the novel *HDAC8* mutation (c.806T>G, p.I269R) in this patient had been predicted to be pathogenic, the predicted results, based on bioinformatics, were not entirely reliable. So, we further assessed the function of the novel missense mutation by using SDS-PAGE and enzymatic activity assay. SDS-PAGE showed that recombinant HDAC8 variant protein had a lower dissolubility compared with recombinant HDAC8 wild-type protein, suggesting that the missense mutation might affect the structure of HDAC8 protein. Enzyme activity assay showed that variant HDAC8 activity was approximately 12% of wild-type HDAC8 activity, indicating that the missense mutation could affect the function of HDAC8.

In conclusion, we found a novel missense mutation (c.806T>G, p.I269R) in the *HDAC8* gene resulting in CdLS, which not only provided strong evidence for definitive diagnosis in this patient, but has also expanded the spectrum of pathogenic mutations for CdLS.

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Disclosure Statement

The authors have no conflict of interests to disclose.

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