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## CLINICAL REPORT

# Ending a diagnostic odyssey: Moving from exome to genome to identify cockayne syndrome

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## ABSTRACT

**Background:** Cockayne syndrome (CS) is a rare autosomal recessive disorder characterized by growth failure and multisystemic degeneration. Excision repair cross-complementation group 6 (*ERCC6* OMIM: \*609413) is the gene most frequently mutated in CS.

**Methods:** A child with pre and postnatal growth failure and progressive neurologic deterioration with multisystem involvement, and with nondiagnostic whole-exome sequencing, was screened for causal variants with whole-genome sequencing (WGS).

**Results:** WGS identified biallelic *ERCC6* variants, including a previously unreported intronic variant. Pathogenicity of these variants was established by demonstrating reduced levels of *ERCC6* mRNA and protein expression, normal unscheduled DNA synthesis, and impaired recovery of RNA synthesis in patient fibroblasts following UV-irradiation.

**Conclusion:** The study confirms the pathogenicity of a previously undescribed upstream intronic variant, highlighting the power of genome sequencing to identify noncoding variants. In addition, this report provides evidence for the utility of a combination approach of genome sequencing plus functional studies to provide diagnosis in a child for whom a lengthy diagnostic odyssey, including exome sequencing, was previously unrevealing.

## KEY WORDS

cockayne syndrome, DNA repair, *ERCC6*, whole-genome sequencing

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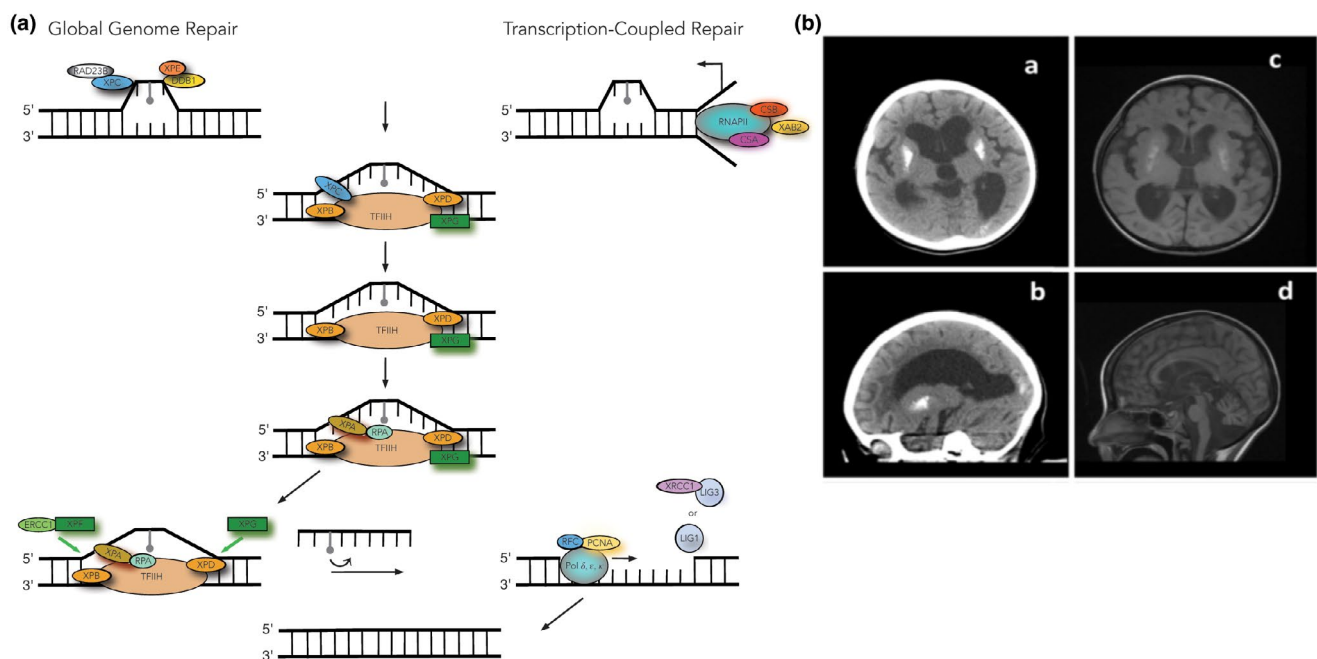
Molecular diagnosis of children with rare neurodegenerative and complex multisystem disease is challenging especially when the phenotypic presentation deviates from what is reported in the literature. Next-generation sequencing (NGS) techniques have improved diagnostic rates by providing an unbiased diagnostic approach guided, but not limited by, phenotype. Nevertheless, for rare disorders for which few variants may be reported and the phenotypic spectrum may not yet be fully elucidated, variants of uncertain significance (VUS) remain a vexing problem for NGS interpretation. Functional studies are often unavailable to investigate VUSs and even when available, are often beyond the diagnostic scope of a clinical testing laboratory.

Cockayne syndrome (CS) is a spectrum diagnosis that is characterized by growth deficiency, premature aging, pigmentary retinal degeneration as well as multiple other neurologic and systemic findings. Standard CS classification is based on the age at onset and severity of symptoms and progression. The classifications are as follows: CS-I, canonical; CS-II (including Cerebro-Oculo-Facio-Skeletal [COFS] syndrome and Pena-Shokeir type 2 syndrome [PS-2]), very severe; CS-III, mild; CS-IV, late onset; and xeroderma pigmentosum–Cockayne syndrome (XP-CS) (Laugel, 2000).

CS-I is the classic form with symptom onset early in life. CS-II is more severe with symptoms evident pre-natally or at birth. The majority of CS cases are caused by mutations

in *ERCC6*OMIM: \*609413) or *ERCC8* (OMIM: \*609412). *ERCC6* mutations are most common in Caucasian CS patients, but in other ethnic groups, *ERCC8* mutations are more prevalent (Wilson et al., 2016). Mutations in *ERCC6* are also seen in cerebro-ocular facial-skeletal syndrome (COFS) (Laugel et al., 2008), XP-CS (Colella et al., 2000), and Ultraviolet (UV)-Sensitive Syndrome (Horibata et al., 2004). Recessive mutations in five additional DNA repair genes may cause CS [*ERCC3* (OMIM: \*133510), *ERCC2* (OMIM: \*126340), *ERCC5* (OMIM: \*133530), *ERCC4* (OMIM: \*133520), and *ERCC1* (OMIM: \*126380)] in association with XP (Kashiyama et al., 2013; Laugel et al., 2010) (Figure 1A). Roughly 65% of patients with CS have mutations in *ERCC6* (Colella et al., 2000). The true prevalence of CS remains unknown. A study in 2008 estimated the incidence at 2.7 per million births in western Europe (Kleijer et al., 2008), though this is likely an underestimate in part due to reliance on the presence of photosensitivity as a diagnostic feature (Kleijer et al., 2008).

All seven genes that if mutated can cause CS are critical for nucleotide excision repair (NER), the pathway responsible for the repair of helix-distorting DNA adducts including UV-induced photolesions (Schärer, 2013). NER consists of two subpathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER) (Figure 1A) (Gillet & Schärer, 2006; Hanawalt & Spivak, 2008). CSA (*ERCC8*) and



**FIGURE 1** (A) Nucleotide Excision Repair (NER) Pathway and brain imaging of patient INE4CC. Global Genome NER (left) and Transcription Coupled NER (right) converge. (B) Brain MRI and CT Scan: CT Scan age 5 (a and b) demonstrates diffuse cerebral and cerebellar atrophy. There is dense calcification of the bilateral globi pallidi and parieto-occipital and left frontal (not shown) cortical calcification. MRI Scan age 6. T1 Images (c and d) show diffuse supra and infratentorial volume loss evidenced by ventriculomegaly, widened cortical sulci, diffuse thinning of the corpus callosum and inferior vermian and brainstem hypoplasia. There was an abnormal basal ganglia signal including mild T2 hyperintensity (not shown) T1 shortening and susceptibility artifact of the lenticular nuclei consistent with mineralization

CSB (*ERCC6*) are key TC-NER factors that participate in the repair of transcription-blocking DNA lesions that stall RNA polymerase II (Schärer, 2013). Once the lesion is recognized in the genome or transcribed areas of the genome, the two subpathways converge, utilizing additional proteins to unwind and stabilize the DNA around the lesion, enabling two endonucleases to excise the lesion as part of a single-stranded oligonucleotide. The gap left after the removal of the damaged oligonucleotide is filled by templated DNA synthesis by replication factors including polymerases  $\delta$ ,  $\epsilon$ ,  $\kappa$ , PCNA, RFC, RPA, and DNA ligase (Schärer, 2013). Generally speaking, mutations in genes needed for GG-NER cause xeroderma pigmentosum, a cancer predisposition syndrome, while mutations in genes needed exclusively for TC-NER cause CS. However, the impact of a particular mutation or sequence variant on protein expression and function complicates this generalization (Laugel et al., 2010).

The only CS symptom clearly caused by defective NER is photosensitivity. Despite decades of research, there is no clear explanation for the mechanistic basis of CS. A variety of roles outside of TC-NER have been proposed for CSA and CSB (Karikkineth et al., 2017; Kumar et al., 2020), though at present none may be conclusively mechanistically linked to the spectrum of symptoms observed in CS patients. Genomic sequencing has allowed expansion of the phenotype as in this case where the presentation is atypical.

The patient (INE4CC) is a 7-year-old female with multi-system disease including: failure to thrive, congenital microcephaly, global developmental delay with motor and language regression, tremor, ataxia, cardiomyopathy, renal dysfunction, chronic lung disease, diabetes, hypothyroidism, and hypertension. Pregnancy was notable for intrauterine growth retardation. Uncomplicated Cesarean section delivery occurred at 37 weeks. Birth weight was 2,126 gm ( $z = -2.37$ ), head circumference was 31 cm ( $z = -2.4$ ), and birth length was 46 cm ( $z = -1.44$ ). Growth remained poor with a steady decline in  $z$ -scores (age 7 years: length  $z = -8$ ; weight  $z = -3.3$ ; head circumference  $z = -3.5$ ). Poor weight gain ultimately led to G-tube placement at 23 months.

Fine motor delays with tremor emerged at 1 year. Pulling to stand occurred at 12 months, but walking was delayed until 29 months. She said her first word at 9 months, but by 33 months, had only a 40-word vocabulary. Regression in motor skills (associated with infection) occurred at age 3 years and again at 4.5 years. Though she never regained independent ambulation, development progressed; at age 7 she spoke in phrases, followed simple commands and walked short distances using a walker.

Course was further complicated by hypothyroidism, insulin-dependent diabetes, cardiomyopathy, kidney fibrosis, chronic lung disease, hypertension, dental caries, hearing impairment, and movement disorder (action-induced tremor and paroxysmal tremulous episodes). There is no relevant

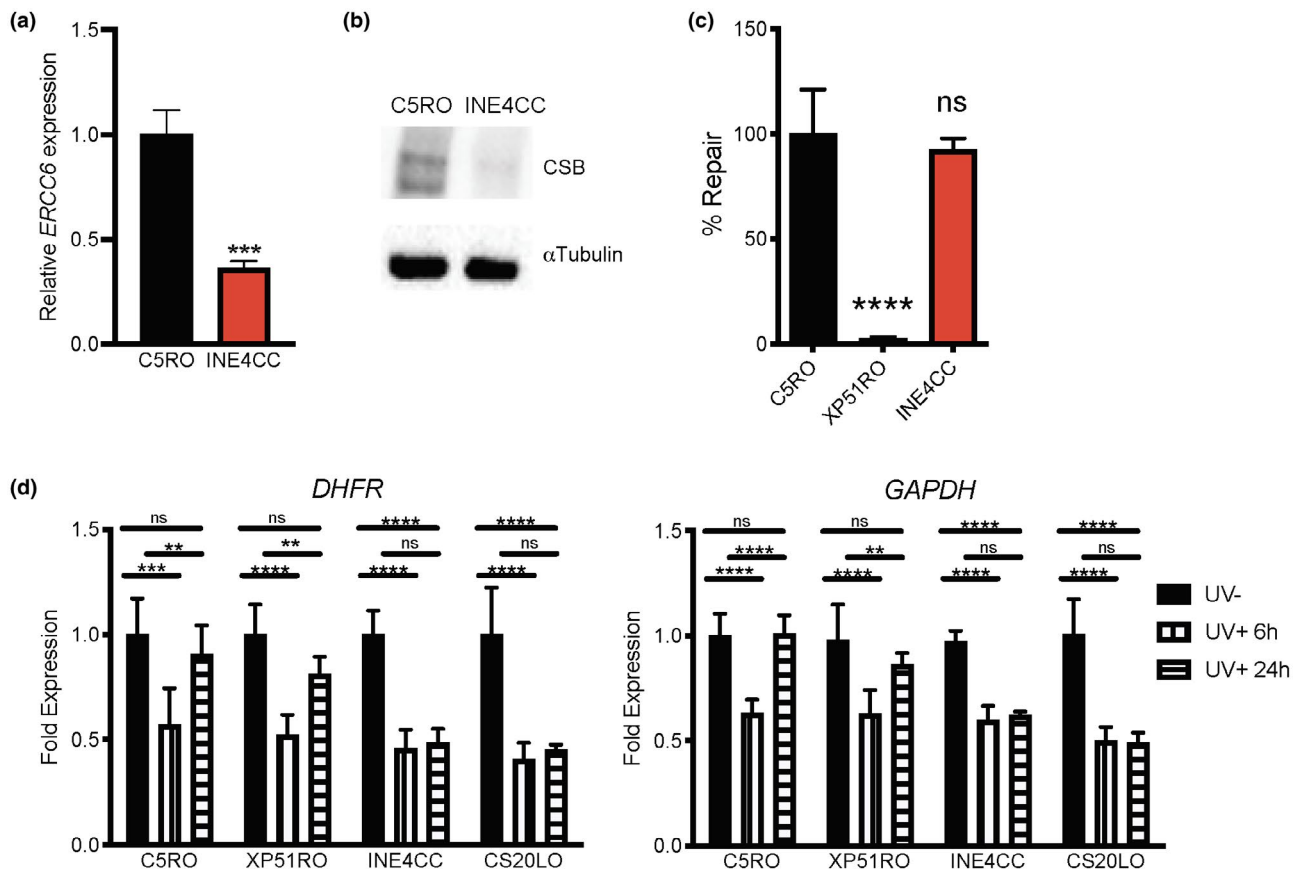
family history or consanguinity. Maternal and paternal country of origin is Korea.

Dilated ophthalmologic evaluation at ages 3 and 4 years showed no cataracts and normal retinas; exam at age 5 years revealed small corneas, prominent Schwalbe's line, severe hyperopia, optic nerve atrophy, foveal hypoplasia, and bilateral reticulated retinal pattern with central vascular sheathing of retinal arterioles and venules. She displayed gaze-evoked nystagmus in all directions. There was increased appendicular tone, hypoactive reflexes, titubation, and tremor with reaching.

Brain MRI (age 2) showed inferior vermian hypoplasia with cerebellar atrophy, diffuse white matter signal abnormality, and mineralization of the basal ganglia. Repeat brain MRI (age four) showed progression of supratentorial volume loss. Computed Tomography Scan (age 5) showed dense calcification of the globi pallidi and lentiform nuclei and calcification of the parietal, occipital and frontal cortices, with stable vermian hypoplasia and supratentorial volume loss (Figure 1B). Radiographs showed diffuse platyspondyly and acetabular dysplasia. Muscle biopsy showed type 2 fiber atrophy of probable central origin. Whole-exome sequencing, right upper and lower motor and sensory nerve conductions and electromyogram of selected muscles of the right lower extremity showed no abnormalities.

Evaluation of this patient was undertaken as detailed in Supplemental Materials. Whole-genome sequencing (WGS) (Farnaes et al., 2018 and Briggs et al., 2018) initially revealed two variants of uncertain significance (VUS) in the *ERCC6* gene: a maternally inherited NM\_00124.3:c.1583G>A, p. Gly528Glu missense variant and a paternally inherited NM\_00124.3:c.-15+3G>T upstream intronic variant (Figure S1). Both variants were absent from the gnomAD population database. At the time of initial analysis, both variants were unreported in the literature. However, *in silico* prediction algorithms were suggestive of pathogenicity with the p. Gly528Glu variant predicted to be damaging by SIFT and Polyphen, and the c.-15+3G>T variant predicted to alter splicing by multiple splicing algorithms utilized by Alamut (Interactive Biosoftware, Rouen, France). Based on the predicted consequence of both variants and the phenotypic overlap between the patient and CS, the family was approached to discuss the possibility of further functional testing. The patient dermal fibroblast line was evaluated for the expression of *ERCC6* and CSB protein levels (Figure 2a,b). *ERCC6* expression was significantly reduced in INE4CC patient cells compared to control C5RO fibroblasts (Figure 2a) and immunoblotting revealed nearly undetectable CSB protein in the patient cells (Figure 2b). These data suggest both *ERCC6* variant alleles contribute to significantly reduced *ERCC6*/CSB levels.

*ERCC6* is required for TC-NER. Thus, diagnosis of CS requires measurement of NER and more specifically TC-NER.



**FIGURE 2** Functional consequences of biallelic *ERCC6* variants in INE4CC cells. (a) *ERCC6* mRNA was quantified in patient (INE4CC) and control (C5RO) dermal fibroblasts by qRT-PCR in triplicate using the  $\Delta\Delta$ Ct method and normalized to *GAPDH* expression. (b) CSB protein levels were detected by immunoblotting of whole-cell extracts with  $\alpha$ Tubulin as control. (c) Measurement of UV-induced unscheduled DNA synthesis (UDS) in dermal fibroblast lines (C5RO: unaffected control; XP51RO: patient with *ERCC4* mutation affecting NER; the patient in this study: INE4CC). Cells were UV-C- or sham-irradiated and then incubated with the thymidine analog EdU to allow time for DNA repair. Alexa Fluor 647 was conjugated to EdU incorporated into the nuclear genome before cell fixation, DAPI staining, and flow cytometric quantification of Alexa Fluor 647 intensity in  $G_1$  cells. UDS was measured in triplicate for each sample and normalized to the control samples. (d) Recovery of RNA synthesis in fibroblasts following UV irradiation. Expression of the house-keeping genes *DHFR* and *GAPDH* was measured after UV-C irradiation of cells from C5RO (normal control), XP51RO (*ERCC4* mutation with a diagnosis of XFE progeroid syndrome), CS20LO (CS caused by mutations in *ERCC1*), and the patient in this study (INE4CC). Expression was measured at baseline (no UV) and at 6 and 24 hr postirradiation and normalized to quantity of *18s* rRNA. Results were plotted as the ratio of expression in irradiated versus sham-irradiated cells. qPCR reactions were performed in triplicate for five independent experiments. Values represent mean  $\pm$  SD, ns  $p \geq 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared using unpaired two-tailed Student's *t* test or one-way ANOVA with Tukey's test

To determine if the patient's sequence variants had a functional impact on NER, unscheduled DNA synthesis (UDS) was measured following UV-irradiation of the patient cells (Mori et al., 2018). UV-induced UDS is a direct measure of GG-NER capacity. Defective GG-NER is pathognomonic for XP and defective TC-NER is pathognomonic for CS, whereas mutations in common components of both GG-NER and TC-NER can cause XP, less commonly trichothiodystrophy, or less commonly CS, COFS, or even Fanconi anemia (Kashiyama et al., 2013). UDS was not impaired in INE4CC patient fibroblasts compared to a normal control (C5RO used to set normal levels of NER at 100%) (Figure 2c). Cells from a patient with XFE progeroid syndrome caused by mutations in *ERCC4* known to have an NER capacity of ~5% (XP51RO)

were utilized as an NER-deficient control (Niedernhofer et al., 2006). The lack of an NER-defect in the patient fibroblasts is consistent with a diagnosis of CS.

Impaired recovery of RNA synthesis (RRS) post-UV irradiation of cells is pathognomonic for a TC-NER defect, which is present in CS patients. Expression of the house-keeping genes *DHFR* and *GAPDH* was measured 6 and 24 hr post-UV irradiation in the patient cells (INE4CC) and compared to C5RO and XP51RO fibroblasts as well as those from a CS patient (CS20LO) (Kashiyama et al., 2013, Mori et al., 2018 and Vélez-Cruz et al., 2013). In all cell lines, *DHFR* and *GAPDH* mRNA were significantly reduced 6 hr post-UV irradiation compared to sham-irradiated cells (Figure 2d). However, in TC-NER-proficient C5RO cells, by 24 hr,



mRNA levels had recovered to levels equivalent to unirradiated cells. Similar results were obtained with XP51RO fibroblasts derived from a NER-defective patient with clinical features distinct from CS. As expected, RNA synthesis recovery was significantly reduced in the CS patient fibroblasts (CS20LO). mRNA expression in the patient (INE4CC) fibroblasts also failed to return to normal levels by 24 hr post-UV irradiations, indicating impaired RRS, consistent with a TC-NER defect and CS (Figure 2d).

Re-curation of the variants after functional testing revealed the p. Gly528Glu was recently reported in a patient with Cockayne syndrome in the compound heterozygous state (Calmels et al., 2018), and the variant was upgraded to pathogenic based on American College of Medical Genetics (ACMG) guidelines (PM2, PP2, PP3, PS3, PP4) (Richards et al., 2015). The c.-15+3G>T remained unreported in the literature. However, with the results from functional studies and re-curation of the variant, the c.-15+3G>T variant was reclassified to likely pathogenic (PM2, PP3, PS3, PM3, PP4). An updated clinical report was generated and the new diagnosis was communicated to the multiple specialists involved in the patient's care and to the family. The c.-15+3G>T variant was published in the ClinVar database (Variation ID: 692034), and the p. Gly528Glu variant has been submitted to ClinVar as well.

The differential diagnosis for patients with neurodegenerative symptoms is broad. Determining etiology is further hampered when classic phenotypic features are absent or have not yet emerged. Next-generation sequencing techniques have addressed this problem by providing an unbiased diagnostic approach guided, but not limited, by phenotype. Our patient displays many features characteristic of CS-II, but several common features that may have led to diagnosis were absent (Table S1). The family did not initially report photosensitivity, though in retrospect, after the diagnosis was made, they noted that the child sunburns easily. In addition, the child did not develop cataracts or characteristic cachexic birdlike facies. These features are present in 62%, 55%, and 70% of CS-II patients respectively (Kou et al., 2018). She does display early signs of pigmentary retinopathy seen in 47% of patients, though full descriptive identification of that retinal finding required examination under anesthesia due to decreased cooperation in keeping with cognitive and behavioral aspects of the disease. To our knowledge, the additional ophthalmologic findings of prominent Schwalbe's line and central retinal vascular sheathing have not been previously reported in Cockayne Syndrome and microcornea has been reported in only a single patient (Nance & Berry, 1992). In addition, she is less severely developmentally delayed than other children with CS-II as she achieved the ability to ambulate and speak in sentences. A previously non-diagnostic trio whole-exome sequence completed at GeneDx further hampered diagnosis in this case.

WGS done as part of a research protocol identified two variants of uncertain significance (VUS) in *ERCC6* including an upstream intronic variant that was not captured by traditional exome sequencing tests due to lack of coverage in the region, and a maternally inherited c.1583G>A, p. Gly528Glu missense variant that was detected, but not reported because it appeared to be a single heterozygous variant of uncertain significance in a gene associated with an autosomal recessive condition. The pathogenicity of these variants identified by WGS was initially uncertain for multiple reasons including: the absence of a classical phenotype, novelty of variants and absence of evidence of a functional impact of the variants on the gene product. Molecular analysis demonstrated impaired *ERCC6* expression and scant abundance of CSB protein in patient fibroblasts (INE4CC) relative to the normal control cell line. Pathogenicity was established based upon functional studies demonstrating normal UDS but reduced recovery of RNA synthesis after UV-irradiation in patient cells, pathognomonic for a diagnosis of Cockayne Syndrome. This case report highlights how WGS in conjunction with functional testing can lead to a clinically unexpected diagnosis. Evaluation and confirmation of pathogenicity of VUS remains a vexing challenge to clinicians and diagnostic sequencing laboratories.

A recent article summarized the molecular and clinical findings of 85 patients with mutations in *ERCC6* (Calmels et al., 2018). The majority of the mutations identified to date are truncation mutations and although no strong genotype-phenotype correlation is observed, a higher proportion of severely affected patients had mutations in *ERCC6* compared to *ERCC8*. Our study demonstrates that intronic variation may account for a yet to be determined percentage of pathogenic variants in *ERCC6*. This study supports the joint approach of molecular analysis in conjunction with robust functional testing and highlights the future promise of the additional value of whole-genome sequencing compared to whole-exome sequencing.

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#### CONFLICTS OF INTEREST

Dr. Friedman holds shares in Illumina and her Spouse is Founder and Principal of Friedman Bioventure, which holds a variety of publicly traded and private biotechnology interests. Other authors have no conflicts.

#### AUTHORS' CONTRIBUTIONS

JF, MJY, LJN, and SC involved in study design, data collection, data interpretation, drafting, and revising the manuscript. LMB and SLR involved in data collection, data

interpretation, and revising the manuscript. RH involved in data collection and revising the manuscript. SAN, DPD, and MAW involved in data collection and data interpretation.

## DATA AVAILABILITY STATEMENT

The variants described in the publication were submitted to ClinVar.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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