Ending A Diagnostic Odyssey: Moving From Exome to Genome to Identify Cockayne Syndrome

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Abstract

Cockayne syndrome is an ultra-rare autosomal recessive disorder characterized by growth failure and multisystemic degeneration. Excision repair cross-complementation group 6 (ERCC6) mutations account for most cases. We report a child with pre- and post-natal growth failure and progressive neurologic deterioration with multi-system involvement who has bi-allelic ERCC6 variants, that were discovered by whole genome sequencing, 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. The study confirms the pathogenicity of a previously undescribed upstream intronic variant, highlighting the power of genome sequencing to identify non-coding 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.

Keywords (3-5)

Cockayne Syndrome; Whole Genome Sequencing; *ERCC6* ; DNA Repair

Molecular diagnosis of children with rare neurodegenerative and complex multi-system 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 lab.

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: CS-I, canonical; CS-II (including C-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* or *ERCC8* .*ERCC6* mutations are most common in Caucasian CS patients, but in other ethnic groups, *ERCC8* mutations are more prevalent (Wilson, 2016). Mutations in *ERCC6* are also seen in cerebro-ocululer facial-skeletal syndrome (COFS) (Laugel, 2008), and Ultraviolet (UV)-Sensitive Syndrome (Horibata, 2004). Recessive mutations in five additional DNA repair genes may cause CS (*ERCC3/XPB, ERCC2/XPD, ERCC5/XPG, ERCC4/XPF* and *ERCC1*) in association with XP (Kashiyama, 2013; Laugel, 2010) (Figure 1A).

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 (Scharer, 2013). NER consists of two sub-pathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER) (Figure 1A) (Gillet, 2006; Hanawalt, 2008). CSA (*ERCC8*) and CSB (*ERCC6*) are key TC-NER factors that participate in the repair of transcription-blocking DNA lesions that stall RNA polymerase II (Scharer, 2013). Once the lesion is recognized in the genome or transcribed areas of the genome, the two sub-pathways 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 the replication machinery. Generally speaking, mutations in genes needed for GG-NER cause xeroderma pigmentosum, a cancer predisposition syndrome, while mutations in genes needed for TC-NER cause CS. However, the impact of a particular mutation or sequence variant on protein expression and function complicates this generalization (Laugel, 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 (Wilson, 2016), though at present none may be conclusively mechanistically linked to the spectrum of symptoms observed in CS patients. Roughly 65% of patients with CS have mutations in*ERCC6* (Laugel, 2000). The true prevalence of CS is unknown. A study in 2008 estimated the incidence at 2.7 per million births in western Europe (Wilson, 2016), though this is likely an underestimate in part due to reliance on the presence of photosensitivity as a diagnostic feature (Kleijer, 2008). 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 Korean female with multisystem disease including: failure to thrive, congenital microcephaly, global developmental delay with motor and language regression, tremor, ataxia, cardiomyopathy, renal dysfunction, chronic lung disease with oxygen requirement, diabetes, hypothyroidism and hypertension. Pregnancy was unremarkable until 36 weeks of gestation when intrauterine growth retardation was identified, prompting an uncomplicated delivery by Cesarean section at 37 weeks of gestation. Birth weight was 2126 grams (z=-2.37) and birth length was 43.2 cm (z=-2.87). Weight gain was appropriate for the first 6 months of life while breast fed. Poor weight gain was noted after transition to formula due to poor oral intake and a G-tube was placed at 23 months. Sitting was attained at 6 months and pulling to stand at 12 months. Delays in development were noted at 1 year of age at which time she had difficulty with fine motor movements due to tremor. Independent ambulation was achieved at 29 months. Though she spoke a single word - “dada” at nine months, by 33 months she had only a 40-word vocabulary and was not combining words. Regression in motor skills was noted at age 3 years in association with a two-week hospitalization for RSV infection, with increased tremor and progressive difficulty with gait and balance. Following a complicated hospitalization at age 4.5 years, including multiple infections and a prolonged mechanical ventilation, she lost the ability to ambulate.

Since that time, she made forward developmental progress and at age 7 was able to walk short distances with a walker and was able to speak in short sentences. She has had poor somatic and cerebral growth with a steady decline in her z-scores for linear growth with most recent measurement at -8. At 7 years her weight z score was ˜3.3, and her head circumference z score was ˜3.5. Her course has been further complicated

by hypothyroidism (onset age 3 years), insulin-dependent diabetes (diagnosed at age 4 years), cardiomyopathy, fibrotic kidney disease, chronic lung disease with oxygen requirement, hypertension, dental caries, and movement disorder characterized by action-induced tremor and paroxysmal tremulous episodes. Audiogram at age 6 showed bilateral absent oto-acoustic emissions and reduced compliance on tympanometry; visual reinforced audiometry and conditioned play audiometry were felt to be unreliable. Dilated ophthalmologic evaluation at ages 3 and 4 years showed bilateral hyperopia with secondary refractive amblyopia, no early cataracts and normal appearing retinas. Dilated ophthalmologic evaluation, with a different pediatric ophthalmologist (SLR), at age 5 in the office setting and later under anesthesia, revealed decreased lacrimation, small corneas with scarring consistent with exposure keratopathy, prominent Schwalbe's line with normal intraocular pressure, severe hyperopia (farsightedness) requiring eyeglass correction, optic nerve atrophy and foveal hypoplasia. Additionally, bilateral reticulated retinal pattern with central vascular sheathing of both retinal arterioles and venules was observed. Neurologic examination at age 7 revealed a small child able to speak in phrases and follows simple commands. She displayed gaze-evoked nystagmus in all fields of gaze. There was increased appendicular tone, hypoactive reflexes, titubation and tremor while reaching for objects. She was able to stand with support.

At age two, brain MRI showed inferior vermian hypoplasia with cerebellar atrophy, diffuse white matter signal abnormality and mineralization of the basal ganglia. Repeat brain MRI at age four showed progression of supratentorial volume loss. Computed Tomography Scan at 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). Radiographic review identified diffuse platyspondyly and acetabular dysplasia. Extensive laboratory evaluations including whole exome sequencing, electromyogram nerve conductions and muscle biopsy were unremarkable.

Whole genome sequencing (WGS) initially revealed two variants of uncertain significance (VUS) in the

ERCC6 gene (NM_00124.3): a maternally inherited c.1583G>A, p.Gly528Glu missense variant and a paternally inherited c.-15+3G>T upstream intronic variant (Supplemental Figure 1). 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 was 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. Patient dermal fibroblast line was evaluated for the expression of *ERCC6* and CSB protein levels (Figure 2A and 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. 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. 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 and trichothiodystrophy, whereas mutations in common components of both GG-NER and TC-NER can result clinically in CS, XP, COFS, or even Fanconi anemia (Kashiyama, 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 mutations in XPF, known to have an NER capacity of ˜5% (XP51RO), were utilized as an NER-deficient control (Niedernhofer, 2006). 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 housekeeping genes *DHFR* and *GAPDH* were measured 6 and 24 hrs 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, 2013). In all cell lines,

DHFR and*GAPDH* mRNA was significantly reduced 6 hr post-UV irradiation compared to sham-irradiated cells (Figure 2D). However, in TC-NER-proficient C5RO cells, by 24 hrs, mRNA levels had recovered to levels equivalent to unirradiated cells. Similar results were obtained with XP51RO fibroblasts derived from a NER-defective patient with no clinical signs of 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 hrs 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, 2018), and the variant was upgraded to pathogenic based on American College of Medical Genetics (ACMG) guidelines (PM2, PP2, PP3, PS3, PP4). 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 re-classified to likely pathogenic (PM2, PP3, PS3, PM3, PP4). The new diagnosis was communicated to the multiple specialists involved in the patient's care and to the family.

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 (Supplemental Table 1). 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, 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, 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 further hampered diagnosis in this case.

Whole Genome Sequencing done as part of a research protocol identified two variants of uncertain significance (VUS) in *ERCC6* including an upstream intronic variant that would not have been captured by traditional exome sequencing tests, or potentially not reported due to lack of variant evidence or patient phenotypic overlap. The pathogenicity of these variants was initially uncertain for multiple reasons including: 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, 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 STATEMENT

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.

DATA AVAILABILITY STATEMENT

The variants described in the publication were submitted to ClinVar.

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FIGURE LEGENDS

Figure 1. A. Nucleotide Excision Repair (NER) Pathway and brain imaging of patient INE4CC. Global Genome NER (left) and Transcription Coupled NER (right) converge. Representative Xeroderma Pigmentosum and Cockayne Syndrome patients shown. Photo Credit: Child with xeroderma pigmentosum in Rukum, Nepal by Lax and Child with Cockayne Syndrome by Eric Bixel / Creative Commons BY 4.0. **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.

Figure 2. Functional consequences of biallelic *ERCC6* variants in INE4CC cells. A.*ERCC6GAPDH* expression. **B** . Whole cell extracts were prepared from the aforementioned cells and CSB protein levels were detected by immunoblotting. [?]Tubulin served as a loading control.**C.** Measurement of UV-induced unscheduled DNA synthesis (UDS) in dermal fibroblast lines (C5RO: unaffected donor; XP51RO: patient with a mutation in *XPF* affecting NER; the patient in this study: INE4CC). Cells were UV-C- or sham-irradiated and afterwards cells were 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.** Measurement 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 (*XPF* mutation with a diagnosis of XFE progeroid syndrome), CS20LO (CS caused by a mutation in *ERCC1*), and the patient in this study (INE4CC). Expression was measured at baseline (no UV) and at 6 and 24 hr post-irradiation and normalized to the amount of *18s* rRNA. The results were plotted as the ratio of expression in irradiated vs. sham-irradiated cells. qPCR reactions were performed in triplicate for five independent experiments. Values represent mean +- SD, ns 0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001 measured by unpaired two-tailed Student's t test or one-way ANOVA with Tukey's test.

Supplemental Figure 1. Sanger sequencing of biallelic *ERCC6* variants

The pathogenic maternally inherited c.1583G>A (p.Gly528Glu) missense variant is shown with the conservation of the amino acid position shown as well as the whole genome sequencing (WGS) data and variant call. **B.** The likely pathogenic paternally inherited c.-15+3G>T upstream intronic variant is shown with the conservation of the base as well as the raw WGS data variant call.

Supplemental Table 1. Characteristics of Cockayne Syndrome and their frequency compared to patient INE4CC. (+) – present; (-) – absent; ns – not specified; * Photesensitivity identified after *ERCC6* variants identified. In retrospect noted to sunburn easily; ** Initial dilated exams showed normal retinae (age 3.8 years); mild changes noted only after dilated examination under anesthesia (age 5.5 years); *** Dental anomalies.