Henian Cao · Christina Williams · Monica Carter
Robert A. Hegele

***CKN1* (MIM 216400): mutations in Cockayne syndrome type A and a new common polymorphism**

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Abstract We found that a subject with Cockayne syndrome type A was a compound heterozygote for two new mutations in *CKN1* (MIM 216400): a missense mutation (A205P) and a nonsense (E13X) mutation. We also identified and characterized a new common single nucleotide polymorphism in *CKN1* in five groups.

Keywords Aging · DNA repair · Developmental delay · Dwarfism · Photosensitivity

Introduction

Cockayne syndrome (CS) refers to a group of very rare autosomal recessive disorders with diverse developmental abnormalities, including cachectic dwarfism with a precociously senile appearance, microcephaly, skeletal and retinal abnormalities, sensorineural hearing loss, dental caries, characteristic 'bird-like' faces, hypogonadism, neurodysmyelination, and mental retardation (Lehmann 1987; Nance and Berry, 1992). Death results from progressive neurological degeneration, usually before age 20 (Lehmann 1987; Nance and Berry 1992). Most CS patients have increased skin photosensitivity but do not develop skin tumors, in contrast to patients with xeroderma pigmentosum (Lehmann 1987; Nance and Berry 1992). The molecular heterogeneity of CS was inferred from cellular complementation experiments (Lehmann 1987). Part of the CS phenotype is a significant delay in the recovery of DNA and RNA synthesis in cultured cells following exposure to UV light, which

helped to define two distinct complementation groups, called CS-A and CS-B (Lehmann 1987). *CKN1* (MIM 216400) encodes the WD-repeat protein that gives rise to CS-A activity (Henning et al. 1995), while *ERCC6* (MIM 133540) encodes the CS-B protein. CS usually arises from mutations in either *CKN1* or *ERCC6*, whose products remove UV-induced lesions in template strands of genes transcribed by RNA polymerase II (RNAP II) by a transcription-coupled repair (TCR) process (Svejstrup 2002). About 20% of mutations in Cockayne syndrome are in *CKN1*, which is also called *ERCC8*, and include four substantial coding sequence deletions, one nonsense mutation (T322X), and one missense mutation (Q106P) (Henning et al. 1995; Cleaver et al. 1999; Ren et al. 2003).

Methods

Study subjects

Cell line of CS proband

Lymphoblasts from CS subject AG07075 were obtained from the Coriell Cell Repositories (Camden, NJ, USA). The proband was an 11-year-old Caucasian girl with photophobia, dwarfism, mental retardation, cataracts, retinopathy, and optic atrophy (additional information can be found at <http://locus.umdj.edu/nia/nia.cgi/display.cgi?AG07075>).

Normal controls

Samples from a DNA archive of 460 normal Caucasian subjects, 50 normal Chinese subjects, 50 normal South Asian subjects, 50 normal African subjects, 50 normal Amerindian subjects, and 50 normal Inuit subjects were used to determine frequencies of the *CKN1* variants.

Cell culture and genomic DNA isolation

Lymphoblasts were cultured in minimal essential medium (Life Technologies, Rockville, MD, USA) supplemented with 20% heat-inactivated fetal bovine serum at 37°C in an atmosphere of 5% CO₂ and 95% air. Genomic DNA from cultured lympho-

H. Cao · C. Williams · M. Carter · R. A. Hegele
Robarts Research Institute, London, Ontario, N6A 5K8, Canada

R. A. Hegele (✉)
Blackburn Cardiovascular Genetics Laboratory,
Robarts Research Institute, 406-100 Perth Drive,
London, Ontario, N6A 5K8, Canada
E-mail: hegele@robarts.ca
Tel.: +1-519-6633461
Fax: +1-519-6633037

blasts was obtained using a commercial method following the manufacturer's instructions (Puregene, Gentra Systems, Minneapolis, MN, USA).

Screening *CKN1* for genomic DNA variants

In order to amplify *CKN1* coding regions and intron-exon boundaries from genomic DNA, primer sets were developed using NCBI sequences NT_006431 and NM_000082. Primers were each designed to anneal at 60°C, which allowed for use of a single amplification apparatus for all reactions. Amplification conditions were 94°C for 5 min, followed by 30 cycles comprised of 30 s each at 94°C, 60°C, and 72°C, ending with a single 10-min extension step at 72°C. All samples were directly sequenced on a Prism 377 Automated DNA sequencer (PE Applied Biosystems, Mississauga, ON, Canada).

Genotyping candidate gene polymorphisms

Rapid genotyping methods included: (1) detection of length of polymorphism by restriction endonuclease digestion followed by gel electrophoresis when a recognition site was altered, or (2) use of mismatched amplification primers to introduce a restriction site whose presence or absence was then dependent on the variant sequence. For the *CKN1* A205P mutation, the exon 7 amplification primers and conditions were used (Table 1), and the resulting 294 bp product was incubated with endonuclease *BseD1*. The allele encoding A205 yielded a single 294 bp fragment after digestion, while the allele encoding P205 yielded two fragments with sizes 162 and 132 bp. For *CKN1* E13X, the mismatched primer was 5'-GGT TTT TGT CCG CAC GCC AAA CGG **GCC** TG (with mismatched nucleotides underlined and in bold), and this was used with reverse primer 5'-ACT GGC CTG TTA GCC AAA AA. The 30 cycle amplification reaction was the same as above, except for a decrease in annealing temperature to 58°C. The resulting 106 bp product was incubated with endonuclease *Bst*N1. The allele encoding E13 yielded two fragments with sizes 79 and 27 bp after digestion, while the allele encoding X13 yielded a single fragment of size 106 bp. For the *CKN1* nt 471T>C single nucleotide

polymorphism (SNP), the mismatched primer was 5'-CTG CAG ATG TAT TTA ATT TTG AGG AAA CAG **TGT** A (with mismatched nucleotide underlined and in bold) and was used with reverse primer 5'-AAA TTG TGA TAT TCC TCT GGG TT. The 30-cycle amplification reaction was the same as the general program, except for a decrease in annealing temperature to 58°C. The resulting 188 bp product was incubated with endonuclease *Rsa*I. The 471T allele yielded a single fragment of size 188 bp after digestion, while the 471C allele yielded two fragments of sizes 156 and 32 bp.

Reverse transcription amplification and subcloning of *CKN1*

In order to determine the chromosomal phase of the two mutations found in subject NG070705, total lymphoblast RNA was isolated with the RNeasy Mini-kit (Qiagen, Mississauga, ON, Canada). First strand cDNA was synthesized from total RNA using an oligo-dT primer (Superscript First-strand Synthesis System, Invitrogen, Burlington, ON, Canada). Next, 2 µl of the first strand were amplified in a total of volume of 30 µl containing specific mRNA for *CKN1* primers using GenBank accession number NM_000082. The forward primer was 5'-CGG TGT GAG GAC ACG ATA TG and the reverse primer was 5'-CAG CTT GTG ACT TTT TCC CA, spanning the coding sequence from exons 1 to 8. Thirty amplification cycles were performed at an annealing temperature of 60°C. The 726 bp PCR product was cloned into the pCR2.1 vector (TA Cloning kit, Invitrogen, Burlington, ON, Canada). After purification of plasmid DNA and *Eco*RI digestion, the positive clones were sequenced on a Prism 377 Automated DNA sequencer (PE Applied Biosystems, Mississauga, ON, Canada) using the above primer pair.

Statistical analysis

SAS version 6.12 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. Chi-square analysis was used to test deviations of genotype frequencies from those predicted by the Hardy-Weinberg equation. The nominal level of statistical significance for all analyses was $P < 0.05$.

Table 1 Amplification primers for *CKN1*

Exon	Primer sequence	Product size
1	F: 5'-CTG AGC TCT GTC ATG GCG AC R: 5'-ACT GGC CTG TTA GCC AAA AA	165 bp
2	F: 5'-CTT GTT AAT TTC TTT CTC CCC C R: 5'-CAG GTT TTA CTG CAT TTC ACT TAG A	190 bp
3	F: 5'-TTG AGT GTT TGC AAA TCA GTT TTT R: 5'-TGC AAT TTT CAT GTT TTG CAT	284 bp
4	F: 5'-TCA TCC TAC CTG AGA CTA CTT GTT R: 5'-TCC TAC AAA GCA ATC TGC AA	257 bp
5	F: 5'-TTT TTG GGG TAT TAC ATT CCT ATG R: 5'-AAA TTG TGA TAT TCC TCT GGG TT	338 bp
6	F: 5'-ATG TCT TGT TTC CTT TTG ATG ATG R: 5'-TCA TGT CAC TTA CAA AGA ATA CAC TG	228 bp
7	F: 5'-TTT GGC CTC ACT TTC TTC AG R: 5'-CAA TAC TTC ATT TTA AAG GAG TGA A	294 bp
8	F: 5'-CGA GAC CTC TGT GTG CCA TA R: 5'-TGC GTT TAT TAT GTG GCT TCA	281 bp
9	F: 5'-CAT CAA ACA TTA AAT ATG TCC TGA A R: 5'-AAA TCA AGT GTA TGT CAC AGA TCC A	256 bp
10	F: 5'-TCT CCT AGG CTT TGT TGT GTT TT R: 5'-CAT ATA ACT GGT CTG GCA AGC	328 bp
11	F: 5'-CCA CAG TAT CAT CTT TGC CTT C R: 5'-GAA ATG CTT TAA AAG TCT CTC TCA CA	206 bp
12	F: 5'-AAA AAC AGA ATC TTC CTT TTT CTT TA R: 5'-GCT GTC AGG AAT AGA CCA TAC AGT T	198 bp

Results

Identification of *CKN1* mutations

We found that the study subject was heterozygous for *CKN1* mutations c.73G>T (E13X) and c.649G>C (A205P). Subcloning of cDNA and sequencing indicated that these mutations were in a *trans* orientation on opposite alleles, indicating that the subject was a compound heterozygote. These mutations were completely absent from the genomes of 460 normal Caucasian, 50 normal Chinese, 50 normal South Asian, 50 normal African, 50 normal Amerindian, and 50 normal Inuit subjects ($P < 0.0000001$). We found the common *CKN1* c.471T>C SNP had 471C allele frequency of 0.27, 0.09, 0.17, 0.02, 0.02, and 0.07 in normal Caucasian, Chinese, South Asian, African, Amerindian, and Inuit subjects, respectively. This SNP is novel and is not present in the NCBI SNP database. None of the observed genotype frequencies deviated from the expectations according to the Hardy-Weinberg equation.

Discussion

We report: (1) primer sets that amplified *CKN1* from genomic DNA and from reverse transcribed cDNA, (2) compound heterozygosity for two *CKN1* mutations (E13X and A205P) in a cell line from a subject with CS, the seventh and eighth mutations reported to date, and (3) a common SNP in *CKN1* and its frequency in five ethnic groups. Subject NG07075 is the second reported compound heterozygote for mutant *CKN1* and A205P is the second reported missense mutation for this gene (Ren et al. 2003). While we have no functional data at present, the two *CKN1* mutations from subject NG07075 are very likely to be pathogenic, since they are

both completely absent from 710 normal genomes of individuals from various ethnic groups ($P < 0.0000001$). Etiological relevance is also strongly suggested for the E13X mutation by its very early truncation and for the A205P mutation by virtue of its preservation in human, hamster, and mouse (data not shown). Codon 205 is within the third Walker repeat domain of *CKN1* (Henning et al. 1995). The amplification primers and the common *CKN1* c.471T>C SNP may prove to be helpful in future association and/or pharmacogenetic studies of developmental and/or aging related phenotypes.

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