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Characterisation of novel mutations in Cockayne syndrome type A and xeroderma pigmentosum group C subjects

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Abstract We report that a subject with Cockayne syndrome type A (CS3BE) was a compound heterozygote for mutations in *CKN1*, the gene encoding the CSA protein (MIM 216400). CS3BE displayed a novel missense mutation (A160V) and a previously described nonsense mutation (E13X). Although residing between the second and third WD-40 repeats characteristic of the CSA protein, A160 is completely conserved in all species that possess a *CKN1* homologue. We also describe a mutation in a previously uncharacterised xeroderma pigmentosum group C subject (XP8CA) in the *XPC* gene (MIM 278720). XP8CA was homozygous for a 2 bp TG deletion in codon 547 resulting in premature termination at codon 572. Immunoblotting of XP8CA extracts confirmed the absence of full-length XPC protein that was present in unaffected cell lines.

Keywords Hyperphotosensitivity · Skin neoplasia · Progeria · Nucleotide excision repair · Xeroderma pigmentosum · Cockayne syndrome

Introduction

The human genome is under perpetual attack from a range of endogenous and exogenous genotoxic agents (Lindahl 1993). Genome stability is maintained by a set of integrated DNA repair pathways, which comprise at least 130 gene products (Wood et al. 2001). Of these

pathways the most versatile is nucleotide excision repair (NER), a highly conserved mechanism that requires at least 30 polypeptides (Aboussekhra et al. 1995). Lesions removed by NER include UV-induced photoproducts. Loss of function of some NER genes is incompatible with life whilst defects in a subset of proteins can result in developmental abnormalities or cancer.

Xeroderma pigmentosum (XP) is characterised by hyperphotosensitivity to the UV constituent of sunlight. The hallmark of XP is a predisposition to skin neoplasia, with a >1,000-fold incidence of UV-induced squamous cell carcinoma and malignant melanoma. Classical XP is caused by mutations in one of seven genes *XPA-XPG*. Cockayne syndrome (CS) patients do not have a predisposition to UV-induced skin neoplasia but exhibit a battery of clinical manifestations, such as segmental progeria, cachectic dwarfism, deafness, retinal degeneration, neurological dysfunction and skeletal-associated abnormalities including osteoporosis and a bird-like face. Death occurs at a mean age of 12 years. Mutations in the *CKN1* (*CSA*) and *CSB* genes are responsible for CS.

Whilst CS and XP cells have defective NER, the striking differences between the syndromes are explained by the fact that the NER mechanism is divided into two distinct sub-pathways. Global genome repair (GGR) facilitates the removal of lesions from the non-transcribed strand and transcriptionally silent regions of the genome. Transcription-coupled repair (TCR) preferentially removes lesions from the transcribed strand of active genes. In GGR, the recruitment of the 106 kDa XPC protein complexed with the 43 kDa hHR23B protein allows interaction with damaged DNA, facilitating the recognition of UV-induced lesions (Sugasawa et al. 1998). Conversely, the arrest of an elongating RNA polymerase II at a lesion on the transcribed strand of an active gene elicits the recruitment of TCR-specific proteins, including CSA. The 44 kDa CSA protein is encoded by the *CKN1* gene and contains multiple WD-40 repeats (Henning et al. 1995). The two NER sub-pathways converge at the point where the presence of a

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lesion is confirmed. Excision, re-synthesis and ligation follow using a common mechanism.

Both *XPC* and *CKN1* genes were cloned as a result of screening cDNA libraries for genes that corrected the UV sensitivity of XP-C and CS-A cell lines. The mutations present in the original CS-A line used for library screening (CS3BE) have not previously been identified (Henning et al. 1995) although it was reported that the cell line appeared to produce no CSA protein (van Gool et al. 1997). Here we report a novel mutation in the *CKN1* gene of this patient and the defect in a previously uncharacterised XP-C cell line, XP8CA.

Materials and methods

Cell lines and culture conditions

MRC-5 foetal lung fibroblasts were purchased from ECACC and were grown in monolayers in Eagle's minimum essential medium with Earle's salts (EMEM, Invitrogen), supplemented with 10% foetal calf serum (FCS; Imperial Laboratories, London), 1× MEM non-essential amino acids (NEAA) without L-glutamine. XP-C (GM02996, XP8CA), CS-A (GM01856, CS3BE) and XP-A (GM05509, XP12BE) diploid primary human fibroblasts were purchased from Coriell and grown as monolayers in EMEM with Earle's salts with 10% FCS, 2× MEM NEAA without L-glutamine, 2× essential amino acids MEM, 2 mM L-glutamine, 2× MEM vitamins. All media were supplemented with penicillin (10,000 U/ml) and streptomycin (10 µg/ml) and cells grown at 37°C in a humidified incubator with 5% CO₂. G418 (400 µg/ml) was used for cells undergoing drug selection.

Telomerase immortalisation of cell lines

The catalytic sub-unit of telomerase, human telomerase reverse transcriptase (hTERT), was transduced into MRC-5, XP8CA, CS3BE and XP12BE primary cells using an amphotropic retroviral vector, pBABE neo hTERT, using protocols previously described (Wyllie et al. 2000).

PCR amplification of *XPC* and *CKN1* gene fragments from isolated genomic DNA

Genomic DNA was isolated and purified using RNase A treatment and proteinase K digestion, followed by a standard phenol/chloroform extraction. PCR primer sets (Table 1), used to amplify *XPC* and *CKN1* coding regions and intron–exon boundaries from XP8CA and CS3BE genomic DNA were devised using NCBI sequences NT_022517.17 and NT_006713.14, respectively. Thermocycling was performed on an MJ Tetrad.

Amplification conditions were 95°C for 12 min, followed by 33–35 cycles of 30 s each at 94°C, the primer specific annealing temperature, and 72°C, ending with a final 10-min extension step at 72°C.

Screening *XPC* and *CKN1* for genomic DNA variants

PCR products were purified using an exonuclease I, shrimp alkaline phosphatase PCR purification protocol (Amersham). PCR amplification products were prepared for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Reaction Ready Kit (Applied Biosystems) and excess dye terminators removed using Montage Seq₉₆ Sequence Reaction Cleanup Kits (Millipore). Sequence analysis of *XPC* and *CKN1* was performed on an ABI PRISM 3100 Genetic Analyser.

Analysis of CS3BE *CKN1* mRNA

Total RNA was isolated using TRIzol and reverse transcribed with SuperScript II (both Invitrogen). Amplification of *CKN1* cDNA was performed using *Taq* polymerase (Promega) using the primers 5'-GGT TTT TGT CCG CAC GCC AA-3' (forward) and 5'-GTG GAG ACC AGG AAA CTG CT-3' (reverse). PCR products were ligated into the TA cloning vector pCR2.1 and transformed into INVαF' *Escherichia coli* One Shot cells (Invitrogen). Individual colonies were expanded and sequenced (as above) in both directions using M13 forward and reverse primers.

Immunoblotting

Whole-cell extracts were prepared, as previously described (Evans et al. 2003). Proteins were separated on 8% (XPC) and 12% (CSA) SDS polyacrylamide gels, transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore) and exposed to either an anti-XPC mouse monoclonal antibody (ab6264; Abcam) or anti-CSA goat polyclonal antibody (W-16: sc-10997; Santa Cruz). Blots were visualised using an appropriate HRP-coupled secondary antibody and an ECL system using Hyperfilm (Amersham). Equal protein loading was confirmed by staining the PVDF membrane with India ink.

Results and discussion

Primary human diploid fibroblasts have a limited lifespan in culture due to telomere-dependent replicative senescence. In order to provide sufficient material for biochemical analysis, we infected MRC-5, CS3BE, XP8CA and XP12BE with a retrovirus expressing the catalytic subunit of telomerase (hTERT) and isolated subclones. The extended lifespan of these lines con-

Table 1 Forward and reverse primer sets for *CKN1* and *XPC* amplification from genomic DNA

Exon	Forward	Reverse	Product size (bp)
<i>CKN1</i>			
1	5'-GAC TCT GCT GTT CCA GTC C	5'-CAA AGC TTA CAG TCA TTG GTC C	220
2	5'-TAC GTT AGG ATG TGT GGT AG	5'-ATG CCA GAT TCA AAA ATG CTA C	500
3	5'-TGA AGT CTG TGT TAT TGA GG	5'-CAG TCA AGT GAA TGG GTC AA	363
4	5'-ATG TGA TCT CGG TTT GGC	5'-GGA TTA AAT TCT CCT TTA TCC TAC	302
5	5'-TGT TTA AGT TGA ATT GCT AAC AGT C	5'-TTG TGA TAT TCC TCT GGG	316
6	5'-TTC TGC ATG GAT ACA GTG AAA ATG	5'-TGA GTC TCA ACA ACC AGC AC	302
7	5'-CAC TTT CTT CAG AAT CAG GC	5'-GGA AGA TTT CTT TTG GTG ACA	367
8	5'-TAA CGA GAC CTC TGT GTG CCA	5'-GAA AGT GAT ATA CGA TGA ATG CC	342
9	5'-GTA ACT CAA GTA GTT GTC CT	5'-ATC AAG TGT ATG TCA CAG ATC C	295
10	5'-AGT ATT TCT CCT AGG CTT TGT TGT G	5'-ACA TAT AAC TGG TCT GGC AAG C	334
11	5'-ATT GGC CTA GTT TCT TTA GGG AG	5'-TAA GCT TTA GAA GTC ACT GTA CC	299
12	5'-TTT TCT TCC AAA ATA ATG TAA TTG CT	5'-CTG TAT GGT CTA TTC CTG AC	235
<i>XPC</i>			
1	5'-TGA CTA GGC CTC CAA CGA AG	5'-TAC GCA GGA GCT TGG ATC G	453
2	5'-ATA AGC TGC ACT GCC TCC AC	5'-GAT CCA ATC TTC CAT GGA CC	371
3	5'-GCT TGA ATG GAA CAC TAG G	5'-TAG TGA TCT GAC TCC AAA CAG	257
4	5'-TGA TTC TGT TCA GTA CAG TAG C	5'-CAA AGT CCT CCT AAG CAG C	308
5	5'-GAG GAG AAG GAA TTG CCT G	5'-AGC ACA AGC TCT TTG CAC C	224
6	5'-CAT GTC TTG ACT TTG GCA GC	5'-CTG TGG AAG TGA CCT GAA CC	325
7	5'-CTT GGC TGG AAA TGA AAA TTC C	5'-GCA CAT GGC TGC CAT TAT C	257
8	5'-TTC TTA GGA TAA CTA TGT TCT TCC	5'-ACT CCG TGA ATA CCA GCT C	232
9.1	5'-CTC TAG CTG GTG ACT TAA CC	5'-CTT GAA GAG CTT GAG GAT GC	453
9.2	5'-GCT CTG ATT TTG AGC TCT CC	5'-CCT GAC TGT GTC TTG GAG C	588
10	5'-GTC TAA GGA TCA TCT CCC TC	5'-TGC TGT CCA GTC AGA TGA GC	344
11	5'-ACG TTC AAG GCT GTT TGC C	5'-GCT CAT CAT CAC TTC TCT GC	344
12/13	5'-TGA GGA ACT GGA TGC CTT TG	5'-TGA AAA TTG GAG CCA CCA GG	558
14	5'-CAC TGT CTT CCA CAA ACT GG	5'-TGT ATT CAG TGC TCG CTC C	333
15	5'-ACT TGG TGT GAA GGA GAG GC	5'-CCT TTC TGA GCT GCA TCT CC	291
16	5'-GAA CTT GCT GCC TCT TCA TGG	5'-TGC CTT CTC AGC AGA GAA GC	416

firmated that hTERT expression had immortalised the cultures (not shown). The repair-defective cell lines also retained their UV sensitivity (not shown). Genomic DNA from these cell lines was isolated and the 12 exons of *CKN1* and 16 exons of *XPC* were amplified by PCR (Table 1). PCR products were sequenced and base changes identified by comparing to consensus cDNA sequences (NM_000082 [*CKN1*]; NM_004628 [*XPC*]).

The original assignment of patient XP8CA to XP complementation group C was made on the basis of cell fusion studies. In XP8CA, a homozygous 2 bp TG deletion, was identified in codon 547 at position 1639. This would result in a frame shift and a predicted protein product of 63.4 kDa. Two further homozygous changes were identified: 2061A>G (R687R) and 2815A>C (K939Q). Immunoblotting using an XPC-specific antibody confirmed the absence of full-length protein in XP8CA. XPC protein was present in CS-A and XP-A and normal MRC-5 cell extracts (Fig. 1). XPC protein levels were slightly reduced in the CS-A cell extract. XPC is an inducible protein (Adimoolam and Ford 2002), and the lower expression in CS3BE cells reflects a different steady state level compared to other cell lines. The presence of a further mutation at K939 is likely to have no effect because firstly, translation terminates prior to the mutation, and secondly, K939 is adjacent to the final amino acid of the normal protein. The majority of previously reported *XPC* mutations result in truncated proteins (Chavanne et al. 2000), and

an identical mutation to that in XP8CA has been reported in the patient XP4PA (Li et al. 1993). The relationship between patients XP8CA and XP4PA is unknown although they were originally identified in different laboratories.

CS3BE, assigned to CS complementation type A, was a compound heterozygote for *CKN1* mutations 37G>T (E13X) and 479C>T (A160V). Immunoblot analysis of CS3BE and MRC-5 cell extracts using an anti-CSA antibody detected a non-specific cross-reacting band (Fig. 1). However, a band corresponding to CSA was present in MRC-5 and absent in CS3BE, confirming the earlier report that the protein was not expressed in the cell line (van Gool et al. 1997). It was confirmed that the mutations were on separate alleles by reverse transcription of CS3BE total RNA followed by PCR. Products were sub-cloned and sequenced, and in clones where 37G>T was present, the 479C>T mutation was absent. No clones were recovered containing the 479C>T mutation although cDNA containing the wild-type 37G appeared to have spliced out the exon containing 479C>T. The instability of mRNA from the second allele is likely to explain the lack of CSA protein in the cells.

To date, the availability of *CKN1* mutation data is limited because although mutations in the *CKN1* gene account for approximately 20% of CS (Stefanini et al. 1996), only eight have been characterised (Cao et al. 2004; Ren et al. 2003). Here we report a third missense

Fig. 1 Loss of expression of XPC and CSA proteins in mutant cell lines. **a** Protein extracts (20 μ g) from a normal (MRC-5) and DNA repair-defective XP-A, XP-C and CS-A cell lines were separated on SDS-PAGE gels, transferred and membranes probed with an anti-XPC antibody. A band of approximately 125 kDa corresponding to XPC protein was absent in XP8CA consistent with the predicted truncated product. **b** Protein extracts (20 μ g) from MRC-5 and CS3BE (CS-A) were separated as above and transferred protein probed with an anti-CSA antibody. CS3BE cell extracts showed no expression of a protein of approximately 44 kDa (*upper band*) that was present in MRC-5 extracts

mutation in *CKN1*. The Utah Genome Center SNP database (<http://www.genome.utah.edu/genesnps>) and a previous study (Cao et al. 2004) indicate that this is not an SNP. The availability of genomes from other species has shown that the CSA protein is highly conserved (data not shown; <http://www.ensembl.org>). The two previously reported missense mutations (Q106P and A205P) lie in the WD-40 regions. The A160V mutation reported here lies between the second and third WD-40 repeats. If undetectably low levels of full-length protein are expressed in CS3BE, this is likely to be inactive as the sites of all three missense mutations (Q106, A205 and A160) are highly conserved in mammalian, chicken and puffer fish CSA protein homologues. Unlike Q106 and A205, A160 is retained in *Schizosaccharomyces pombe* and *Arabidopsis thaliana*. The role of CSA in DNA repair is still poorly understood, and the existence

of this highly conserved region suggests a possible avenue for elucidating the function of this protein.

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