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Reduced *XPC* DNA repair gene mRNA levels in clinically normal parents of xeroderma pigmentosum patients

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Xeroderma pigmentosum group C (XP-C) is a rare autosomal recessive disorder. Patients with two mutant alleles of the *XPC* DNA repair gene have sun sensitivity and a 1000-fold increase in skin cancers. Clinically normal parents of XP-C patients have one mutant allele and one normal allele. As a step toward evaluating cancer risk in these *XPC* heterozygotes we characterized cells from 16 XP families. We identified 15 causative mutations (5 frameshift, 6 nonsense and 4 splicing) in the *XPC* gene in cells from 16 XP probands. All had premature termination codons (PTC) and absence of normal XPC protein on western blotting. The cell lines from 26 parents were heterozygous for the same mutations. We employed a real-time quantitative reverse transcriptase–PCR assay as a rapid and sensitive method to measure *XPC* mRNA levels. The mean *XPC* mRNA levels in the cell lines from the XP-C probands were 24% ($P < 10^{-7}$) of that in 10 normal controls. This reduced *XPC* mRNA level in cells from XP-C patients was caused by the PTC that induces nonsense-mediated mRNA decay. The mean *XPC* mRNA levels in cell lines from the heterozygous XP-C carriers were intermediate (59%, $P = 10^{-4}$) between the values for the XP patients and the normal controls. This study demonstrates reduced *XPC* mRNA levels in XP-C patients and heterozygotes. Thus, *XPC* mRNA levels may be evaluated as a marker of cancer susceptibility in carriers of mutations in the *XPC* gene.

Abbreviations: CypE, cyclophilin E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGR, global genomic repair; HCR, host cell reactivation; NER, nucleotide excision repair; NMD, nonsense-mediated mRNA decay; PTCs, premature termination codons; QRT–PCR, quantitative reverse transcriptase–PCR; UDS, unscheduled DNA synthesis; XP, Xeroderma pigmentosum; XP-C, Xeroderma pigmentosum group C.

Introduction

Xeroderma pigmentosum (XP) is an autosomal recessive disorder with defective nucleotide excision repair (NER) (1,2). This DNA repair system eliminates sunlight induced cyclobutane pyrimidine dimers and 6-4 photoproducts in DNA. The *XPC* gene encodes a 940 amino acid protein, which is part of a heterotrimeric complex, with HHR23B and centrin 2, that is involved in the recognition of the UVR-induced DNA lesions during global genomic repair (GGR) but not transcription-coupled repair (3–8).

Strong evidence of the role of DNA repair in cancer susceptibility is derived from studies of XP patients who have an incidence of skin cancers ~1000 times that of the general population (9,10). The frequency of clinically normal individuals who are XP heterozygotes (~1 in 500) is much higher than XP patients (homozygotes or compound heterozygotes) (~1 in 10^6 in the US). Whether individuals who are heterozygous for a mutation in the *XPC* gene are at increased risk of malignancy is not well understood. The only study to date of cancer risk in XP heterozygotes was published in 1979, before the XP genes were cloned (11). The study mentioned above was conducted on the pedigrees of XP families and suggested that carriers of one mutated XP allele have an elevated incidence of skin cancer. Mice that have a homozygous knockout of the *XPC* gene have markedly increased susceptibility to UV induction of skin cancer (12–19) and *XPC* heterozygous mice have increased cancer susceptibility after prolonged UV exposure (15,19).

Xeroderma pigmentosum group C (XP-C) is one of the more common forms of XP in the United States (1,2). We have studied a series of 16 XP-C kindreds from throughout the world and characterized their causative mutations. By employing real-time quantitative reverse transcriptase–PCR (QRT–PCR) assays (20) we found low levels of *XPC* mRNA in all XP-C probands and intermediate levels in XP-C heterozygotes.

Materials and methods

Cell lines, culture conditions and DNA/RNA isolation

Fibroblast (F) and lymphoblastoid (L) cell cultures from 16 XP-C families were studied: A: XP100TMA (L) (21) (GM15709, GM15710), XP101TMA (L) (GM15715, GM15716) and XPH102TMA (GM15717, GM15718); B: XP87BE (F) (GM16381), XPH88BE (F) (GM16380), XPH89BE (F) (GM16379); C: XP105BE (L) (GM16467), XPH106BE (L) (GM16468), XPH107BE (L) (GM16469); D: XP25TA (F) (22) (KR04047), XPH26TA (F) (KR04048), XPH27TA (F) (KR04049); E: XP24BE (L) (GM11637), XPH206BE (L) (GM11639), XPH207BE (L) (GM13296); F: XP23BE (F)

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(23) (AG10032), XPH294BE (F) (AG10034); G: XP314VI (F), XPHM312VI (F), XPHF313VI (F); H: XP69TMA (L) (GM14870), XPH93TMA (L) (GM15702), XPH94TMA (L) (GM15704); I: XP1BE (L) (24–26) (GM02246), XPH291BE (L) (GM04237), XPH292BE (L) (GM04238); J: XP16VI (F), XPF16VI (F), XPM16VI (F); K: XP664VI (F), XP665VI (F), XPHF662VI (F), XPHM663VI (F); L: XP132BE (L) (GM16692), XPH133BE (L) (GM16690); M: XP30BE (L) (KR05792), XPH298BE (L) (GM15451); N: XP67TMA (F) (27) (GM14867), XPH96TMA (F) (GM15707), XPH97TMA (F) (GM15721); O: XP54BE (L) (GM17861), XPH311BE (L) (GM17860); and P: XP25BE (L) (KR04490); XPH310BE (L) (KRO4491). The patients were studied under a protocol approved by the NCI institutional review board. Normal SV40-transformed fibroblasts (GM00637), normal primary skin fibroblasts (AG04349, AG04659, AG05186, AG13145, AG13153, AG05247E) and normal lymphoblastoid cells (GM00130, KR06057, KR06058, KR06059, KR06060) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). SV40-transformed XP-C (XP4PA-SV-EB) cells, as described by Daya-Grosjean *et al*. (28), were a gift from Dr R.Legerski (MD, Anderson Hospital, Houston, TX). Cell culture and separation of RNA and DNA was performed as described previously (21).

DNA repair measurement and complementation group assignment

DNA repair abnormalities were assessed as increased post-UV growth inhibition (29), as reduced post-UV unscheduled DNA synthesis (UDS) (30,31) or as reduced post-UV host cell reactivation (HCR) (21,29) using the pCMVLuc reporter gene plasmid (a generous gift from M.Hedayati and L.Grossman, Johns Hopkins University, Baltimore, MD). HCR was also used to assign XP cells to a specific complementation group as described previously (21,32).

Mutation detection by PCR amplification, sub-cloning and nucleotide sequencing

All 16 *XPC* gene exons, including splice donor and acceptor sites, splice lariat branch point sequences and pyrimidine tracts, were PCR amplified using intronic primers flanking these sequences and sequenced as described previously (20).

Real-time QRT–PCR for XPC and housekeeping gene mRNA levels

XPC mRNA was quantified using gene specific primer pairs, employing real-time QRT–PCR as described previously (20). The allele-specific primer pairs, oVMM-21/oVMM-22 and CCB-331/CCB-337, were used to measure the amount of *XPC* mRNA including exons 4 and exon 12, respectively. Real-time QRT–PCR assays were carried out on either the Bio-Rad iCycler iQ or MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using intercalation of SYBR Green I as the fluorescence reporter. Amplicon synthesis was monitored continuously by SYBR Green I dye binding to double stranded DNA during PCR amplification of the exon 4 or exon 12 regions of the XPC gene. Specificity was verified by amplicon melting temperatures. All real-time QRT–PCR data are expressed as fg of a full length wild-type *XPC* cDNA clone (20).

XPC mRNA expression in the cells were normalized to cyclophilin E (CypE), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin using the method of Vandesompele *et al*. (33) as implemented in the Bio-Rad Gene Expression Macro. We measured the levels of CypE, GAPDH and β-actin mRNA expression by real-time QRT–PCR using fluorescence dye SYBR Green I as the fluorescence reporter. The sequences of the sense and antisense primers used for the PCR amplification of these housekeeping genes were: CypE; sense (oCCB-529), 5′-GACTGTGGGGAGTACGTGTG-3′, antisense (oCCB-530), 5′-GAAGGGCACATATCCCAAAT-3′; GAPDH; sense (oCCB-388), 5′-TCTCCTCTGACTTCAACAGC-3′, antisense (oCCB-389), 5′-GAAATGAGCTTGACAAAGTG-3′; β-actin; sense (oFY-4), 5′-GCTTGCTGATCCACATCTGC-3′, antisense (oFY-5), 5′-TGGA-CATCCGCAAAGACCTG-3′. For the quantification of CypE, the PCR was performed utilizing 1 μl cDNA (corresponding to 50 ng RNA), 300 pM primers and iQ SYBR Green supermix (Bio-Rad) per reaction in triplicates of 25 μl volume, following the manufacturer's protocol. Two step PCR (denaturation at 95°C for 15 s and annealing/extension at 58°C for 1 min) was carried out on either the Bio-Rad iCycler iQ or the MyiQ Real-Time PCR Detection System, with the data collection and the analysis carried out during the combined annealing and extension step. The quantification of GAPDH and β-actin mRNA levels were performed in a similar way to CypE except that we used 1 μl diluted cDNA (1:25 dilution) per 25 μl reaction and 60°C annealing/extension temperature.

DNA sequence information analysis

Sequences were scanned with the donor and acceptor individual information weight matrices and the identified sites were displayed as described previously (34,35) and at http://www.lecb.ncifcrf.gov/~toms/delilaserver.html

XPC protein detection by western blotting

Protein extraction and western blot analysis with chemiluminescent detection were as described (36). The following antibodies and dilution factors were used: mouse anti-XPC (ab6264, Abcam, Inc., 1:500), rabbit anti-actin (H-196, Santa Cruz, 1:1000) and anti-mouse peroxidase-conjugated immunoglobulin G (IgG-HRP, Santa Cruz, 1:1000). The X-ray films were scanned and the intensity of the bands was assessed using image J 1.30v (Wane Rasband, NIH, http://rsb.info.nih.gov/ij/). The XPC protein was calculated as the ratio of XPC/β-actin. The results were expressed in percentage using the level of normal XPC protein as 100%.

Results

Clinical findings

The age of the XP-C patients ranged from 7 to 49 years (Table I). The origin of the families varied widely and included Turkey, Israel, Morocco, Algeria, Poland, Hungary, France, Honduras, African American and Native American ancestry. All patients had freckling, telangiectasia and/or actinic keratoses and began developing skin lesions at an early age on sun-exposed sites. All patients had at least one skin cancer. Several had >100 skin cancers including basal cell carcinomas, squamous cell carcinomas and melanomas. Four XP patients also had primary internal cancers: XP24BE (brain astrocytoma), XP23BE (spinal cord astrocytoma) (23), XP1BE (endocervical adenocarcinoma of the uterus) (25) and XP664VI (brain glioma). None of the XP patients had XP type neurological degeneration (37). Their parents, who are obligate XP heterozygotes, appeared to be clinically normal except that the father in family M, XPH298BE, had a basal cell carcinoma of his eyelid at the age 30.

Assignment of XP cells to complementation group C

The cells from the XP patients showed reduced post-UV cell survival, post-UV HCR or UDS (Figure 1A and B and data not shown). The cells from the parents that were tested showed normal or nearly normal post-UV cell survival, UDS or HCR (Figure 1A and B, (21) and data not shown). The UV irradiated reporter gene plasmid was transfected into the patients' cells along with plasmids expressing cloned wild-type XP group A, C or D cDNA. Only co-transfection with a plasmid containing the *XPC* cDNA led to a markedly increased post-UV HCR in these patients' cells thus assigning these cells to the XP complementation group C (Figure 1C; 21,22,27,38 and data not shown).

Mutation in the XPC gene: genotype–phenotype correlations in the XP-C patients

We characterized the causative mutations in the *XPC* gene in the cell lines derived from these 16 XP probands and their parents, who were obligate XP heterozygotes (Table I, Figures 2 and 3). There were 15 mutations found. Ten of the patients were homozygous for *XPC* mutations. Six patients were compound heterozygotes with different *XPC* mutations in each parent. We were able to identify all the 10 mutations in the homozygous patients and 9 of the 12 mutations in the compound heterozygous patients. No large mutational hotspots were apparent. The most frequent mutation was observed in 4 families (J, K, L and M) and the next most frequent was seen in two families (C and D), and 13 of the mutations were found in one family each.

Five of the inactivating changes were frameshift mutations resulting from the deletion of two bases (4 mutations) or four bases (1 mutation) within exons. One mutation was an

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Table I. XPC mutations in 16 Xeroderma pigmentosum probands

FAM	XP NUM	Age[1] (year)/sex	Proband origin	Proband genotype	Allele 1—maternal DNA mutation	Allele 2—paternal DNA mutation	Allele 1—mRNA	Allele 2—mRNA
A	XP100TMA	14/M	Turkey	Homozygous	Intron 3–9 T→A Lariat	Homozygous	Exon 4 Del→Stop 3 codons downstream	Homozygous
B	XP87BE	14/M	USA/Israel	Compound heterozygote	Exon 4 del TGAG 525–8[a]	Exon 8 A 1669T[a]	fs 140→Stop 6 codons downstream	AAA (Lysine) 522→ UAA Stop
C[2]	XP105BE	24/F	USA/Israel	Homozygous	Exon 5.1 del AT 669–70	Homozygous	fs188→Stop 10 codons downstream	Homozygous
D	XP25TA	24/F	Israel/Poland	Homozygous	Exon 5.1 del AT 669–70	Homozygous	fs188→Stop 10 codons downstream	Homozygous
E	XP24BE	31/F	USA/Hungary	Compound heterozygote	Intron 5.1 A→G at −2[a]	Exon 4 C568T[a]	83 BP insertion of intron 5.1→ Stop 34 codons downstream and Exon 5.2 Del—Stop 7 codons downstream	CGA (Arginine) 155→ UGA Stop
F	XP23BE	d 31/M	Native American	Homozygous	Intron 5.1 A→C at −2[a]	Homozygous	83 BP insertion of intron 5.1→ Stop 34 codons downstream	Homozygous
G	XP314VI	10/M	France	Compound heterozygote	Unknown	Exon 8 C1348T[a]	Unknown	CGA (Arginine) 415→ UGA Stop
H	XP69TMA	10/M	Turkey	Homozygous	Exon 8 Del TAAAGA 1395–1400[a]	Homozygous	Del 430→UAG Stop	Homozygous
I	XP1BE	d 49/F	USA	Homozygous	Exon 8 Del AA 1396–7	Homozygous	fs 431→Stop 5 codons downstream	Homozygous
J[2]	XP16VI	19/F	Algeria	Homozygous	Exon 8 Del TG1744-5	Homozygous	fs 547→Stop 25 codons downstream	Homozygous
K	XP664VI	9/M	Morocco	Homozygous	Exon 8 Del TG1744–5	Homozygous	fs 547→Stop 25 codons downstream	Homozygous
L	XP132BE	8/M	Honduras	Compound heterozygote	Exon 8 Del TG1744–5	Unknown	fs 547→Stop 25 codons downstream	Unknown
M	XP30BE	30/M	USA	Compound heterozygote	Exon 8 Del AA 1208–9	Exon 8 Del TG 1744–5	fs 368→Stop 5 codons downstream	fs 547→Stop 25 codons downstream
N	XP67TMA	d 8/M	Turkey	Homozygous	Exon 8 C1840T[a]	Homozygous	CGA (Arginine) 579→UGA Stop	Homozygous
O	XP54BE	12/M	USA	Compound heterozygote	Exon 10 A2179T[a]	Unknown	AAG (Lysine) 692→UAG Stop	Unknown
P	XP25BE	7/F	African American	Homozygous	Intron 11 Del −1, −2 AG and Insertion of CC between −6, −7[a]	Homozygous	Exon 12 Del→Stop 1 codons downstream or Exon 12 Del 44 BP (2356–99)→ Stop 32 codons downstream or retains intron 11 (82 bp)→Stop 74 codons downstream	Homozygous

[a]Mutation newly reported in this paper.
[1]Age at death.
[2]Box encloses families with one or more identical mutations.

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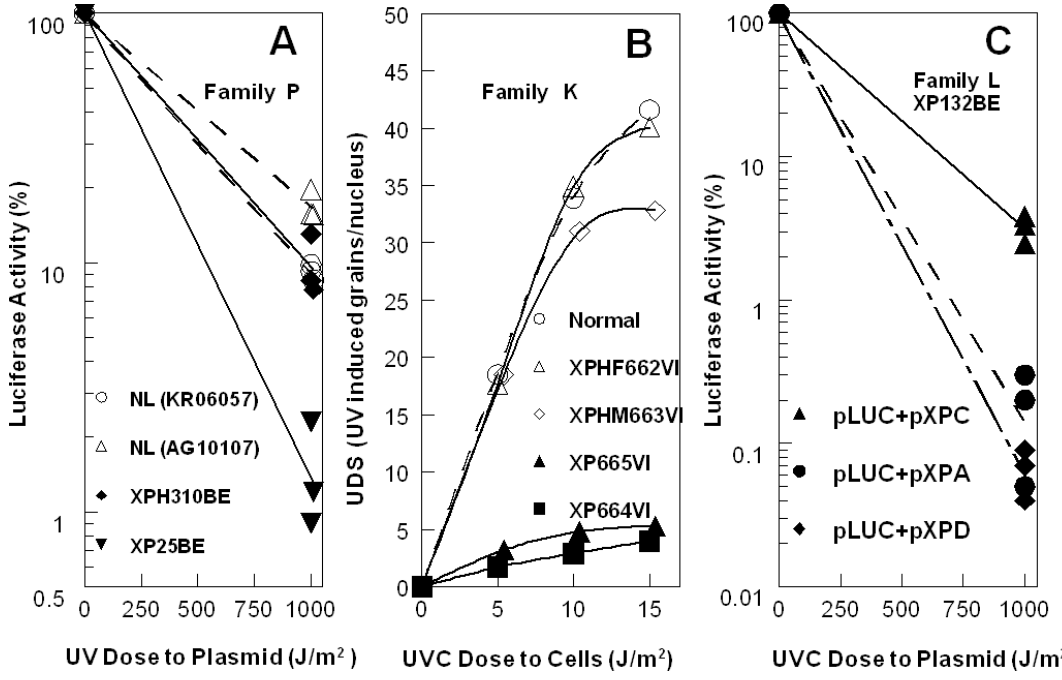


Fig. 1. DNA repair studies and XP complementation group assignment. (**A**) The relative luciferase activity of UV-treated pLUC was much lower in the cells from the proband of family P (XP25BE) than from the normal donors (KR06057 and AG10107). The activity in the cells from her mother (XPH310BE) was within the normal range. Each symbol represents the relative reporter gene activity in an independent transfection experiment 48 h after transfection compared with the corresponding unirradiated control reporter gene plasmid. (**B**) The UDS level was measured in family K as the average number of grains per nucleus as a function of UV dose to the cells. The UDS from the XP patients (XP664VI and XP665VI) was 10–15% of that of a normal donor and their parents (XPHF662VI and XPM663VI). (**C**) UV-treated reporter gene plasmid (pLUC) was co-transfected with XP cDNA containing plasmid [pXPC, pXPA or pXPD] into cultures of lymphoblasts from XP132BE, the proband of family L. The correction was achieved only by co-transfection of pXPC, indicating that these cells are in the XP complementation group C.

out-of-frame deletion of six bases within exon 8 that creates a stop codon. The deletion of AT residues in *XPC* exon 5.1 at positions 669–70 was found in both alleles of XP105BE and XP25TA. There was a deletion of AA in exon 8 at position 1396–7 in both the alleles of XP1BE. This same deletion was part of a 6 base deletion (TAAAGA) at 1395–1400 that was found in both alleles of XP69TMA suggesting a role of neighboring sequences in the generation of these mutations. There was a TG deletion in exon 8 at position 1744–5 that was found in both alleles of XP16VI and in the maternal alleles of XP132BE and XP30BE. The paternal allele of XP30BE had an AA deletion in exon 8 at 1208–9. Some of these deletions occurred in runs of identical bases presumably resulting from replication slippage. All of these deletions lead to premature termination of the encoded protein (Table I).

Cells from a 14-year-old boy (XP87BE) in family B with severe XP symptoms, including multiple skin neoplasms including melanoma, were compound heterozygote with a frameshift and a nonsense mutation. The maternal allele had a deletion of 4 bases (TGAG) in exon 4 at 525–8, resulting in a frameshift with a new termination site 6 codons downstream. The paternal allele had a nonsense mutation (A1669T) in exon 8, converting the AAA codon of lysine at amino acid 522 to a UAA stop codon.

There were three other cell lines with nonsense mutations. The paternal allele of XP24BE in family E had a C568T transition leading to Arg155X in exon 4. A nonsense mutation consisting of a C1840T transition in exon 8 (Arg579X) was observed in both alleles of the patient XP67TMA in family N. These C to T transitions at CpG sites are likely to be a consequence of the demethylation of 5-methylcytosine to thymine. The maternal allele of XP54BE in family O had A2179T leading to Lys692X in exon 10.

There were four XP patient cell lines with splice mutations. The *XPC* intron 5.1 splice acceptor sequence ends with 'cag' in DNA from normal cells. We found single nucleotide changes: cag to cCg in XP23BE (homozygous) in family F, and cag to cGg in XP24BE in family E (maternal allele, compound heterozygote) (Figure 2A, B and C). These single base changes eliminated the canonical AG dinucleotide that is found as the last two nucleotides of the vast majority of eukaryotic introns. Information theory can be used to provide a measure of the ability of a sequence to code for splice elements. Measured on a logarithmic scale, the higher values have a greater splicing ability (34). The mutations reduced the information content of the natural intron 5.1 splice acceptor from 17.4 bits in the normal cells (20) to 10 bits in XP23BE and 9.3 bits in XP24BE. The XP23BE and XP24BE cells and those from their mothers expressed an alternatively spliced *XPC* mRNA isoform with an 83 bp insertion from the 3′ end of intron 5.1 (Figure 2B). This alternatively spliced isoform is generated by the activation of a cryptic alternative acceptor splice site 83 bases upstream in intron 5.1 that has an information content of 7.7 bits.

In the cell line from another XP-C patient (XP25BE) in family P, there was a homozygous (or hemizygous) deletion of AG (at −1 and −2 bp) of intron 11 and insertion of CC between −6 and −7 bases at the 3′ end of intron 11. There was no detectable full length *XPC* mRNA in the XP25BE cells. The deletion and insertion mutations in *XPC* intron 11 at the 3′ end in the XP25BE cells reduce the information content of the native splice acceptor site in intron 11 from 5.1 bits to 0.3 bits

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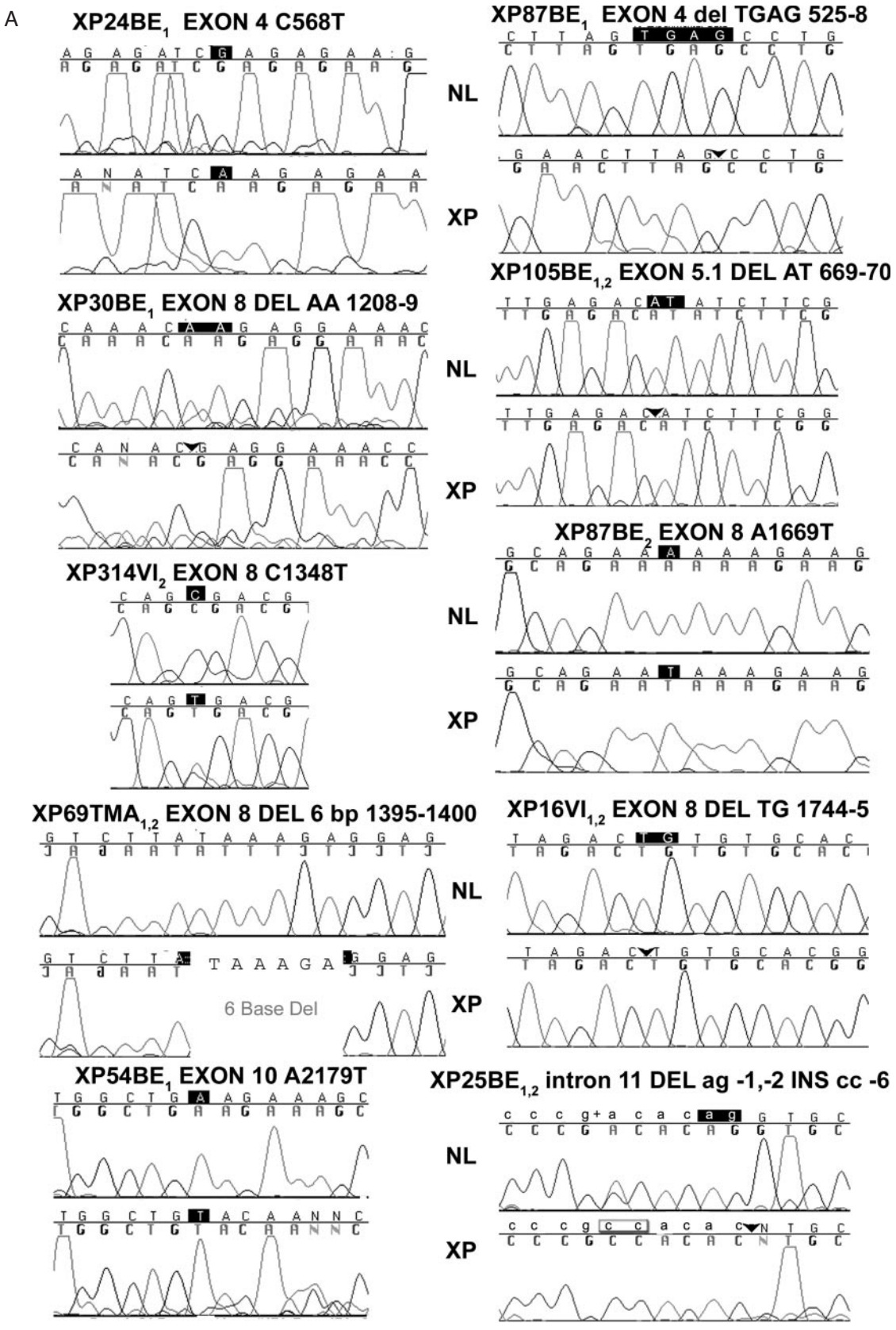


Fig. 2. XPC mutations and *XPC* mRNA isoforms in cells from XP patients. (**A**) Non-radioactive sequencing of genomic DNA. In each section, the upper sequence is for the normal DNA and the lower sequence is for the patient DNA. For XP24BE, XP87BE, XP30BE and XP54BE subcloned DNA was sequenced. For XP105BE, XP314VI, XP69TMA, XP16VI and XP25BE uncloned DNA was sequenced. (**B**) Alternatively spliced *XPC* mRNA isoforms from family F (XP23BE) and E (XP24BE). The normal cells and that of the father (XPH206BE) in family E have a single 274 bp band. The proband of family F (XP23BE) has a single band that is 83 bp larger. The proband for family E and both mothers have both the bands. (**C**) Radioactive DNA sequencing of uncloned DNA (XP23BE) and subcloned DNA (XP24BE) show a change of a –2 c and a –2 g, respectively, near the intron 5.1 splice acceptor site.

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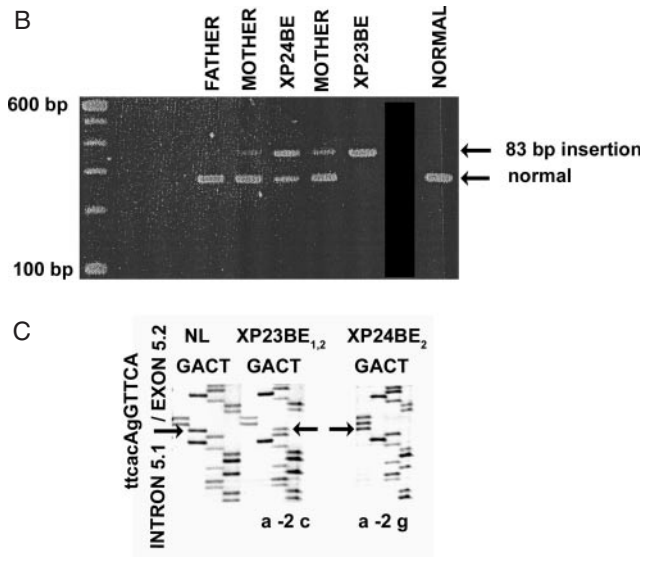


Fig. 2. (continued)

and leads to the generation of three alternatively spliced *XPC* mRNA isoforms: (i) with exon 12 skipped, (ii) with deletion of 44 bases at the 5′ end of exon 12 due to activation of a cryptic splice acceptor site in exon 12 with an information content of 6.2 bits and (iii) with retention of intron 11. The cells from XP100TMA in family A contain a homozygous point mutation in *XPC* intron 3 (−9 T to A), located within the splice lariat branchpoint sequence and express exclusively an XPC mRNA isoform with deleted exon 4 (21).

XPC mRNA levels in the cells from XP-C patients and heterozygotes

A real-time QRT–PCR assay (20) with allele-specific primers that detect *XPC* mRNAs containing either exon 4 (Figure 4) or exon 12 (data not shown) was used to measure the levels of *XPC* mRNA in cells from the XP-C patients, their heterozygous parents and normal controls. In our families these assays measured both wild-type full length *XPC* mRNA as well as mutant *XPC* mRNA, except in families A and P. For RNAs from family A with a splice lariat mutation in intron 3, the exon 4 inclusion assay measures only full length wild-type *XPC* mRNA while the exon 12 inclusion assay measures both wild-type *XPC* mRNA as well as *XPC* mRNA skipping exon 4. Likewise for RNAs from family P with a mutation in intron 11, the exon 12 inclusion assay measures only full length wild-type *XPC* mRNA, while the exon 4 inclusion assay measures both wild-type *XPC* mRNA as well as all alternatively spliced *XPC* mRNAs.

The cell lines from the 16 XP-C patients had a mean *XPC* mRNA level of 49 fg using the exon 4 inclusion assay. This represents 24% ($P < 10^{-7}$) of the mean value for the 10 normal controls (209 fg). All 16 XP cell lines had *XPC* mRNA levels (maximum 84 fg) that were less than those of the normal controls (range 159–279 fg) (Figure 4). It is important to note that the *XPC* mRNA detected in the XP-C cell lines, with the possible exception of XP23BE and XP24BE (Figure 2B), is mutant and unable to code for the full length wild-type XPC protein. Cell lines from 27 patients with XP-A, XP-D, XP-G, XP variant, trichothiodystrophy or Cockayne syndrome had normal *XPC* mRNA levels (mean 236 fg) (data not shown).

The mean *XPC* mRNA level in cell lines from 26 XP-C heterozygotes was intermediate between that of the normal cells and that of the XP patients (Figure 4). The mean value was 123 fg for the exon 4 inclusion assay, representing 59% ($P < 10^{-4}$) of the values for the normal controls. In XPH102TMA in family A this value cannot be compared directly with the other heterozygotes since the splice lariat mutation results in skipping of exon 4 (21) and thus the exon 4 inclusion assay measures only full length wild-type *XPC* mRNA. However, the exon 12 inclusion assay detects both wild-type and mutant *XPC* mRNA from XPH102TMA and gave a value that was 41% of the mean from normal controls (data not shown). Of the 26 XP-C heterozygote cell lines 22 had *XPC* mRNA values that were below the 99% confidence interval for the 10 normal control donors for exon 4 inclusion. There were no significant differences between the values for lymphoblasts or fibroblasts within each donor class.

In order to assess the possible confounding effects of variations in input mRNA or degradation of the mRNA we performed an additional analysis of 26 samples (8 normals, 7 XP patients, and 11 XP heterozygotes). Using real-time QRT–PCR we measured the mRNA levels of *XPC* and three housekeeping genes (CypE, GAPDH and β-actin) as internal standards. We then normalized *XPC* mRNA to all three internal standards as described in Ref. (33). There were only small differences between the normalized and the not normalized measurements of the *XPC* mRNA (data not shown). Furthermore, the quantification of *XPC* mRNA by real-time QRT–PCR using RT reactions from duplicate RNA samples from each individual was in close agreement between the samples. In addition, frozen cDNA samples re-assayed after 4 years had similar relative values of the *XPC* mRNA.

Two XP families (Families C and D) were homozygous for the same mutation in exon 5.1 (del AT 669–70), and four families (Families J, K, L and M) had the same mutation in exon 8 (del TG 1744–5) in at least one allele (Table I and Figures 2 and 3). The *XPC* mRNA levels in the XP patients with the same mutations were similar (Figure 4). All the 10 heterozygote cell lines in these families had *XPC* mRNA levels that were lower than the normal.

XPC protein levels in the cells from XP-C patients, parents and normal donors

To measure the XPC protein levels in the cells from XP-C patients and their parents, western blot analysis was performed on total cellular extracts using XPC-specific monoclonal and anti-β-actin polyclonal antibodies. XPC protein was readily measured in the normal cells (Figure 5 Lanes A1, B1, C1, D1, 8). In contrast, neither full length nor truncated XPC protein was detectable in the lymphoblasts or fibroblasts from any of the XP-C patients (Figure 5 Lanes A2, 4, 7; B2, 4; C2, 4 and data not shown). The level of XPC protein for the heterozygous parents was lower than the normal controls (Figure 5 Lanes A 3, 5, 6, 8; B 3, 5; C 3, 5, 6; D 2, 3, 4, 5, 6, 7). The ratio of XPC/β-actin density for the heterozygous parents was 12–100% of that of the normal controls.

Discussion

XP-C is one of the more common forms of XP (1,2,9). All the patients had at least one skin cancer. Skin cancers in XP patients are predominately the direct effect of sunlight induced

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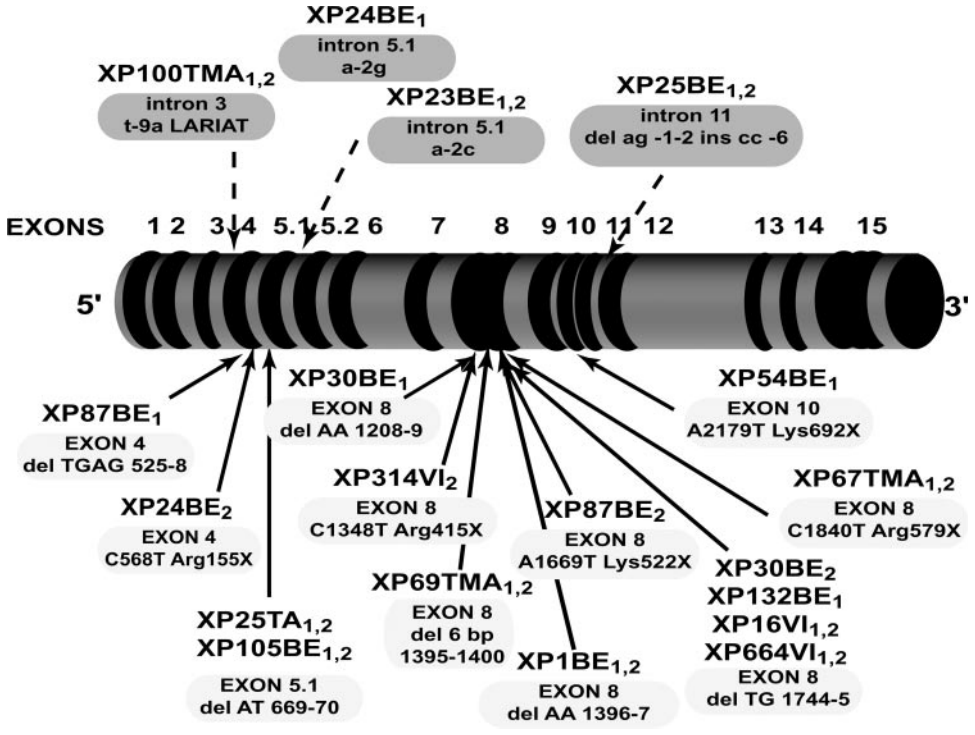


Fig. 3. Location of mutations in the *XPC* gene in 16 kindreds. The 16 exons of the XPC gene are indicated by black bands. Mutations in introns are indicated above the gene with dashed arrows and mutations in exons are indicated with solid arrows below. For each proband the maternal allele is indicated by a subscript 1 and the paternal allele by a subscript 2.

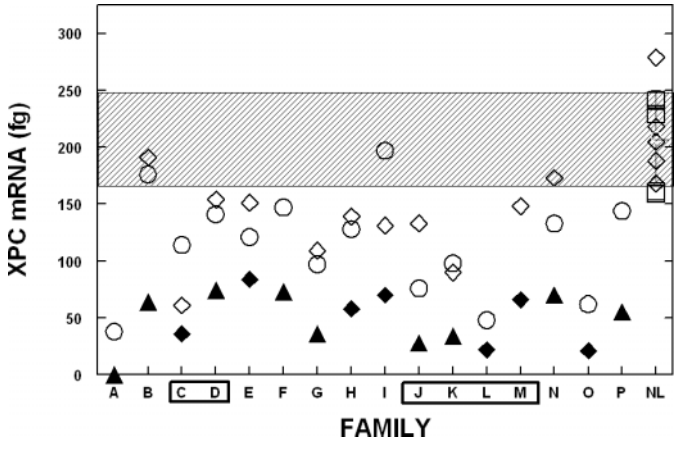


Fig. 4. Level of *XPC* mRNA in 16 XP families. The amount of *XPC* mRNA measured by real-time QRT–PCR (exon 4 inclusion assay) is indicated for the probands (solid symbols) and parents (open symbols) for each kindred. Probands: fibroblasts (solid triangles) and lymphoblasts (solid diamond). Parents: mothers (open circles) and fathers (open diamonds). The 99% confidence interval for the five fibroblasts (open squares) and five lymphoblasts (open diamonds) from normal donors is indicted by a shaded rectangle. All probands had low levels of *XPC* mRNA and the values for the parents were significantly lower than that of the normal donors.

DNA damage, as assessed by the characteristic C to T and CC to TT 'signature mutations' in the p53 tumor suppressor gene (39). Four of the probands had internal cancers in addition to skin cancers. These included three cancers of the nervous system (astrocytomas or gliomas of the brain and spinal cord) and an adenocarcinoma of the uterus. Another XPC patient had a glioma of the thalamus (40). An increased frequency of internal cancers of the nervous system has previously been reported in XP (10). Internal tumors in XP patients may be associated with types of lesions in the p53 gene that could be caused by oxidative damage (39,40).

Mutations in XP-C patients

There have been few reports of mutations in the *XPC* gene that are responsible for the clinical phenotype of XP (21,22, 24,27,38,41,42). These 17 inactivating mutations included 9 frameshift, 3 nonsense and 3 splice site mutations. There were only two inactivating missense mutations: Proline 334 Histidine in XP1MI (24) and Tryptophan 690 Serine in XP13PV (41). The A to C change in exon 15 of XP8BE (24) and the C to A change in the intron 11 acceptor site 6 bp upstream from the intron 11/exon 12 junction that leads to a low frequency of deletion of exon 12 in XP9PV (41) are common polymorphisms (20,22,43). Three mutations in exon 8 (del AA 1396–7, del AA 1208–9 and del TG 1744–5) (24,41), the t-9a splice lariat mutation in intron 3–kindred A (21) and the del AT 669–70 in exon 5.1–kindred D (22) were reported previously. Thus 5 nonsense, 2 frameshift and 3 splice mutations in the *XPC* gene that result in clinical disease are newly described in the current paper. Most of the mutations described in these reports have been found in one or two kindreds. One mutation (del TG 1744–5 in exon 8) found in four kindreds in our study (J, K, L and M) was previously reported in XP4PA (24,28) and in XP26PV (41). This may represent a hotspot for polymerase slippage mutations, since the deleted TG occurs in a run of 3 TGs in exon 8 (Figure 2A), and/or a founder mutation (since two of the families and XP4PA were from North Africa).

XPC mRNA

The creation of premature termination codons (PTCs) in the *XPC* gene suggests that the expression of low levels of mutant *XPC* message in cell lines from XP-C patients may involve the nonsense-mediated mRNA decay (NMD) pathway (44–48).

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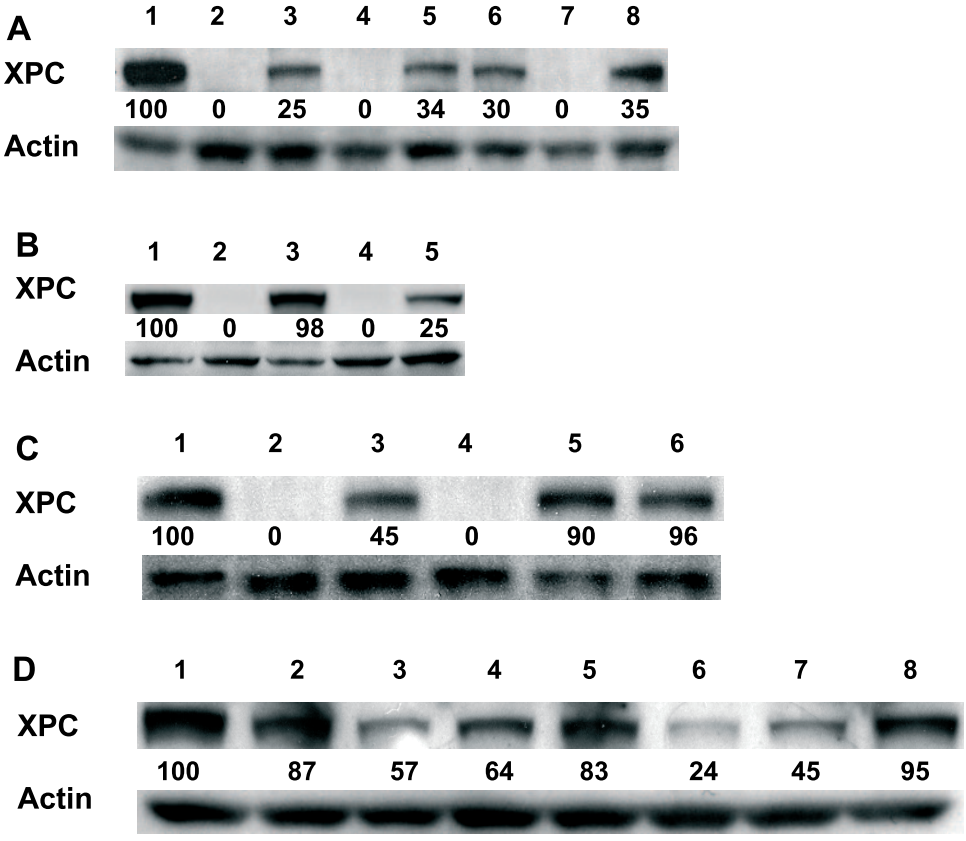


Fig. 5. *XPC* protein levels in cells from XP patients, parents and normal controls. Western blotting was performed on the extracts from lymphoblasts (**A**, **B**) and fibroblasts (**C**, **D**) and probed with the antibody for XPC (upper bands) and β-actin (lower bands). The normalized ratio (%) of the intensity of the XPC band to that of the β-actin band is indicated between the bands in each lane. (A) Lane 1, normal (KR06057); Lane 2, proband family M (XP30BE); Lane 3, father family M (XPH298BE); Lane 4, proband family H (XP69TMA); Lane 5, father family H (XPH93TMA); Lane 6, mother family H (XPH94TMA); Lane 7, proband family P (XP25BE); Lane 8, mother family P (XPH310BE). (B) Lane 1, normal (KR06057); Lane 2, proband family L (XP132BE); Lane 3, mother family L (XPH133BE); Lane 4, proband family O (XP54BE); Lane 5, mother family O (XPH311BE). (C) Lane 1, normal (AG06239); Lane 2, proband family (XP23BE); Lane 3, mother family F (XPH294BE); Lane 4, proband family B (XP87BE); Lane 5, father family B (XPH88BE); Lane 6, mother family B (XPH89BE). (D) Lane 1, normal (AG13145); Lane 2, father family B (XPH88BE); Lane 3, mother family B (XPH89BE); Lane 4, father family K (XPH662VI); Lane 5, mother family K (XPH663VI); Lane 8, normal (AG04349).

The decay of *XPC* transcripts containing pre-termination signals by the NMD pathway is a protective mechanism that prevents the expression of deleterious truncated proteins. By use of the real-time QRT–PCR method (20) we found that the *XPC* mRNA levels were significantly reduced to a level $<25\%$ of the normal ($P < 10^{-7}$) in the XP-C patient cell lines (Figure 4). Legerski and Peterson (49) and Khan *et al*. (38) reported very low levels of *XPC* mRNA in cells from XP-C patients by use of Northern blots. In contrast, Chavanne *et al*. (41) measured *XPC* mRNA in XP patients from Italy by use of the northern blot analysis and found only a slight reduction in the levels of the *XPC* transcript with values ranging between 60 and 80% of the normal. Our real-time QRT–PCR assay (20) has greater sensitivity than northern blotting, allows more accurate quantification over a broader dynamic range, and the isoform-specific primers permit quantification of individual isoforms that might co-migrate on gels.

The proband XP100TMA in family A from Turkey (Table I) and his 16-year-old sister (XP101TMA) had extremely severe XP skin disease beginning at age 3, with multiple skin cancers. They both had a mutation in the splice lariat branchpoint in the XPC intron 3 and had undetectable levels of normal full length *XPC* mRNA [Figure 4 and (21)]. In contrast three siblings from another Turkish family (XP72TMA, XP73TMA and XP123TMA) had a mild disease with only one skin cancer by 18 years of age. They had a different splice lariat branchpoint mutation in the *XPC* intron 3 and all three had 3–5% of the normal *XPC* mRNA (21). Thus, affected siblings had similarly reduced levels of *XPC* mRNA and a small amount of normal *XPC* mRNA appeared to provide some protection against skin cancers.

Sunlight exposure also plays a major role in the expression of the XP phenotype. In family D, XP12TA began developing skin cancers at age 2 and died at age 10 with multiple skin cancers, while XP25TA, a sibling with the same XPC mutation [del AT 669–70 in exon 5.1; Table I, Figures 2 and 3 and (22)], had greater sun protection, began to develop skin cancers at a later age (10 years) and was alive at age 24 with multiple skin cancers (22). In family C, XP105BE had the same homozygous mutation as XP25TA in family D (Table I and Figures 2 and 3). Yet the much greater sun protection in XP105BE was associated with greatly delayed onset of her first skin cancer until age 24. In the case of XP314VI in family G, the sun exposure he received during the first 2 years of his life, before the disease was detected, was enough to induce three skin tumors in the following 6 years. However, starting at the age of 2 years, he was completely protected from UV exposure, and now at age 10 his skin is almost normal (some hyperpigmentation and freckles are still visible) and he has been tumor free for the past 2 years.

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XPC protein levels

Measurement of XPC protein levels by western blotting showed a clearly measurable amount in the cells from normal donors and no detectable XPC protein in the cells from the XP-C probands (Figure 5). This finding is in agreement with the previous report by Chavanne *et al*. (41) of absent XPC protein in cells from XP-C patients. *XPC* is not an essential gene for cell proliferation or viability. The mutations characterized in the *XPC* gene are expected to cause protein truncations as a result of nonsense mutations, frame-shifts, deletions or aberrant splicing, as previously reported in other nonessential DNA repair genes, such as CSB and XP-A (42,50). These mutant XPC proteins would not retain the COOH-terminus which contains domains that recruit TFIIH for NER and would not be expected to be functional. No full length or truncated XPC proteins were detected by western blotting in the cells from the XP-C patients (Figure 5), however, the binding epitope for the XPC antibody we used is not known. In contrast, the XPD protein is a component of the basal transcription factor, TFIIH, as well as a part of the NER pathway and is essential to life. The causative *XPD* mutations are all missense mutations, which preserve some function (42,51).

The XPC protein is required for the initial phases of GGR pathways of NER (3–5). DDB2 binds to DNA photoproducts and attracts XPC associated with hHR23B to initiate GGR and recruits other NER factors via protein–protein interactions (52). Low levels of XPC mRNA expression in normal human cells (~10 *XPC* mRNA molecules per cell, assuming 100% efficiency of the RT reaction) have been reported (20). Faulty repair of oxidative damage by impaired interaction of XPC-hHR23B and thymine DNA glycosylase (53) or by inability of GGR to repair bulky oxidative lesions, such as cycloadenine (54,55), may contribute to the elevated level of central nervous system cancers seen in XP-C patients (9,10,39,40).

XPC heterozygotes

XPC heterozygotes who are carriers of one mutated *XPC* allele and one normal allele are generally clinically normal without the marked sun sensitivity and greatly increased skin cancer susceptibility observed in XP patients. A 1979 study of cancer risk in XP heterozygotes, performed before the genes were cloned, examined 31 XP families and included >2500 blood relatives and spouse controls (11). XP heterozygotes, estimated using indirect methods, had an ~2.3-fold increased risk of developing non-melanoma skin cancer and the risk in 4 of the 31 families was elevated by 16-fold.

The study of Chavanne *et al*. (41) using Northern blots to assess *XPC* mRNA levels reported no significant differences between the parents of the Italian XP patients and the normal donors. In contrast to Chavanne *et al*. (41) we could detect the expression of abnormally spliced *XPC* mRNA in cells from the parents of some patients (Figure 2B), indicating that these mutated alleles were expressed. By using real-time QRT–PCR assay we previously found that the XPH102TMA cells from the mother in family A had a low level of *XPC* mRNA (21). This appears to be a more general finding (Figure 4). The real-time QRT–PCR assay we used for exon 4 inclusion does not distinguish between the normal and the mutant *XPC* mRNA (except in family A). Low levels of *XPC* mRNA in XP-C heterozygotes may be due to a combination of NMD of the *XPC* mRNAs expressed from the mutated allele and a gene dosage effect for the normal allele. It should be noted that nonsense-mediated mRNA decay is dependent on the presence of a nonsense codon >50–55 nucleotides 5′ from an exon–exon junction (47). Although the mutations identified in this manuscript generate appropriate nonsense mutations, there are missense XPC mutations that would not be expected to cause nonsense-mediated mRNA decay. There was also a reduction in the level of XPC protein in the cells from the XP-C heterozygotes, as measured by western blotting (Figure 5).

The effect of NMD on mRNA from the mutant allele is evident from the XP patients in families B–P, where only the mutant *XPC* mRNA is expressed (Figure 4). The effect of gene dosage can be demonstrated for families A and P. For XPH102TMA (family A), only *XPC* mRNA from the normal allele can be detected by the exon 4 inclusion assay, which was expressed at only 18% of the normal levels. Likewise, for XPH310BE (family P), the exon 12 inclusion assay detects only *XPC* mRNA from the normal allele, which was expressed at 41% of the normal levels (data not shown). This suggests a mechanism for the hypothesis that XP heterozygotes may be at a higher risk for the occurrence of sun-induced skin cancers. Haploinsufficiency, in which a half dose of the *XPC* gene product is insufficient for full XPC function in the maintenance of genomic integrity, might give rise to an increased mutation rate in the heterozygous cell. UV damage of partially corrected XP-C cells having subnormal expression of the XPC protein results in normal repair of cyclobutane pyrimidine dimers, but only minimal repair of 6–4 photoproducts (36). Cells from XP heterozygotes have a reduced ability to repair chromosome damage induced by X-radiation in comparison with the cells from normal donors (56).

Elevated cancer risks in association with heterozygosity for mutations have been reported for other recessive DNA repair disease genes, such as breast cancer in ataxia-telangiectasia (57) and colon cancer in Bloom syndrome (58). A common single nucleotide polymorphism in the *XPC* gene (20,43) has been associated with decreased DNA repair and increased risk of squamous cell carcinoma of the head and neck (SCCHN) (59) and of lung cancer (60) in the general population. Reduced levels of *XPC* mRNA has also been associated with increased risk for SCCHN (61).

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