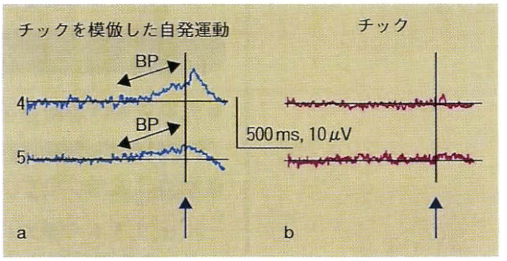
●花粉症の患者でかゆい眼をこすり続けたり，瞬きを繰り返した後，花粉症が治癒しても瞬きがチックとして持続することがある．何らかの感覚運動ゲーティングの異常を示唆する．

●また強迫性障害が基盤にあり，不合理とわかっていてもしっくりくるまでやり続けるという "just-right" phenomena の側面もある．

●TS の患者で上肢のチックとそれを模倣した自己ペースでの随意運動の運動誘発電位（MRCP）をみる（❶）と，チックでは運動準備電位が出現していない[1)*4, *5]．

❶ 2 人の Tourette 症候群でのチック（上肢屈曲，b）およびそれを模倣した場合の運動誘発電位（a）



↑：筋収縮開始．

（Karp BI, et al. 1996[1)] より引用，一部改変）

●われわれが自己ペースで随意的に身体を動かす 1 秒以上前から両側補足運動野および運動前野は活性化され，意識下で運動準備が始まる．

●自己ペースでの随意運動とはいえ「随意性」の中には意識下での過程も含まれることになる．

●チックでその運動準備にかかわる過程が記録できない場合があるということは，その「随意性」が自己ペースでの随意運動とは異なるということである．

●この「勝手に動く」チックと先のイチローの「身体が勝手に反応する」打撃技術とはどこが異なるのであろうか．

●「習慣化」しているという点では共通であるが，その獲得過程を学習という面からみると強化学習の過程に違いがあるのかもしれない．

●またチック自体は高度の集中を要する行為[*6]，歌唱やスポーツの最中には通常起こりにくい．

●彦坂は，チックは本来は報酬がないはずの行動に基底核のレベルで報酬としての信号が入り，それが強化されて繰り返されることにより起こるのではないかと述べている[2)]．

●なぜ不合理とわかっていても儀式のように同じ行為・行動を繰り返すのであろうか，また何を打ち消したいのか，その繰返し行為そのものが目的なのか，または単に習慣化しただけなのか．

●ヒトの正常な（生理的な）行為・行動と心理・情動機制の仕組みの中にその本態はすべてあると思われるが．

●チックの頻度が抗精神薬[*7]で減少することから，TS ではドパミン神経伝達亢進が示唆される．

●野村[3)] は TS の主病態を黒質と腹側被蓋野のドパミン神経の線条体への投射の低活性が発達過程に生じ[*8]，基底核間接路ではドパミン伝達過剰状態が生じ，皮質-基底核-視床-皮質回路[*9]が機能変容を起こす点に求めている．

●TS の病態には不快な前兆とチック発現[*10]，その習慣化[*11]，併発症発現[*12]，一部でのチック固定化など，随意運動を修飾する多様な系の発達期の異常が基盤にある．

BP：bereitschaftspotential（運動準備電位）

MRCP：movement related cortical potentials

＊4
運動準備電位がチックにも出現した例も同論文に報告されている．

＊5
この運動準備電位は自己ペースでの筋収縮に先立つ緩やかな陰性電位から成り立ち，成人で 1 秒以上前から出現し，その早期成分は両側補足運動野および運動前野，後期成分は反対側運動野および運動前野が起源だとされる．

＊6
goal-directed behavior.

＊7
ハロペリドールやリスペリドンなどドパミン D_2 受容体阻害薬．

＊8
受容体過感受性が基盤にあり，発達過程に出現する経年齢性減衰変化の加速された状態．

＊9
運動系では補足運動野，感覚運動野，非運動系では眼窩前頭皮質，前帯状回が重要．

＊10
感覚運動ゲーティング，ドパミン伝達過剰．

＊11
変容した強化学習．

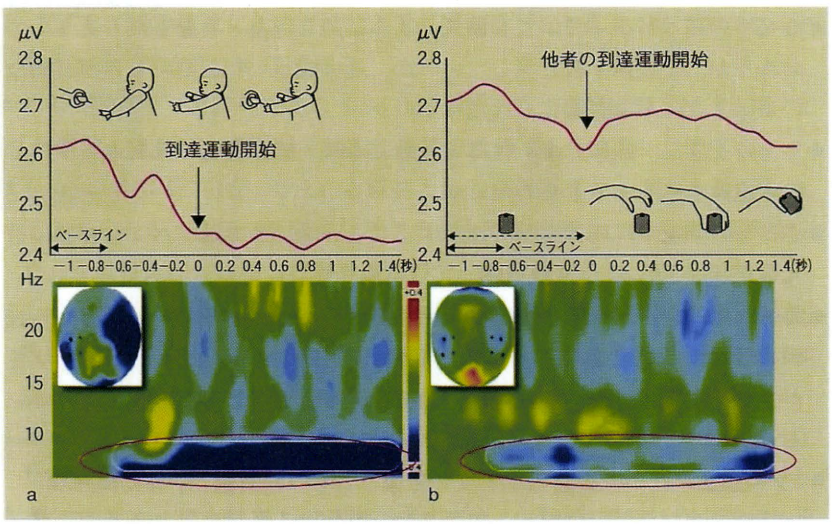
＊12
非運動系回路の変容．

- われわれの随意運動のなかには本来的に，意識した制御の及ばない要素があるということをTSの病態は指し示してくれている．

乳児における随意運動と他者の運動の観察

- 乳児における随意運動は1歳までに劇的に変化するが，その「随意性」と「反射性」の解析は容易ではない．
- Southgateら[4]は9か月乳児での到達運動の際の脳波の周波数変化，とくにαリズムの減衰に着目して解析を行った．
- ❷aに示すように乳児の手の到達運動開始前からαリズムの減衰が始まっている．
- また他者の手の到達運動を観察したときにもα領域の減衰が認められる(❷b)[*13]．
- 9か月乳児での自身の到達運動や他者の到達運動の観察の際のαリズムの減衰は年長児・成人における同様の条件でのμリズムの減衰・消失に等価であると推定できる．
- μリズムは感覚運動皮質に起源を有し，ミラーニューロンシステム[*14]（下前頭回，上側頭溝，下頭頂小葉）から制御を受ける．
- 減衰したαリズムの起源は不明だが，μリズムと同様に考えることも可能である．
- また手の到達運動開始前からαリズムが減衰したことは，その運動準備性の側面も推定させる．
- つまり乳児の物に対する到達運動は，少ない運動パターンの中で随意的[*15]に行われたとみなしてよい．
- それを視覚情報として受け取った場合には，すでに獲得された運動レパートリーの運動表象[*16]へ自動的に変換されるということも表している．

❷ 9か月乳児の手の到達運動および他者の到達運動観察時のα波の減衰



a：手の到達運動，b：他者の到達運動観察．

(Southgate V, et al. 2009[4]より引用，一部改変)

*13
これは何度か課題を繰り返すうちに認められたもので，行為の予測や期待とも関連する．
これらの減衰は6か月の乳児では認められていない．
年長児・成人においても他者の運動の観察のみでμリズムの減衰・消失が起こる．

*14
リゾラッティはミラーニューロンシステムの第一の役割を「他者の行為の意味の理解」とし「行為の共有空間を生み出す」もので，認知的側面とは異なるとしている[5]．
また見る側があたかも自分が行っているかのように観察できるかどうかは観察者本人の運動レパートリーによって決まり，観察した行為を運動の言語でコードし，その行為を再現可能にするという，模倣における根本的な役割を果たしているとした[5]．

*15
目標志向性に，goal-directed.

*16
motor representation.

- 随意性という場合，熟練するとどこまで意識されているかを問えないのは乳児でも同じかもしれない．

表情筋における随意性の解離

- 笑いはほぼヒトに特有の表情であり，その中枢性支配は複雑である．
- 時に脳血管障害において症例の❸に示すように笑えるにもかかわらず，「イー」という発声を命じると顔片側下半分の収縮ができない解離した状態に遭遇する．
- これは顔面の随意的収縮と笑いでは機能している中枢神経系の回路が異なるために起こる．
- サルでは，皮質から脳幹顔面神経核へは5つの回路がある．これによると顔面上半分は補足運動野（M2），吻側帯状運動皮質（M3）から両側支配を受け，下半分は対側一次運動皮質（M1），腹側外側運動前野（LPMCv），尾側帯状運動皮質（M4）から片側支配を受ける（❺）*17．
- M2，M3，M4は前大脳動脈の支配を受け，M1，LPMCvは中大脳動脈の支配を受けるため，障害された部位により臨床症状に差異が生まれる．
- ヒトでもほぼ相同と考えられ，M1，LPMCvは対側顔面下半分を強力に支配するためこの部分の皮質や皮質下白質が障害されると随意運動（たとえば「イーと発声する」）での当該部位の収縮が障害される*18．
- ところが笑ったときの表情はM1，LPMCv以外の関与（とくにM4）が大きいため，たとえM1，LPMCvが障害されても他が障害を免れていれば❸左写真のように保たれる．
- 2つの帯状運動皮質（M3，M4）は辺縁系からの強力な入力があり，笑いという情動と不可分な行為は単純な随意運動とは別経路でなされているの

*17
Morecraftら[6]の研究による．

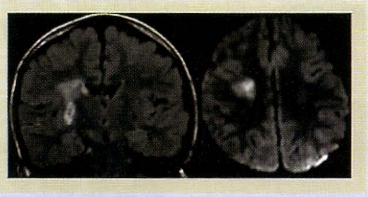
*18
❸の右写真での左鼻唇溝消失．

症例

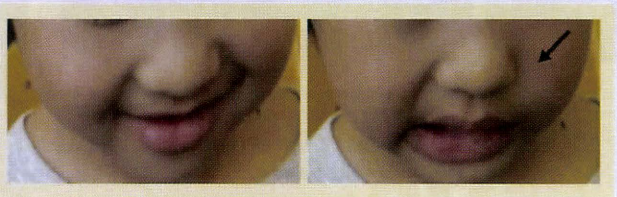
7歳，男児（❸）．

診断：原因不明の脳梗塞．転倒後に発症．左上肢の巧緻性低下，左下肢の軽度痙性と顔面筋収縮に笑いと随意運動で解離を認めた．MRI上右深部白質，右島，右内包後脚，右大脳脚外側に信号異常あり（❹）．

❹ 症例の頭部MRI

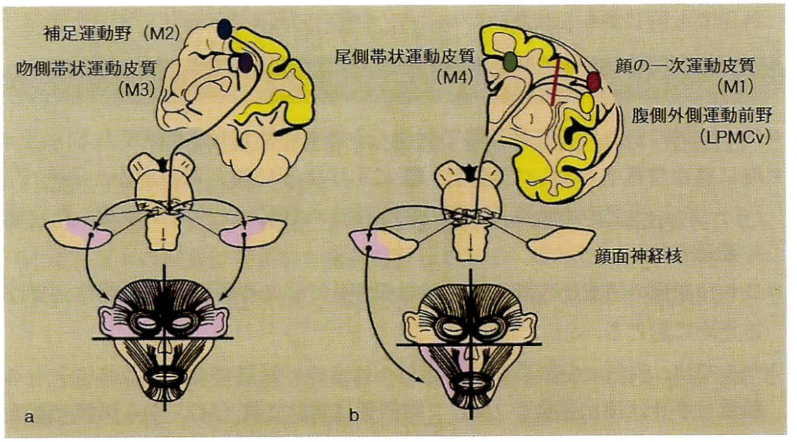


❸ 表情筋の随意性の解離



笑ったときには顔面筋は対称に収縮するが，「イー」という発声では左表情筋は収縮せず非対称となる（矢印）．

❺ 顔面筋の中枢からの支配様式



a：顔上半分の運動の皮質支配，b：顔下半分の運動の皮質支配．
症例1，2における病変（—）．

（Morecraft RJ, et al. 2001[6] より引用，一部改変）

であろう*19．

- Iwase ら[7] は PET を用いた研究で，コミックをみて笑ったときの笑いの程度は両側補足運動野と左被殻の活性が優位に相関したが，一次運動皮質の活性とは相関しなかったと報告した．
- これに対し単純な顔の随意運動では一次運動皮質と補足運動野の活性化を認めている．
- こういう運動の情動性支配は表情筋だけなのであろうか．
- Morecraft らの図[6] では表情筋と同様に MI 以外に M2，M3，M4 に体部位局在性をもって刺激により上下肢を収縮させるニューロンがある．
- 通常の随意運動では M1 の支配が絶大で前面に出ることはなく，情動刺激による運動では表情の影に隠れて目立たないが上下肢の情動性運動はあるのではないだろうか*13．
- 表情筋を中心とした運動の情動性支配は，四肢の目標志向的な goal-directed 随意運動とは異なる発生と発達を示す．

*19
臨床的には M1，LPMCv が保たれて M3，M4 が障害された場合，逆に単純な顔の随意運動は可能だが笑えないという状態になる．

PET：positron emission tomography

*13
いわゆる「火事場のばか力」も，情動性出力も含めて皮質を総動員した結果かもしれない．

■文献

1) Karp BI, et al. Simple motor tics may be preceded by a premotor potential. J Neurol Neurosurg Psychiatry 1996；61：103-6.
2) 彦坂興秀ほか．彦坂興秀の課外授業　眼と精神．東京：医学書院；2003.
3) 野村芳子．チック．神経内科 2007；66：145-51.
4) Southgate V, et al. Predictive motor activation during action observation in human infants. Biol Lett 2009；5：769-72.
5) ジャコモ・リゾラッティほか．ミラーニューロン．東京：紀伊国屋書店；2009.
6) Morecraft RJ, et al. Cortical innervation of the facial nucleus in the non-human primate：a new interpretation of the effects of stroke and related subtotal brain trauma on the muscles of facial expression. Brain 2001；124(Pt 1)：176-208.
7) Iwase M, et al. Neural substrates of human facial expression of pleasant emotion induced by comic films：a PET study. Neuroimage 2002；17：758-68.

XPA Gene Mutations Resulting in Subtle Truncation of Protein in Xeroderma Pigmentosum Group A Patients with Mild Skin Symptoms

Yoshito Takahashi[1], Yoko Endo[1], Yoshinori Sugiyama[1], Shintaro Inoue[1], Masahiro Iijima[2], Yasushi Tomita[3], Satoshi Kuru[4], Masahiro Takigawa[5] and Shinichi Moriwaki[5,6]

Comparisons of the clinical manifestations with gene mutations in patients with xeroderma pigmentosum group A (XPA) have suggested that those with mutations closer to the C-terminal coding region of the *XPA* gene have milder neurological and cutaneous symptoms. Here we report on four middle-aged, newly diagnosed Japanese XPA patients whose unusually mild symptoms, especially those affecting the skin, implicate a reduced association of a subtle defect in the C-terminus of XPA protein with skin lesions. All patients had a heterozygous G→C transversion at the splice acceptor site of *XPA* intron 3. We identified previously unreported heterozygous mutations in exon 6: a single-base insertion (690insT) in one patient and a four-base insertion (779insTT and 780insTT) in the other patients. These mutations led to the frameshift that created new premature termination codons, resulting in the production of truncated XPA proteins. They were longer than any previously reported truncated XPA protein, suggesting that the minimal cutaneous symptoms in these patients are due to a higher residual level of XPA protein activity and that the subtle defect in the C-terminus of XPA protein is more closely related to neurological impairment than to cutaneous abnormalities.

Journal of Investigative Dermatology (2010) **130,** 2481–2488; doi:10.1038/jid.2010.137; published online 24 June 2010

INTRODUCTION

Xeroderma pigmentosum (XP) is an autosomal recessive disease characterized by hypersensitivity to sunlight, abnormal pigmentation, and a predisposition to skin cancers, especially on sun-exposed areas (Kraemer *et al.*, 1994; Cleaver and Kraemer, 1995). It results from defective nucleotide excision repair (NER), the system responsible for the repair of UV-induced DNA damage (de Boer and Hoeijmakers, 2000). Cultured cells derived from XP patients have a reduced DNA repair capacity for UV-induced DNA damage and are hypersensitive to being killed by UV (Protic-Sabljic and Kraemer, 1985). Besides the cutaneous manifestation, neurological abnormalities such as loss of hearing, loss of tendon reflexes, walking impairment, and intellectual impairment are observed in about 20% of XP patients (Kraemer *et al.*, 1987). These symptoms are due to progressive degeneration of the central nervous system (Robbins *et al.*, 1991), thought to be caused by a defective repair of lesions that are produced in neurons by reactive oxygen species (Reardon *et al.*, 1997; Rass *et al.*, 2007).

Cell fusion analysis has identified seven complementation groups (A–G) of excision-repair-deficient cells, and there is also a variant form that is proficient in excision repair (Kraemer, 1993; Cleaver and Kraemer, 1995). Patients with xeroderma pigmentosum group A (XPA; OMIM #278700) generally show the most severe symptoms (Takebe *et al.*, 1987) and in most cases die in their second or third decade (Sidwell *et al.*, 2006). The human gene complementing the defect in *XPA* is located on chromosome 9q34.1 and is composed of six exons. The protein consists of 273 amino acids (a.a.; Cleaver *et al.*, 1999) and is required for the early stages of NER, with a role in damage verification and stabilizing of other NER proteins (Berneburg and Lehmann, 2001).

In Japan, the most frequent type of XP is group A (Moriwaki and Kraemer, 2001), and approximately 80% of Japanese XPA patients are homozygous for the G→C transversion mutation of the *XPA* gene at the splice site of intron 3 (IV3: –1 G to C) (Satokata *et al.*, 1990). The other common mutations found in Japanese XPA cases are a nonsense mutation of exon 3, which alters the 116th Tyr codon (Y116stop), and a nonsense mutation of exon 6, which

[1]*Innovative Beauty Science Laboratory, Kanebo Cosmetics, Odawara, Japan;* [2]*Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [3]*Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [4]*Department of Neurology, Suzuka National Hospital, Suzuka, Japan;* [5]*Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan and* [6]*Department of Dermatology, Osaka Medical College, Takatsuki, Japan*

Correspondence: Shinichi Moriwaki, Department of Dermatology, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki, Osaka 569-8686, Japan. E-mail: der002@poh.osaka-med.ac.jp

Abbreviations: NER, nucleotide excision repair; RFLP, restriction fragment-length polymorphism; TCR, transcription-coupled repair; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum; XPA, xeroderma pigmentosum group A

Received 25 December 2009; revised 22 February 2010; accepted 25 March 2010; published online 24 June 2010

alters the 228th Arg codon (R228stop; Nishigori *et al.*, 1993). Including these three mutations, more than 20 mutation sites in the *XPA* gene have been reported worldwide (States *et al.*, 1998; Maeda *et al.*, 2000; Tanioka *et al.*, 2005; Sidwell *et al.*, 2006). The majority are located in exons 3–5, the DNA binding region, at which the mutations tend to occur homozygously in patients with severe manifestations. In contrast, most milder cases have at least one allele with a mutation in exon 6, which interacts with transcription factor IIH (TFIIH) (Park *et al.*, 1995; Nocentini *et al.*, 1997). In extensive comparisons between the distribution of mutations, the various functional regions of the XPA protein, and the severity of clinical symptoms, it was pointed out that the C-terminal domain of the protein has less importance in overall function (States *et al.*, 1998).

In this study, we describe four XP patients with unusually mild cutaneous abnormalities and minimal or late-onset neurological impairment. Surprisingly, they were assigned to complementation group A, despite their surviving to middle age with no skin cancer. In mutational analysis, we found two previously unreported mutations in exon 6 of the *XPA* gene.

RESULTS

Case reports

Case 1 (XP17HM), a 35-year-old man, was referred to the Hamamatsu University School of Medicine, University Hospital, Hamamatsu, Japan. Case 2 (XP21HM), a 30-year-old woman, was referred to the Nagoya University Hospital, Nagoya, Japan. Case 3 (XP42HM), a 40-year-old woman, and case 4 (XP43HM), a 45-year-old man, were referred to the Suzuka National Hospital, Suzuka, Japan. XP42HM and XP43HM are siblings. Each of these patients presented for consultation because he or she had a mild neurological manifestation and a history of moderate sun sensitivity. Their clinical characteristics are summarized in Table 1. Mild dermatological abnormalities, such as mild freckling and telangiectasia on the face, were present in all of the patients (Figure 1). No skin cancer has developed. All of them have microcephaly, intellectual impairment, ataxia dysarthria, involuntary movement, and hyporeflexia (or areflexia). These neurological abnormalities are minimal or were of late onset but are progressing slowly and gradually.

Classification into a mild form of XP on the basis of UV survival and UV-induced unscheduled DNA synthesis assays

UV survival and UV-induced unscheduled DNA synthesis (UDS) of primary fibroblasts from the patients were

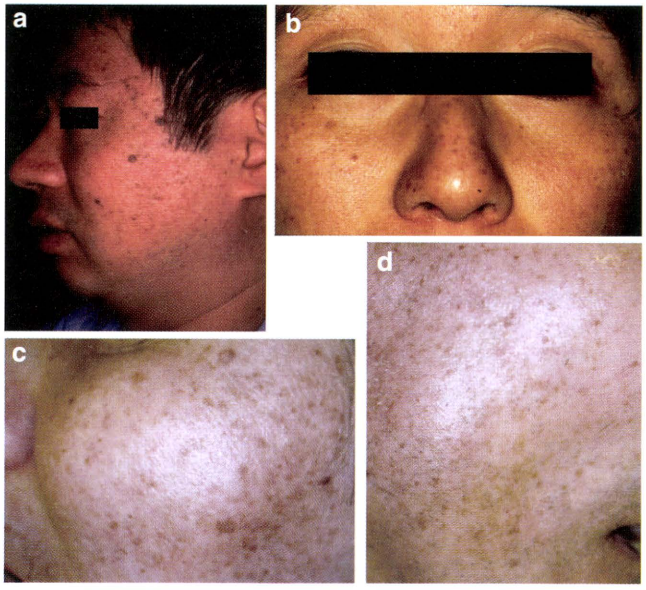


Figure 1. Unusually mild cutaneous features of four patients with xeroderma pigmentosum. (**a**) XP17HM; (**b**) XP21HM; (**c**) XP42HM; (**d**) XP43HM. Each patient's cutaneous symptoms consist only of mild freckling and few, if any, telangiectasias on the face.

Table 1. Clinical and cellular characteristics of patients in this study as well as a typical severe XPA case and normal subjects

Case	Age/Sex	Abnormality in pigmentation	Skin cancer	Neurological abnormality[1]	Fibroblast strain	UDS (%)[2]	UV survival (D_{37}; J m^{-2})[3]
1	35/M	Mild	–	+	XP17HM	17	0.9
2	30/F	Mild	–	+	XP21HM	21	1.4
3[4]	40/F	Mild	–	++	XP42HM	21	0.8
4[4]	45/M	Mild	–	++	XP43HM	32	0.9
XPA[5]	12/F	Severe	–[6]	+++	XP12HM	3	0.4
Normal	42/M	None	–	–	N-42	100	5.9

Abbreviations: F, female; M, male; UDS, unscheduled DNA synthesis; XPA, xeroderma pigmentosum group A.
[1]All the patients have microcephaly, intellectual impairment, ataxia dysarthria, involuntary movement, and hyporeflexia/areflexia. + Mental retardation with minimal, if any, abnormal neurological reflexes; ++ mental retardation and abnormal neurological reflexes; +++ inability to walk because of severe neurological abnormality.
[2]A global ability of nucleotide excision repair system.
[3]UV survival was determined on the basis of colony-forming ability.
[4]Cases 3 and 4 are siblings.
[5]Typical severe XPA patients.
[6]This patient began to protect herself from UV by using sunscreen after she was diagnosed with XPA at the age of 3 years.

compared, according to colony-forming ability (Table 1), with those from a normal subject and a typical XPA patient (XP12HM). The D_{37} (dose that results in 37% cell survival) of cells from the four patients was 0.8–1.4 J m^{-2}, which was much lower than that of cells from a normal subject (5.9 J m^{-2}); however, these cells were less sensitive than those in a typical XPA patient (0.3–0.4 J m^{-2}). The levels of UV-induced UDS in these cells were 14–24% of those of cells from normal subjects, similar to the levels for an intermediate group of XP patients.

Assignment to XP complementation group A

Complementation analysis was carried out with a host-cell reactivation assay in which cells were cotransfected with a UV-damaged luciferase gene expression vector, with expression vectors harboring cloned wild-type XP complementary DNA (cDNA). Increased luciferase activity was observed when the *XPA* gene was transfected into cells from these patients, but luciferase activity was very low in cotransfection with the other *XP* genes (Figure 2a). Thus, only the *XPA* gene complemented the DNA repair defect. This result indicates assignment of these cells to XP complementation group A.

Heterozygous mutations in the *XPA* gene common to Japanese patients

To characterize the mutation of the *XPA* gene in these patients, we first confirmed the presence of the most common Japanese mutation (IV3: –1 G to C) by *Alw*NI PCR-restriction fragment-length polymorphism (RFLP) analysis in amplified DNA fragments of exon 3, including the flanking intron. DNA from these patients showed three bands, whereas two bands appeared in the severe XPA patient, who has this mutation homozygously (Figure 2b). This observation indicates that the patients with mild symptoms had the heterozygous mutation common to Japanese patients. We also examined the other common Japanese mutations (Y116stop and R228stop) using RFLP analysis (Nishigori *et al.*, 1994). However, these mutations were not found (data not shown).

Two insertion mutations in exon 6 of the XPA gene heterozygously

To identify the mutation sites that could not be detected by RFLP analysis, we performed a sequence analysis on each exon of the genomic DNA from these patients. In XP17HM, the heterozygous sequence signal that started from nucleotide 690 in exon 6 was detected (Figure 3a), showing that there was an insertion of a nucleotide (690insT). In the other three patients, the heterozygous sequence signal that started from nucleotide 779 in exon 6 was detected (Figure 3b), showing that there was an insertion of four nucleotides (779insTT 780insTT). These previously unreported mutations are located closer to the C-terminus than any previously reported mutations that cause truncation of the XPA protein

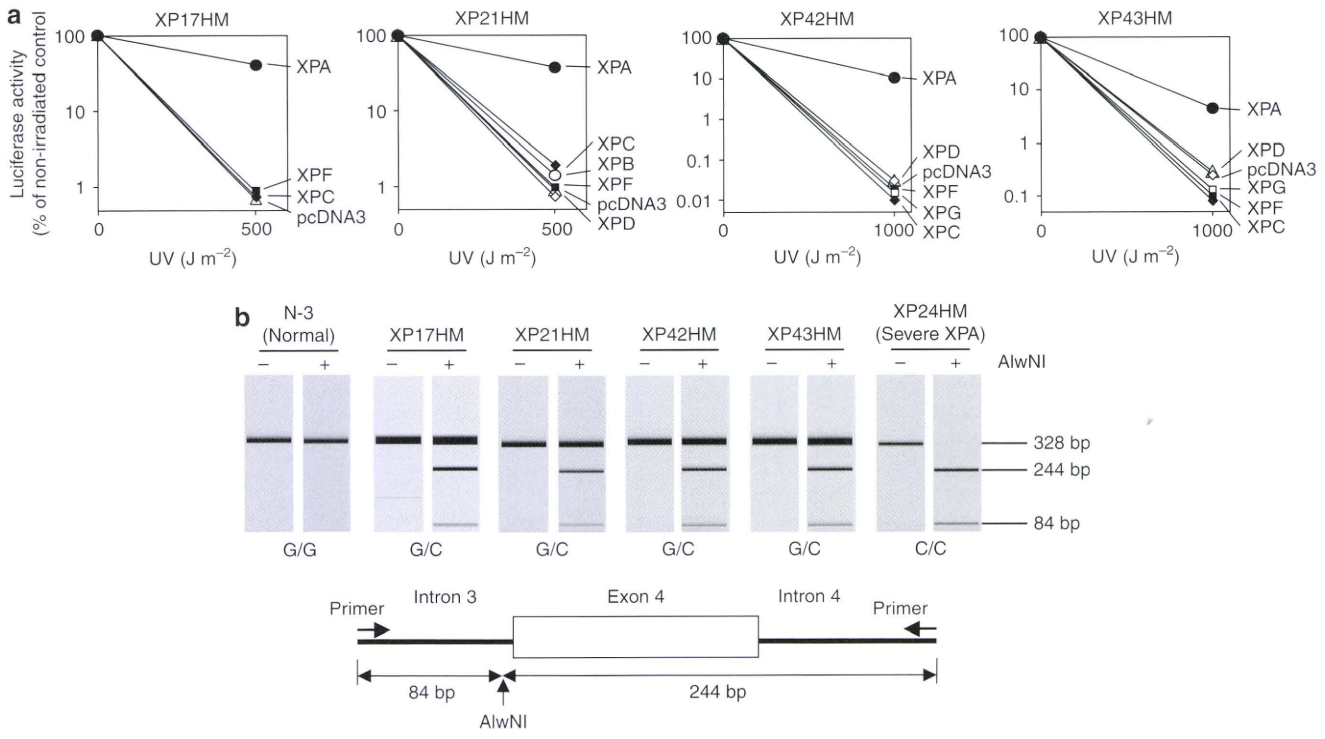


Figure 2. Each patient was diagnosed with xeroderma pigmentosum group A (XPA) and had a common Japanese mutation heterozygously. (**a**) Host-cell reactivation assay was performed via cotransfection of the UV-C-treated reporter plasmid (pGL2Luc) with xeroderma pigmentosum complementary DNA (*XPA* (closed circle), *XPB* (open circle), *XPC* (closed diamond), *XPD* (open diamond), *XPF* (closed square), or *XPG* (open square)) or control plasmid pcDNA3 (open triangle) into fibroblasts from patients. DNA repair capacity was defined as the percentage of residual luciferase activity after repair of UV-irradiated DNA compared with that of nonirradiated DNA. Values taken from two independent experiments are depicted. (**b**) DNA extracted from fibroblasts was analyzed by restriction fragment-length polymorphism as described in Materials and Methods. The polymorphism of patients with a mild phenotype (genotype G/C) showed three bands, whereas normal subjects (genotype G/G) displayed one band and severe XPA patients (genotype C/C) had two.

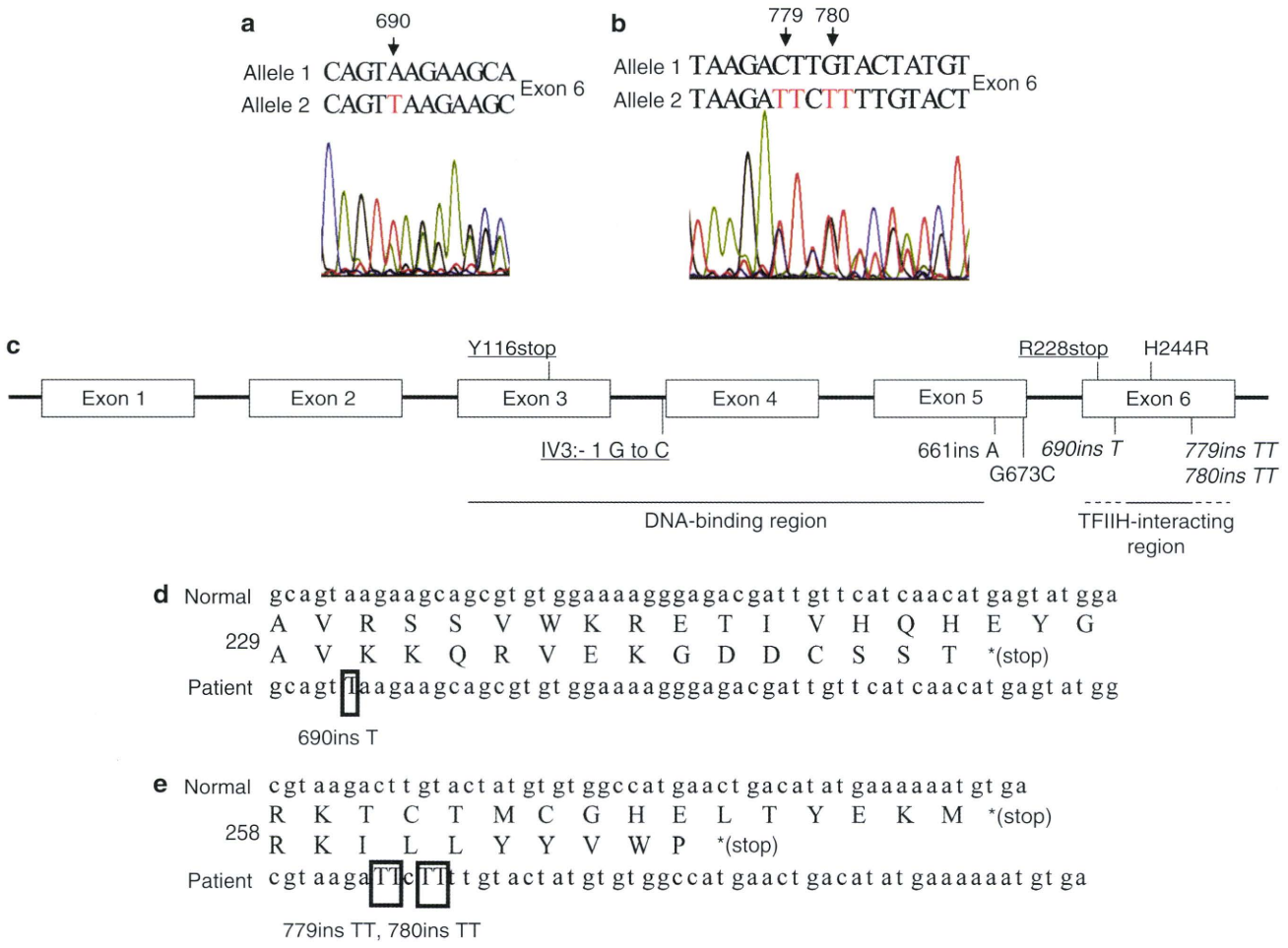


Figure 3. Two insertion mutations were identified in exon 6 of the xeroderma pigmentosum group A (XPA) gene heterozygously. (**a**) The genomic sequencing of exon 6, showing a single-base insertion at nucleotide 690. (**b**) The genomic sequencing of exon 6, showing a four-base insertion at nucleotides 779 and 780. (**c**) Map of *XPA* gene and mutations. The mutations close to the C-terminus reported in the mild cases are indicated. The three Japanese common mutations are underlined. Mutations identified in this study are indicated by italics. Exon 6 is responsible for interacting with transcription factor IIH. (**d**) The base sequences of genomic DNA with coding amino acids corresponding to a normal subject and to a patient with the 690insT mutation. (**e**) The base sequences of genomic DNA with coding amino acids corresponding to a normal subject and to patients with the 779insTT, 780insTT mutation.

(Figure 3c). Mutation 690insT caused a frameshift at amino acid position 231, resulting in a stop codon 15 amino acids downstream (Figure 3d). Another mutation, 779insTT 780insTT, caused a frameshift at amino acid position 260, resulting in a stop codon eight amino acids downstream (Figure 3e). The predicted sizes of XPA proteins are 244a.a. in XP17HM and 267a.a. in the other three patients.

Expression of the truncated XPA protein

We next determined whether the predicted frameshift XPA protein is indeed expressed in cells from these patients. Western analysis revealed two bands as XPA proteins in the cell extract from normal subjects (Figure 4a), consistent with a previous report (Miura *et al.*, 1991). No XPA protein was detected in the severe XPA patient. In each mild XPA patient, a significant band level was detected at a position lower than that for normal protein. The band sizes were estimated at 91% of that of normal protein in XP17HM and 97% of that in the other three patients. These estimated sizes were almost consistent with the predicted size, which was 89% (244/273a.a.) for XP17HM and 98% (267/273a.a.) in the other three patients. To confirm these estimates, we analyzed another patient (XP41HM) who has a nonsense mutation (R228stop) with truncated XPA protein, the predicted size of which is 83% (227/273a.a.) of the normal protein. The estimated size according to western analysis was 82%, showing that the mutations identified in this study cause the truncation of XPA protein. These findings suggest that the unusually mild symptoms might be due to the higher residual level of functional activity of truncated XPA proteins.

Reduced levels of XPA mRNA

To investigate whether the expression levels of XPA proteins correlate with the amounts of XPA mRNA in these patients, we performed northern analysis. Two bands of about 1.3–1.4 kb and 1.0–1.1 kb were detected in the extract from cells of a normal subject (Figure 4b), consistent with a previous study (Tanaka *et al.*, 1990). In the mild XPA patients,

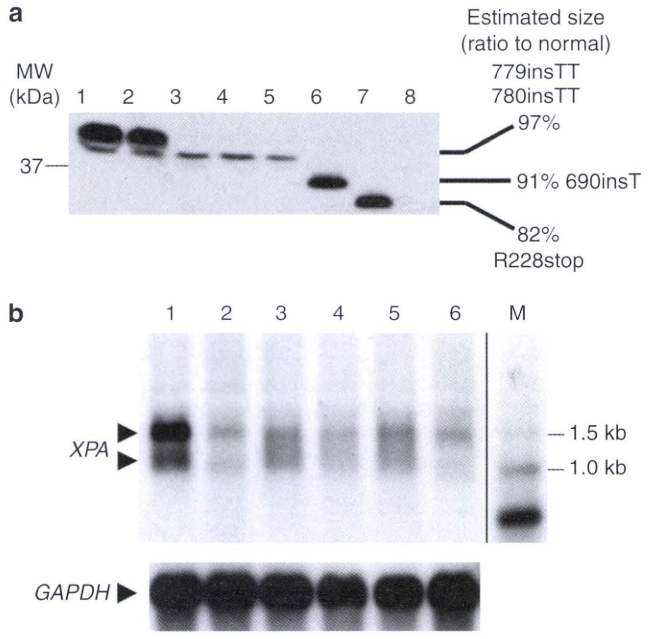


Figure 4. A significant expression of truncated XPA protein and a reduced expression of XPA mRNA in each patient. (**a**) A volume of 50 μg of total protein from N-3 (normal, lane 1), N-75 (normal, lane 2), XP21HM (lane 3), XP42HM (lane 4), XP43HM (lane 5), XP17HM (lane 6), XP 41 HM (mild XPA, lane 7), and XP24HM (severe XPA, lane 8) was analyzed for expression of the XPA protein by western blotting. Equal loading/transfer was confirmed by amido-black staining of membranes. (**b**) A volume of 1 μg of poly $(A)^+$ RNA from N-3 (normal, lane 1), XP17HM (lane 2), XP21HM (lane 3), XP42HM (lane 4), XP43HM (lane 5), XP24HM (severe XPA, lane 6) was subjected to electrophoresis and analyzed by northern blotting with DIG-labeled XPA and GAPDH probe. The GAPDH mRNA is shown as the internal standard. The size of marker RNA is indicated (lane M).

reduced levels of XPA mRNA were detected; these were comparable to those of the smaller mRNA in cells from XP24HM, who has the common Japanese mutation homozygously, suggesting a destabilization of XPA mRNA because of premature termination codons. Therefore, the expression levels of the truncated XPA protein were not relevant to the amounts of XPA mRNA.

DISCUSSION

We have characterized two previously unreported mutations in the *XPA* gene via genetic analysis of four middle-aged XPA patients (XP17HM, XP21HM, XP42HM, and XP43HM). An unexpected finding was that these patients were assigned to XP complementation group A, because, despite being of middle age, they manifested an unusually mild phenotype for XPA, the severest form of XP. Ordinarily, XPA patients are diagnosed on the basis of their severe photosensitivity and developmental delay starting in the first decade of life. The patients in this study have late-onset progressive neurological abnormalities but no cutaneous features other than mild freckles and telangiectasia. Hence, the definitive diagnosis of XP had not been made earlier in their lives.

Thus far, more than 20 mutations of the *XPA* gene have been identified. It is thought that the severity of symptoms of XPA patients depends on the residual activity of the abnormal XPA protein. The typical XPA patients showing both severe neurological abnormalities and extreme photosensitivity have mutations within exons 3–5, almost all of which are deletions or missplicings seriously disrupting the structure. Because these exons encode the DNA binding domain, the severe manifestations are thought to result from the loss of pivotal NER function. Some patients with missplicing in the DNA binding domain show less severe symptoms. It is more likely that these mutations allow the production of a small amount of normal protein by alternative splicing (States *et al.*, 1998).

On the other hand, four mutations close to the C-terminus of the XPA protein have been reported to be associated with milder symptoms (Figure 3c). Two are located in exon 5, but outside the DNA binding region, and the others are in exon 6 (Satokata *et al.*, 1992b; Nishigori *et al.*, 1993; Cleaver *et al.*, 1995; Maeda *et al.*, 1995; Sato *et al.*, 1996). The mutations in exon 5 are an insertion mutation (661insA) and the G→C transversion (G673C) that may allow the production of truncated XPA protein; the protein expression was actually confirmed in a later mutation. The mutations in exon 6 are a nonsense mutation (R228stop) and a missense mutation (H244R). The former is one of the common Japanese mutations. Almost all patients with the above-mentioned mutations close to the C-terminus of the XPA protein were reported to show mild cutaneous symptoms and minimal or late-onset neurological signs. Given that exon 6 interacts with TFIIH, these mutations may cause a decrease in the ability to bind to TFIIH. Therefore, this C-terminal domain is thought to have lesser importance for overall function.

The mutations identified in this study are located closer to the C-terminus than are any previously reported mutations that cause truncation of the XPA protein. They were all compound heterozygote with the most common Japanese mutation (IV3: –1 G to C), which results in severe disruption of the XPA protein, indicating that the newly identified mutations are causative of very mild symptoms. Specifically, the cutaneous symptoms were limited to freckles and telangiectasia and the neurological signs were of late onset and slow to progress. These observations and previous findings suggest that the minimal cutaneous symptoms in these patients are due to the higher residual level of functional activity of XPA protein that has partially lost its ability to interact with TFIIH. In fact, the truncated XPA protein was significantly expressed in each patient, and the truncation of these proteins was much less than that of any XPA protein previously reported (Mimaki *et al.*, 1996; Sato *et al.*, 1996). Thus, the subtle defect in the C-terminus of XPA protein seems to be more closely related to neurological impairment than to cutaneous abnormalities. This possibility merits investigation, e.g., by comparing the ability of truncated XPA proteins to bind to TFIIH in mild cases.

Northern analysis revealed that the amount of XPA mRNA was markedly reduced in all cases in this study, suggesting that XPA mRNA was unstable because of premature termination. However, in XP17HM (690insT), considerable expression of truncated XPA protein was observed, whereas its expression levels were reduced in the

other patients (779insTT, 780insTT). The truncated protein derived from the mutation of 690insT may be difficult to degrade. The reason that XP17HM is not less sensitive than the other patients might be that a shorter length of the truncated protein offsets the greater amount. Whatever the reason, no correlation between the expression levels of the truncated XPA protein and amounts of XPA mRNA was found, implying that each type of truncated XPA has a different level of stability. Therefore, the ability to bind with TFIIH might also be different for each type of mutant XPA protein.

Neurological abnormalities observed in XP patients are postulated to result from the insufficient repair of oxidative DNA lesions in the central nervous system, in which the production rate of reactive oxygen species is higher (Rass *et al.*, 2007). However, a direct correlation between unrepaired oxidative DNA damage and NER deficiency has not been identified. NER consists of two pathways: global genome repair and transcription-coupled repair (TCR). The former repairs nontranscribed DNA lesions throughout the genome; the latter repairs lesions in actively transcribed DNA more rapidly (Moriwaki and Takahashi, 2008). Nondividing neurons in the central nervous system are among the most transcriptionally active cells in the body, suggesting that TCR predominates. Cockayne syndrome, in which TCR is deficient, is known to have neurological defects as a clinical feature but is not characterized by a predisposition to skin cancers (Moriwaki and Kraemer, 2001). These observations imply that TCR is more defective than global genome repair in the studied cases. Therefore, any correlations between the extent of truncation of the C-terminal region and the binding activity of XPA protein to TFIIH may give us insight into the pathogenic mechanisms of neurological defects in XP patients from the view of differences between global genome repair and TCR.

Recently, previously unreported mutations in the *XPA* gene that are associated with the mild phenotype have been identified one after another, including the mutations in this study, thanks to the development of advanced molecular diagnosis techniques. Patients with the mild phenotype show neither skin cancer (this study) nor neurological manifestations (Tanioka *et al.*, 2005; Sidwell *et al.*, 2006) and are unlikely to be diagnosed with XPA in the absence of genetic analysis. The patients in this study are compound heterozygotes for a common Japanese mutation (IV3: –1 G to C) and for previously unreported insertion mutations (690insT or 779insTT, 780insTT). It is known that the homozygote for the nonsense mutation in exon 6 (R228stop) shows a milder phenotype than the heterozygote for this mutation (Satokata *et al.*, 1992a). Therefore, if the homozygote for the mutations we identified exists, it may account for the extremely mild symptoms. It is interesting to analyze the allele frequency of these mutations from the point of view that these mutations may increase the risk for skin and neural aging in subjects who are not clinically recognized as XP patients. If these mutations are found at a comparatively high frequency, they may be used to assess the risk factor of photoaging in a manner similar to the use of single-nucleotide polymorphisms. By contrast, laboratory investigations based on NER abnormality have failed to show clinical and biological differences between XP carriers (heterozygote) and normal subjects (Moriwaki *et al.*, 1993). However, a recent study, in which large-scale screening of Japanese carriers of the founder mutation (IV3: –1 G to C) was performed, revealed that the ratio of Japanese XPA heterozygotes carrying the founder mutation is nearly 1% (Hirai *et al.*, 2006), which is threefold higher than previously estimated (Maeda *et al.*, 1997). In this regard, further analysis of XPA patients with the mild phenotype may provide clues to the relationship between XP mutations and the risk for aging of cutaneous and nervous tissues.

MATERIALS AND METHODS

Cells and media

Cultured fibroblasts designated as XP17HM, XP21HM, XP42HM, and XP43HM derived from cases 1, 2, 3, and 4, respectively, were all established from skin biopsy specimens from the patients. We also used cultured fibroblasts as controls, N-42 and N-75 derived from normal subjects, XP12HM and XP24HM derived from typical XPA patients with the homozygous Japanese common mutation (IV3: –1 G to C), and XP41HM derived from a mild XPA case with a nonsense mutation (R228stop). Analyses were performed with a previously established fibroblast strain (N-3; Moriwaki *et al.*, 1992) and with the nine cell strains mentioned above, after obtaining written informed consent from the donors. The study was approved by the Institutional Review Board of Osaka Medical College and conducted according to the Declaration of Helsinki Principles. The cells were maintained in DMEM (Sigma, St Louis, MO) supplemented with 15% fetal bovine serum (JRH Biosciences, Lenexa, KS) at 37 °C in a 5% CO_2 atmosphere.

Assessment of UV survival

Cells were seeded on 60-mm dishes at 5×10^2–2×10^4 cells per well. After incubation for 18 hours, cells were irradiated by UV and incubated for 1–2 weeks until colonies were formed (with more than 50 cells in a colony). Irradiation was performed with germicidal lamps (GL-10, Toshiba, Tokyo, Japan; predominantly 254 nm) at a dose of up to 20 J m^{-2}, as measured using a UV radiometer (UVR-1, Topcon, Tokyo, Japan). After fixation with formalin and staining with crystal violet, the colonies were counted.

Measurement of UV-induced UDS

Cells were seeded on a glass coverslip in 35-mm dishes. After incubation for 18 hours, cells were UV irradiated with germicidal lamps at a dose of 30 J m^{-2}, followed by incubation with 10 μCi ml^{-1} of methyl-[^{3}H]-thymidine (GE Healthcare, Buckinghamshire, UK) for 3 hours. After labeling, cells were fixed with Carnoy's solution and washed with 5% trichloroacetic acid. The glass coverslips were dipped in nuclear track emulsion, NTB3 (Eastman Kodak, Rochester, NY, USA), for autoradiography. The number of grains per interphase nucleus was scored for 100 nuclei in each specimen. UDS was determined by the percentage of net count when the net count of cells from a normal subject is 100%. Net count is determined by subtracting the mean grain count of nonirradiated cells from that of UV-irradiated cells.

Host-cell reactivation and assignment of XP complementation group

The reporter plasmid, pGL2Luc (Promega, Madison, WI), which harbors the luciferase gene, was used to measure post-UV DNA repair capacity by host-cell reactivation. The reactivation was performed as described previously (Takahashi *et al.*, 2005), with slight modification. Briefly, fibroblasts were seeded on 24-well plates at 2×10^4 cells per well. After incubation for 18 hours, the cells were transfected with the UV-irradiated or nonirradiated plasmid (0.2 μg DNA per well) with the Effectene transfection reagent (Qiagen, Hilden, Germany). After incubation for 48 hours, the luciferase activity in the cell lysate was measured using the PicaGene luciferase assay system (Toyo Ink, Tokyo, Japan). DNA repair capacity was expressed as the percentage of residual luciferase activity after repair of UV-irradiated DNA compared with nonirradiated DNA. In order to assign the patients' fibroblasts to a specific XP complementation group, simultaneous cotransfection was performed with pcDNA3 expression vectors containing different XP cDNAs (*XPA*, *XPB*, *XPC*, *XPD*, *XPF*, or *XPG*) along with the reporter plasmid (Fujimoto *et al.*, 2005).

PCR-RFLP analysis

For detection of the common Japanese mutation (IV3: –1 G to C), PCR-RFLP analysis was performed as described previously (Nishigori *et al.*, 1994). Briefly, DNA was extracted from cells using a QIAamp DNA isolation kit (Qiagen) and amplified by PCR with EX Taq DNA Polymerase (Takara Bio, Shiga, Japan) and primers as follows: sense primer 5′-GGGAATTCTTGCTGGGCTATTTGCAAAC-3′ and antisense primer 5′-GGGGATCCGCCAAACCAATTATGACTAG-3′. The PCR steps consisted of 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 59 °C for 30 seconds, and elongation at 72 °C for 1 minute. Thereafter, the 328 bp PCR product was digested by restriction endonuclease *Alw*NI (New England Biolabs, Beverly, MA) for 3 hours at 37 °C. The G→C substitution creates a new cleavage site for *Alw*NI. The *Alw*NI cuts the 328 bp fragment, resulting in two fragments (84 and 244 bp). The digested PCR products were analyzed by Agilent 2100 Bioanalyzer using a DNA 1000 kit (Agilent Technologies, Santa Clara, CA).

Nucleotide sequence analysis

All six exons and flanking introns of the *XPA* gene in DNA extracted from cells were amplified by PCR with EX Taq DNA Polymerase (Takara Bio), and the PCR products were sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA). The PCR steps consisted of 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 50 °C for 20 seconds, and elongation at 72 °C for 1 minute. Sequencing primers are listed in Supplementary Table S1 online.

Western analysis

The nuclear fraction was extracted from cells using a ReadyPrep Protein Extraction Kit (Bio-Rad Laboratories, Richmond, CA), and the protein concentrations were assayed using a DC Protein Assay kit (Bio-Rad Laboratories). Electrophoresis, transfer, and chemiluminescent detection were performed as described previously (Takahashi *et al.*, 2005). A volume of 50 μg per lane of each extract was run. The transfer membrane (Polyscreen, NEN Life Science Products, Boston, MA) was incubated with 1:1,000 diluted anti-XPA (Clone 12F5, Neomarkers, Fremont, CA) as a primary antibody, followed by incubation with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (H + L; ICN Biomedicals, Aurora, OH).

Northern analysis

RNA was extracted from cells using a Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA). RNA samples were separated by electrophoresis on a 0.8% formaldehyde/agarose gel and transferred to a nylon membrane (Roche, Basel, Switzerland). Preparation of DIG-labeled RNA probe, hybridization, and chemiluminescent detection were performed as described previously (Sayo *et al.*, 2002), with slight modification. The cDNA of human *XPA* genes was generated using reverse transcriptase–PCR with the following primers: sense primer 5′-CATCATTCACAATGGGGTGA-3′ and anti-sense primer 5′-GTCAGTTCATGGCCACACAT-3′. The expected cDNA fragments were ligated into the TA-cloning site of pGEMTeasy (Promega). *In vitro* transcription was performed with cloned cDNA to synthesize antisense RNA probes using a DIG RNA labeling kit (Roche). Membranes were rehybridized with a DIG-labeled glyceraldehyde-3-phosphate dehydrogenase antisense RNA probe prepared from commercially available human glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech, Palo Alto, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Makoto Tomida, Koichiro Onuma, and Masayo Kaji for technical assistance. We also thank all the patients and their families.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Berneburg M, Lehmann AR (2001) Xeroderma pigmentosum and related disorders: defects in DNA repair and transcription. *Adv Genet* 43: 71–102

Cleaver JE, Charles WC, Thomas GH *et al.* (1995) A deletion and an insertion in the alleles for the xeroderma pigmentosum (XPA) DNA-binding protein in mildly affected patients. *Hum Mol Genet* 4:1685–7

Cleaver JE, Kraemer KH (1995) Xeroderma pigmentosum and Cockayne syndrome. In: *The Metabolic and Molecular Bases of Inherited Disease* (Scriver CR, Beaudet AL, Sly WS, Valle D, eds). McGraw-Hill: New York, 4393–419

Cleaver JE, Thompson LH, Richardson AS *et al.* (1999) A summary of mutations in the UV-sensitive disorders: xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy. *Hum Mutat* 14:9–22

de Boer J, Hoeijmakers JH (2000) Nucleotide excision repair and human syndromes. *Carcinogenesis* 21:453–60

Fujimoto M, Leech SN, Theron T *et al.* (2005) Two new XPD patients compound heterozygous for the same mutation demonstrate diverse clinical features. *J Invest Dermatol* 125:86–92

Hirai Y, Kodama Y, Moriwaki S *et al.* (2006) Heterozygous individuals bearing a founder mutation in the XPA DNA repair gene comprise nearly 1% of the Japanese population. *Mutat Res* 601:171–8

Kraemer KH (1993) Heritable diseases with increased sensitivity to cellular injury. In: *Dermatology in General Medicine* (Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KH, eds). McGraw-Hill: New York, 1974

Kraemer KH, Lee MM, Andrews AD *et al.* (1994) The role of sunlight and DNA repair in melanoma and nonmelanoma skin cancer. The xeroderma pigmentosum paradigm. *Arch Dermatol* 130:1018–21

Kraemer KH, Lee MM, Scotto J (1987) Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. *Arch Dermatol* 123:241–50

Maeda T, Sato K, Minami H *et al.* (1995) Chronological difference in walking impairment among Japanese group A xeroderma pigmentosum (XP-A) patients with various combinations of mutation sites. *Clin Genet* 48:225–31

Maeda T, Sato K, Minami H *et al.* (1997) PCR-RFLP analysis as an aid to genetic counseling of families of Japanese patients with group A xeroderma pigmentosum. *J Invest Dermatol* 109:306–9

Maeda T, Sato K, Tanaka T *et al.* (2000) Compound heterozygous group A xeroderma pigmentosum patient with a novel mutation and an inherited reciprocal translocation. *Br J Dermatol* 143:174–9

Mimaki T, Nitta M, Saijo M *et al.* (1996) Truncated XPA protein detected in atypical group A xeroderma pigmentosum. *Acta Paediatr* 85:511–3

Miura N, Miyamoto I, Asahina H *et al.* (1991) Identification and characterization of xpac protein, the gene product of the human XPAC (xeroderma pigmentosum group A complementing) gene. *J Biol Chem* 266:19786–9

Moriwaki S, Kraemer KH (2001) Xeroderma pigmentosum—bridging a gap between clinic and laboratory. *Photodermatol Photoimmunol Photomed* 17:47–54

Moriwaki S, Nishigori C, Horiguchi Y *et al.* (1992) Amyloidosis cutis dyschromica. DNA repair reduction in the cellular response to UV light. *Arch Dermatol* 128:966–70

Moriwaki S, Nishigori C, Teramoto T *et al.* (1993) Absence of DNA repair deficiency in the confirmed heterozygotes of xeroderma pigmentosum group A. *J Invest Dermatol* 101:69–72

Moriwaki S, Takahashi Y (2008) Photoaging and DNA repair. *J Dermatol Sci* 50:169–76

Nishigori C, Moriwaki S, Takebe H *et al.* (1994) Gene alterations and clinical characteristics of xeroderma pigmentosum group A patients in Japan. *Arch Dermatol* 130:191–7

Nishigori C, Zghal M, Yagi T *et al.* (1993) High prevalence of the point mutation in exon 6 of the xeroderma pigmentosum group A-complementing (XPAC) gene in xeroderma pigmentosum group A patients in Tunisia. *Am J Hum Genet* 53:1001–6

Nocentini S, Coin F, Saijo M *et al.* (1997) DNA damage recognition by XPA protein promotes efficient recruitment of transcription factor II H. *J Biol Chem* 272:22991–4

Park CH, Mu D, Reardon JT *et al.* (1995) The general transcription-repair factor TFIIH is recruited to the excision repair complex by the XPA protein independent of the TFIIE transcription factor. *J Biol Chem* 270:4896–902

Protic-Sabljic M, Kraemer KH (1985) One pyrimidine dimer inactivates expression of a transfected gene in xeroderma pigmentosum cells. *Proc Natl Acad Sci USA* 82:6622–6

Rass U, Ahel I, West SC (2007) Defective DNA repair and neurodegenerative disease. *Cell* 130:991–1004

Reardon JT, Bessho T, Kung HC *et al.* (1997) *In vitro* repair of oxidative DNA damage by human nucleotide excision repair system: possible explanation for neurodegeneration in xeroderma pigmentosum patients. *Proc Natl Acad Sci USA* 94:9463–8

Robbins JH, Brumback RA, Mendiones M *et al.* (1991) Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form. *Brain* 114(Part 3):1335–61

Sato M, Nishigori C, Yagi T *et al.* (1996) Aberrant splicing and truncated-protein expression due to a newly identified XPA gene mutation. *Mutat Res* 362:199–208

Satokata I, Tanaka K, Miura N *et al.* (1990) Characterization of a splicing mutation in group A xeroderma pigmentosum. *Proc Natl Acad Sci USA* 87:9908–12

Satokata I, Tanaka K, Miura N *et al.* (1992a) Three nonsense mutations responsible for group A xeroderma pigmentosum. *Mutat Res* 273: 193–202

Satokata I, Tanaka K, Yuba S *et al.* (1992b) Identification of splicing mutations of the last nucleotides of exons, a nonsense mutation, and a missense mutation of the XPAC gene as causes of group A xeroderma pigmentosum. *Mutat Res* 273:203–12

Sayo T, Sugiyama Y, Takahashi Y *et al.* (2002) Hyaluronan synthase 3 regulates hyaluronan synthesis in cultured human keratinocytes. *J Invest Dermatol* 118:43–8

Sidwell RU, Sandison A, Wing J *et al.* (2006) A novel mutation in the XPA gene associated with unusually mild clinical features in a patient who developed a spindle cell melanoma. *Br J Dermatol* 155:81–8

States JC, McDuffie ER, Myrand SP *et al.* (1998) Distribution of mutations in the human xeroderma pigmentosum group A gene and their relationships to the functional regions of the DNA damage recognition protein. *Hum Mutat* 12:103–13

Takahashi Y, Moriwaki S, Sugiyama Y *et al.* (2005) Decreased gene expression responsible for post-ultraviolet DNA repair synthesis in aging: a possible mechanism of age-related reduction in DNA repair capacity. *J Invest Dermatol* 124:435–42

Takebe H, Nishigori C, Satoh Y (1987) Genetics and skin cancer of xeroderma pigmentosum in Japan. *Jpn J Cancer Res* 78:1135–43

Tanaka K, Miura N, Satokata I *et al.* (1990) Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc-finger domain. *Nature* 348:73–6

Tanioka M, Budiyant A, Ueda T *et al.* (2005) A novel XPA gene mutation and its functional analysis in a Japanese patient with xeroderma pigmentosum group A. *J Invest Dermatol* 125:244–6