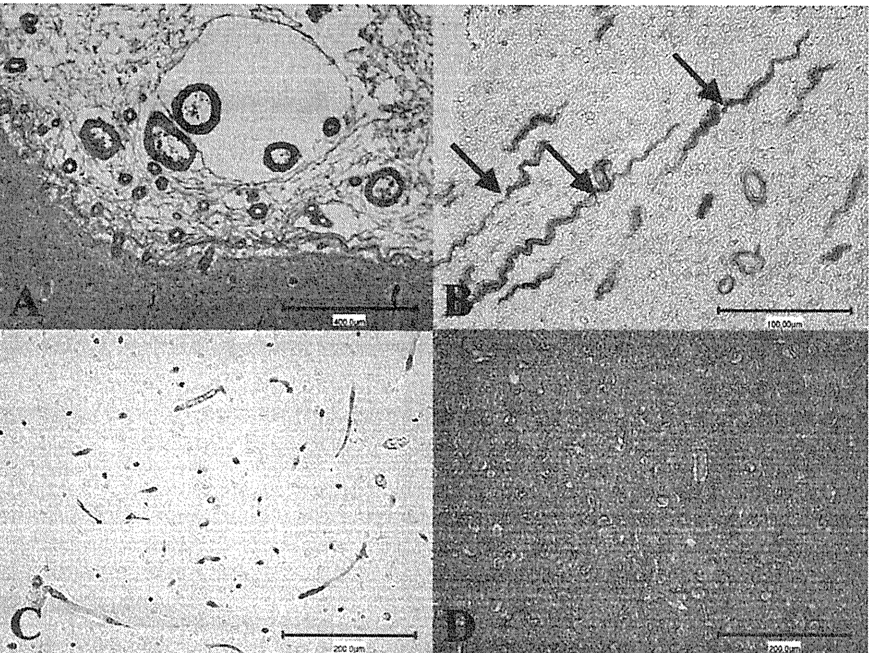

Fig. 1 Vascular changes in the brain of Cockayne syndrome (CS) patients. (A) Small arteries had increased subarachnoid space in CS case 1, elastica van Gieson staining. Bar = 400 μm. (B) Small twisted capillaries (arrows) were identified by immunostaining for collagen type IV in the cerebral white matter in CS case 4. Bar = 100 μm. (C) Vessels immunoreactive for CD34 in the middle temporal cortex in CS case 2. Bar = 200 μm. (D) Astrocyte processes were diffusely visualized by immunohistochemistry for aquaporin 4 in the cerebral white matter in CS case 4. Bar = 200 μm.

in either XP-A patients or controls (Table 1 and Fig. 1B). Twisted capillaries had more than five undulations, and were differentiated from functioning capillaries. In controls, the density of CD34-immunoreactive vessels in the frontal and temporal cortex of aged subjects was one-and-half times higher than that in teenagers, suggesting that the increase was age-dependent (Table 1). The density of CD34-immunoreactive vessels in CS cases aged less than 20 years was over 40 (Fig. 1C), which was equal to that in aged controls. The mean (SD) was 32 (4) in controls aged from 9 to 36 years, 47 (3) in CS cases, and 33 (5) in XP-A cases in the superior frontal cortex, respectively. The mean (SD) was 27 (4) in controls aged from 9 to 36 years, 45 (3) in CS cases, and 31 (5) in XP-A cases in the middle temporal cortex, respectively. The density of CD34-immunoreactive vessels in CS cases was significantly higher than those in controls and XP-A patients ($P < 0.05$). Furthermore, in CS patients, the number of capillaries increased around the calcified foci in the basal ganglia (data not shown). The vessel wall calcification was not found in the cerebral cortex, white matter or subarachnoid space. AQP4 immunostaining visualized the astrocyte processes around the vessels in all subjects (Fig. 1D) and there were no pathological changes in either CS or XP-A patients.

DISCUSSION

Cockayne syndrome and XP-A patients had severe brain atrophy (Table 1) and fibrosis in the enlarged subarachnoid space. However, the number of arteries and arterioles increased only in CS patients, suggesting excessive branching in the cortical arteries in CS. Similarly, in the subarachnoid space, increased density of CD34-immunoreactive vessels in the frontal and temporal cortex was observed in CS but not in XP-A patients. The reduction of brain area due to brain atrophy possibly leads to the apparent increase in the density of vessels. Nevertheless, since there was no increase in density in XP-A patients, the increased density of vessels in CS patients was not caused by the brain atrophy. Subdural hemorrhage is usually caused by tearing of the bridging cortical veins after head trauma, but traumatic aneurysms in the cortical arteries are a rare cause of such hemorrhages.[8] The increased number of subarachnoid and/or intracortical arteries may be a risk factor for subdural hemorrhage, which has been reported in CS patients.[6] A recent study showed that *CSB* mutant cells did not exhibit a normal reaction to hypoxia; these cells did not activate hypoxia-inducible factor-1 on the promoter gene due to which downstream events such as transcription factor IIB (TFIIB) recruitment did not occur.[9] Insufficient hypoxic response may disturb the induction of growth factors such as VEGF, suggesting the possible involvement of angiogenesis in CS. The analysis of brain vessels in fetal autopsy cases of CS might reveal the disturbances of angiogenesis during brain development.

Rapin *et al.* reported twisted microvessels consistent with so-called string vessels in the brains of adult CS patients.[5] We observed a similar morphological change in CS patients in our analysis. The absence of twisted microvessels in XP-A patients is noteworthy, and deficient NER is unlikely to have direct relationships with the

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vascular changes in CS patients. Brooks *et al.* stressed that in addition to brain vascular lesions, there is an overlap in neurological symptoms, such as dysmyelination and brain calcification between CS and Aicardi–Goutières syndrome. They proposed that the vascular changes probably occur due to alterations in gene expression and may play a role in the generation of neurological abnormalities in both the diseases.[10]

CS is considered to be a progeroid condition since many symptoms of CS resemble premature aging. It is intriguing that arteriosclerosis was absent in the brain vessels in our CS patients, although this change has been pointed out in CS cases reported in the literature. In good accordance of our findings, the absence of atherosclerotic changes in the systemic arteries was reported in a 40-year-old patient with CS of probable type III.[11] Furthermore, neither senile plaques nor vascular beta-amyloid depositions were identified in the temporal lobe in three patients with CS of probably type I or II, two aged 2 and one aged 6 years, respectively.[12] In Werner syndrome (WS) associated with supposed accelerated aging, patients rarely show age-associated neuropathology and lack amyloid deposition, indicating the absence of extension of WS-associated aging in the CNS.[13] Although the increased occurrence of arteriosclerosis in the heart, aorta and kidney is a definite characteristic of CS and WS, further analysis of many autopsy cases is required to verify the facilitation of brain arteriosclerosis in both the disorders.

Mouse models for CS-A and CS-B show a TCR defect and increased photosensitivity in the skin. However, growth failure and neurological abnormalities are not predominant.[14,15] $Csb^{m/m}/Xpa^{-/-}$ double mutant animals show post natal growth retardation, ataxia, abnormal locomotor activity, progressive weight loss and early death.[16] However, in these model animals, brain vascular changes have not been examined in detail. Complete inactivation of NER by deletion of *XP-A* gene in animals does not cause CS-like neurodevelopmental and progeroid features, and it has been proposed that some of the CS features may be the outcome of defects in the transcription function of transcription/repair factor TFIIH and/or defective repair of oxidative DNA lesions.[17] We have investigated the involvement of oxidative stress in the brains of XP-A and CS autopsy cases.[2] Lipid peroxidation and protein glycation markers were found in the perivascular calcification areas in the globus pallidus and cerebellum more predominantly in CS than in XP-A patients. We found a similar deposition of oxidative stress markers in the calcification areas in the brain vessel walls in cases of pseudohypoparathyroidism and Fahr disease.[18] Since increased oxidative stress is known to cause vascular calcifications in bone and kidney diseases,[19,20] it is possible that oxidative stress may be involved in the generation of brain vascular changes in CS.

Our findings suggest that vascular changes in the brain may be involved in neurological disturbances in CS. A detailed investigation of the brain vessels may help us clarify the pathogenesis of neurological abnormalities.

REFERENCES

1. Kraemer KH, Patronas NJ, Schiffmann R, Brooks BP, Tamura D, Digiovanna JJ. Xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome: a complex genotype–phenotype relationship. *Neuroscience* 2007; **145**: 1388–1396.
2. Hayashi M. Role of oxidative stress in xeroderma pigmentosum. *Adv Exp Med Biol* 2008; **637**: 120–127.
3. Martin GM. Genetic modulation of senescent phenotypes in Homo sapiens. *Cell* 2005; **120**: 523–532.
4. Nance MA, Berry SA. Cockayne syndrome: review of 140 cases. *Am J Med Genet* 1992; **42**: 68–84.
5. Rapin I, Weidenheim K, Lindenbaum Y *et al.* Cockayne syndrome in adults: review with clinical and pathologic study of a new case. *J Child Neurol* 2006; **21**: 991–1006.
6. Shimoizumi H, Matsui M, Ito S, Miyao M, Kobayashi S. Cockayne syndrome complicated by acute subdural hemorrhage. *Brain Dev* 1995; **17**: 376.
7. Itoh M, Hayashi M, Shioda K *et al.* Neurodegeneration in hereditary nucleotide repair disorders. *Brain Dev* 1999; **21**: 326–333.
8. Cho WS, Batchuluun B, Lee SJ, Kang HS, Kim JE. Recurrent subdural hematoma from a pseudoaneurysm at the cortical branch of the middle artery after mild head injury: case report. *Neurol Med Chir (Tokyo)* 2011; **51**: 217–221.
9. Filippi S, Latini P, Frontini M, Palitti F, Egly JM, Proietti-De-Santis L. CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response. *EMBO J* 2008; **27**: 2545–2556.
10. Brooks PJ, Cheng TF, Cooper L. Do all of the neurologic diseases in patients with DNA repair gene mutations result from the accumulation of DNA damage? *DNA Repair* 2008; **7**: 834–848.
11. Inoue T, Sano N, Ito Y *et al.* An adult case of Cockayne syndrome without sclerotic angiopathy. *Intern Med* 1997; **36**: 565–570.
12. Woody RC, Harding BN, Brumback RA, Leech RW. Absence of β-amyloid immunoreactivity in mesial temporal lobe in Cockyane's syndrome. *J Child Neurol* 1991; **6**: 32–34.
13. Mori H, Tomiyama T, Maeda N, Ozawa K, Wakasa K. Lack of amyloid plaque formation in the central nervous system of a patient with Werner syndrome. *Neuropathology* 2003; **23**: 51–56.

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14. van der Horst GT, Meira L, Gorgels TG *et al.* UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. *DNA Repair* 2002; **1**: 143–157.
15. van der Horst GT, van Steeg H, Berg RJ *et al.* Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. *Cell* 1997; **89**: 425–435.
16. Murai M, Enokido Y, Inamura N *et al.* Early postnatal ataxia and abnormal cerebellar development in mice lacking seroderma pigmentosum group A and Cockayne syndrome group B DNA repair genes. *Proc Natl Acad Sci U S A* 2001; **98**: 13379–13384.
17. Andressoo JO, Weeda G, de Wit J *et al.* An Xpb mouse model for combined xeroderma pigmentosum and Cockayne syndrome reveals progeroid features upon further attenuation of DNA repair. *Mol Cell Biol* 2009; **29**: 1276–1290.
18. *Neurodegenerative Diseases*, edited by Shamim I. Ahmad, from Landes Bioscience and Springer Science (ISBN: 978-1-4614-0652-5). http://www.landesbioscience.com/books/special/id/4242/
19. Mody N, Parhami F, Sarafian TA, Demer LL. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Biol Med* 2001; **31**: 509–519.
20. Massy ZA, Maziere C, Kamel S *et al.* Impact of inflammation and oxidative stress on vascular calcifications in chronic kidney disease. *Pediatr Nephrol* 2005; **20**: 380–382.

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

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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.

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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

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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

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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

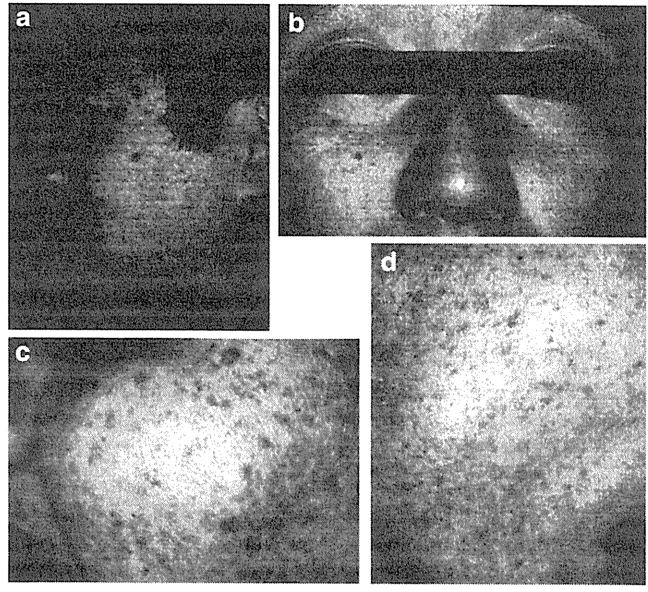

Figure 1. Unusually mild cutaneous features of four patients with xeroderma pigmentosum. (**a**) XP17HM; (**b**) XP21HM; (**c**) XP42HM; (**d**) XP43HM. Each patient's cutaneous symptoms consist only of mild freckling and few, if any, telangiectasias on the face.

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

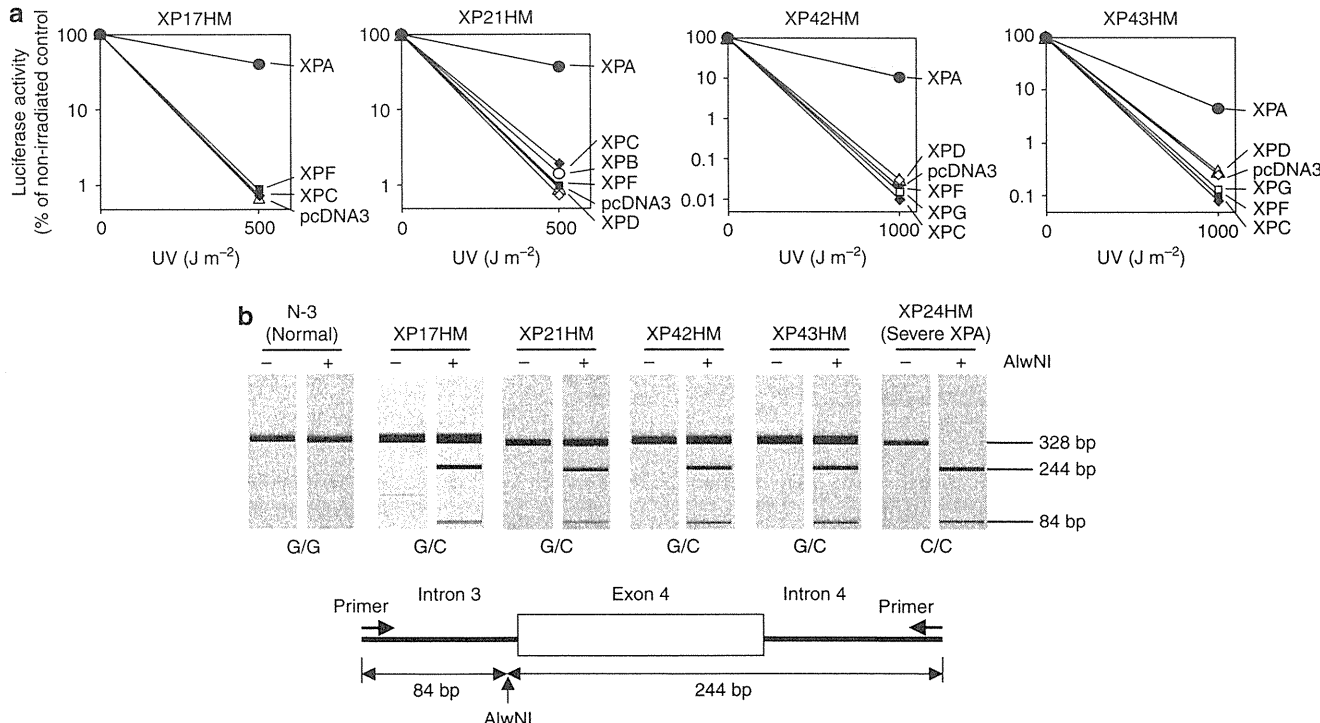

Figure 2. Each patient was diagnosed with xeroderma pigmentosum group A (XPA) and had a common Japanese mutation heterozygously. (**a**) Host-cell reactivation assay was performed via cotransfection of the UV-C-treated reporter plasmid (pGL2Luc) with xeroderma pigmentosum complementary DNA (*XPA* (closed 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.

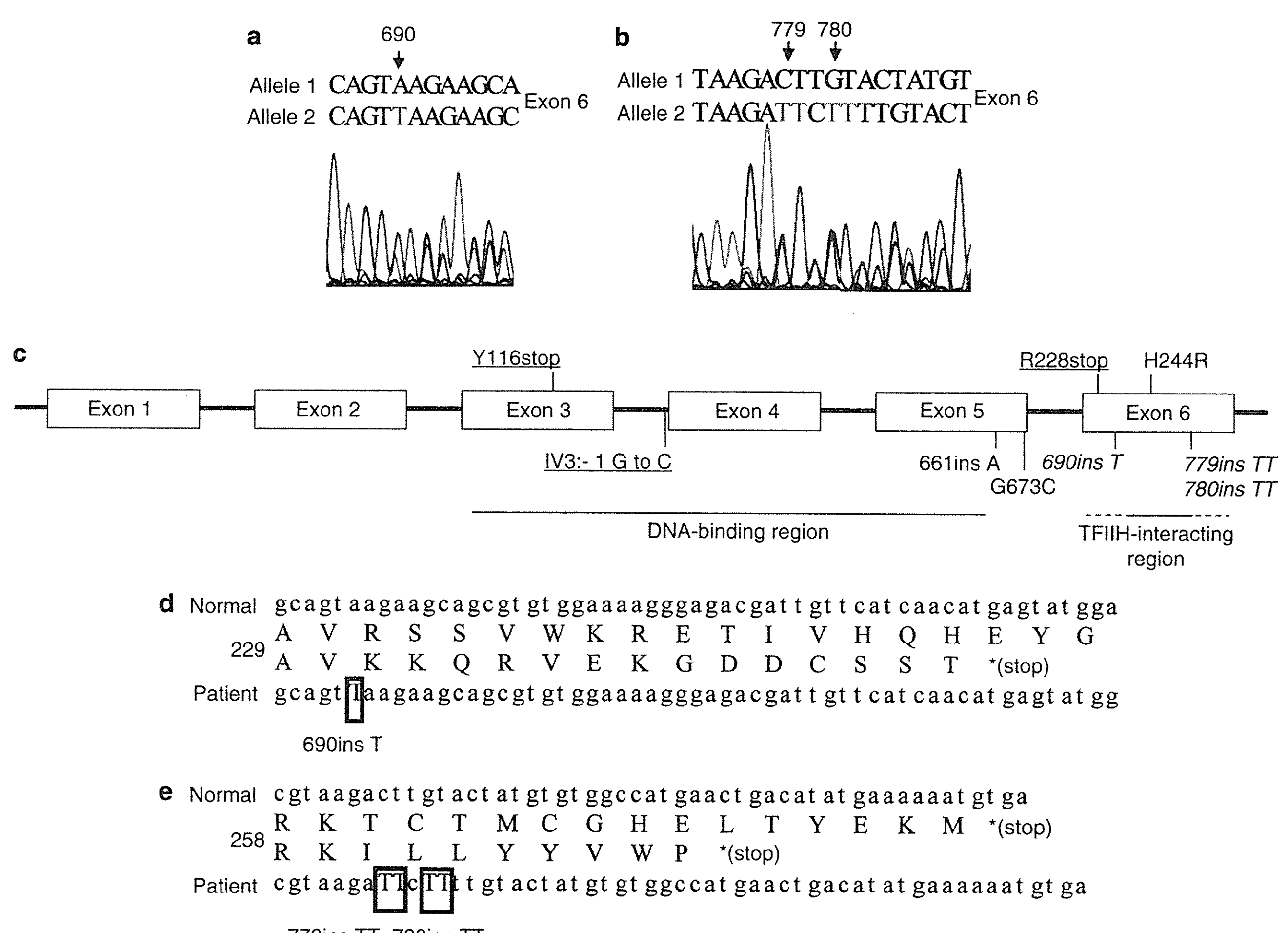

Figure 3. Two insertion mutations were identified in exon 6 of the xeroderma pigmentosum group A (XPA) gene heterozygously. (**a**) The genomic sequencing of exon 6, showing a single-base insertion at nucleotide 690. (**b**) The genomic sequencing of exon 6, showing a four-base insertion at nucleotides 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,

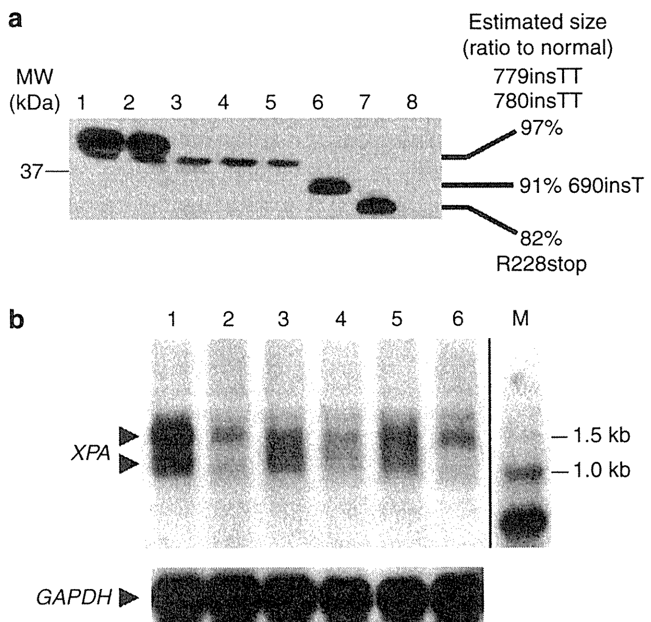

Figure 4. A significant expression of truncated XPA protein and a reduced expression of XPA mRNA in each patient. (**a**) A volume of 50 μg of total protein from N-3 (normal, lane 1), N-75 (normal, lane 2), XP21HM (lane 3), XP42HM (lane 4), XP43HM (lane 5), XP17HM (lane 6), XP 41 HM (mild XPA, lane 7), 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.

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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

遺伝性光線過敏症と患者家族会活動

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遺伝性光線過敏症と患者家族会活動

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1. はじめに

筆者のグループでは1998年11月より紫外線により生じたDNA損傷の修復システムの先天性異常で発症する遺伝性光線過敏症の診断センターを維持している．代表的な対象疾患は色素性乾皮症（xeroderma pigmentosum；XP），コケイン症候群（Cockayne syndrome；CS）であり，2011年3月現在，全国から依頼，紹介のあった381症例を細胞生物学的，分子遺伝学的に解析し，新たに121例のXP，21例のCSを確認した．このように多くの遺伝性疾患の症例を直接経験する過程の中で，我々は患者のみならず患者家族や彼らをとりまく社会とも直接接触する機会が多い．

一般に光線過敏症患者のquality of life（QOL）は有意に低下する[1]．遺伝性の光線過敏症では「生涯にわたる遮光」のため患者のQOLはさらに低下する．XP，CSでは疾患特異的な生命予後に関わる合併症が加わるため，患者のみならず家族共々ますますQOLは低下する．すなわちXP，CSでは「生涯にわたる厳重な紫外線防御」，「障害児（進行性脳神経障害）」，「難病（治療法がない）」，そして「遺伝の悩み」という四重苦を背負っており，患者・家族の肉体的，精神的，経済的なストレスは多大である．

しかし，このような疾患をもつ患者へのケアや家族，社会への対応は医師のみでは充分には行えない．そのような際には，ひとつの家族内だけで悩まないで疾病の知識，日常生活の中での工夫や情報を共有しよう，楽しみも分かち合おう，より良い医療環境を目指して社会へこれらの稀な疾患の存在を認知させよう，というコンセプトのもとに立ち上げられた患者家族会の存在意義は非常に大きいものである．患者家族会の活動はもちろん患者家族主導であるが，我々医師も情報提供などを通じて前向きな支援を行っている．

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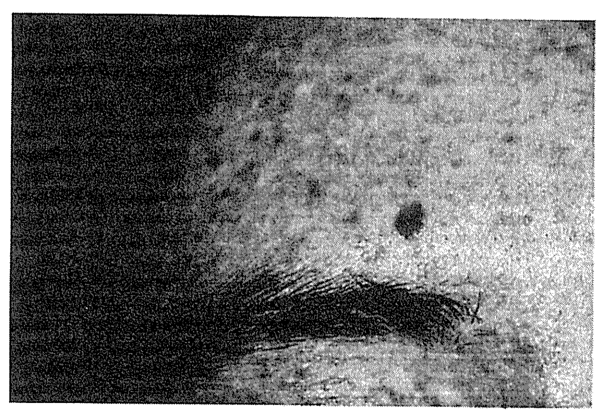


図1　色素性乾皮症患者に生じた皮膚癌（基底細胞癌）

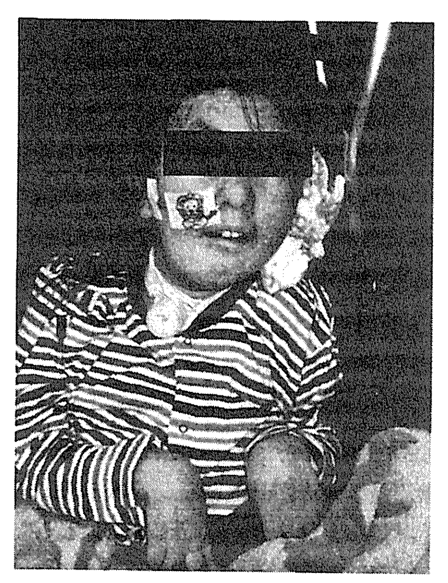
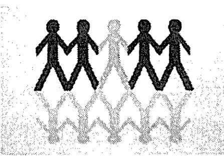


図2　色素性乾皮症患者にみられる進行性の神経障害

2. 色素性乾皮症（xeroderma pigmentosum；XP）とコケイン症候群（Cockayne syndrome；CS）

XPは主として紫外線性DNA損傷に対するヌクレオチド除去修復機構に関わる因子の単一遺伝子変異で発症する常染色体劣性遺伝性疾患で，本邦での頻度は数万人にひとりである．乳幼児期からの激しい光線過敏症で発見され，露光部皮膚は高発がん性であり（図1），本邦患者では原因不明，進行性の中枢性・末梢性神経障害が過半数にみられる（図2）．XPは遺伝的に異



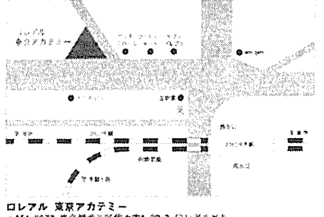
「コケイン症候群（CS）の集い2010」開催のご案内

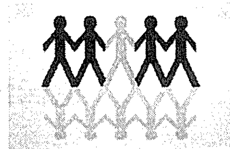
コケイン症候群（以下CS）は100万人に1人に発症する珍しい遺伝病です。現在、日本ではCS患者さんは30数人いるといわれていますが、人数が少ないために医師やその他の医療従事者の間でもCSという病気の存在が認知されていないのが現状です。病気の特徴は早くに年をとる。紫外線に弱く皮膚に炎症がおきやすい、耳が聞こえなくなる、目が見えなくなる、歩くのが困難になって車椅子での生活を余儀なくされる、などの特徴を有し、ほとんどの子供たちは20歳までに亡くなっていきます。残念ながら現在の医療ではCSの治療法はありません。

今から15年前に日本で、CSという同じ痛みを共通の思いとして子供達、家族同士で分かち合い、その活動を支援する会としてCS家族会（「分かち合いと助け合い日本コケイン症候群ネットワーク」）ができました。この家族会はこれまでに色々な活動をしてきましたが、もっと多くの方にこの病気の存在を知って頂きたいと思い、このたび日本ロレアル株式会社からの多大なるご支援とご協力により「CSの集い2010」を開催させて頂く事となりました。
また、今回、会社の方のご厚意によりCSの子供達、お母様に日焼け止めクリームをたくさんプレゼントして頂きました。クリームと一緒に届いた暖かい気持ちのプレゼントを受け取り、毎日、子供達とお母様、ご家族は一生懸命に生きておられます。どうぞそんな子供達とご家族にぜひ会いに来てください。

日時　2010年10月30日（土曜日）　医師・医療関係者対象　13時開始
　　　31日（日曜日）　一般の皆様対象　10時開始

セミナー会場





コケイン症候群（CS）の集い2010

2010年10月30日（土）－10月31日（日）

＜医師・医療関係者向けセミナー＞

2010年10月30日（土曜日）13:00～18:00

セミナー　13:00～16:15

大阪医科大学感覚器機能形態医学講座皮膚科学　教授　森脇 真一 先生
国立成育医療研究センター神経内科　星野 英紀 先生
東京都神経科学総合研究所神経発達・再生（臨床神経病理）　林 雅晴 先生
千葉大学教育学部養護教育学基礎医科学部門　教授　杉田 克生 先生

懇親会　16:30～18:00（軽食をご用意しています）

＜一般の皆様向けセミナー＞

2010年10月31日（日曜日）10:00～15:00

セミナー　10:00～12:30

日本コケイン症候群ネットワーク　■■■ 代表
日本コケイン症候群ネットワーク　患者保護者数名

懇親会　12:45～15:00（軽食をご用意しています）
日本コケイン症候群ネットワークによるポスター・パネル展示など
日本ロレアル 100 Love Hands Project によるハンドマッサージ

事前のお申し込みは必要ございませんので、直接会場にお越しください。
開場は両日ともにセミナー開始30分前とさせていただきます。
セミナー内容の詳細などにつきましては 03-6911-8572 増井・望月までお問い合わせいただきますようお願いいたします。

図3　CSの集い2010　開催案内
2日間で95名（CS患者家族，XP患者家族，学生ボランティア7含む）が参加した．

なるA～G群とバリアント（V）の計8種類に分類されるが，症状の進行度，重症度や予後は各群で異なる．本邦では最も重篤で神経難病とされるXPA群が過半数を占め，生命予後に直結する神経症状への対応が医療上の問題点となっている．患者は生涯，厳重な紫外線防御が必要となるが，遮光生活を強いられる事は日常の生活に規制がかかり，患者家族のQOLは著しく低下する．幼児期より屋外行動の際はサンスクリーン使用や防護服着用のため，野外活動，プールなどの制限がかかり，活発な行動が行いにくい．また，小児患者では疾患が同じ学校の学童や教師，周りの親などに正しく理解されず差別や過度な対応に気を遣い悩む事も少なくない．中学校進学の頃からは難聴，歩行障害，言語障害，知能低下などが徐々にみられるようになり特別養護学校に就学する場合がほとんどである．

CSはXP同様紫外線性DNA損傷の修復機構に異常をもつ稀な疾患であるが，特にDNAの転写領域の損傷がうまく修復できないことにより発症する常染色体劣性遺伝性疾患である．光線過敏症に加え，特有の顔貌，著明な発育低下，網膜色素変性，小頭症，栄養不良，難聴，精神障害，失調，四肢の硬直など多彩な症状を示し，重症度という面ではXPより上であると言える．臨床的には思春期頃まで生存できる古典型，乳幼児期で死亡する重症型，成人発症の遅発型の3型がある．XP同様に厳重な遮光が必要であるが，著明な発育不良に加え学童期から思春期の間に失明し聴力を失う場合が多く，腎機能の低下が予後に直結する．XPとは違い，CS患児は親とのコミュニケーションがほとんどないまま20歳前後で生涯を終える．

3．XP患者家族会のあゆみと歴史

1986年大阪大学医学部附属病院皮膚科にて診療を受けていた患者家族により「XPふれっくるの会」が結成された．その後，1991年に東京医科歯科大学医学部附属病院小児科，皮膚科を受診していた患者家族が中心となって「XPひまわりの会」が，また同年，神戸大学医学部附属病院皮膚科の患者家族を中心とした「XPつくしんぼの会」が結成された．3団体はそれぞれ個別に活動をしていたが，2004年東京都立神経研の主催で

初の全国規模のXPシンポジウムが開催されたのを機に3つの患者家族会がひとつになり2005年XP連絡会が設立された．2006年からは国の難病認定を目標に積極的な署名活動（60万筆以上）を展開し，映画やテレビドラマの製作にも協力した努力が実を結び，2007年3月念願であった厚生労働省難治性疾患克服研究事業対象疾患に指定された．2008年には厚生労働省の研究対象疾患指定記念の全国大会が愛知県にてXP患者家族170名(49家族)，医師，研究者，教育関係者など65名，ボランティア150名の参加のもとに盛大に開催された．2009年には正式なホームページ(http://www.xp-japan.net/)が完成し，128人の患者(110家族)(2010年10月現在)のさらなる充実した医療環境の整備を目指して活動中である[2]．

4. XP患者家族会への医療人としての関わり

XPは全身疾患であるため，患者に関わる医療関係者は皮膚科医のみならず，小児科医，神経内科医，整形外科医，リハビリ専門医，歯科医，基礎研究者，看護師，臨床心理士，医療ボランティアなど多岐にわたる．これまで難病指定を目指した署名活動を積極的に応援し，広報活動の際にはマスコミと対応し，年一度のお泊まり会(サマーキャンプ)，勉強会においては疾患の概念，遺伝，治療の展望，遮光を含む患者ケア，療養生活などについての情報発信などを行ってきた．皮膚がん予防のための紫外線防御，紫外線防護服の製作，紫外線カット用品の協同購入の際の情報提供は皮膚科医の責務である．患者をXPと診断した場合に患者家族会の存在は伝えるが，それに参加するかどうかはもちろん患者家族の自主性に任される．患者家族会活動を通して，同じ疾患をもつ患者家族間で情報交換ができ，同じ痛みを分かち合えるという大きな利点もあるが，一方で患児の親は時間と共に自分の子供が将来そういう経路をたどるという現実を目のあたりにする事になり，いずれ生じてくる脳神経症状を子供の成長と共に受け入れざるを得ないのもまたこの疾患の問題である．親や患者本人の精神的な苦悩も考慮して心のケアを十分に行うことが必要である．

5. CS患者家族会のあゆみと歴史

CS患者家族会（日本CSネットワーク）は“Share & Care”(「分かち合いと助けあい」)をポリシーとして長崎県在住のアメリカ人宣教師夫妻（Cathy & Robert Garrott）と数名の患者家族により1995年に結成された．これまで25患者(23家族)が会に参加したが現在家族会に関わっているCSの生存患者はわずかに7名である．まず疾患の説明のための小冊子を作成し，次いで情報誌「架け橋」を発刊，1998年からは患者家族での宿泊旅行が毎年実施され，医師など医療関係者を招いての疾患，紫外線防御，遺伝，治療，療養生活についての勉強会も積極的に開催している．2007年より公式ホームページが開設され(http://jpcsnet.com/)，現在XPで実現した厚生労働省難治性疾患克服研究事業対象疾患への指定を目指して積極的な署名活動が行われている．2010年には疾患の存在を社会に知ってもらおうという趣旨でCSの集い2010が開催された（図3）．

6. CS患者家族会への医療人としての関わり

CSは重篤な疾患であるが，発症頻度がきわめて低く患者数も少ないため，社会的にはほとんど認知されておらず，CS自体を知らない医療関係者も多いのが実情である．これまで患者家族が中心となって厚生労働省難治性疾患克服研究事業対象疾患への指定を目指して30万筆以上の署名を集めたが未だ難病認定には至っていない．患者家族会の今後の更なる署名活動への協力，マスコミ，医療関係者，教育関係者などに働きかけてCSという疾患を知ってもらい患者家族が快適に生活できるような社会の実現のための支援活動をこれからも継続していく必要があろう．

2009年，CSに有効とされるプロダルサンという薬剤が米国FDAにより認可され小児CSを対象とした臨床試験もすでに終了し現在データ解析の段階にある．低分子化合物でDNA損傷を減少させる安全性の高い新薬（内服薬）であるため，CS患者の医療に携わる医療人としてCS患者のために今後の同薬の動向には注目していく予定である．

文献

1) Ling TC, Richards HL, Janssens AS, et al: Seasonal and latitudinal impact of polymorphic light eruption on quality of life, *J Invest Dermatol*, 2006; 126: 1648–1651.
2) 塩沢治江：色素性乾皮症患者家族の思い，*Visual Dermatology*，2011；10：504.

Biosci. Biotechnol. Biochem., 73 (6), 1318–1322, 2009

The Roles of HSP27 and Annexin II in Resistance to UVC-Induced Cell Death: Comparative Studies of the Human UVC-Sensitive and -Resistant Cell Lines RSa and AP^{r}-1

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We have reported that heat shock protein 27 (HSP27) and annexin II are involved in the protection of human cells against UVC-induced cell death. In this study we tried to confirm the combined roles of HSP27 and annexin II in cell death after UVC irradiation. In RSa cells with sensitivity to UVC, expression of annexin II decreased after UVC irradiation, but not in AP^{r}-1 cells with increased resistance to UVC. HSP27 siRNA-transfected AP^{r}-1 cells were sensitized to UVC lethality and showed decreased annexin II expression after UVC irradiation. In contrast, transfection of RSa cells with *HSP27* cDNA increased their resistance to UVC lethality and caused increased annexin II expression. Furthermore, over-production of annexin II in RSa cells resulted in increased resistance to UVC lethality. This study indicates the involvement of cellular HSP27 expression in the UVC susceptibility of human cells, which occurs in association with regulation of annexin II expression.

Key words: HSP27; annexin II; human cells; UVC

Heat shock proteins (HSPs) are expressed in response to a wide variety of physically and chemically induced stress conditions in prokaryotes and eukaryotes, and the increased expression of HSPs is involved in protection against stress-induced cellular injury.[1–3] We have found that one HSP, HSP27, may be involved in the resistance of human cells to UVC-induced cell death.[4] This finding was based on a comparison of gene expression profiles using a cDNA array between human RSa cells and their variant AP^{r}-1 cells. RSa cells were established from human embryo-derived fibroblastic cells by double infection with Simian virus 40 and Rous sarcoma virus.[5] The high sensitivity of RSa cells to UVC-induced cell death (at a principal wavelength of 254 nm) has been reported.[6] AP^{r}-1 cells, which are more resistant to UVC-induced cell death, were established from RSa cells by mutagenesis with ethyl methanesulfonate followed by UVC irradiation.[7] The expression of HSP27 was found to be lower in the RSa cells than in the AP^{r}-1 cells.[4] RSa cells transfected with *HSP27* cDNA showed slightly lower sensitivity to UVC-induced cell death than the control cells transfected with empty vector alone, and much lower sensitivity than RSa cells transfected with antisense *HSP27* cDNA.[4]

An important function of HSP27 is to act as a molecular chaperone. Under stressful conditions, HSP27 proteins form complexes with a variety of cellular proteins, such as Akt,[8] cytochrome *c*,[9] actin microfilament,[10] and eIF4G,[11] resulting in inhibition of apoptosis induction,[8,9] cytoskeleton disruption,[10] and translation initiation[11] respectively. Hence, we next evaluated the potential roles of HSP27 in the UVC response by determining which proteins interact with HSP27 and are involved in the UVC resistance of AP^{r}-1 cells.[12] Annexin II was identified as one of HSP27-interacted proteins from AP^{r}-1 cell lysates by pull-down with GST-fused HSP27 proteins and subsequent molecular mass analysis.[12] Moreover, AP^{r}-1 cells transfected with annexin II siRNA were found to have increased susceptibility to UVC-induced cell death.[12]

Annexin II is a phospholipid-binding protein implicated in several membrane related-events, including regulation of the exocytotic and endocytotic pathways, and it functions as a plasminogen receptor.[13] Increased expression levels of annexin II have been reported in some cancer cell lines,[14] but the relationship between HSP27 and the functions of annexin II in the susceptibility of human cells to UVC-induced cell death remains unknown.

In the present study, we tried to confirm the involvement of both proteins in the difference in resistance to UVC-induced cell death between RSa and AP^{r}-1 cells by transfecting RSa cells with *HSP27* cDNA and *annexin II* cDNA.

Materials and Methods

Cell culture conditions and UVC irradiation. Cells were cultured in Eagle's MEM (EMEM) (Nissui, Tokyo) medium supplemented with 10% (v/v) calf serum (CS) (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO_2. UVC was generated by a 6-W National germicidal lamp (Panasonic, Osaka, Japan). The

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* These authors contributed equally to this study.

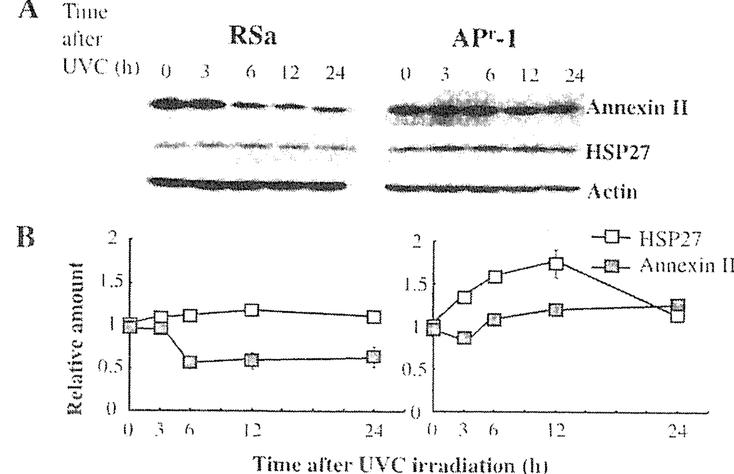

Fig. 1. Annexin II and HSP27 Expression Levels after UVC Irradiation in RSa Cells and APʳ-1 Cells.

A, Cells were irradiated with UVC (8 J/m^2) and harvested at the indicated times after UVC irradiation. Whole cell lysates were separated in 12% SDS gel and the protein levels were examined using antibodies against the respective proteins by Western blotting, as described in "Materials and Methods." The protein levels of actin were also analyzed as the loading control. B, The protein levels are expressed as levels relative to those at time 0 (immediately after UVC irradiation). Data are the means ± SD for three experiments.

intensity of UVC was 1 J/m^2/s, measured with a UV radiometer, UVR-254 (Tokyo Kogaku Kikai, Tokyo). Cells were irradiated with UVC as previously described,[7] and mock-irradiated cells were treated in the same manner but without UVC irradiation.

Western blotting. Immunoblotting analysis was carried out as described previously.[4] In brief, cells were washed twice with phosphate-buffered saline and whole cells were lysed with SDS sampling buffer and boiled for 5 min. Whole cell lysates were separated by 12% SDS gel and transferred onto a Millipore membrane. HSP27 protein was detected using mouse anti-HSP27 monoclonal antibody (mH3)[15,16] (1:1,000 dilution) and annexin II protein was detected using rabbit anti-annexin II antibody (sc-9061) (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1,000 dilution). The antigen-antibody complexes were detected using horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Biosciences, Buckinghamshire, UK) and anti-rabbit IgG antibody (GE Healthcare, Bucks) following the ECL system (Amersham Biosciences). The protein levels of actin were also analyzed using mouse anti-actin antibody (ICN Biomedicals, Costa Mesa, CA) (1:10,000 dilution) as the loading control. The intensity of the protein signals was quantified using Multi Gauge Ver2.2 image analyzing software (Fuji Foto Film, Tokyo) and were expressed as values relative to that of actin.

Construction of expression vectors. Full-length human *HSP27* cDNA was prepared as described previously.[4] The cDNA was ligated into pQE-30 plasmid (Qiagen, Valencia, CA) using Sac I and Pst I restriction sites. The construction was confirmed by sequencing and was then digested with EcoR I and Hind III. The digested fragment was gel-purified and then ligated into pcDNA3.1(−) (Invitrogen) using EcoR I and Hind III restriction sites to construct a His-tagged HSP27 (His-HSP27) expression vector (His-HSP27/pcDNA3.1(−)), and the construction was confirmed by sequencing. Human *annexin II* cDNA was prepared by PCR using forward (5′-GCAGGTACCGGATTTCA-CTCTCTACCCGGAG-3′) and reverse (5′-TCACTCGAGTTCAGT-CATCTCCACCACACA-3′) primers from a cDNA library of human SW837 cells, and PCR products were inserted into pcDNA3.1(+) (Invitrogen) using Kpn I and BamH I restriction sites to construct the annexin II expression vector (ANX II/pcDNA3.1(+)). The construction was confirmed by sequencing.

Plasmid transfection. RSa cells grown to 60–80% confluence in 60-mm dishes were transiently transfected with the indicated expression plasmids using FuGENE HD transfection reagent (Roche Applied Science, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions. To obtain cell lines with stable overexpression of annexin II, RSa cells were transfected with ANX II/pcDNA3.1(+) and pcDNA3.1(+) as described above, and cultured in G418-containing medium, followed by cloning the G418-resistant cells. Two cell lines with overexpression of annexin II, ANXII-11, and ANXII-13, and two cell lines with the empty vectors, V1 and V2, were obtained after analysis of annexin II expression by immunoblotting.

Knockdown of HSP27. Duplex small interfering RNA (siRNA) against human HSP27 (HSP27 siRNA) was synthesized based on the nucleotide sequence. The sequences of the duplex were as follows: 5′-GCUGCAAAAUCCGAUGAGACTT-3′/3′-TTCGACGUUUUAGG-CUACUCUG-5′. A negative control for HSP27 siRNA (NC siRNA) with random sequences designed as follows: 5′-GAUCCGACCUGC-AAGAUGAATT-3′/3′-TTCUAGGCUGGACGUUCUACUU-5′. These siRNA duplexes were synthesized by FASMAC (Atsugi, Japan). The siRNAs for HSP27 (100 nM) were transfected into APʳ-1 cells for 5 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two d after transfection, the cells were irradiated with UVC and harvested at the indicated times after UVC irradiation, and the levels of annexin II and HSP27 proteins were analyzed by immunoblotting. To analyze the susceptibility of the cells transfected with HSP27 siRNA, the cells were replated for colony survival assay 2 d after transfection.

Cell survival assay. The susceptibility of the cells to UVC-induced cell death was measured by colony survival assay and methylthiazole tetrazolium (MTT) metabolic viability assay, as reported previously.[17]

Results and Discussion

To determine the relative roles of HSP27 and annexin II in cell survival after UVC irradiation of human cells, we analyzed the expression of HSP27 and annexin II after UVC irradiation (up to 24 h) in RSa cells and APʳ-1 cells. The levels of HSP27 immediately after irradiation (time 0) were lower in RSa cells than in APʳ-1 cells (Fig. 1A). The levels of HSP27 increased in APʳ-1 cells after UVC irradiation, but hardly increased in RSa cells (Fig. 1A, B). In APʳ-1 cells, the levels of HSP27 at 12 h were 1.7-fold higher than those at time 0, and the peak levels (at 12 h) decreased at 24 h towards the basal levels (Fig. 1A, B). The reason for the decrease at 24 h remains unclear. HSP27 has been reported to

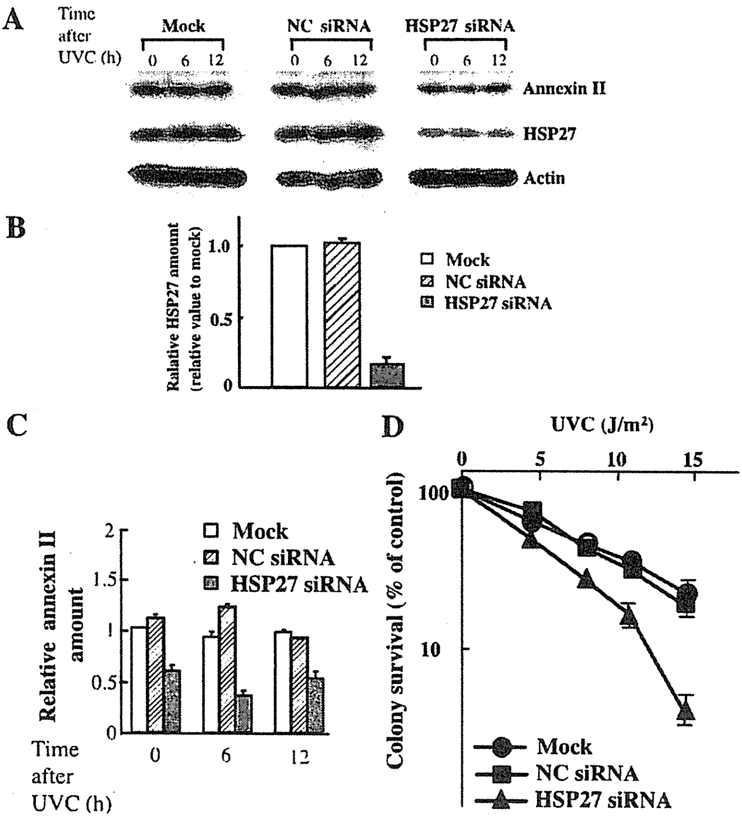

Fig. 2. Reduction of Annexin II after UVC Irradiation and UVC Sensitization in Association with Knockdown of HSP27 by siRNA in AP^r-1 Cells.
A, Forty-eight h after transfection with mock, NC siRNA, or HSP27 siRNA, AP^r-1 cells were irradiated with UVC (8 J/m^2) and harvested at the indicated times after UVC irradiation. Whole cell lysates were prepared, and the protein levels of HSP27, annexin II and actin were analyzed using antibodies against the respective proteins by Western blotting, as described in "Materials and Methods." B, The HSP27 protein levels in HSP27 siRNA- and NC siRNA-transfected cells without UVC irradiation are expressed as levels relative to those in the mock-transfected cells. C, The annexin II protein levels in the HSP27 siRNA- and NC siRNA-transfected cells and mock-transfected cells after UVC irradiation are expressed as levels relative to those in the mock-transfected cells at time 0 (immediately after UVC irradiation). D, Colony survival activity after UVC irradiation of HSP27 siRNA-, NC siRNA-, and mock-transfected cells was analyzed by colony formation assay, as described in "Materials and Methods." Data are shown as percentages of colony numbers relative to those of non-irradiated cells. All data (B–D) are means ± SD for three independent experiments.

translocate from the cytoplasm to the nucleus after UVB irradiation in human cells.[18] However, the decrease in HSP27 observed in this analysis might not have been due to translocation of HSP27, because whole cell lysates were used. Oxidative stress also induced HSP27 expression in human cells; the peak protein levels decreased slightly with exposure time, and the change in the protein levels did not always correspond with that in the mRNA levels.[19] Therefore, posttranslational regulatory mechanisms have been involved in the regulation of HSP27 protein levels. Annexin II expression decreased in RSa cells after UVC irradiation, and at 6 h after irradiation the levels were approximately 60% of those at time 0 (Fig. 1A, B). In contrast, annexin II expression did not decrease but increased in AP^r-1 cells after UVC irradiation (Fig. 1A, B).

HSP27 functions as a chaperone by interaction with target proteins under stress conditions and assists protein stabilization, modification, and translocation.[20,21] Taking this together with the results presented in Fig. 1, we hypothesized that HSP27 acts as a molecular chaperone for annexin II in resistance to UVC-induced cell death, thus regulating annexin II expression after UVC irradiation. Hence, we examined the effect of HSP27 expression levels on UVC-induced reduction in annexin II protein levels by knockdown of HSP27 in AP^r-1 cells and overexpression of HSP27 in RSa cells.

In HSP27 siRNA-transfected AP^r-1 cells, the levels of HSP27 protein were lower than in mock- and NC siRNA-transfected AP^r-1 cells without UVC irradiation (Fig. 2A, B). The HSP27 levels increased in mock- and NC siRNA-transfected AP^r-1 cells after UVC irradiation (up to 12 h), but did not increase in HSP27 siRNA-transfected AP^r-1 cells (Fig. 2A). The levels of annexin II protein were also lower in HSP27 siRNA-transfected AP^r-1 cells than in the mock- and NC siRNA-transfected cells without UVC irradiation (Fig. 2A). After UVC irradiation, the levels of annexin II protein in HSP27 siRNA-transfected AP^r-1 cells decreased; the levels at 6 h were approximately 50% compared with those at time 0, and then recovered at 12 h to the basal levels (Fig. 2A, C). In contrast, the levels did not change as much after UVC irradiation in the mock- and the NC-transfected cells (Fig. 2A, C). In addition, the sensitivity of the HSP27 siRNA-transfected cells to UVC-induced cell death was higher than that of the mock- and NC siRNA-transfected cells (Fig. 2D). To induce HSP27 overexpression in RSa cells, we transfected His-HSP27/pcDNA3.1(−) into the cells. The transfectant showed higher expression of His-HSP27 protein than the control

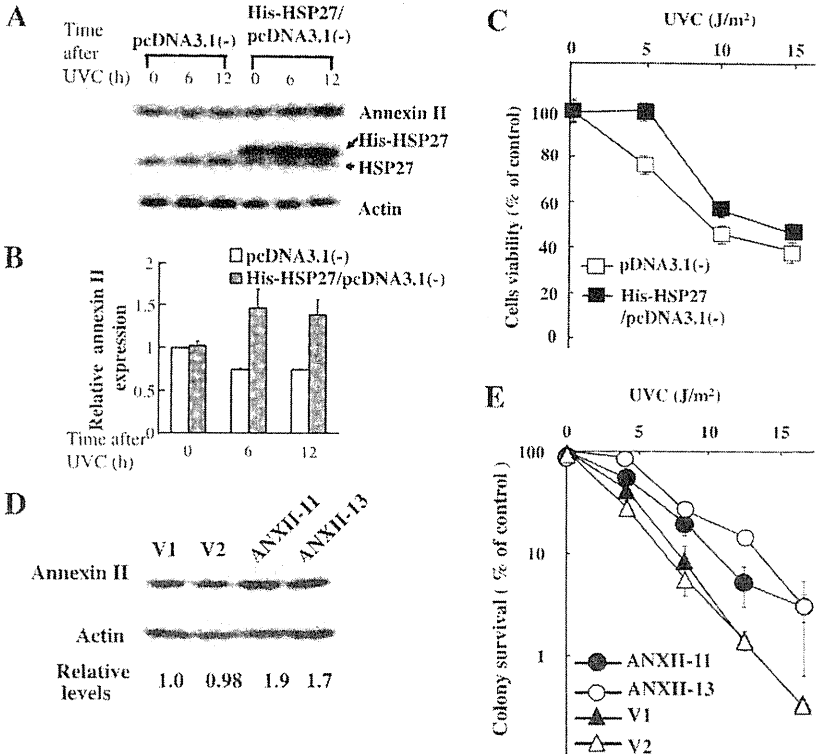

Fig. 3. Suppression of a Decrease in Annexin II Expression in RSa Cells Transfected with *HSP27* cDNA, and Increased Resistance to UVC-Induced Cell Death in Cells with Annexin II Overexpression.

A, Twenty-four h after transfection with His-HSP27/pcDNA3.1(−) or pcDNA3.1(−), RSa cells were irradiated with UVC (8 J/m^2) and were harvested at the indicated times after UVC irradiation. Whole cell lysates were prepared, and the protein levels of His-HSP27 and endogenous HSP27 were analyzed using anti-HSP27 antibody, and those of annexin II and actin were analyzed using the respective antibodies by Western blotting, as described in "Materials and Methods." B, Annexin II protein levels in the transfected cells after UVC irradiation are expressed as levels relative to those in the control cells transfected with the empty vector at time 0 (immediately after UVC irradiation). C, Twenty-four h after transfection with His-HSP27/pcDNA3.1(−) or pcDNA3.1(−), the cells were irradiated with UVC, and susceptibility to UVC irradiation was analyzed by MTT assay as described in "Materials and Methods." Data are shown as percentages of viability relative to those of non-irradiated cells. D, The annexin II protein levels in V1, V2, ANXII-11, and ANXAII-13 cells were analyzed by Western blotting, and are expressed as levels relative to those in the V1 cells. E, Colony survival activity after UVC irradiation of V1, V2, ANXII-11, and ANXAII-13 cells was analyzed by colony formation assay, as described in "Materials and Methods." Data are shown as percentages of colony numbers relative to those of non-irradiated cells. All data (B–E) are means ± SD for three independent experiments.

RSa cells transfected with empty vector (Fig. 3A). Furthermore, the His-HSP27-expressing cells showed no reduction in annexin II protein expression, but showed an increase in expression after UVC irradiation (Fig. 3B). On the other hand, the RSa cells transfected with empty vector showed a decrease in annexin II expression after UVC irradiation (Fig. 3A, B), as shown in Fig. 1. The His-HSP27-expressing cells showed higher resistance to UVC-induced cell death than the control cells transfected with empty vector (Fig. 3C and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site), similarly to the stable transfectants with HSP27 cDNA, as described previously.[4]

To confirm the role of annexin II expression levels in the resistance to UVC-induced cell death, we established cells with stable overexpression of annexin II protein by transfection of RSa cells with *annexin II* cDNA. The transfected cells, ANXII-11 and ANXAII-13, showed higher levels of annexin II than the control RSa cells transfected with empty vector, V1 and V2 (Fig. 3D). There was little difference in cell proliferation rates among the four cell lines; the doubling time of V1 cells was approximately 24 h and that of the other three cells was approximately 23 h. The ANXII-11 and ANXAII-13 cells showed increased resistance to UVC-induced cell death as compared to V1 and V2 cells (Fig. 3E).

The present findings suggest that up-regulation of HSP27 prevents a decrease in annexin II expression after UVC irradiation (Fig. 2A, C and Fig. 3A, B) and UVC-induced cell death in association with the regulation of annexin II expression. The mechanisms underlying the involvement of annexin II in UVC resistance and by which HSP27 regulates the levels of annexin II protein remain unknown. Recently, HSP27 has been reported to increase the stability of the Her2 protein[20] and androgen receptor[21] by affording protection against proteasome-mediated degradation. In this study, the changes in annexin II protein levels were a result of the balance between synthesis and degradation activities. The UVC-induced decrease in the annexin II protein levels in the cells with low HSP27 expression, RSa (Fig. 1A, B), may have been due to enhanced degradation activity, overcoming synthesis activity. On the other hand, the UVC-induced increase in the annexin II protein levels in the cells with high HSP27 expression, AP^r-1 cells and His-HSP27-expressing RSa cells (Fig. 1A, B and Fig. 3A, B), may have been associated with greater synthesis activity than degradation activity. Therefore, HSP27 may regulate annexin II protein levels after UVC irradiation *via* stimulation of synthesis

and/or prevention of degradation. Even in the HSP27 siRNA-transfected AP^r-1 cells, the UVC-induced decrease in annexin II recovered at 12 h (Fig. 2A, C). This recovery might also have been due to the balance between protein synthesis and degradation activities, which appeared to be affected by factors other than HSP27, because HSP27 expression was still suppressed at the time. Our preliminary analysis, using a protein synthesis inhibitor, cycloheximide, suggested that UVC irradiation enhanced the rate of annexin II protein degradation in the RSa cells (data not shown). More detailed analysis of the change in annexin II protein levels after UVC irradiation is underway, using protein synthesis inhibitors and protease inhibitors.

In addition, HSP27 enhances not only the stability but also the shuttling to the nucleus and the transcriptional activity of the androgen receptor in human LNCaP cells.[21] In the AP^r-1 cells, the formation of a complex between HSP27 and annexin II increased in the nuclear fraction after UVC irradiation.[12] Annexin II is reported to function mainly in the cytoplasm,[13] but part of the protein localizes to and functions in the nucleus.[22,23] Thus HSP27 might enhance induction, stability, and translocation into the nucleus of annexin II after UVC irradiation, and might regulate the function of annexin II to protect against UVC-induced DNA damage. To prove these hypotheses, a pair of RSa and its variant AP^r-1 cell lines should be valuable.

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References

1) Richards FM, Watson A, and Hickman JA, *Cancer Res.*, **48**, 6715–6720 (1988).
2) Polla BS, Kantengwa S, Francois D, Salvioli S, Franceschi C, Marsac C, and Cossarizza A, *Proc. Natl. Acad. Sci. USA*, **93**, 6458–6463 (1996).
3) McDuffee AT, Senisterra G, Huntley S, Lepock JR, Sekhar KR, Meredith MJ, Borrelli MJ, Morrow JD, and Freeman ML, *J. Cell. Physiol.*, **171**, 143–151 (1997).
4) Wano C, Kita K, Takahashi S, Sugaya S, Hino M, Hosoya H, and Suzuki N, *Exp. Cell Res.*, **298**, 584–592 (2004).
5) Kuwata T, Oda T, Sekiya S, and Morinaga N, *J. Natl. Cancer Inst.*, **56**, 919–926 (1976).
6) Suzuki N and Fuse A, *Mutat. Res.*, **84**, 133–145 (1981).
7) Isogai E, Ishijima S, Sonoda T, Kita K, Suzuki H, Hasegawa R, Yamamori H, Takakubo Y, and Suzuki N, *Mutat. Res.*, **403**, 215–222 (1998).
8) Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, and Klein JB, *J. Biol. Chem.*, **278**, 27828–27835 (2003).
9) Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, and Garrido C, *Nat. Cell Biol.*, **2**, 645–652 (2000).
10) Kindas-Mugge I, Rieder C, Frohlich I, Micksche M, and Trautinger F, *Cell Biol. Int.*, **26**, 109–116 (2002).
11) Cuesta R, Laroia G, and Schneider RJ, *Genes Dev.*, **14**, 1460–1470 (2000).
12) Tong XB, Kita K, Karata K, Zhu CL, Sugaya S, Ichimura Y, Satoh M, Tomonaga T, Nomura F, Jin YH, and Suzuki N, *Photochem. Photobiol.*, **84**, 1455–1461 (2008).
13) Gerke V and Moss SE, *Physiol. Rev.*, **82**, 331–371 (2002).
14) Qi YJ, Wang LD, Jiao XY, Feng XS, Fan ZM, Gao SS, He X, Li JL, and Chang FB, *Ai Zheng*, **26**, 730–736 (2007).
15) Okubo MA, Chiba S, Nishikata T, Matsuno A, and Hosoya H, *Dev. Growth Differ.*, **41**, 381–389 (1999).
16) Hino M, Kurogi K, Okubo MA, Murata-Hori M, and Hosoya H, *Biochem. Biophys. Res. Commun.*, **271**, 164–169 (2000).
17) Zhai L, Kita K, Wano C, Wu Y, Sugaya S, and Suzuki N, *Exp. Cell Res.*, **305**, 244–252 (2005).
18) Shi B, Grahn JC, Reilly DA, Dizon TC, and Isseroff RR, *Exp. Dermatol.*, **17**, 108–111 (2008).
19) Yu AL, Fuchshofer R, Birke M, Kampik A, Bloemendal H, and Welge-Lüssen U, *Invest. Ophthalmol. Vis. Sci.*, **49**, 5403–5411 (2008).
20) Kang SH, Kang KW, Kim KH, Kwon B, Kim SK, Lee HY, Kong SY, Lee ES, Jang SG, and Yoo BC, *BMC Cancer*, **8**, 286 (2008).
21) Zoubeidi A, Zardan A, Beraldi E, Fazli L, Sowery R, Rennie P, Nelson C, and Gleave M, *Cancer Res.*, **67**, 10455–10465 (2007).
22) Jindal HK, Chaney WG, Anderson CW, Davis RG, and Vishwanatha JK, *J. Biol. Chem.*, **266**, 5169–5176 (1991).
23) Liu J, Rothermund CA, Ayala-Sanmartin J, and Vishwanatha JK, *BMC Biochem.*, **4**, 10 (2003).