A New Disorder in UV-Induced Skin Cancer with Defective DNA Repair Distinct from Xeroderma Pigmentosum or Cockayne Syndrome

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We report the characterization of a Japanese woman who exhibited many freckles and skin cancers in sun-exposed areas, but displayed no photosensitivity. Fibroblasts (KPSX7) derived from this patient showed similar UV sensitivity to that of normal human fibroblasts. The KPSX7 cells showed normal levels of unscheduled DNA synthesis, recovery of RNA synthesis, recovery of replicative DNA synthesis, protein-binding ability to UV-damaged DNA, and post-translational modification of xeroderma pigmentosum (XP) C. These results indicate that the patient had neither XP nor Cockayne syndrome. Although these results suggest that the KPSX7 cells were proficient in nucleotide excision repair activity, host-cell reactivation (HCR) activity of KPSX7 cells was reduced. Furthermore, introduction of UV damage endonuclease into the cells restored repair activity in the HCR assay to almost normal levels. These results indicate that KPSX7 cells are defective for some types of repair activity in UV-damaged DNA. In summary, the patient had a previously unknown disorder related to UV-induced carcinogenesis, with defective DNA repair.

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INTRODUCTION

Exposure to UV radiation has several different effects on human skin. Acute effects include erythema, tanning, and immunosuppression, and long-term effects include photoaging and carcinogenesis (Daya-Grosjean and Sarasin, 2005). During the evolution of UV-induced carcinogenesis, a variety of alterations and instability in genomic DNA occur through the accumulation of DNA lesions. UV irradiation produces two predominant types of DNA lesions: cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) (Lippke *et al.*, 1981; Mitchell and Nairn, 1989). If not repaired through nucleotide excision repair (NER), these UV-induced lesions lead to mutations during replication, specifically C to T or tandem CC to TT transitions (Afaq *et al.*, 2005), and lead to variable instabilities, including epigenetic alterations of oncogenic, tumor suppressive, and cell-cycle control signaling pathways (Hussein, 2005).

The widely conserved NER process is an important repair system, and is responsible for removing several kinds of DNA lesions, particularly those induced by UV irradiation that distort the DNA helix, CPD, and 6-4PP (Prakash and Prakash, 2000). Defects in NER result in rare autosomal-recessive diseases, known as xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Hebra and Kaposi first described XP in 1874. Defining characteristics include photosensitivity, pigment changes, and neoplasia in the skin (Kraemer and Slor, 1985; Kraemer *et al.*, 1994). Some patients with XP also have neurological complications. Seven XP complementation groups (XP-A to -G) with defective NER are recognized (Hanawalt, 2002), and a variant form (XP-V) with normal NER, but defective translesion DNA synthesis, also occurs (Masutani *et al.*, 1999). CS is an inherited syndrome characterized by short stature, mental deficiency, photosensitivity, disproportionately large hands, feet, and ears, ocular defects, and extensive demyeliation (Nance and Berry, 1992). CS is distinguished from XP by these physical characteristics and lack of cutaneous malignancies (Lehmann, 2003).

To diagnose cells derived from photosensitive patients in our laboratory, a simple method has been established that examines unscheduled DNA synthesis (UDS), recovery of RNA synthesis (RRS), and recovery of replicative DNA synthesis (RDS) (Itoh *et al.*, 1996). XP-A through G cells (except for XP-E) are characterized by reduced UDS, whereas CS cells have normal UDS and reduced RRS. XP-V cells show

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Abbreviations: 6-4PP, pyrimidine (6-4) pyrimidone photoproduct; AP site, abasic site; CPD, cyclobutane pyrimidine dimers; CS, Cockayne syndrome; DDB, UV-damaged DNA-binding protein; HCR, host-cell reactivation; MMC, mitomycin C; MMS, methyl methanesulfonate; RDS, recovery of replicative DNA synthesis; RRS, recovery of RNA synthesis; UDS, unscheduled DNA synthesis; UVDE, UV-damage endonuclease; XP, xeroderma pigmentosum

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normal UDS and RRS, but reduced RDS. XP-E cells show normal USD, RRS, and RDS, but exhibit reduced UV-damaged DNA-binding protein (DDB) activity (Feldberg and Grossman, 1976; Kataoka and Fujiwara, 1991) (Table 2). The host-cell reactivation (HCR) assay is a simple method to diagnose the DNA repair capacity in cells (Qiao *et al.*, 2002). Moreover, this analysis of the DNA repair capacity can be confirmed by a complementation assay, introducing UV-damage endonuclease (UVDE) into the cells. UVDE introduces a nick immediately 5′ to CPD, 6-4PP, or abasic sites (AP sites) and initiates an excision repair pathway (Freyer *et al.*, 1995; Yasui and McCready, 1998; Kanno *et al.*, 1999).

In this study, we diagnosed a Japanese woman having several skin tumors in sun-exposed areas who also exhibited a new disorder with defective DNA repair. The patient showed no clinical or cellular hypersensitivity to UV light, and although cells established from this patient showed impaired repair activity for UV-damaged DNA, the cells did not appear to be like those from XP or CS patients in terms of UDS, RRS, and RDS.

RESULTS AND DISCUSSION

Clinical state of the patient

KPSX7, a 67-year-old Japanese woman, gave a history of freckling in sun-exposed areas, which had developed since her teens. Otherwise, she had a typical sun exposure history with an indoor occupation, and she had never suffered any other symptoms of sunburn. There was no risk of exposure to UV or other DNA-damaging agents in her job. She had developed several verrucae and undiagnosed skin tumors since her 20s, and many actinic keratoses, basal cell carcinomas, and squamous cell carcinomas since her 50s in sun-exposed areas. She had more than 50 tumors on her hands, arms, and legs, but no tumors on her face (Figure 1). A skin biopsy was taken from each developing tumor, and if the histological findings revealed malignancy (Figure 2), the entire lesion was subsequently excised and the defect was closed with a skin graft. Her parents were not consanguineous. All her siblings had similar skin lesions. Physical examinations, including neurological status, were negative (Table 1). Results of blood chemistries, urinalysis including urinary porphyrin, fecal porphyrin analyses, and minimal erythema dose (99 mJ/cm^2) were normal.

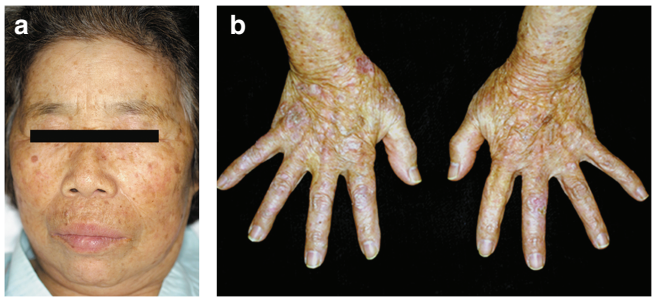

Figure 1. Clinical manifestations of patient KPSX7. Photograph of the face (**a**) and hands (**b**) of a 67-year-old Japanese woman.

Normal UV sensitivity of fibroblasts from the patient

We established primary fibroblasts from the patient and named them KPSX7. The UV sensitivity of KPSX7 cells was measured by a colony-forming assay in the presence or absence of caffeine after UV irradiation compared with Turu and Mori cells (normal primary fibroblasts). KPSX7 cells showed similar survival to Turu cells when the cells were irradiated with a low dose of UV (Figure 3a). The presence of caffeine in the medium did not affect post-UV survival. Moreover, KPSX7 showed similar sensitivity to Turu and Mori cells when the cells were irradiated with a high dose of UV (Figure 3b).

KPSX7 cells differ from XP and CS cells

As the clinical features of the patient resembled those of XP, despite the absence of photosensitivity (Table 1), we examined UDS and RRS in the KPSX7 cells and compared them with normal and XP-A primary fibroblasts. Turu cells showed numerous dots in their nuclei after UV irradiation (Figure 4a). In contrast, KPSX3 (XP-A) cells displayed few dots, indicating that normal fibroblasts have robust UDS activity, whereas XP-A fibroblasts have impaired UDS activity. KPSX7 cells showed a similar number of dots to Turu cells, indicating that KPSX7 cells have normal UDS activity. We then examined RRS in KPSX7 cells after UV

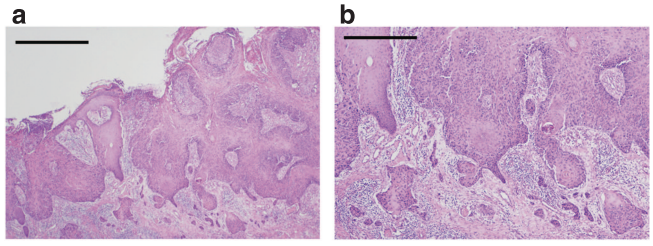

Figure 2. Histological findings of the squamous cell carcinoma from patient's hand. The lesion showed proliferation of atypical squamous cells with hyperkeratosis and dyskeratotic cells. (**a**) Bar = 1 mm; (**b**) 500 μm.

Table 1. Clinical features of XP, CS, and the patient

Clinical features	XP	CS	Patient
Skin			
Acute sunburn	+	+	–
Skin tumors	+	–	+
Freckling	+	–	+
Others			
Neurological findings	±[1]	+	–
Cachectic dwarfism	–	+	–
Pupillary abnormalities	–	+	–
Osteoporosis	–	+	–

CS, Cockayne syndrome; XP, xeroderma pigmentosum.
[1]XP complementation groups C, E, F, and V do not show neurological findings.

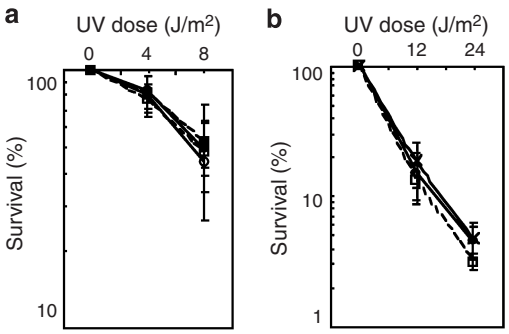

Figure 3. UV survival of KPSX7 cells established from the patient. Appropriate numbers of cells were inoculated into 100-mm dishes. After UV irradiation at (**a**) 0–8 J/m^2 (**b**) or 0–24 J/m^2, cells were incubated in the presence or absence of 1 mM caffeine for 14 days, fixed, and stained with Giemsa. Each point represents the average of three experiments (mean ± SD). Turu cells (○) and Mori cells (×) were used as normal controls. KPSX7 cells (□); Turu cells with 1 mM caffeine (●); KPSX7 cells with 1 mM caffeine (■).

irradiation (Figure 4b). The number of dots in KPSX7 cells decreased dramatically at 3 hours post-UV irradiation, and had recovered at 24 hours post-irradiation, indicating that KPSX7 cells have normal RRS activity. Since KPSX7 cells showed normal UDS and RRS, we concluded that KPSX7 cells were different from both XP-A through G cells (except for XP-E) and CS cells (Table 2).

Since reduced sensitivity after UV irradiation with caffeine is a biological characteristic of XP-V cells (Broughton *et al.*, 2002), KPSX7 cells apparently differ from XP-V cells (Figure 3a). However, it is still possible that KPSX7 cells are XP-V cells with unusual properties. XP-V cells show normal NER, but are defective in translesion synthesis. We next measured RDS and performed post-replication repair assay in KPSX7 cells to ascertain whether they were similar to XP-V cells. We determined RDS by counting the number of S-phase cells in cells irradiated with UV *versus* that in non-irradiated cells (Figure 4c) (Itoh *et al.*, 1996). The DNA synthesis rate after

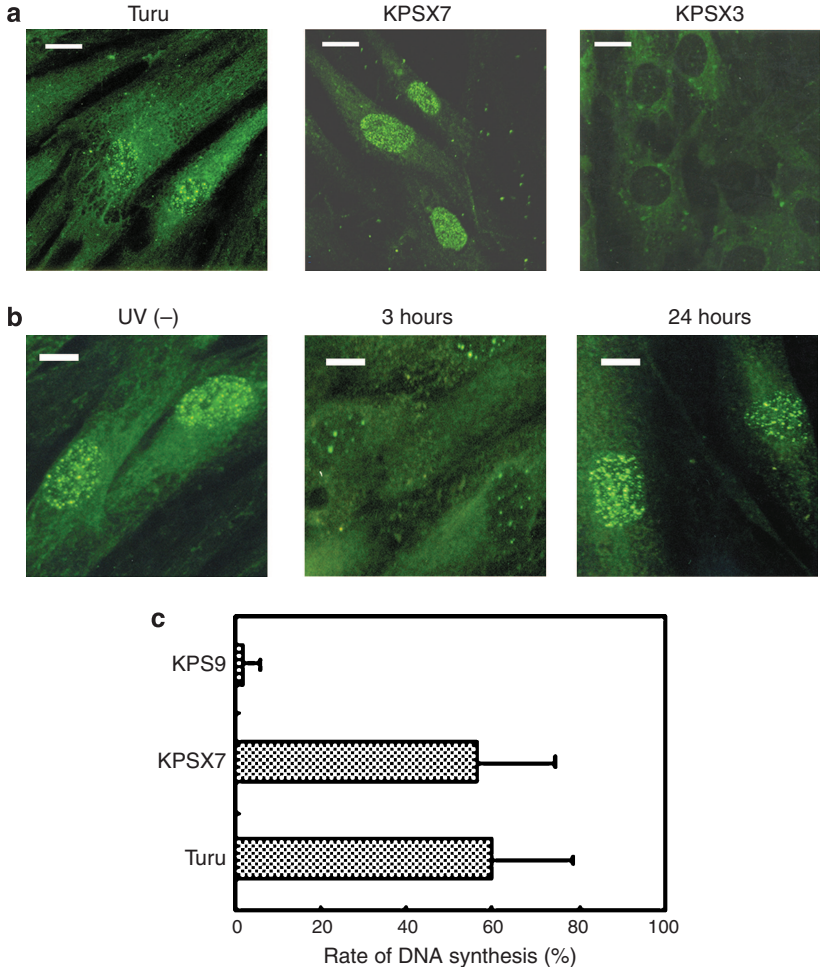

Figure 4. Normal levels of UDS, RRS, and RDS in KPSX7 cells. (**a**) Normal UDS of KPSX7 cells. Turu (normal fibroblasts), KPSX7, or KPSX3 (XP-A) cells were irradiated with UV at 30 J/m^2, incubated for 1 hour with BrdU, and processed for immunostaining. Bar = 20 μm (**b**) Normal RRS in KPSX7 cells. KPSX7 cells were irradiated with UV at 30 J/m^2 and incubated for 3 or 24 hours. The cells were microinjected with BrU into the cytoplasm, incubated for 0.5 hour, and processed for immunostaining for BrU. Bar = 20 μm (**c**) Normal RDS in KPSX7 cells. The cells were irradiated with UV at 30 J/m^2, incubated for 2 hours, labeled with BrdU for 1 hour, and processed for immunostaining. RDS was calculated as the percentage of BrdU-positive cells with *versus* without UV irradiation. Each point represents the average of three experiments (mean ± SD).

Table 2. Biological Characteristics of XP, CS, and the patient

Laboratory findings	XP (without E and V)	XP-E	XP-V	CS	Patient
UV sensitivity	+	–	–	+	–
Enhancement of UV Sensitivity by caffeine	–	–	+	–	–
Depress of UDS	+	–	–	–	–
Depress of RRS	±[1]	–	–	+	–
Depress of RDS	ND	–	+	ND	–
Depress of DDB activity	–	+	–	–	–
Depress of HCR	+	ND	ND	+	+

CS, Cockayne syndrome; DDB, UV-damaged DNA-binding protein; HCR, host-cell reactivation; ND, not determined; RDS, recovery of replicative DNA synthesis; RRS, recovery of RNA synthesis; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum.
[1]XP complementation group C does not depress.

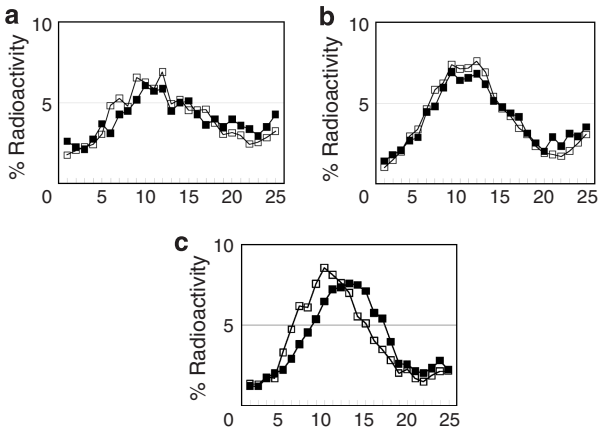

Figure 5. Proficient postreplication repair of KPSX7 cells. Cells were irradiated with UV at 5 J/m^2, grown for 1 hour, and then pulse labeled with [^{3}H]thymidine. Centrifugation was performed for 90 minutes at 50,000 r.p.m. The top of gradient is to the right. □, unirradiated cells (control); ■, UV-irradiated cells. (**a**) Turu cells; (**b**) KPSX7 cells; (**c**) KPS9 cells.

UV irradiation of KPSX7 cells was similar to that of Turu cells (normal fibroblasts), whereas it was reduced to less than 5% in KPS9 cells (XP-V cells). Moreover, in the post-replication repair assay, KPSX7 cells had the same amount of size DNA synthesized *de novo* after UV irradiation as non-irradiated cells, as in Turu cells (Figure 5a and b). In contrast, KPS9 cells synthesized less DNA *de novo* after UV irradiation than non-irradiated cells (Figure 5c). Therefore, we concluded that KPSX7 cells differ from XP-V cells.

KPSX7 cells are different from XP-E cells

As XP-E cells show normal UDS, RRS, and RDS but are defective in DDB activity, we examined protein-binding ability to UV-damaged DNA by gel mobility shift assays to ascertain whether KPSX7 cells are similar to XP-E cells. Turu

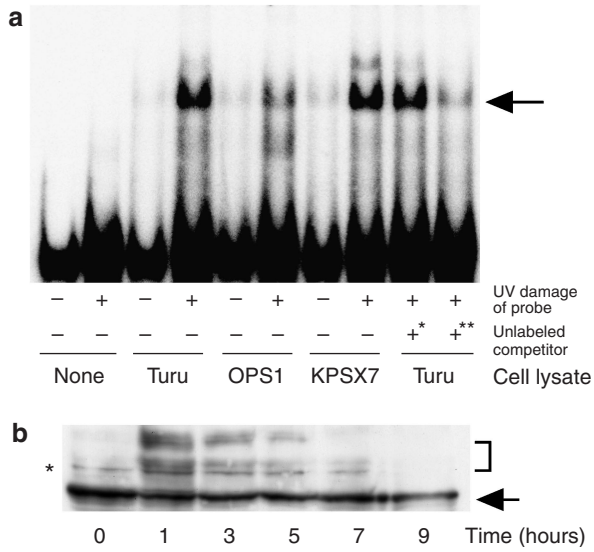

Figure 6. Normal DDB activity in KPSX7 cells. (**a**) Normal protein-binding ability to UV-irradiated DNA in KPSX7 cells. Whole-cell extract from Turu (normal), KPSX7, or OPS1 (XP-E) cells was incubated with ^{32}P-labeled UV-irradiated DNA as a probe, in the presence or absence of unlabeled competitor DNA (*non-irradiated and **UV-irradiated), and was then processed for electrophoresis to separate the protein-bound DNA fraction. The arrow indicates the protein-bound damaged DNA fraction. (**b**) Normal post-translational modification of XPC protein in KPSX7 cells. Cells were irradiated with UV at 10 J/m^2 and incubated at 37°C in the presence of 0.1 mM cycloheximide for the indicated times. Whole-cell extracts were prepared and subjected to immunoblotting with an anti-XPC antibody. The arrow and bracket indicate unmodified and polyubiquitinylated forms of XPC, respectively. The asterisks indicate nonspecific crossreacting bands.

cells showed robust protein-binding ability to UV-damaged DNA, whereas OPS1 cells (XP-E) showed impaired protein-binding ability (Figure 6a). KPSX7 cells showed similar binding activity to that of Turu cells. These results indicate that KPSX7 cells are different from XP-E cells. The XPC protein is known to be polyubiquitinated in normal fibroblasts upon UV irradiation, although XPC is not ubiquitinated in XP-E cells (Sugasawa *et al.*, 2005). As shown in Figure 6b, KPSX7 cells exhibited polyubiquitination of XPC upon UV irradiation. These results also indicate that KPSX7 cells are different from XP-E cells.

KPSX7 cells are defective in some DNA repair mechanism(s)

Although KPSX7 cells are different from XP and CS cells, it is possible that the KPSX7 cells have defects in some type of DNA repair because the patient exhibited considerable actinic keratosis, basal cell carcinomas, and squamous cell carcinomas in sun-exposed areas. It is widely accepted that DNA repair plays an important role in preventing genetic susceptibility to cancer. Thus, we tested DNA repair capacity in KPSX7 cells by a HCR assay with UV-irradiated plasmids containing a luciferase reporter gene. KPSX7 cells showed similar luciferase activity to those of Turu and Mori cells (NER-proficient fibroblasts) when plasmid DNA irradiated with 250 J/m^2 of UV was used. However, KPSX7 cells showed lower luciferase activity than those of normal fibroblasts

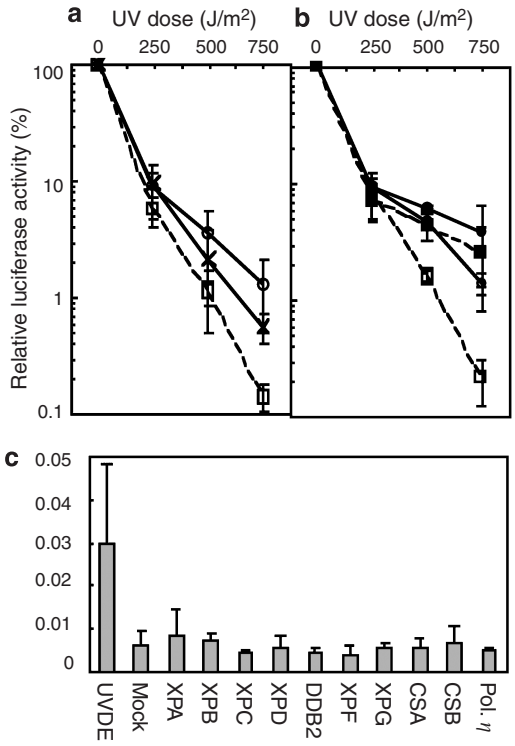

Figure 7. Defective HCR in KPSX7 cells. (**a**) KPSX7 (□) cells and normal fibroblasts, Turu (○), and Mori (×), were transfected with plasmids that were irradiated with the indicated amounts of UV, and then incubated for 48 hours. Lysate from the cells were subjected to an assay for the reporter gene product (luciferase activity). Each point represents the average of three experiments (mean±SD). (**b**) Restoration of HCR in KPSX7 cells by expression of UVDE. Turu with mock plasmid (○), Turu with UVDE expression plasmid (●), KPSX7 with mock plasmid (□), or KPSX7 with UVDE expression plasmid (■). Cells were treated by the same method as (**a**) except that the cells were co-transfected with the UVDE expression plasmid or the mock plasmid. (**c**) Restoration of HCR in KPSX7 cells by introduction of NER factors expression vector.

when plasmid DNA irradiated with 750 J/m^2 of UV was used (Figure 7a). Since high UV dose induce some single- or double-stranded DNA breaks and interstrand cross-links, we examined HCR of KPSX7 cells when a plasmid containing single- or double-stranded DNA breaks and interstrand cross-links was used (Figures S1b–d). KPSX7 cells showed similar luciferase activity to that in normal fibroblasts suggesting a normal repair activity of strand breaks and interstrand cross-links in KPSX7 cells. To confirm that KPSX7 cells are defective in some type of DNA repair, we examined whether the defective luciferase activity in the HCR assay could be restored by introduction of UVDE into the cells. Indeed, luciferase activity in KPSX7 cells was restored to the same level as that in Turu cells (normal fibroblasts) by introducing a plasmid expressing UVDE (Figure 7b). Because UVDE is known to incise DNA containing CPD, 6-4PP, or AP sites (Freyer *et al.*, 1995; Yasui and McCready, 1998; Kanno *et al.*, 1999), we next examined whether KPSX7 cells exhibited HCR when a plasmid containing AP sites was used. KPSX7 cells showed similar luciferase activity to that in normal fibroblasts (Figure S1a), suggesting a normal repair activity of AP sites in KPSX7 cells.

These results suggested that KPSX7 cells had a new form of DNA repair defect, although a possibility still existed that KPSX7 cells possessed a form similar to XP or CS, but with unusual properties. To examine further this possibility, we assessed whether expression of NER factors or polymerase η could restore the defective HCR activity in KPSX7 cells. The expression of NER factors or polymerase η did not correct the defective HCR activity in KPSX7 cells (Figure 7c).

Efficient repair of 6-4PP and CPD in KPSX7 cells

To confirm that 6-4PP and CPD defects were efficiently repaired in KPSX7 cells, we measured the amount of the photoproducts by ELISA after UV irradiation. KPSX7 cells showed a similar pattern of repairing 6-4PP and CPD to that of MSU2 cells (normal fibroblasts) (Figure 8), indicating that KPSX7 cells had normal efficiency in repairing the photoproducts.

In this study, we established KPSX7 cells from an elderly Japanese woman who exhibited many freckles, actinic keratosis, basal cell carcinomas, and squamous cell carcinomas. KPSX7 cells showed similar UDS, RDS, RRS, and DDB activity to those of wild-type human fibroblasts, suggesting that KPSX7 cells are different from those of XP and CS. KPSX7 cells showed only reduced HCR activity. It was reported that the HCR repair capacity for UV-induced DNA damage is reduced in elderly human cells (Takahashi *et al.*, 2005) and HCR activity seems to be decreased by *in vitro* as cultured cells age (Moriwaki *et al.*, 1996), although this is controversial (Merkle *et al.*, 2004). KPSX7 cells did show reduced HCR activity when a high-dose UV-irradiated plasmid was used in the assay, and we used KPSX7 cells at less than 17 passages and Turu cells (normal fibroblasts) at 16–24 passages. Moreover, the patient had exhibited skin lesions since her 20s. Thus, we concluded that aging was not responsible for the KPSX7 cells showing reduced HCR activity.

We suggest two possibilities as to why KPSX7 cells apparently have defective repair of high-dose UV-damaged DNA; first, we suspect that some minor DNA lesions, other than 6-4PP, CPD, or AP sites, were not repaired in KPSX7 cells and that these minor lesions may have induced skin cancer in the patient. Data supporting the possibility are as follows. The HCR activity recovered when UVDE was expressed in the cells, and UVDE is known to repair DNA containing 6-4PP, CPD, or AP sites (Freyer *et al.*, 1995; Yasui and McCready, 1998; Kanno *et al.*, 1999). In KPSX7 cells, however, 6-4PP and CPD were repaired with almost equal efficiency to that seen in normal human fibroblasts, as assessed by ELISA. Moreover, KPSX7 cells showed normal HCR activity when a plasmid containing AP sites was used in the assay. Thus, KPSX7 cells were apparently able to repair 6-4PP, CPD, and AP sites, suggesting that other lesions were not repaired in these cells.

Alternatively, KPSX7 cells may be defective in a minor subpathway of NER, which proceeds via at least two alternative pathways. One is transcription-coupled DNA

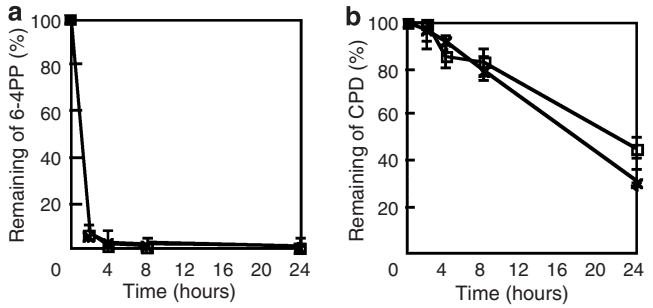

Figure 8. Ability to repair CPD and 6-4PP in genomic DNA. KPSX7 (□) cells or MSU2 (×), were irradiated with 10 J/m^2 UV and incubated for the indicated periods. At each time point, genomic DNAs were purified from the cells and used for quantitation of 6-4PP (**a**) or CPD (**b**) by ELISA. Each point represents the average of three experiments (mean ± SD).

repair, which removes lesions only in the actively transcribed DNA strand, and another is global genome repair (GGR), which removes lesions in any sequence of the genome (Hanawalt, 2002). We suggest the existence of another possible subpathway of NER, different from transcription-coupled DNA repair or GGR, which normally repairs 6-4PP or CPD in specific regions of the genome, but which is non-functional in KPSX7 cells.

Although XP patients commonly have skin cancers, CS patients, defective in transcription-coupled DNA repair, do not (Lehmann, 2003). This difference between XP and CS may be caused by faulty repair of particular types of photoproducts in a specific region of the genome that provokes chromosome instability (Parris and Kraemer, 1993; Bridges, 1998; Cleaver, 2005). The skin cancers of sun-exposed areas are commonly induced by several conditions other than XP. Nishigori *et al.* (1996) reported that unrepaired UV-induced DNA damage in the skin activates the production of cytokines that downregulate the immune response. Halliday (2005) reviewed how UV causes inflammation, gene mutation, and immunosuppression in the skin through oxidative damage, and these processes contribute to photocarcinogenesis. To ascertain whether KPSX7 cells are defective for any other DNA damage repair than NER, we investigated the sensitivities of KPSX7 cells to X-rays, methyl methanesulfonate (MMS), mitomycin C (MMC), or H_2O_2 (Figure S2). However, KPSX7 cells showed normal sensitivity to these agents. We suggest that faulty repair of a lesion in a specific region of the genome or unknown lesions was a reasonable cause of the skin cancers in our patient.

Finally, we recommend that the HCR assay be used to evaluate the UV-damaged DNA repair capacity in patients who have multiple skin cancers in sun-exposed areas, similar to our patient, and that a group including patients with defective HCR, but normal function in other DNA repair tests, be collected. A new pathological model may then be established by investigating the genetic backgrounds of these patients. Further study of these cells may provide a clue to the mechanism of UV-induced skin carcinogenesis.

MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee of the University of Kumamoto and was conducted according to the Declaration of Helsinki Principles. Informed consent was obtained from the patient.

Cells and culture conditions

We cultured primary human fibroblasts KPSX7 (established from our patient), KPSX3 (XP-A), OPS1 (XP-E), KPS9 (XP-V), MSU2 (a generous gift from Dr Toshio Mori, Nara Medical University), Mori, and Turu (wild-type, NER-proficient) in Dulbecco's modified Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) at 37°C and 5% CO_2.

UV survival assay

Appropriate numbers of cells were seeded into 10-cm dishes. After 10 hours, cells were washed with phosphate-buffered saline (PBS) and UV-irradiated at various doses. Cells were then incubated for 2 weeks. Colonies were fixed with 50% methanol, stained with 5% Giemsa's solution, and counted under a microscope.

UDS

Cells were seeded on coverslips and incubated until subconfluent. Cells were washed with PBS, irradiated with UV at 30 J/m^2, labeled for 1 hour in medium containing 20 μM BrdU, and fixed with 70% ethanol. The samples were denatured with 2 M HCl and washed with PBS. The cells were incubated with diluted anti-BrdU antibody (PharMingen, San Diego, CA) in PBS for 1 hour at room temperature. We then washed the cells in PBS and incubated them with the FITC-conjugated anti-mouse secondary antibody. After washing the cells on the coverslips, they were mounted on glass slides and examined under a fluorescence microscope.

RRS

Cells on coverslips were washed with PBS, irradiated with UV at 30 J/m^2, and incubated for various times. We then microinjected BrU into the cytoplasm of the cells, incubated them for 0.5 hour, and fixed the samples. The BrU incorporated into RNA was then detected by the method described for UDS except for the denaturing step.

RDS

Cells on coverslips were treated with or without UV radiation at 30 J/m^2, incubated for 2 hours, and labeled for 1 hour in medium containing 20 μM BrdU. Samples were fixed and denatured. The BrdU incorporated into DNA was detected by the method described for UDS.

Post-replication repair assay

Semiconservative DNA replication after UV irradiation was measured by a method described elsewhere (Itoh *et al.*, 1995). Briefly, cells were irradiated with UV at 15 J/m^2 and incubated for 60 minutes before being labeled with [^{3}H]thymidine (10 μCi/ml) for 30 minutes. Cells were lysed with (0.2 M NaOH with 0.02 M EDTA) and X-ray-irradiated at 20 Gy on ice. Cell suspensions were layered onto 5–20% alkaline sucrose gradients with 0.1 M NaOH and centrifuged at 50,000 r.p.m. for 90 minutes at 4°C in a P56ST rotor (Hitachi, Tokyo, Japan). After centrifugation, drop fractions were

collected on Whatman Grade 17 paper strips, and the acid-insoluble radioactivity was determined in a liquid scintillation counter.

Gel mobility shift assay

A gel shift assay was performed as described elsewhere (Chu and Chang, 1988; Keeney *et al.*, 1992; Itoh *et al.*, 1999), with some modifications. Briefly, ^{32}P-labeled 66-base pair duplex DNA (0.2 ng) irradiated with 6 kJ/m^2 was incubated with whole-cell extracts (3 μg) for 15 minutes at 30°C. Samples were subjected to 5% polyacrylamide gel electrophoresis in Tris–borate/EDTA buffer. Dried gels were analyzed using a Fujix Bio-Analyzer BAS-2000 (Fuji Photo Film, Kanagawa, Japan).

Immunoblot

Whole-cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated with anti-XPC antibody and followed by the ECL Western blotting detection system (GE Healthcare, Buckinghamshire, UK).

Preparation of plasmids containing specific DNA lesions

The plasmid pRL-CMVluc (4,797 bp) containing the cytomegalovirus immediate-early enhancer and promoter, along with a luciferase gene, was used for the HCR assay. Treatment of the plasmid DNA was as described elsewhere (Spivak and Hanawalt, 2006), with some modifications. Briefly, to introduce photoproducts, plasmid in Tris–EDTA buffer on ice was irradiated with UVC light (254 nm) from a germicidal lamp for various times. To introduce AP sites, plasmid DNA was mixed with 9 vol of sodium citrate buffer (10 mM sodium citrate, 100 mM NaCl, adjusted to pH 4.0 with 10 mM citric acid) and incubated at 60°C for various times. Samples were mixed with an equal volume of 10 × endonuclease III reaction buffer (Trevigen, Gaithersburg, MD) and chilled on ice. Single-strand breaks were generated by treatment of AP site-containing plasmid with T4 endonuclease V for 60 minutes at 37°C. Double-strand breaks were generated by treatment of plasmid with restriction enzyme, *Ehe*1. To introduce interstrand cross-links, plasmid was reacted with mitomycin C and sodium borohydride. The cross-linking reaction was initiated by the addition of 1 vol% of 20 mM $NaBH_4$ for 10 minutes at 37°C.

HCR assay

Cells were plated in 24-well dishes, incubated overnight, and transfected with the plasmids indicated using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C for 48 hours, and then were lysed in reporter lysis buffer (Promega, Madison, WI). Supernatant of the cell extract was mixed with Luciferase Assay Substrate (Promega) at room temperature, and luciferase activity was measured with a Mini Lumat LB9506 (Perkin-Elmer, Wellesley, MA).

In vivo repair assay

Cells were irradiated with 10 J/m^2 UVC light (254 nm) using a germicidal lamp and incubated for various times. Genomic DNA was purified with a DNeasy kit (Qiagen, Valencia, CA), and the amount of CPD or 6-4PP in the DNA was determined by ELISA using specific mAbs (TDM-2 or 64M-5, respectively), as described previously (Mori *et al.*, 1991; Wakasugi *et al.*, 2002).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Normal HCR in KPSX7 with variety damaged plasmid.

Figure S2. Normal sensitivity in KPSX7 cells to DNA-damaging agents.

REFERENCES

Afaq F, Adhami VM, Mukhtar H (2005) Photochemoprevention of ultraviolet B signaling and photocarcinogenesis. *Mutat Res* 571:153–73

Bridges BA (1998) UV-induced mutations and skin cancer: how important is the link? *Mutat Res* 422:23–30

Broughton BC, Cordonnier A, Kleijer WJ, Jaspers NG, Fawcett H, Raams A *et al.* (2002) Molecular analysis of mutations in DNA polymerase eta in xeroderma pigmentosum-variant patients. *Proc Natl Acad Sci USA* 99:815–20

Chu G, Chang E (1988) Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. *Science* 242:564–7

Cleaver JE (2005) Cancer in xeroderma pigmentosum and related disorders of DNA repair. *Nat Rev Cancer* 5:564–73

Daya-Grosjean L, Sarasin A (2005) The role of UV induced lesions in skin carcinogenesis: an overview of oncogene and tumor suppressor gene modifications in xeroderma pigmentosum skin tumors. *Mutat Res* 571:43–56

Feldberg RS, Grossman L (1976) A DNA binding protein from human placenta specific for ultraviolet damaged DNA. *Biochemistry* 15:2402–8

Freyer GA, Davey S, Ferrer JV, Martin AM, Beach D, Doetsch PW (1995) An alternative eukaryotic DNA excision repair pathway. *Mol Cell Biol* 15:4572–7

Halliday GM (2005) Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat Res* 571:107–20

Hanawalt PC (2002) Subpathways of nucleotide excision repair and their regulation. *Oncogene* 21:8949–56

Hussein MR (2005) Ultraviolet radiation and skin cancer: molecular mechanisms. *J Cutan Pathol* 32:191–205

Itoh T, Fujiwara Y, Ono T, Yamaizumi M (1995) UV^s syndrome, a new general category of photosensitive disorder with defective DNA repair, is distinct from xeroderma pigmentosum variant and rodent complementation group 1. *Am J Hum Genet* 56:1267–76

Itoh T, Mori T, Ohkubo H, Yamaizumi M (1999) A newly identified patient with clinical xeroderma pigmentosum phenotype has a non-sense mutation in the DDB2 gene and incomplete repair in (6-4) photoproducts. *J Invest Dermatol* 113:251–7

Itoh T, Ono T, Yamaizumi M (1996) A simple method for diagnosing xeroderma pigmentosum variant. *J Invest Dermatol* 107:349–53

Kanno S, Iwai S, Takao M, Yasui A (1999) Repair of apurinic/apyrimidinic sites by UV damage endonuclease: a repair protein for UV and oxidative damage. *Nucleic Acids Res* 27:3096–103

Kataoka H, Fujiwara Y (1991) UV damage-specific DNA-binding protein in xeroderma pigmentosum complementation group E. *Biochem Biophys Res Commun* 175:1139–43

Keeney S, Wein H, Linn S (1992) Biochemical heterogeneity in xeroderma pigmentosum complementation group E. *Mutat Res* 273:49–56

Kraemer KH, Lee MM, Andrews AD, Lambert WC (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, Slor H (1985) Xeroderma pigmentosum. *Clin Dermatol* 3: 33–69

Lehmann AR (2003) DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* 85:1101–11

Lippke JA, Gordon LK, Brash DE, Haseltine WA (1981) Distribution of UV light-induced damage in a defined sequence of human DNA: detection of alkaline-sensitive lesions at pyrimidine nucleoside-cytidine sequences. *Proc Natl Acad Sci USA* 78:3388–92

Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M *et al.* (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature* 399:700–4

Merkle TJ, O'Brien K, Brooks PJ, Tarone RE, Robbins JH (2004) DNA repair in human fibroblasts, as reflected by host-cell reactivation of a transfected UV-irradiated luciferase gene, is not related to donor age. *Mutat Res* 554:9–17

Mitchell DL, Nairn RS (1989) The biology of the (6-4) photoproduct. *Photochem Photobiol* 49:805–19

Mori T, Nakane M, Hattori T, Matsunaga T, Ihara M, Nikaido O (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4) photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. *Photochem Photobiol* 54:225–32

Moriwaki S, Ray S, Tarone RE, Kraemer KH, Grossman L (1996) The effect of donor age on the processing of UV-damaged DNA by cultured human cells: reduced DNA repair capacity and increased DNA mutability. *Mutat Res* 364:117–23

Nance MA, Berry SA (1992) Cockayne syndrome: review of 140 cases. *Am J Med Genet* 42:68–84

Nishigori C, Yarosh DB, Ullrich SE, Vink AA, Bucana CD, Roza L *et al.* (1996) Evidence that DNA damage triggers interleukin 10 cytokine production in UV-irradiated murine keratinocytes. *Proc Natl Acad Sci USA* 93:10354–9

Parris CN, Kraemer KH (1993) Ultraviolet-induced mutations in Cockayne syndrome cells are primarily caused by cyclobutane dimer photoproducts while repair of other photoproducts is normal. *Proc Natl Acad Sci USA* 90:7260–4

Prakash S, Prakash L (2000) Nucleotide excision repair in yeast. *Mutat Res* 451:13–24

Qiao Y, Spitz MR, Guo Z, Hadeyati M, Grossman L, Kraemer KH *et al.* (2002) Rapid assessment of repair of ultraviolet DNA damage with a modified host-cell reactivation assay using a luciferase reporter gene and correlation with polymorphisms of DNA repair genes in normal human lymphocytes. *Mutat Res* 509:165–74

Spivak G, Hanawalt PC (2006) Host cell reactivation of plasmids containing oxidative DNA lesions is defective in Cockayne syndrome but normal in UV-sensitive syndrome fibroblasts. *DNA Repair (Amst)* 5:13–22

Sugasawa K, Okuda Y, Saijo M, Nishi R, Matsuda N, Chu G *et al.* (2005) UV-induced ubiquitylation of XPC protein mediated by UV-DDB–ubiquitin ligase complex. *Cell* 121:387–400

Takahashi Y, Moriwaki S, Sugiyama Y, Endo Y, Yamazaki K, Mori T *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

Wakasugi M, Kawashima A, Morioka H, Linn S, Sancar A, Mori T *et al.* (2002) DDB accumulates at DNA damage sites immediately after UV irradiation and directly stimulates nucleotide excision repair. *J Biol Chem* 277:1637–40

Yasui A, McCready SJ (1998) Alternative repair pathways for UV-induced DNA damage. *Bioassays* 20:291–7