Homozygous R788W Point Mutation in the *XPF* Gene of a Patient with Xeroderma Pigmentosum and Late-Onset Neurologic Disease

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The second Caucasian xeroderma pigmentosum patient (XP42RO) belonging to complementation group F (XP-F) is described. Mild ocular photophobia was present from childhood, and acute skin reactions occurred upon exposure to sunlight. Basal and squamous cell carcinomas developed after his twenty-seventh year. In his late forties progressive neurologic symptoms emerged, which included intellectual decline, mild chorea and ataxia, and marked cerebral and cerebelar atrophy. Such neurologic abnormalities are very unusual in XP-F. Similar symptoms have been described in only one of 17 other XP-F individuals. His ≈5-fold reduced activity of nucleotide excision repair in cultured cells, combined with moderately affected cell survival and DNA replication after UV exposure, are typical of XP-F. The recent cloning of the *XPF* gene allowed a molecular genetic analysis of this unusual patient. XP42RO, representing the second case studied in this respect, turned out to be homozygous for a point mutation in the *XPF* gene, causing an R788→W substitution in the encoded protein. Surprisingly, this mutation had also been found in one allele of the other unrelated Caucasian XP-F case. The amount of mutated XPF protein is strongly reduced in cells from XP42RO, presumably due to a conformational change. Biochemical, genetic, and clinical data all indicate the presence of considerable residual repair activity, strongly suggesting that the R788W mutation is leaky. ***Key words: ERCC1/nucleotide excision repair/skin cancer. J Invest Dermatol 110:832–836, 1998***

The autosomal recessive disease xeroderma pigmentosum (XP) is clinically manifested by photosensitivity of skin and eyes and early onset of freckling and other lesions on sun-exposed skin, culminating in a high propensity to neoplasms, especially carcinomas. Progressive neurologic degeneration occurs in some XP patients, varying from central nervous system abnormalities to, in rare cases, additional peripheral nerve dysmyelination characteristic of Cockayne syndrome (Cleaver and Kraemer, 1994). The photosensitivity of XP patients is due to defective nucleotide excision repair (NER), a process responsible for the removal of DNA damage induced by UV and diverse chemical mutagens. NER is a complex mechanism, requiring the correct interplay of about 30 different genes (Aboussekhra *et al*, 1995; Mu *et al*, 1995). Seven of these, named *XPA* to *XPG*, were found to be defective in XP patients. The XPA, XPC, and XPE proteins are probably involved in damage recognition, whereas XPB and XPD mediate subsequent local unwinding of the DNA helix. XPF and XPG are required for strand cleavage at either side of the DNA lesion. In the final stages of NER, the damaged patch is removed and replaced using enzymes from the DNA replication machinery.

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Abbreviations: NER, nucleotide excision repair; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum.

Most NER defective XP patients are affected in *XPA* or *XPC*, followed by *XPD*. *XPF* and *XPG* defects are less frequent and only rare cases with XP-B and XP-E have been described. A patient with a mutated *XPF* gene, first identified in 1979 by genetic complementation analysis (Arase *et al*, 1979), showed relatively mild cutaneous symptoms and no neurologic involvement. This picture is characteristic of the XP-F patients reported later on, all from Japan (Nishigori *et al*, 1986; Yamamura *et al*, 1989) (see **Table II**) except for one from Europe (Norris *et al*, 1988). One exceptional Japanese case was identified having neurologic abnormalities (Moriwaki *et al*, 1993). Here we describe a European XP-F patient with multiple carcinomas and other mild oculocutaneous symptoms, who developed marked neurodegeneration after his late forties. In view of this unusual clinical picture, we conducted detailed biologic and biochemical studies of his NER capabilities. After the recent cloning of the *XPF* gene (Sijbers *et al*, 1996a), molecular genetic analysis has also become possible. Here we report the second XP-F patient studied in this respect. To our surprise, we found a homozygous mutation, identical to one of the *XPF* alleles of the first patient.

MATERIALS AND METHODS

Case report Patient XP42RO, aged 62 y, is one of nine siblings from nonconsanguineous parents of Dutch origin. From about 10 y of age he developed irregularly pigmented macules and telangiectasia on cheeks, temples, and dorsa of hands. From his fifteenth to twenty-seventh year he worked outdoors as a farm laborer, experiencing frequent acute sunburn reactions. Since his thirties, nine basal or squamous cell carcinomas have been removed from sun-exposed areas. Mild photophobia and occasional conjunctival vasoinjection were present from childhood. At age 47, his 24 h and 72 h MED were 0.08

and 0.04 J per cm^2 of UV light, respectively, corresponding to the most sensitive type I skin individuals.

After the age of 47, neurologic symptoms started to appear in the form of progressive dysarthria, clumsiness, restlessness, and a mild chorea. Ataxia with unsteady gait, dysmetria of the limbs, and saccadic dysmetria of ocular movement are now present. Nuclear magnetic resonance showed a corresponding marked atrophy of the cerebelum and cerebral cortex. Electroencephalogram, reflexes, and audiogram were normal. Signs of central nerve conduction impairment were noted in somatosensory and motor evoked potentials. Electromyography findings suggested mild axonal polyneuropathy. Neurophysiologic testing revealed deficiencies in attention, concentration, and recall as well as visuospatial and constructional deficits. 1-chloro-2,4-dinitrobenzene testing (Bleumink *et al*, 1974) revealed a normal cell-mediated immune response. The patient is married without offspring, due to gonadal dysplasia. The family history revealed that sun sensitivity was also noted in the father, but without the development of severe skin lesions and neoplasms. None of the patient's siblings are known to have similar neurologic or cutaneous problems.

Cell strains and culture Primary skin fibroblasts were cultured routinely in Ham's F10 medium supplemented with 15% fetal bovine serum and antibiotics. Cell strains used were XP42RO (patient), 95RD28 (patient's father), XP25RO (XP-A), XP11BE (XP-B), XP20RO, XP21RO and XP3MA (XP-C), XP1BR and XP2CS (XP-D), XP2RO (XP-E), XP2YO and XP126LO (XP-F), XP3BR (XP-G), and C5RO (normal control).

DNA repair studies The UV source was a standard germicidal mercury tube emitting UVC rays of predominantly 254 nm. NER activity was assayed in cultured fibroblasts as unscheduled DNA synthesis (UDS), by measuring UV induced incorporation of tritiated thymidine in autoradiograms, as described elsewhere (Vermeulen *et al*, 1994a). The number of grains above the nuclei represents the cellular repair activity. The short-term effect of UV on nucleic acids synthesis was similarly assayed by [^{3}H]thymidine incorporation 16 h after irradiation (Hamel *et al*, 1996). A simplified assay was used for cellular survival (Hamel *et al*, 1996). In short, sparsely seeded, 2 d old cultures were exposed to graded UV doses. Three to five days after irradiation, the number of proliferating cells was estimated by liquid scintillation counting of tritiated thymidine incorporated during a 3 h pulse. For complementation analysis, XP cells were fused using Sendai virus and assayed for UDS after 24–48 h, as described (Keijzer *et al*, 1979).

General biochemical procedures Nucleic acids purification, restriction enzyme digestions, gel electrophoresis, and immunoblot analysis were performed according to standard procedures (Sambrook *et al*, 1989). Coupled *in vitro* transcription and translation reactions using the TNT reticulocyte lysate system were performed as described by the manufacturer (Promega, Madison, WI). Labeled translation products were analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Mutational analysis The *XPF* gene was amplified in two overlapping segments by polymerase chain reaction from cDNA prepared from total cell RNA isolated from cultured fibroblasts using random hexamers. Products were digested with different sets of restriction enzymes and 5′ end labeled with ^{32}P-phosphate (Sijbers *et al*, 1996a). Labeled single strands were separated based on conformation in nondenaturing polyacrylamide gels containing 5% glycerol and run at 4°C, as described (Liu and Sommer, 1995). Amplified genomic or cDNA fragments were directly sequenced (USB).

Microinjection *XPF* cDNA subcloned in a mammalian expression vector pcDNA3 (Invitrogen, Leek, the Netherlands) was microneedle-injected into one nucleus of XPF deficient XP126LO homopolykaryons. After a 24 h expression period, UV-UDS was measured in the injected cells (Vermeulen *et al*, 1994a).

RESULTS

DNA repair studies The overall activity of NER was assayed in cultured skin fibroblasts as UDS, i.e., UV-induced incorporation of tritiated thymidine was measured by autoradiography. In the patient, NER after exposure to 10 J per m^2 was reduced to 22% of normal levels (**Table I**, *top panel*) and varied between 15% and 30% on other occasions (not shown). NER in the cells of the father was in the normal range (see **Table I**). Genetic complementation analysis was performed to identify the gene defect. After fusion of XP42RO fibroblasts with one or two known representatives from all seven XP groups A through G (for strains see *Materials and Methods*), normal or near-normal UDS levels were obtained in all cases except for the XP-F strain XP126LO where UDS remained low in the heterokaryons

Table I. Correction of NER activity in XP42RO

Sample tested	Grains per nucleus[a]	Repair activity[b]
Standard NER assay		
XP42RO (patient)	12.9 ± 0.6 (50)	22
95RD28 (father)	55.4 ± 2.5 (50)	96
C5RO (normal control)	58.7 ± 2.7 (50)	–
Microinjection of XPF cDNA		
XP126LO uninjected	5.5 ± 0.6 (24)	27
XP126LO injected R788W cDNA	12.0 ± 0.7 (27)	58
XP126LO injected WT cDNA	22.9 ± 0.9 (27)	112
C5RO uninjected	20.6 ± 0.5 (29)	–

[a]Mean ± SEM (number of nuclei).
[b]Percentage of normal.

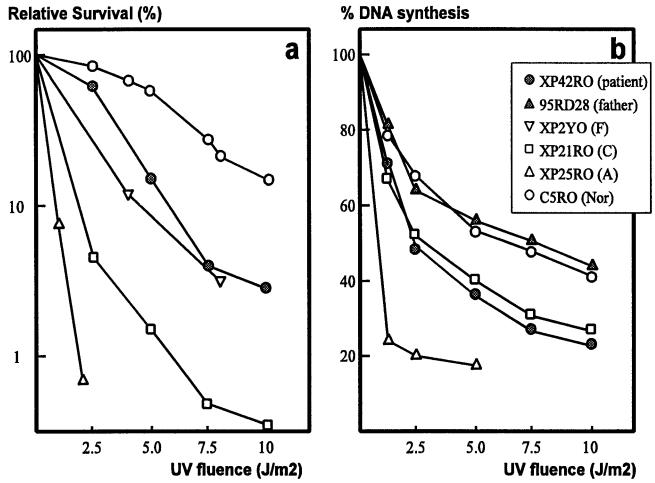

Figure 1. Cellular responses to UV exposure. (*a*) Survival assay (semilog scale); (*b*) inhibition of DNA synthesis measured 16 h after various doses of UV (linear scale). All points represent averaged duplicate measurements.

(data not shown). This result assigns the patient to XP group F. Cell survival studies showed that the relatively low NER activity resulted in only moderate UV cytotoxicity, much less marked than in XP cells from group A (**Fig 1*a***). This behavior is characteristic of XP-F as shown here for XP2YO (**Fig 1*a***) and for other XP-F patients elsewhere (Arase *et al*, 1979; Nishigori *et al*, 1986) (**Table II**). Also the consequences for the overall rates of DNA synthesis, measured 16 h after UV exposure, were only moderate: the inhibition was much less pronounced than in XP-A cells, and similar to an XP-C strain (**Fig 1*b***), known to be intermediate in this respect. The cells from the patient's father showed a normal behavior in the latter two assays (only DNA synthesis is shown in **Fig 1*b***).

Molecular genetic studies Recently, the *XPF* gene was cloned (Sijbers *et al*, 1996a), which allows mutational analysis of the affected gene in this patient. Initial restriction enzyme fingerprint analysis by single-strand conformation-sensitive gel electrophoresis indicated the presence of specific sequence abnormalities in the downstream end of cDNA amplified from XP42RO mRNA. These were partly similar to the ones observed in XP126LO, another XP-F individual (**Fig 2*a***). XP126LO had been shown earlier to carry a 4nt deletion in one allele, coding for a truncated protein, 803 aa in length, and a C→T transition in the other, changing amino acid residue 788 from arginine to tryptophan (R788W) (Sijbers *et al*, 1996a). Subsequent sequence analysis of the region in XP42RO revealed that this patient is homozygous for the R788W mutation (**Fig 2*b***). Investigation of genomic DNA verified the homozygous (or hemizygous) state of the patient and established the father as carrier (**Fig 2*b***). Material from the mother was not available for analysis.

Table II. Overview of clinical, photobiologic, and repair characteristics of published XP-F cases

Patient description				Skin characteristics							
Code	Sex	Age	Ref.[e]	Acute erythema	med/ peak	Freckles (AoO)	Tumors, no and type (AoO)[f]	Ocular	Neuro	%NER	UV^S
XP23OS	F	45	1			+ (6)	–		no	10	4.3x
XP101OS	F	49	2	+[d]	low	+	1 MF (30), 2 BCC, 1 KA (47)	no	no	15	2x
XP2YO[a]	F	64	3, 4			+	1 SSC (62), BDC († 65)		no	17	3.4x
XP3YO[a]	M	29	3, 4			+	1 KA (26)		no	15	2.8x
XP25KO	F	8	5, 6	+	low/24 h	+ (1.5)	–	no	no	10–15	3x
XP27KO[b]	F	11	5, 6	+	nor/24 h	+ (3)	–	no	no	10–15	3x
XP28KO[b]	F	8	5, 6	+		+ (3)	–	no	no	10–15	3x
XP41KO[b]	M	5	5, 6	+		+ (3)	–	no	no		
XP38KO	F	44	6, 7	+	nor/24 h	+	–	no	no	20–25	2.3x
XP46KO	F	61	6	+	nor/24 h	+ (14)	–	no	no	12	2.8x
XP7KA	F	42	6, 8	+	nor/24 + 72 h	+	1 BCC (40)		no	19	
XP90TO[c]	M	42	9	+	nor/48 h	+ (10)	6 BCC (42)	±	no	12	3x
XP92TO[c]	F	41	9	+	nor/48 h	+ (5)	1 BCC (41)	no	no	12	3x
XP107TO	F	73	3	+	low/48 + 72 h	+ (5)	2 BCC, 1 TF (72)		no	19	2.3x
Kps6	F	18	10	±		+ (11)	1 BCC (18)	no	no	20.5	1.3x
MNHN	M	48	11			+ (6)	1 BDC († 50)	no	yes	7	3.1x
XP126LO	F	22	12	+	nor/24 h	+ (10)	–	no	no	13	
XP42RO	M	62	this paper	+	nor/24 + 72 h	+ (10)	8 BCC, 1 SCC, 1 KA (27)	±	yes	15–30	2x

[a]Family relationship.
[b]Family relationship.
[c]Family relationship.
[d]After amoxicilin trihydrate treatment, not after UV.
[e]1, Arase *et al* (1979); 2, Nishigori *et al* (1986); 3, Nishigori *et al* (1991); 4, Takebe *et al* (1980); 5, Fujiwara *et al* (1985b); 6, Yamamura *et al* (1989); 7, Fujiwara *et al* (1985a); 8, Arase *et al* (1988); 9, Kondo *et al* (1989); 10, Itoh *et al* (1995); 11, Moriwaki *et al* (1993); 12, Norris *et al* (1988).
[f]AoO, age of onset; BCC, basal cell carcinoma; SCC, sqamous cell carcinoma; KA, keratoacanthoma; TF, trichofolliculoma; MF, mammary fibroadenoma; BDC, bile duct cancer.

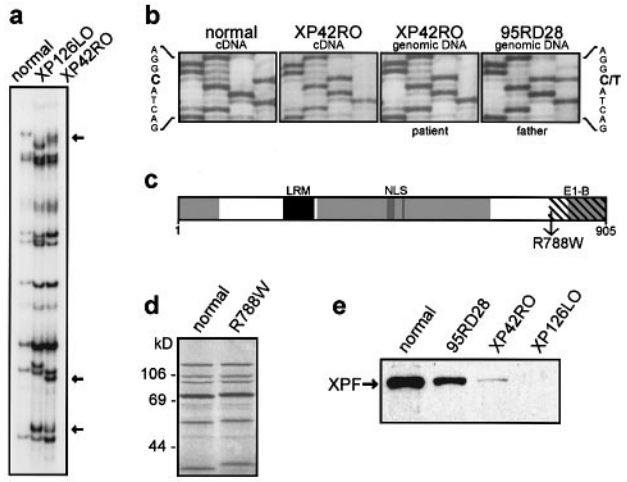

Figure 2. Mutational analysis of XP42RO. (*a*) Restriction enzyme fingerprint analysis. The restriction enzyme fingerprint is shown of the 3′ end of cDNA from normal, XP126LO, and XP42RO cells after digestion with EaeI, SacI, and StuI. *Arrows* indicate aberrant bands. (*b*) Sequence showing C→T transition in cDNA and genomic DNA. (*c*) Schematic structure of XPF protein with R788W mutation. Domains shown in white are highly conserved; LRM, leucine-rich motif; NLS, putative nuclear location signal; E1-B, region required for ERCC1 binding. (*d*) Migration of XPF (R788W) and normal XPF *in vitro* translated proteins in a denaturing gel. Bands smaller than the full-length protein of 115 kDa represent N-terminally truncated species, caused by translational starts on internal AUG sites. Aberrant slower mobility of mutated XPF is more readily visible in the shorter *C*-terminal products consistent with the mutation being situated in the *C*-terminal part of the protein. (*e*) Immunoblot of equal amounts of cell-free protein extracts with anti-XPF anti-serum (Sijbers *et al*, 1996a).

Consequences of the R788W mutation To determine the relevance of the observed change for *in vivo* NER, an *XPF* cDNA carrying the mutation was cloned in a mammalian expression vector with a strong promoter and microinjected into the nuclei of XP-F strain XP126LO. This overexpressed XPF species could correct the NER defect only very partially, whereas the nonmutated cDNA produces fully normal levels of NER upon microinjection (**Table I**, *bottom panel*) (Sijbers *et al*, 1996a); however, significant residual activity was evident, which shows that the R788W substitution is not a null-mutation. On the protein level, the mutation causes an aberrant electrophoretic mobility detected in denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *in vitro* translated polypeptides (**Fig 2*d***), indicative of a considerable conformational change. In XP42RO cell extracts, the amount of XPF protein is strongly reduced, as measured by immunoblotting (**Fig 2*e***). The levels were also low in XP126LO but were intermediate in the father (**Fig 2*e***), in agreement with his heterozygous state.

DISCUSSION

Patients with a mutated *XPF* gene are mostly found in Japan, where it is the most common form of XP after XP-A. Outside Japan, XP-F is rare. This patient XP42RO represents only the second Caucasian case ever reported. The first case was an English patient XP126LO (Norris *et al*, 1988). Characteristic of XP-F are mild cutaneous manifestations, relatively late onset of cutaneous carcinomas, and the absence of neurologic abnormalities. Clinical, photobiologic, and repair hallmarks of published XP-F cases are summarized in **Table II**.

Acute sun sensitivity reactions were reported in 14 of 18 patients. The subnormal MED peak at 24 h in our patient corresponded with that in seven of 10 other XP-F patients, whereas it was low in only three. Delayed or morphologically abnormal photosensitivity reactions have been reported in the majority of XP patients, consistent with the delayed reaction at 72 h seen in our patient.

Malignant skin tumors first appeared between 27 and 47 y of age, which is relatively late for XP patients in general, but early among the nine of 18 XP-F cases where tumors have arisen. The prevalence of only basal and squamous carcinomas, characteristic for XP-F, is also lower than in XP-A and XP-D patients. Obviously, tumor risk also depends on total UV exposure and medical care. Our patient, who had been extensively exposed to sunlight for at least 12 y, contrasts with nine tumor-free patients of whom four were still very young (**Table II**).

Residual repair activity is similar to the other XP-F patients. This 4–6 times reduction of overall NER rate seems to be in contradiction with an only moderate UV cytotoxicity and nucleic acids synthesis inhibition, a unique feature, typical for the XP-F group, also found in our patient. This behavior is thought to be caused by a partially impaired NER activity that proceeds longer and is less reduced at later times after exposure (Zelle *et al*, 1980; Arase *et al*, 1990). The rather mild cutaneous and ocular manifestations of XP-F are in line with these repair characteristics.

Whereas the UV-sensitive phenotype of our patient is rather typical of XP-F, the neurologic abnormalities that started to develop in the patient's late forties are unusual and have been reported in only one exceptional Japanese case so far (Moriwaki *et al*, 1993), with a type and onset of neurologic symptoms grossly similar to our case. Five of the other patients were much younger at the time of report, but in three aged 50–73 there was no evidence of neurologic problems. In general, central nervous system dysfunction is not uncommon in XP; however, usually only cases with the most severe photosensitivity are affected, such as those in groups A and D (Andrews *et al*, 1978), where the problems develop at an early age. Robbins *et al* (1993) reported neurologic degeneration in a mildly sensitive XP-C patient who was in her forties; however, in our case with comparable mild NER impairment the origin may be distinct and specifically related to the *XPF* defect as the course of the neurologic disease was different from that of the XP-C patient. Peripheral nervous system problems such as dysmyelination, which are not present in our case, are found in another repair disorder, Cockayne syndrome, and in rare cases that combine the features of XP and Cockayne syndrome (Nance and Berry, 1992). Such patients occur in XP groups B, D, and G only (Hamel *et al*, 1996; Vermeulen *et al*, 1997). Therefore, these abnormalities were proposed to be associated with subtle disturbances in basal transcription, a process requiring the proper functioning of the XPD and XPB proteins (Vermeulen *et al*, 1994b).

The nonstandard clinical picture of our patient prompted us to perform a more detailed molecular genetic analysis. The assignment to group F by complementation analysis was confirmed on the molecular level by the identification of a homozygous mutation in the *XPF* gene. A C→T transition (probably due to deamination of a methylated cytosine at a CpG site) at nucleotide 2377 changes the basic arginine residue 788 of the encoded protein into a hydrophobic tryptophan. This mutation is physiologically relevant and most likely responsible for the patient's NER defect, because overexpression of microinjected mutated cDNA cannot fully correct UDS in XP-F cells. Surprisingly, the same R788W mutation had been found earlier in one of the alleles of the unrelated English patient XP126LO (Sijbers *et al*, 1996a). Both this point mutation and the *C*-terminal truncation encoded by the second allele of XP126LO affect the region of the XPF protein that is required for stable physical interaction with another repair enzyme called ERCC1 (our unpublished data, see **Fig 2*c***). Uncomplexed, free XPF and ERCC1 proteins are unstable *in vivo* (Sijbers *et al*, 1996b). Therefore, the strongly reduced levels of XPF protein that we observed in both patients' cells (Sijbers *et al*, 1996a) and the deficiency of XP-F cells in ERCC1 protein (Biggerstaff *et al*, 1993; van Vuuren *et al*, 1995; Yagi *et al*, 1997) would be readily explained by this interaction failure; however, *in vitro*, the R788W protein could still bind ERCC1 (our unpublished data). It follows that the apparent XPF protein instability *in vivo* is related to the conformational changes that we detected in a denaturing gel.

In the process of NER, the XPF-ERCC1 protein complex catalyzes strand incision at the 5′ side of the DNA lesion (Sijbers *et al*, 1996a; Bessho *et al*, 1997). Because this structure-specific endonuclease activity is essential for reconstitution of NER *in vitro* (Aboussekhra *et al*, 1995; Mu *et al*, 1995), the presence of significant residual repair in the XP-F cells suggests that in most patients the mutations are leaky. Intrinsic residual activity in the R778W mutated protein was apparent from its capability to partially restore repair in microinjected XP-F cells that overexpress it.

In conclusion, a homozygous R788W mutation in the *XPF* gene underlies the NER defect of patient XP42RO. The mutation causes strongly reduced but still detectable levels of XPF protein. The biochemical defect results in clinical and cellular UV responses that are mild and typical of other XP-F cases reported so far. Whether the R788W substitution is also responsible for the unusual late-onset neurologic symptoms in the patient remains to be seen, because the other European XP-F patient with the same mutation and showing no neurologic symptoms (Norris *et al*, 1988) was still young (22 y) at the time of report. Moreover, extended case finding and mutational analysis, currently in progress, will also shed light on the possibility that R788W represents a Caucasian founder mutation.

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