

**Table 3**

Effect of sample storage on *Pig-a* assay measurements. For each of 2 experiments, blood was collected from 1 vehicle control and 1 mutagen-treated rat. Blood samples collected and processed for flow cytometric analysis on the same day as harvest are referred to as “Day 0” specimens. These results were used to evaluate the effect of storage over time for samples leukodepleted on the day of collection and stored at 4 °C for several days before further processing (“Lympholyte” samples), as well as whole blood stored in K<sub>2</sub>-EDTA tubes at 4 °C for several days. Each sample/condition was labeled and analyzed as 3 technical replicates per day. Reticulocyte (RET) values are percentages, and mutant phenotype red blood cell (RBC) and mutant phenotype RET data are expressed as the number per 10<sup>6</sup> RBCs or RETs, respectively.

Experiment 1										
Measurement	Treatment	Day 0			Day 2 (Lympholyte)			Day 2 (K <sub>2</sub> -EDTA)		
		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
%RETs	Vehicle	2.2	2.2	2.4	2.5	2.4	2.4	2.3	2.2	2.3
	Mutagen	1.9	2.0	2.0	2.1	2.1	2.1	2.0	2.1	2.0
Mutant RBCs × 10 <sup>-6</sup> RBCs	Vehicle	0.9	0.8	0.7	0.9	0.6	1.0	0.9	0.6	0.7
	Mutagen	205.7	202.4	168.5	211.3	219.2	190.7	212.1	200.2	213.4
Mutant RETs × 10 <sup>-6</sup> RETs	Vehicle	1.7	1.3	0.8	1.0	0.8	5.7	0.0	1.7	0.3
	Mutagen	62.1	52.8	54.3	64.7	56.8	52.0	65.7	57.7	67.1

Experiment 2										
Measurement	Treatment	Day 0			Day 7 (Lympholyte)			Day 7 (K <sub>2</sub> -EDTA)		
		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
%RETs	Vehicle	2.0	2.0	2.0	2.2	2.2	2.2	2.1	2.1	2.2
	Mutagen	4.8	4.8	4.7	4.9	5.0	4.6	5.0	5.0	4.9
Mutant RBCs × 10 <sup>-6</sup> RBCs	Vehicle	0.5	0.5	0.4	0.3	0.3	0.3	0.4	0.4	0.3
	Mutagen	51.4	65.7	71.1	60.7	63.0	57.8	79.0	61.2	61.9
Mutant RETs × 10 <sup>-6</sup> RETs	Vehicle	0.7	1.3	1.5	0.0	0.3	0.3	0.4	0.7	0.0
	Mutagen	13.5	19.8	20.2	15.5	14.6	17.0	18.6	19.3	18.4

be somewhat different (see Table 2), it is important to define *a priori* which parameter(s) is(are) considered the primary endpoint(s) in a given study. As stated before, the two endpoints (RET and RBC mutant frequency) should be analyzed separately for the purpose of statistical analysis, but for evaluating biological relevance, the results should be considered together and in the context of the expected mutant manifestation kinetics.

Results that do not meet the above criteria are considered negative in the assay; however, for evaluating the biological relevance of a negative result, exposure of the bone marrow to the test agent or its metabolite(s) should be confirmed. Toxicity to the bone marrow (e.g., a reduction in the %RETs in the peripheral blood of treated animals) can be used as evidence of bone marrow exposure. When the results do not meet the criteria for a positive or negative response, the response should be declared equivocal.

### 3. Conclusions and recommendations

The formation of this international Workgroup on the *Pig-a* assay indicates the degree of interest in developing a practical method for measuring *in vivo* gene mutation as an alternative to the existing TGR mutation assays. The Workgroup notes that the *Pig-a* assay currently has limitations in terms of tissue coverage and the challenge of directly proving the mutational basis for the response in erythrocytes. Even so, the Workgroup recognizes the assay's potential for integration with other *in vivo* tests, its potential for clinical translation, its remarkable sensitivity to mutagenic agents, its relatively low cost, and, above all, the nature of the endpoint that it measures (phenotypic mutation in an endogenous mammalian gene as opposed to DNA damage, non-heritable chromosomal changes, or mutation in a bacterial transgene). Based on these positive attributes, the Workgroup strongly recommends adoption of the assay to supplement existing *in vivo* genetic toxicology assays.

The *Pig-a* assay has made remarkable progress in its short history; however, the Workgroup believes that further research in various aspects of the assay will enhance its broader acceptance in the regulatory as well as the regulated communities. To foster this development, the Workgroup makes several recommendations for

research needed in the short term and work that would be desirable for the long term.

#### 3.1. Future research: short-term

##### 3.1.1. Further work on the confirmation of the *Pig-a* mutant genotype

While it is desirable to obtain this information from erythroid lineage cells, development of robust data based on other hematopoietic cells should enhance confidence in the presumed genotypic change in mutant erythrocytes. The ideal data would demonstrate mutations in the *Pig-a* gene of individual cells identified as mutants by flow cytometry (direct linkage of phenotype with genotype). However, the Workgroup suggests that demonstrating the anticipated mutation spectra in animals treated with a small number of different agents (e.g., 4) having distinctive mutation spectra would be useful and perhaps provide sufficient circumstantial evidence that the assay is detecting mutations. The Workgroup further suggests that next generation sequencing or deep sequencing of pools of mutant cells might be a practical approach for generating such spectra.

##### 3.1.2. Analyze the effect of methylation/demethylation on assay performance

The assay uses a reporter gene on the X chromosome; thus males have only one copy of the *Pig-a* gene, while females have one functional copy and one copy that is nonfunctional because of silencing by methylation. Although the X-linked *Hprt* gene has been used successfully as a reporter of mutation in males and females, there is still a question of how test agent treatments, animal age, diet, etc. might affect the *Pig-a* mutant frequencies detected in females vs. males. The Workgroup suggests conducting studies to evaluate any sex-related differences.

##### 3.1.3. Analyze additional test agents

The Workgroup recommends analyzing additional test agents, especially those not expected to induce a mutagenic response in the assay either because they are not genotoxic, they are not expected to reach the bone marrow, or because they produce DNA damage not easily recovered by an X-linked gene as gene mutations

(i.e., they are primarily clastogens/aneugens/recombinogens). Among non-genotoxicants, the Workgroup recommends testing agents known to perturb the erythropoietic system by different mechanisms.

### 3.2. Future research: longer-term

Support the ongoing HESI effort to develop a framework for the adoption of new test methods by using *Pig-a* assay development as a case study.

Examine the influence of longer treatments on assay performance (e.g.,  $\geq 90$  days).

Standardize robust testing protocols for laboratory/investigational species other than rats (e.g., human, mouse, dog, swine).

Develop robust testing protocols for other hematopoietic cell types (e.g., T lymphocytes, granulocytes).

Explore the utility of the assay for measuring mutation in cells from solid tissues (e.g., liver).

Develop analogous *in vitro* cell culture based assays to facilitate follow-up investigations by testing hypotheses more efficiently. In addition, an *in vitro* flow-based assay, when adequately validated, may prove to be a cost-effective and reliable replacement/addition to the currently available selection-based, labor-intensive, *in vitro* mammalian cell mutation assays such as the CHO/*Hprt* or mouse lymphoma forward mutation assays.

Explore the assay's utility for measuring mutation in germ cells.

Develop additional methods for increasing the number of cells interrogated for mutation in the assay.

### Conflict of interest

S.D.D. is an employee of Litron Laboratories, which holds patents covering flow cytometric methods for scoring GPI anchor-deficient erythrocytes and sells kits based on this technology (*In Vivo MutaFlow*<sup>®</sup>). L.F.S. Jr. works for BioReliance, which offers the *Pig-a* assay as a commercial service. The authors declare no additional potential conflicts of interest.

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## ORIGINAL ARTICLE

# Constructive rescue of TFIIH instability by an alternative isoform of XPD derived from a mutated XPD allele in mild but not severe XP-D/CS

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Mutations in *XPD* cause xeroderma pigmentosum (XP), XP and Cockayne syndrome (CS) crossover syndrome (XP/CS), trichothiodystrophy and cerebro-oculo-facio-skeletal syndrome (COFS). COFS represents the most severe end of the CS spectrum. This study reports two Japanese patients, COFS-05-135 and COFS-Chiba1, who died at ages of <1 year and exhibited typical COFS manifestations caused by *XPD* mutations p.[I619del];[R666W] and p.[G47R];[I619del], respectively. Two other cases of severe XP-D/CS (XP group D/CS), XP1JI (p.[G47R];[0]) and XPCS1PV (p.[R666W];[0]), died at ages <2 years. On the other hand, two cases of mild XP-D/CS, XP1NE (p.[G47R];[L461V;V716\_R730del]) and XPCS118LV (p.[L461V;V716\_R730del];[R666W]), lived beyond 37 years of age. p.I619Del and p.[L461V;V716\_R730del] are functionally null; therefore, despite the differences in clinical manifestations, the functional protein in all of these patients was either p.G47R or p.R666W. To resolve the discrepancies in these *XPD* genotype–phenotype relationships, the p.[L461V;V716\_R730del] allele was analyzed and we found that p.[L461V;A717G] was expressed from the same allele as p.[L461V;V716\_R730del] by authentic splicing. Additionally, p.[L461V;A717G] could partially rescue the loss of *XPD* function, resulting in the milder manifestations observed in XP1NE and XPCS118LV. *Journal of Human Genetics* advance online publication, 26 February 2015; doi:10.1038/jhg.2015.18

## INTRODUCTION

Cerebro-oculo-facio-skeletal syndrome (COFS) is an autosomal recessive disorder characterized by severe mental retardation, cataracts, optic atrophy, progressive joint contractures and postnatal growth deficiency.<sup>1</sup> *XPD* is one of the genes mutated in COFS; such mutations confer a DNA repair deficiency.<sup>2</sup> In addition to COFS, mutations in *XPD* cause xeroderma pigmentosum (XP)<sup>3,4</sup> and the XP and Cockayne syndrome (CS) crossover syndrome (XP/CS).<sup>5</sup> CS is characterized by abnormalities in physical and neurological development, with dysmyelination but no predisposition to skin cancer.<sup>6</sup> Both COFS and CS are dysmorphology syndromes, and COFS shares many clinical and cellular similarities with CS.<sup>7</sup> Therefore, COFS is considered to represent the most severe end of the CS spectrum;<sup>8</sup> the phenotypic differences between COFS and CS are caused by variations in the mutations that occur in the responsible genes. The *XPD* protein is involved in nucleotide excision repair (NER),<sup>9</sup> a versatile DNA repair system that removes a wide range of DNA lesions, including those induced by UV damage. Because *XPD* is one of the components of basal transcription factor TFIIH and has a dual role in transcription and NER,<sup>10–12</sup> the various manifestations observed in COFS, XP and

XP/CS patients are attributed to complex abnormalities in both DNA repair and transcription.<sup>13–17</sup> In this study, we describe two Japanese COFS patients with compound heterozygous mutations in *XPD*, including the novel *XPD* p.I619del mutation in both patients. We further analyzed the genotype–phenotype relationships between these patients and other XP-D/CS (XP group D/CS) patients who exhibited mild or severe XP-D/CS clinical features, and found that a third mutated *XPD* protein, p.[L461V;A717G], was expressed because of authentic splicing in patients who exhibited mild XP-D/CS clinical features. Additionally, we showed that the protein encoded by the p.[L461V;A717G] allele could form TFIIH and partially rescue the UV hypersensitivity of an *XPD*-deficient cell line. These findings suggest that p.[L461V;A717G] can restore some aspects of *XPD* function, resulting in milder XP-D/CS symptoms.

## MATERIALS AND METHODS

Ethical approvals from The Life Sciences Committee at Osaka University and Chiba Children's Hospital were obtained for all studies. The research was conducted according to the Principles of the Declaration of Helsinki. Informed consent for the studies was obtained from both patients' relatives.

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### UV survival assay

Cells were inoculated into 10-cm dishes at a density of 1000–2000 cells per dish. After 6 h, cells were washed with phosphate-buffered saline and UV irradiated. The cells were then incubated for 2–3 weeks. The colonies that formed were fixed with 10% formalin, stained with 0.1% crystal violet and counted under a binocular microscope.

### Sequencing analysis of XPD cDNA and genomic DNA

Total RNA was prepared from fibroblast cell lines using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). XPD cDNA was amplified using the Qiagen OneStep RT-PCR Kit (Qiagen) with the following primers: sense, 5'-GTC GACCCGCTGCACAGTC-3'; antisense, 5'-CAGGAGTCAACAGGAAC CGTTTATG-3'. Genomic DNA was prepared from fibroblast cell lines using the QIAamp DNA Mini Kit (Qiagen). Genomic DNA fragments containing XPD mutations were amplified by PCR using the following primers: p.G47R sense, 5'-AAGACCACTCAACAAGGAGGAGGC-3'; p.G47R antisense, 5'-TT CCCAAACACCCCGAAAG-3'; p.I619del sense, 5'-GCTCTCTGGAACA CCTGCCCG-3'; p.I619del antisense, 5'-GAAATGAACGGGAAACAGCCTG G-3'; p.R666W sense, 5'-CTACACACAGCCGCATCTCA-3'; p.R666W antisense 5'-GTCAGGTTGAGGTTGGCATCTG-3'.

The DNA sequences of the reverse transcription-PCR (RT-PCR) and PCR products were analyzed using the Big Dye Terminator Cycle Sequencing Kit (Life Technologies Japan, Tokyo, Japan) on an ABI PRISM 3130 Genetic Analyzer (Life Technologies Japan).

### Unscheduled DNA synthesis after UV irradiation

Unscheduled DNA synthesis after UV-irradiation (UV-UDS) tests were performed as described previously<sup>18</sup> with some modifications. Polycarbonate beads with diameters of 0.7 or 2 µm were added to each type of cells at 50% confluence in 10-cm dishes. Three days after incubation, cells were trypsinized and washed five times with phosphate-buffered saline. Cells carrying large beads and small beads were mixed at 1:1 ratio, and then seeded on poly-L-lysine-coated coverslips in a 6-well culture plate (Corning Japan, Tokyo, Japan). UV-C light was irradiated at a dose of 20 J m<sup>-2</sup> after overnight incubation. Cells were then labeled for 3 h in Dulbecco's modified Eagle's medium containing 50 µCi ml<sup>-1</sup> [<sup>3</sup>H]thymidine, and then fixed with methanol-acetic acid (3:1). Coverslips were mounted on glass slides, dipped in Kodak NTB-3 emulsion and exposed for 96 h at 4 °C. The slides were then imaged using developer and medical X-ray film fixer solution (Fujifilm, Tokyo, Japan) and stained with 2% Giemsa. Autoradiographic grains above the nuclei were counted under the microscope; S-phase cells were excluded. UV-UDS efficiency was calculated as the average number of grains in cells carrying large and small beads.

### RT-PCR/restriction fragment length polymorphism analysis

The XPD cDNA fragment containing the c.2150C>G mutation (found in XP1NE) was amplified from total RNA purified from fibroblasts using the Qiagen OneStep RT-PCR Kit (Qiagen) and the following primers: sense, 5'-CCATCAGGGGCAAGACGGAC-3'; antisense, 5'-GTGGAAGGGCTGTGC CATCTG-3'. The amplified DNA fragments were digested with the restriction enzyme *MscI* (Takara Bio Inc., Otsu, Japan), and then subjected to electrophoresis on a 2% agarose gel.

### Immunoprecipitation of XPD protein

The N-terminally HA- and C-terminally FLAG-tagged cDNA of XPD p.WT was cloned into the expression vector pCAGGS. p.[L461V;A717G] and p.[L461V;V716\_R730del] cDNAs were prepared from XP1NE cells by RT-PCR and subcloning. Other mutant XPD cDNAs were prepared using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Tokyo, Japan), and then recloned into pCAGGS. XP6BESV cells stably expressing N-terminally HA- and C-terminally FLAG-tagged cDNA of p.WT, p.G47R, p.L461V, p.[L461V;A717G], p.[L461V;V716\_R730del], p.I619del, p.R666W, p.A717G or p.V716\_R730del were established. The protein complexes were immunoprecipitated from whole-cell extracts using anti-FLAG M2-conjugated agarose. Bound proteins were eluted with the FLAG peptide.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously. Whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes in blotting buffer (25 mM Tris-HCl (pH 8.3), 0.2 M glycine). After blocking, the blot was incubated for 1 h with specific antibodies, followed by the ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd., Buckinghamshire, UK). Anti-XPB (S-19), anti-p62 (Q-19) and anti-XPG (8H7) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-p44 (TF2-1H5) and anti-HA (3F10) antibodies were purchased from Euromedex (Souffelweyersheim, France) and Roche Diagnostics K. K. (Tokyo, Japan), respectively.

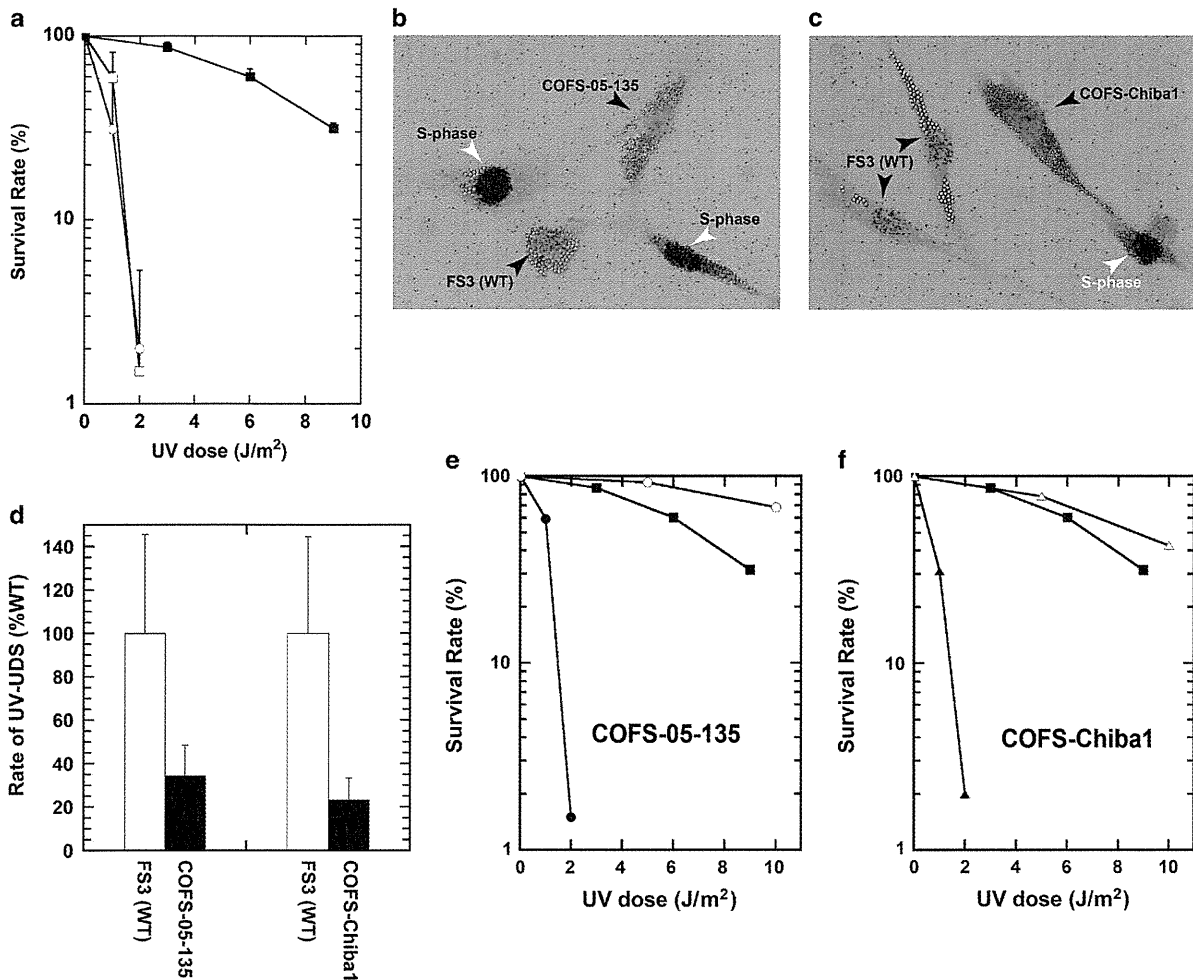
## RESULTS

### Clinical report of COFS-05-135

Here we report a 10-month-old girl with COFS. A Japanese couple, both healthy and unrelated, was referred to us during their third pregnancy because of a past history of two abnormal pregnancies. The first pregnancy ended in early spontaneous abortion, and the second pregnancy resulted in a boy with COFS who died at 5 months. During the third pregnancy, fetal ultrasound at 33 weeks showed severe intrauterine growth retardation, and subsequent amniocentesis revealed a chromosomal complement of 46,XX. The girl was delivered vaginally at 39 weeks of gestation. The patient had a birth weight of 1850 g, a length of 42 cm and a head circumference of 29.3 cm. Apgar scores were 7 at 1 min and 9 at 5 min. Physical examination revealed microcephaly, microphthalmia, deep-set eyes with prominent nasal root, large ear pinnae, micrognathia, camptodactyly, moderate flexion contractures in the elbows and knees and kyphosis. Ophthalmologic examination revealed hypoplasia of the macula lutea and microcornea, and audiological evaluation revealed profound bilateral hearing impairment. Based on the phenotype and family history, the girl was diagnosed as having COFS. The patient exhibited profound growth and developmental delay, with no head control at 6 months. At 7 months, she had episodes of seizures compatible with a diagnosis of West syndrome. At 9 months, she developed a cutaneous erythematous lesion, later diagnosed by biopsy as Langerhans cell histiocytosis. She died of respiratory insufficiency at the age of 12 months.

### Clinical report of COFS-Chiba1

This male infant was born after 37 weeks of gestation as the fourth offspring of non-consanguineous normal Japanese parents with no history of miscarriage or stillbirth. The patient exhibited a prenatal growth deficit (birth weight, 1820 g) as well as classic COFS facial features, including microcephaly, bilateral microphthalmia with deep-set eyes, prominent nasal root, prominent large ear pinnae and micrognathia. In addition, the patient had bilateral simian creases and widely set nipples. He manifested failure to thrive, progressive multiple joint contractures, no developmental progress, infantile spasm and obvious photosensitivity. He died before 1 year of age from pneumonia, at which time his weight was 3000 g. His eldest brother, who died at 5 months of age with leukocytosis and proteinuria, had similar features: intrauterine growth retardation, microcephaly, bilateral microphthalmia, failure to thrive, progressive multiple joint contractures, no developmental progress and obvious photosensitivity. In both siblings, chromosomes of peripheral lymphocytes revealed a normal 46,XY male karyotype. Two other male siblings were normal.



**Figure 1** Cellular characteristics of COFS-05-135 and COFS-Chiba1 cells. (a) After UV treatment, survival of WI38VA13 cells (closed squares), COFS-05-135 cells (open squares) and COFS-Chiba1 cells (open circles). Both patients' cell lines exhibited UV hypersensitivity. (b and c) UV-UDS test of COFS-05-135 cells (b) and COFS-Chiba1 cells (c). S-phase cells are indicated by open arrowheads. (d) Quantitative measurement of UV-UDS tests in (b and c). Grains from  $n=55$  cells were counted under the microscope. (e) After UV treatment, survival of p.WT/COFS-05-135 cells (that is, COFS-05-135 cells expressing XPD p.WT) (open circles), COFS-05-135 cells (closed circles) and WI38VA13 cells (closed squares). (f) After UV treatment, survival of p.WT/COFS-Chiba1 cells (open triangles), COFS-Chiba1 cells (closed triangles) and WI38VA13 cells (closed squares). Error bars represent the s.d. from the mean of three independent experiments.

#### Cellular characterization of COFS cells

UV survival assay revealed that primary fibroblast cell lines derived from both COFS-05-135 and COFS-Chiba1 were hypersensitive to UV irradiation (Figure 1a), indicating that both patients were defective in nucleotide excision repair (NER) of UV damage. Two subpathways exist in NER:<sup>9</sup> transcription-coupled NER, which preferentially removes DNA lesions from the transcribed strand of active genes that block ongoing transcription, and global genome NER, which removes lesions throughout the genome. To determine which pathway was defective in these patients, we analyzed the level of UV-UDS, a hallmark of global genome NER. Cells from both COFS patients exhibited reduced levels of UV-UDS relative to control cells (Figures 1b–d), indicating that both patients were defective in global genome NER. Because mutations in the *XPB*, *XPD* and *XPG* genes have been associated with XP/CS, we analyzed these three genes and detected *XPD* mutations in both patients: c.[1855\_1857del];[1996C>T] in COFS-05-135, resulting in XPD p.[I619del];[R666W]; and c.[139G>A];[1855\_1857del] in COFS-Chiba1, resulting in XPD p.

[Gly47Arg];[Ile619del] (see Supplementary Information). Stable expression of wild-type XPD (p.WT) in COFS-05-135 and COFS-Chiba1 cells (p.WT/COFS-05-135 and p.WT/COFS-Chiba1) complemented the UV hypersensitivity of COFS-05-135 and COFS-Chiba1 cells (Figures 1e and f). Therefore, we concluded that COFS in both these patients was caused by mutations in the *XPD* gene.

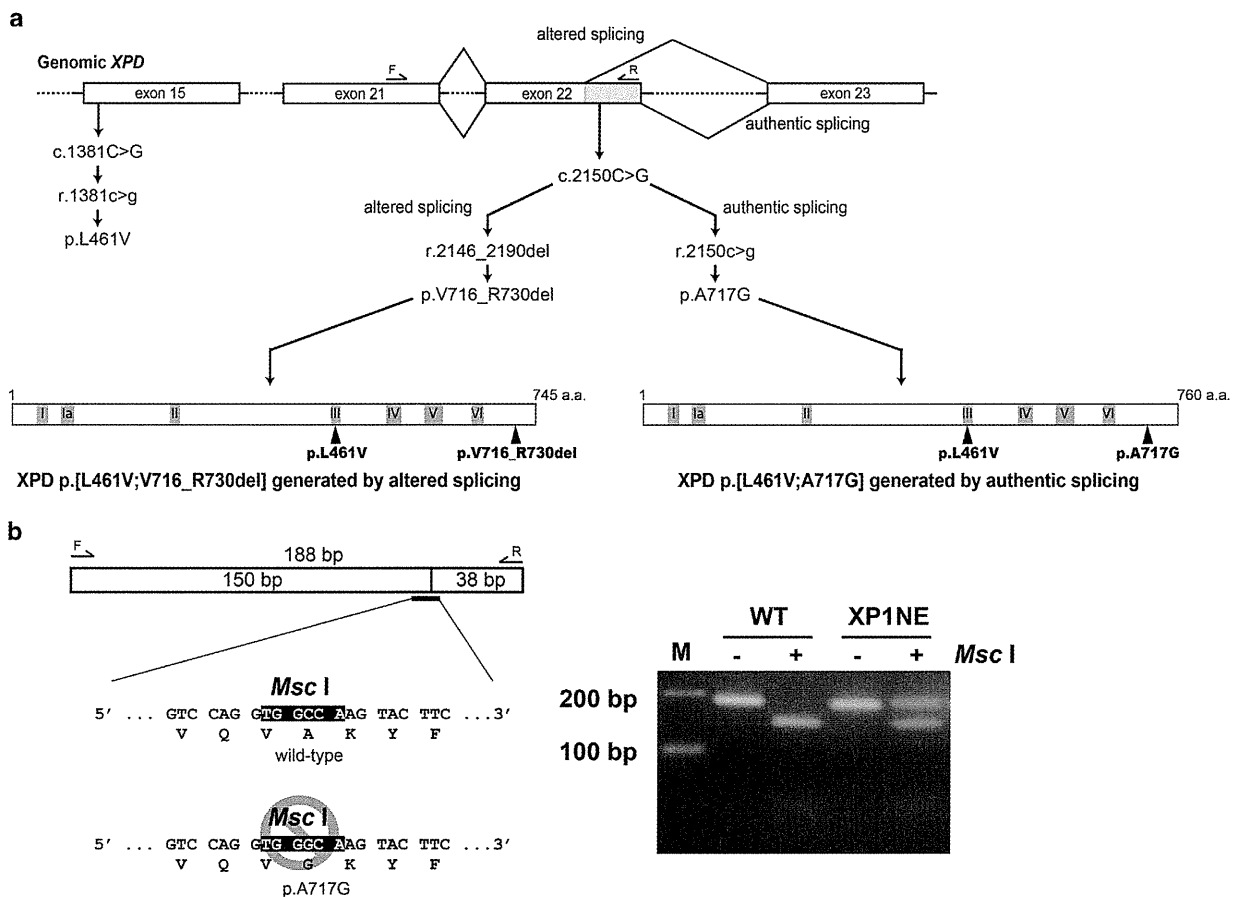
#### Genotype–phenotype relationships in XP-D/CS patients

Compound heterozygous XP-D/CS patients with p.G47R or p.R666W are summarized in Table 1. In XP1JI and XPCS1PV, the only expressed allele was p.G47R and p.R666W, respectively.<sup>19,20</sup> p.[L461V;V716\_R730del], found in XP1NE and XPCS118LV, is functionally null in NER and TFIIH.<sup>3,21,22</sup> Because p.I619del could not form the TFIIH complex, as in the case of p.[L461V;V716\_R730del] (Figure 3c, lanes 5 and 6), we considered p.I619del to be functionally null. These observations mean that the only functional XPD protein in these patients was p.G47R or p.R666W; however, XP1NE and XPCS118LV exhibited considerably milder

**Table 1** Summary of genotype–phenotype relationships of the *XPD* alleles used in this study

Patients	Diagnosis	Age at death	<i>XPD</i> mutation in allele 1	<i>XPD</i> mutation in allele 2	Reference
XP1NE	XP-D/CS (mild)	43 years	p.G47R	p.L461V;V716_R730del p.L461V;A717G	3,9,21, this paper
XP1JI	XP-D/CS (severe)	24 months	p.G47R	p.0	19,20
COFSS-Chiba1	COFS	5 months	p.G47R	p.I619del	This paper
XPCS118LV	XP-D/CS (mild)	>37 years	p.L461V;V716_R730del	p.R666W	20,23, this paper
XPCS1PV	XP-D/CS (severe)	15 months	p.0	p.R666W	20
COFSS-05-135	COFS	12 months	p.I619del	p.R666W	This paper

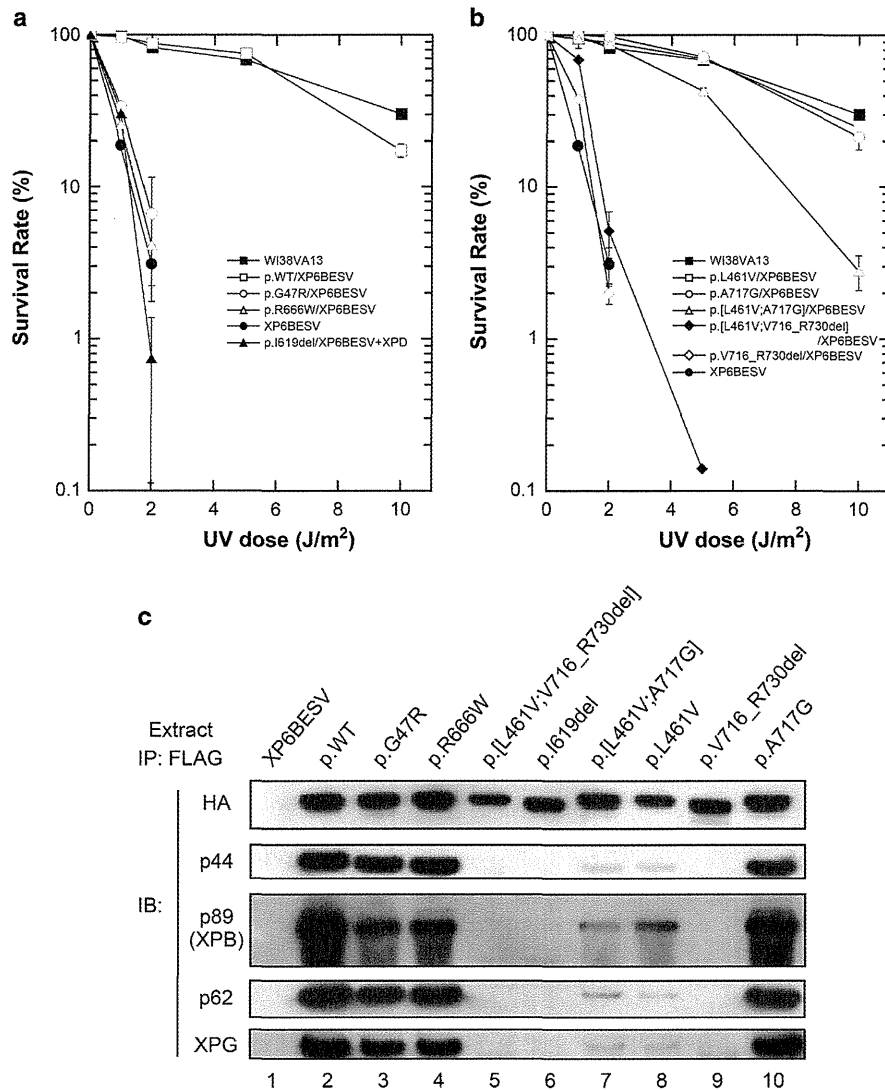
Abbreviations: COFS, cerebro-oculo-facio-skeletal syndrome; XP-D/CS, xeroderma pigmentosum group D and Cockayne syndrome crossover syndrome.



**Figure 2** Analyses of *XPD* alleles of severe and mild XP-D/CS patients. (a) In the *XPD* allele of mild XP-D/CS patients, a c.1381C>G mutation was located on exon 15, and a c.2150C>G mutation was located on exon 22, resulting in a novel splice donor. This new splice donor gives rise to altered splicing, resulting in the expression of p.[L461V;V716\_R730del]. Additionally, *XPD* mRNA r.2150c>g, the alternative isoform arising from the c.1381C>G mutation, was generated by authentic splicing, resulting in the expression of p.[L461V;A717G]. (b) RT–PCR/restriction fragment length polymorphism (RFLP) analysis of the c.2150C>G mutation. The mutation site in the *XPD* cDNA of XP1NE corresponds to the recognition site of the restriction enzyme *MscI*. *XPD* cDNA containing the mutation site was amplified by RT–PCR using primers located on exons 21 and 22, shown by arrowheads on the upper side of (a), and then digested with *MscI*. The RT–PCR products derived from WI38VA13 cells were cut with *MscI*, generating 150- and 38-bp fragments, whereas the *XPD* cDNA containing the c.2150C>G heterozygous mutation from XP1NE was not cut; this is visible as the 188-bp fragment that appears in addition to the digested 150- and 38-bp fragments.

symptoms and survived more than 10 times longer than XP1JI, XPCS1PV, COFS-05-135 and COFS-Chiba1 (Table 1). To resolve this discrepancy in the genotype–phenotype relationships, we analyzed the allele encoding p.[L461V;V716\_R730del]. In addition to the c.1381C>G mutation resulting in p.L461V, the c.2150C>G mutation on exon 22 generated a splice donor. The novel splice site gave rise to altered splicing, resulting in r.[1381c>g;2146\_2190del], which is

translated into p.[L461V;V716\_R730del] (Figure 2a).<sup>20,21</sup> Based on the presence of this c.2150C>G mutation, we predicted that authentic splicing could generate a normal-length mutated *XPD* mRNA, r.[1381c>g;2150c>g], which would be translated into p.[L461V;A717G] (Figure 2a). Because the c.2150C>G mutation site corresponds to the recognition site of the restriction enzyme *MscI*, we were able to perform RT–PCR/restriction fragment length polymorphism



**Figure 3** Functional analyses of mutated XPDs. (a) After UV treatment, survival of WI38VA13 (closed squares), p.WT/XP6BESV (open squares), p.G47R/XP6BESV (open circles), p.R666W/XP6BESV (open triangles), XP6BESV (closed circles) and p.I619del/XP6BESV (closed triangles). (b) After UV treatment survival of p.L461V/XP6BESV (open squares), p.A717G/XP6BESV (open circles), p.[L461V;A717G]/XP6BESV (open triangles), p.[L461V;V716\_R730del]/XP6BESV (closed diamonds) and p.V716\_R730del/XP6BESV (open diamonds). (c) Immunoprecipitation of XPD protein. N-terminally HA- and C-terminally FLAG-tagged XPD proteins were stably expressed in each XP6BESV-derived cell line shown in (a) and (b). The protein complexes were immunoprecipitated from whole-cell extracts with anti-FLAG M2-conjugated agarose. Elutants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, using the antibodies indicated on the left side of (c). Error bars represent the s.d. from the mean of three independent experiments.

analysis. As shown in Figure 2b, the RT–PCR products derived from WT cells were fully cut by *MscI*, generating 150- and 38-bp fragments, whereas the RT–PCR products derived from XP1NE primary fibroblasts generated both cut and uncut fragments. Additionally, full-length c.[1381C>G;2150C>G] cDNA was subcloned from XP1NE cells and used in subsequent experiments. These results indicated that p.[L461V;A717G] was expressed from the p.[L461V;V716\_R730del]-encoding allele as a result of authentic splicing.

#### Functional analysis of mutated XPDs

To examine the NER activity of each mutant XPD protein found in these six patients, we established stable transfectants expressing N-terminal HA- and C-terminal FLAG-tagged XPD proteins, including

WT, in the XPD-deficient cell line XP6BESV (p.WT/XP6BESV, p.G47R/XP6BESV, p.L461V/XP6BESV, p.[L461V;V716\_R730del]/XP6BESV, p.[L461V;A717G]/XP6BESV, p.I619del/XP6BESV, p.R666W/XP6BESV, p.V716\_R730del/XP6BESV and p.A717G/XP6BESV). We then subjected these transfectants to UV survival assays. p.WT/XP6BESV, p.L461V/XP6BESV and p.A717G/XP6BESV cells exhibited the same level of UV resistance as WI38VA13 (WT) (Figures 3a and b). By contrast, p.G47R/XP6BESV, p.[L461V;V716\_R730del]/XP6BESV, p.I619del/XP6BESV, p.R666W/XP6BESV and p.V716\_R730del/XP6BESV exhibited the same UV hypersensitivity as XP6BESV cells. Interestingly, p.[L461V;A717G]/XP6BESV exhibited UV hypersensitivity intermediate between that of WI38VA13 and XP6BESV (Figures 3a and b). These results indicated that p.G47R,



p.[L461V;V716\_R730del], p.I619del, p.R666W and p.V716\_R730del were completely defective in NER of UV-induced DNA damage. Additionally, although p.L461V and p.A717G possessed full NER activity (comparable to p.WT), the combined mutant p.[L461V;A717G] possessed only partial NER activity.

#### TFIIH-forming activity of mutated XPDs

The results of the UV survival assays shown in Figures 3a and b prompted us to investigate the ability of the XPD variants to form the TFIIH complex. To this end, we subjected whole-cell extracts from XP6BESV cells expressing various XPD proteins to immunoprecipitation with anti-FLAG antibodies. p.G47R, p.R666W and p.A717G could bind components of TFIIH, including XPG, as efficiently as p.WT (Figure 3c, lanes 2–4 and 10), whereas p.I619del, p.V716\_R730del and p.[L461V;V716\_R730del] could not (Figure 3c, lanes 5, 6 and 9). Additionally, p.L461V and p.[L461V;A717G] co-immunoprecipitated with components of TFIIH, including XPG, but only weakly (Figure 3c, lanes 7 and 8). These results indicated that p.G47R, p.R666W and p.A717G could form TFIIH complex normally, whereas p.I619del, p.V716\_R730del and p.[L461V;V716\_R730del] could not. Additionally, p.L461V and p.[L461V;A717G] formed the TFIIH complex to some extent, but they could not do so as efficiently as the WT protein.

#### DISCUSSION

We describe here two Japanese patients who exhibited COFS symptoms caused by mutations in the *XPD* gene. *XPD* gene mutations give rise to XP and XP/CS in addition to COFS. On the basis of *XPD* genotype–phenotype relationships, different clinical symptoms were strongly associated with various mutations in the *XPD* gene.

COFS-Chiba1 and COFS-05-135, described in this report, and XP-D/CS patients XP1JI and XPCS1PV, described previously,<sup>19,20,23</sup> had very severe symptoms and died at ages of 5 months to 2 years (Table 1). COFS represents the most severe end of the CS spectrum,<sup>8</sup> therefore, we refer to these cases here as severe-XP-D/CS. p.I619Del, found in the two COFS patients described here, did not bind to the core complex of TFIIH (Figure 3c, lane 6) and could not rescue the NER defect of XP1BESV cell lines (Figure 3b). From these findings, we conclude that p.I619del is functionally null, and that only p.G47R or p.R666W was expressed as functional XPD in these COFS patients; single expression of p.G47R and p.R666W was the cause of severe XP-D/CS in XP1JI and XPCS1PV, respectively.<sup>19,20</sup> Both p.G47R and p.R666W could bind and form TFIIH complex components, including XPG (Figure 3c, lanes 3 and 4), but neither protein could rescue the NER defect of XP6BESV cell lines (Figure 3a), indicating that both p.G47R and p.R666W completely lack NER activity. Previous biochemical examination revealed that p.G47R is defective in ATPase, helicase, DNA repair synthesis and dual-incision activities.<sup>24</sup> There is no detailed biochemical information regarding human p.R666W, but structural and biochemical analyses have been performed on the *Sulfolobus acidocaldarius* XPD homolog.<sup>25</sup> The *Sulfolobus acidocaldarius* XPD mutants p.G34R and p.R514W, which, respectively, correspond to human XPD p.G47R and p.R666W, are defective in ATPase and helicase activities. These biochemical results indicate that p.G47R and p.R666W have lost the ability to engage in basal transcription; nevertheless, both mutants can still form TFIIH complex (Figure 3c, lanes 3 and 4). Based on these observations, we conclude that sole expression of p.G47R or p.R666W caused the severe manifestations found in these patients.

On the other hand, XP1NE and XPCS118LV exhibited typical XP features along with neurological disease, and both patients survived beyond 30 years of age: XP1NE and XPCS118LV died at 43 and 37 years, respectively.<sup>19,20,23</sup> Here we refer to these milder symptoms as mild XP-D/CS. A previous report indicated that p.[G47R];[L461V;V716\_R730del] and p.[L461V;V716\_R730del];[R666W] are the causes of XP1NE and XPCS118LV, respectively.<sup>20,21</sup> However, we observed that authentic splicing resulted in the p.A717G amino-acid change, and that consequently, p.[L461V;A717G] was expressed from the p.[L461V;V716\_R730del]-encoding allele (Figure 2). Therefore, it is clear that three kinds of mutated XPD protein are expressed in XP1NE (p.[G47R];[L461V;A717G;V716\_R730del]) and XPCS118LV (p.[L461V;A717G;V716\_R730del];[R666W]), as shown in Table 1. The p.[L461V;V716\_R730del] mutation cannot rescue lethality in a *rad15* mutant of *Schizosaccharomyces pombe*.<sup>21</sup> Furthermore, biochemical analysis revealed that the single p.V716\_R730del mutant protein lacks the ability to bind to p44 (ref. 22) and has a defect in basal transcription.<sup>24</sup> Additionally, p.L461V has NER activity comparable to that of p.WT, although the p.L461V mutant forms a loose conformation of TFIIH (Figures 3b and c). These observations indicate that p.[L461V;V716\_R730del] is functionally null because of the p.V716\_R730del mutation. p.A717G formed TFIIH as competently as p.WT (Figure 3c, lane 10) and rescued the UV hypersensitivity of XP6BESV as well as p.WT (Figure 3b), indicating that p.A717G might possess full XPD function. In the case of compound-mutated XPD, that is, p.[L461V;A717G], the ability to bind core TFIIH was reduced, as in the case of p.L461V (Figure 3c, lane 10), suggesting that the p.L461V mutation affected the binding ability of p.A717G. Interestingly, p.[L461V;A717G] had partial NER activity (Figure 3b). These observations suggest that the low TFIIH-binding capacity of p.[L461V;A717G] might affect basal transcription and cause the mild XP-D/CS clinical features, although the basal transcription ability of p.[L461V;A717G] remains unknown.

The p.[L461V;V716\_R730del]-encoding allele has been considered to be functionally null.<sup>20,21,23</sup> However, we conclude that coexpression of p.[L461V;A717G] because of authentic splicing in the mild XP-D/CS patients partially rescues the functional defect of p.G47R or p.R666W, resulting in a more than 10-fold increase in lifespan in mild XP-D/CS patients relative to patients with severe XP-D/CS.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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