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ARTICLE

Abnormal *XPD*-induced nuclear receptor transactivation in DNA repair disorders: trichothiodystrophy and xeroderma pigmentosum

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***XPD* (*ERCC2*) is a DNA helicase involved in nucleotide excision repair and in transcription as a structural bridge tying the transcription factor IIH (TFIIH) core with the cdk-activating kinase complex, which phosphorylates nuclear receptors. Mutations in *XPD* are associated with several different phenotypes, including trichothiodystrophy (TTD), with sulfur-deficient brittle hair, bone defects, and developmental abnormalities without skin cancer, xeroderma pigmentosum (XP), with pigmentary abnormalities and increased skin cancer, or XP/TTD with combined features, including skin cancer. We describe the varied clinical features and mutations in nine patients examined at the National Institutes of Health who were compound heterozygotes for *XPD* mutations but had different clinical phenotypes: four TTD, three XP, and two combined XP/TTD. We studied TFIIH-dependent transactivation by nuclear receptor for vitamin D (VDR) and thyroid in cells from these patients. The vitamin D stimulation ratio of *CYP24* and *osteopontin* was associated with specific pairs of mutations (reduced in 5, elevated in 1) but not correlated with distinct clinical phenotypes. Thyroid receptor stimulation ratio for *KLF9* was not significantly different from normal. *XPD* mutations frequently were associated with abnormal VDR stimulation in compound heterozygote patients with TTD, XP, or XP/TTD.**

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INTRODUCTION

Trichothiodystrophy (TTD) (MIM no. 601675) is a rare autosomal recessive multisystem disorder characterized by sulfur-deficient brittle hair, neurological abnormalities, ichthyosis, and cutaneous photosensitivity without skin cancer.[1] TTD may be manifested when both alleles are affected by mutations in one of the three DNA repair genes (*XPB (ERCC3)* MIM no. 133510, *XPD* (*ERCC2*) MIM no. 126340, *TTDA* (*GTF2H5*) MIM no. 608780),[2] or in *TTDN1 (C7orf11)* MIM no. 609188,[3] a gene of unknown function. Interestingly, mutations in *XPB* and *XPD* can also cause xeroderma pigmentosum (XP) (MIM no. 278730),[4–7] with sunlight-induced freckle-like skin pigmentation and >10 000-fold increased risk of skin cancer.[8] TTD and XP have an incidence of about 1 per million live births in the United States and Western Europe.[9] Very rare XP/TTD patients have clinical features of both XP and TTD[10,11] and carry an increased cancer risk.

The XPD, XPB, and TTDA proteins are associated with transcription factor IIH (TFIIH), a 10 protein complex involved in both initiation of transcription by RNA polymerase II and nucleotide excision repair.[12–15] XP and TTD patients may have different, although overlapping, *XPD* mutations, suggesting that the site of mutation influences the clinical phenotype.[10,16–20] Cells from XP and TTD patients with *XPD* mutations differ in the rate of repair of ultraviolet-induced DNA photoproducts and in the time course of localization and removal of nucleotide excision repair proteins to sites of UV damage.[10]

Nuclear receptors are hormone-dependent transcription factors that play essential roles in development, differentiation, and metabolism by controlling the expression of specific networks of genes.[21,22] *XPD* mutations reduce basal transcription activity in TTD cells and inhibit nuclear receptor transactivation in XP as well as in TTD cells.[23–25] Using our unique collection of fibroblast strains established from the TTD, XP, and XP/TTD patients we examined at the National Institutes of Health (NIH) over the past three decades,[8,26–28] we selected cells from nine patients with *XPD* mutations (four TTD, two XP/TTD, and three XP), with varied and distinct clinical features. We tested two types of nuclear receptor, vitamin D receptor (VDR) and thyroid receptor, which are important for bone growth and maintenance, development, and innate and adaptive immunity.[21,22] We hypothesized that these patients would have abnormal nuclear receptor transactivation.

MATERIALS AND METHODS

Clinical protocol, cells, maintenance culture conditions, and mutation analysis

Patients were studied under protocol 99-C-0099 approved by the National Cancer Institute Institutional Review Board, which adhered to the Declaration

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of Helsinki Principles. Skin biopsies were obtained and sent to the Human Genetic Mutant Cell Repository (Camden, NJ, USA) or to NCI-Frederick (Frederick, MD, USA) for establishment of fibroblast cell cultures. Fibroblast cultures from four TTD, two XP/TTD, three XP patients (Table 1), and normal controls AG04438 and AG13145 were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 4 mM glutamine and 10% fetal calf serum (FCS) (Hyclone Laboratories Inc., Logan, UT, USA) in an 8% CO_2 humidified incubator at 37 °C as described previously.[10] Mutation analysis was performed as described previously.[10] The *XPD* GenBank sequences used were NM_0000400.3 (*XPD* cDNA) with numbering based on +1 as the A of the ATG initiation codon and NP_000391.1 (XPD protein).

Hormone stimulation and DNA/RNA isolation

Nuclear receptor activation was performed as described previously.[25] In brief, fibroblasts were plated at 50–60% confluence in complete media for 24 h. The media were changed to a phenol red-free media containing 10% charcoal-treated FCS and the cells were incubated for 12 h with either 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; VD) (Sigma-Aldrich, St Louis, MO, USA) or triiodothyronine (T_3) (Sigma-Aldrich). Total cytoplasmic RNA was isolated from cells using the RNAqueous small-scale phenol-free total RNA isolation kit (Ambion Inc., Austin, TX, USA). DNA was isolated using DNAzol reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time RT–PCR

Quantitative real-time RT-PCR (qPCR) was performed as described.[25] Total RNA was treated with DNA-free DNase (Ambion Inc., Austin, TX, USA) and then converted to cDNA using Superscript II (Invitrogen, Carlsbad, CA, USA) as described.[29] A measure of 2 μl (corresponding to 25 ng RNA) of RT reaction was used as template for each PCR reaction. qPCR assays were carried out on a Bio-Rad iCycler iQ system (Bio-Rad Laboratories Inc., Hercules, CA, USA) using intercalation of SYBR Green (iQ SYBR Green Supermix; Bio-Rad) as the fluorescence reporter. Two-step PCR (denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 1 min) was performed with data collection and analysis during the combined annealing and extension step. Melt-curve analyses were performed on all PCR reactions to rule out nonspecific amplification. The following forward and reverse primer sequences were used for the target genes: *CYP24* MIM no. 126065 (VD target gene) – 5′-TCTTGACAAGGCAACAGTTC-3′, 5′-AAGCCAACGTTCAGGTCTAA-3′ (PCR fragment: 132 bp); *osteopontin* (*OPN*) (*SPP1*) MIM no. 166490 (VD target gene) – 5′-TATGATGGCCGAGGTGATAG-3′, 5′-AGGTGATGTC CTCGTCTGTA-3′ (PCR fragment: 86 bp); *KLF9* MIM no. 602902 (T_3 target gene) – 5′-CCAAGAGCTTGTTGGACCTGAACA-3′, 5′-TCGGATCCCATA TCCTCATCTGGACT-3′ (PCR fragment: 96 bp); intercellular adhesion molecule 1 (ICAM1) MIM no. 147840 (not a VD target gene) – 5′-T TTTCTATCGGCACAAAAGC-3′, 5′-AATGCAAACAGGACAAGAGG-3′ (PCR fragment: 232 bp) (product number VHPS-4429 from RealTimePrimers.com); and *18S* RNA (internal control) – 5′-CGGACAGGATTGACAGATTG-3′, 5′-T GCCAGAGTCTCGTTCGTTA-3′ (PCR fragment: 98 bp). Ct values were normalized relative to 18S RNA expression.

Data analysis and statistics

The results are the mean of at least two independent experiments performed with duplicate samples. All data are presented as fold change against each control (specified as either mean of non-stimulated cells or mean of normal cells under the same conditions). Student's *t*-test with a two-tailed distribution was used to analyze the data. Statistical significance was judged to be a *P*-value of <0.05.

RESULTS

Clinical features differ markedly among TTD, XP, and XP/TTD patients with *XPD* mutations

Patient TTD354BE was a Caucasian female with TTD (Figures 1a–c) who was compound heterozygous for a previously unreported *XPD* mutation (p.G459-T460ins34aa) and p.A725T, which was present in another TTD patient (TTD421BE)[30] (Table 1 and Figure 2). TTD354BE had features of severe TTD,[1,31] including tiger tail banding of her hair (Figure 1c), short stature (<3%-ile), congenital cataracts, microcephaly, brain dysmyelination, global developmental delay, including central osteosclerosis and peripheral osteopenia of her bones, elevated hemoglobin A2 (HbA2) levels, and a low mean corpuscular volume. She had recurrent ear infections and pneumonias and died at the age of 9 years from acute infection following hip surgery.[27]

Patient TTD412BE was a 7-year-old Caucasian male with TTD (Figure 1d and e)[27] with p.A725P mutation in one *XPD* allele and p.L461V plus p.V716-R730del in the other allele, resulting from a g.18339C>G splice mutation (Table 1 and Figure 2). These same mutations have been reported previously in another patient (TTD183ME).[32] Interestingly, while TTD183ME was acutely sun sensitive, TTD412BE did not have this same sun sensitivity.

Patient TTD351BE (Table 1 and Figure 2) was a Caucasian male with a unique p.R378H mutation and the TTD-specific mutation, p.R722W.[20] A mouse engineered to have this mutation was shown to have some clinical features of TTD.[33] The TTD351BE cells had normal DNA repair.[10] The patient died at the age of 15 years owing to infection and respiratory failure following hip surgery.[27]

Patient TTD404BE[27] was a 11-year-old Caucasian male with a novel p.R112C mutation at the same site as a more common p.R112H mutation reported in other TTD patients[11,16] (Table 1 and Figure 2). He also had a p.D681N mutation, which was present in a patient with cerebro-ocular-facial-skeletal (COFS) syndrome[34] and in XP patient XP17BE.[10] He had long hair with tiger tail banding under polarized microscopy.

Patient XPTTD268BE was a 25-year-old Caucasian male who had the commonly reported TTD mutation, p.R112H, and a unique p.C663R mutation[10] (Table 1 and Figure 2). He had long hair and TTD features of tiger tail banding on polarized microscopy, ichthyosis, cataracts, leukodystrophy, and recurrent infections. He also had XP-type freckle-like pigmentation supporting the diagnosis of XP/TTD.[11]

Patient XPTTD306BE was a 14-year-old Caucasian male with XP/TTD who had a novel mutation, p.S51F, and a commonly reported TTD-specific mutation, p.R722W, in the XPD protein[10] (Table 1 and Figure 2). He had XP features of acute sun sensitivity and freckle-like skin pigmentation but no skin cancers. In addition, he had the characteristic TTD features of 'tiger tail' banding of his hair on polarized microscopy, ichthyosis, short stature, microcephaly, and brain dysmyelination and recurrent pneumonias.[6]

Patient XP17BE[10] was an African-American male with the p.R683W mutation commonly seen in XP patients,[10,17,19,20] as well as in XP29BE and XP34BE. He also had a p.D681N mutation, which was present in a COFS patient[34] and in a TTD patient TTD404BE (Table 1 and Figure 2).

Patient XP29BE was a Caucasian male with severe XP phenotype with neurological abnormalities (Figure 1f) who was compound heterozygous for p.R683W in one *XPD* allele and a premature stop codon p.Q452X mutation in the other allele[25,35,36] (Table 1 and Figure 2). Besides severe sunburning on minimal sun exposure, and extensive freckle-like pigmentation, he had >300 skin cancers documented, including 24 cutaneous melanomas, 284 basal cell carcinomas, and 12 squamous cell carcinomas, despite treatment with oral retinoids.[35] He died at the age of 39 years owing to neurological degeneration.

Patient XP34BE was a Caucasian male with a mild XP phenotype (Figure 1g) who had a p.R683W mutation from one *XPD* allele and a p.I199Pfs*52 mutation from the other allele, which arose from a splice

Table 1 Clinical features and laboratory abnormalities of trichothiodystrophy (TTD) and xeroderma pigmentosum (XP) patients studied

Diagnosis	*TTD*	*TTD*	*TTD*	*TTD*	*XP/TTD*	*XP/TTD*	*XP*	*XP*	*XP*
Patient	TTD354BE	TTD412BE	TTD351BE	TTD404BE	XPTTD268BE	XPTTD306BE	XP17BE	XP29BE	XP34BE
Complementation group	XP-D	XP-D	XP-D	XP-D	XP-D	XP-D	XP-D	XP-D	XP-D
Age[a]/sex	d9y/F	7y/M	d15y/M	11y/M	25y/M	16y/M	d26/M	d37y/M	32y/M
Clinical features									
Skin and Hair									
Acute skin sun sensitivity	Yes	Normal	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tiger tail banding[b] of hair	Yes	Yes	Yes	Yes	Yes	Yes	Not tested	Normal	Normal
Freckle-like pigmentation	Normal	Normal	Normal	Normal	Yes	Yes	Yes	Yes	Yes
Ichthyosis	Severe	Mild	Yes	Normal	Yes	Moderate	Normal	Normal	Normal
Skin cancers (number)	0	0	0	0	0	0	25–50	300 + [c]	0[d]
Stature	<3%-ile	<3%-ile	<3%-ile	10%-ile	<3%-ile	<5%-ile	Normal	Normal	Normal
Cataracts	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
Neurological									
Brain abnormalities	Dysmyelination (MRI)	Dysmyelination (MRI)	Hypomyelination, enlarged ventricles (MRI)	Diffuse cerebral hypomyelination (MRI)	Leukodystrophy, enlarged ventricles (MRI)	Dysmyelination (MRI)	Low IQ (50)	Diffuse cerebral atrophy (CT)	Normal
Microcephaly	Yes	Normal	Normal	Yes	Yes	Yes	Normal	Not tested	Normal
Bone abnormalities	Yes[e]	Yes[f]	Yes	Yes[m]	Yes[n]	Not tested	Normal	Normal[g]	Not tested
Recurrent infections	Yes[h]	Yes[h]	Yes[h]	Yes[h]	Yes[h]	Yes[h]	Normal	Normal	Normal
Laboratory tests									
Low red cell mean corpuscular volume	Yes	Yes	Yes	Yes	No	Yes	Yes	Normal	Normal
Elevated HbA2	Yes	Yes	Yes	Yes	Yes	Not Tested	Not Tested	Not Tested	Not Tested
DNA repair	Decreased[i]	Decreased[i]	Normal[j]	Decreased[i]	Decreased[k]	Decreased[k]	Decreased[k,l]	Decreased[k]	Decreased[k]
XPD mutations[o]									
Allele 1	c.2173G>A p.A725T	c.2173G>C p.A725P	c.2164C>T p.R722W	c.334C>T p.R112C	c.335G>A p.R112H	c.2164C>T p.R722W	c.2047C>T p.R683W	c.2047C>T p.R683W	c.2047C>T p.R683W
Allele 2	c.1378-1G>T c.1377–1378ins102 p.G459-T460ins34aa	c.1381C>G p.L461V + g.18339C>G c.2146_2190del p.V716-R730del	c.1133G>A p.R378H	c.2041G>A p.D681N	c.1987T>C p.C663R	c.152C>T p.S51F	c.2041G>A p.D681N	c.1354C>T p.Q452X	c.595-10G>A c.594–595insCCCCCCAG p.I199Pfs*52
Mutations reported	This report	This report	Boyle *et al*[10]	This report	Boyle *et al*[10]	Boyle *et al*[10]	Boyle *et al*[10]	Ueda *et al*[25]	Boyle *et al*[10]

[a]Age at last observation or age at death (d).
[b]Alternating dark and light bands when viewed with polarized microscopy.[37]
[c]Histological documentation of 284 basal cell carcinomas, 12 squamous cell carcinomas, and 24 melanomas.[25]
[d]Extensively sun-protected from an early age.
[e]TTD354BE had delayed bone age, central osteosclerosis, and peripheral osteopenia, flattened femoral head.
[f]TTD412BE had low–normal bone age, bilateral coxa valgus, central osteosclerosis, peripheral osteopenia, and avascluar necrosis of femoral head.
[g]XP29BE had retinoid-induced hyperostoses of ligaments and tendons,[36] vertebral osteosclerosis, femoral osteopenia, compression of mid-thoracic vertebra, and mild scoliosis of thoracic spine.
[h]TTD354 and XPTTD268BE had recurrent ear infections and pneumonias; TTD412BE had recurrent pneumonias and GI infections; TTD351BE died of infection with respiratory failure following hip surgery; and XPTTD306BE had recurrent pneumonias.
[i]Reduced post-UV fibroblast survival (this report).
[j]Normal unscheduled DNA synthesis TTD351BE 115%.[10]
[k]Reduced post-UV unscheduled DNA synthesis (UDS). XP29BE 11% (this report); and TTD351BE (115%), XP34BE 19%, XP17BE 33%, XPTTD268BE 8%, and XPTTD306BE 28%.[10]
[l]Reduced post-UV cell survival XP17BE.[38]
[m]TTD404BE had mild, diffuse central osteosclerosis.
[n]XPTTD268BE had central osteoclerosis, cannot exclude peripheral osteopenia.
[o]From NC_000019.9 (ERCC2 genomic); NM_000400.3 (ERCC2 cDNA); and NP_000391.1 (XPD protein).

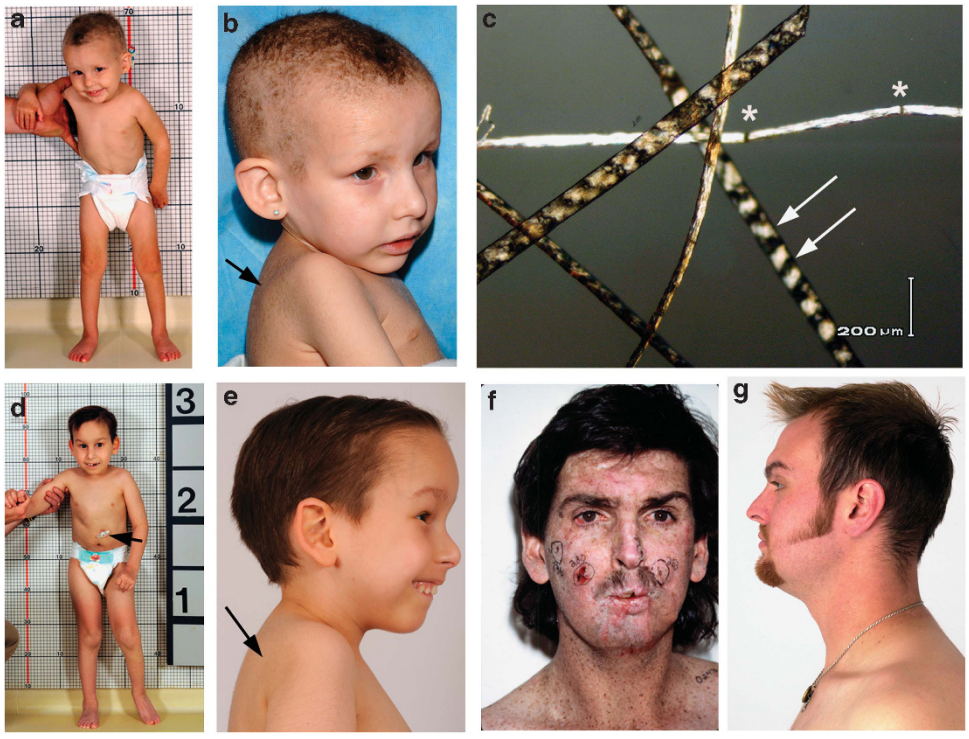

Figure 1 TTD and XP patients studied. (**a**) TTD patient TTD354BE at age 3 years. She has extremely short stature (70 cm, <3%-ile), diminutive features, and cheerful affect, typical of TTD. She was unable to stand without assistance. Note relative size of her parent's hand. (**b**) TTD patient TTD354BE at age 7 years. Her hair is extremely brittle and breaks easily. She never needs a haircut. There is loss of the lateral portion of her eyebrows. Ichthyosis is present on her back (arrow) and chest. (**c**) TTD patient TTD354BE hair. Examination with polarized microscopy shows prominent alternating dark and light 'tiger tail' banding characteristic of TTD (arrows) and trichoschisis defects of the hair shaft (*). (**d**) TTD patient TTD412BE at age 7 years. He had short stature (96 cm, <3%-ile) and a cheerful affect. He was unable to stand without assistance. He utilized a g-tube (arrow) for feeding. (**e**) TTD patient TTD412BE has long hair, micrognathia (small jaw), and mild ichthyosis (arrow) with cheerful affect. (**f**) XP patient XP29BE at age 21 years. He has freckle-like pigmented lesions on his chest, neck, and face. There are numerous scars on his face from multiple surgical procedures for removal of skin cancers. Several lesions suspicious for skin cancer are present (circles). Note pterygium in the lateral conjunctiva of his right eye. (**g**) XP patient XP34BE at age 24 years. Despite severe acute sun sensitivity, he has used sun protection and shows minimal pigmentary changes of his skin.

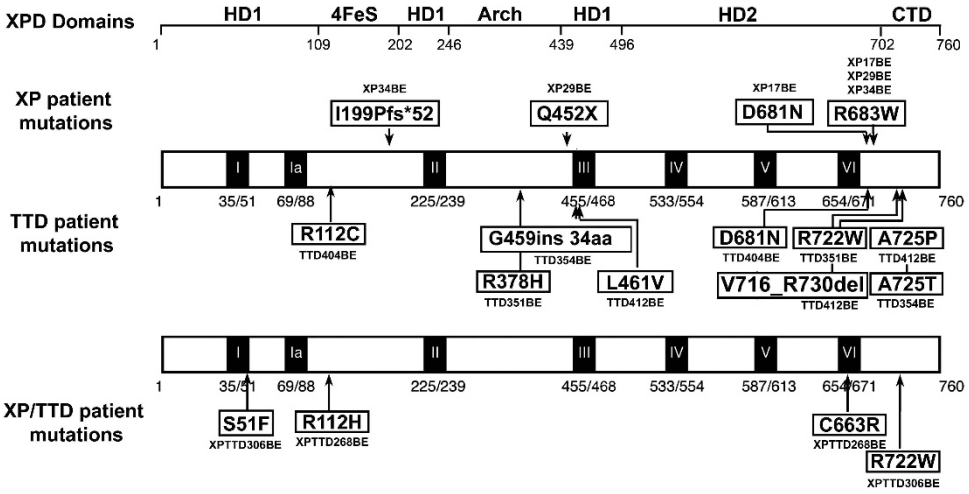

Figure 2 XPD domains, functional helicase motifs, and mutation sites. Relative positions of the XPD domains are shown above a linear schematic of the human XPD protein. The XPD catalytic core of *Sulfolobus acidocaldarius* XPD (SaXPD) is comprised of four domains: two Rad51/RecA-like helicase domains (HD1 and HD2) with two additional domains (4FeS and Arch domains) inserted into HD1.[18] The helicase motifs (labeled with Roman numerals) are conserved from SaXPD to human XPD.[18] However, human XPD has an extended C-terminal domain (CTD) that has been proposed to be the TFIIH p44 interface.[23] The relative positions of the mutations found in XP patients (XP17BE, XP29BE, and XP34BE), TTD patients (TTD351BE,TTD354BE, TTD404BE, andTTD412BE), and XP/TTD patients (XPTTD268BE and XPTTD306BE) are shown. XP patient mutations are presented above the protein schematic, and TTD patient mutations and XP/TTD patient mutations are presented below.

mutation that gave rise to an insertion of eight nucleotides in the mRNA and a predicted truncated protein (Table 1 and Figure 2). He had a sister (XP35BE) who had the same mutations and a similar mild XP phenotype.[10,25] He never developed skin tumors and had no evidence of neurological abnormalities by age 32 years.

Eight of the nine patients had decreased DNA repair as measured by post-UV unscheduled DNA synthesis or post-UV fibroblast survival[10,25] (Table 1). The only exception was patient TTD351BE.

Abnormal *CYP24* induction in *XPD* mutant fibroblasts indicates abnormal transactivation of the VDR

We analyzed by real-time quantitative RT–PCR the expression of two genes known to be under the control of VDR. One of these target genes, *CYP24*, encodes the cytochrome *P*450 enzyme vitamin D_3 hydroxylase that catalyzes the inactivation of vitamin D (VD). We used normal cells to determine the time and dose–response curves for *CYP24* expression. The peak of *CYP24* mRNA expression after vitamin D treatment was reached after about 12 h and then did not show a significant change, suggesting that it had reached the expression limit for that concentration of vitamin D (100 nM) (Figure 3a). A similar time course was present in the TTD cells, although at lower peak levels (Supplementary Figure 1). *CYP24* expression increased with vitamin D concentration in a dose-dependent manner (Supplementary Figure 2b). The fold increase in *CYP24* mRNA of treated relative to untreated control (induction ratio) was in the same range as that described previously for normal fibroblasts[39] (Figure 3a and Supplementary Figure 2a). Given the strong *CYP24* mRNA expression obtained from incubating the wild-type cells for 12 h with 100 nM vitamin D, we chose to use these same conditions for the patients' cells to allow comparison.

The response to vitamin D treatment varied with different cell lines and appears to be influenced by both alleles in these compound heterozygous patients. TTD cells TTD354BE, TTD412BE, TTD404BE, and XPTTD306BE had a significantly ($P<0.05$) reduced induction ratio when compared with the induction ratio of normal fibroblasts ($\sim$13 000-fold in AG04438 and $\sim$16 000-fold in AG13145) (Figure 3b). XPTTD306BE had the greatest decrease, with only about a $\sim$300-fold induction ratio. By comparison, XP29BE and XP34BE had $\sim$3000- and $\sim$4000-fold induction ratios, respectively. This reduction is in accord with a previous study of *CYP24* mRNA expression.[25] Of note, TTD404BE and XP17BE both have the same D681N allele and both have reduced *CYP24* induction ratios. Interestingly, these same six TTD, XP/TTD, and XP cell strains also had elevated relative *CYP24* baseline levels compared with the normal control (Supplementary Figure 2c, lower panel). In contrast, the TTD and XPTTD cells (TTD351BE, XPTTD306BE) with the same p.R722W mutation in one allele (Figure 2) had opposite responses (Figure 3b and Supplementary Figure 2c). TTD351BE, with an elevated *CYP24* response, is the only TTD cell line with normal DNA repair (Table 1 and Boyle *et al*[10]). In accord with previous studies,[25] these results suggest that the second mutated allele (R378H in TTD351BE and S51F in XPTTD306BE) may also influence the response. Also in agreement with the earlier study,[25] XP cells XP17BE, XP29BE, and XP34BE have the same R683W mutation, but differ in their responses to vitamin D (Figure 3b and Supplementary Figure 2c) and have different second mutated alleles.

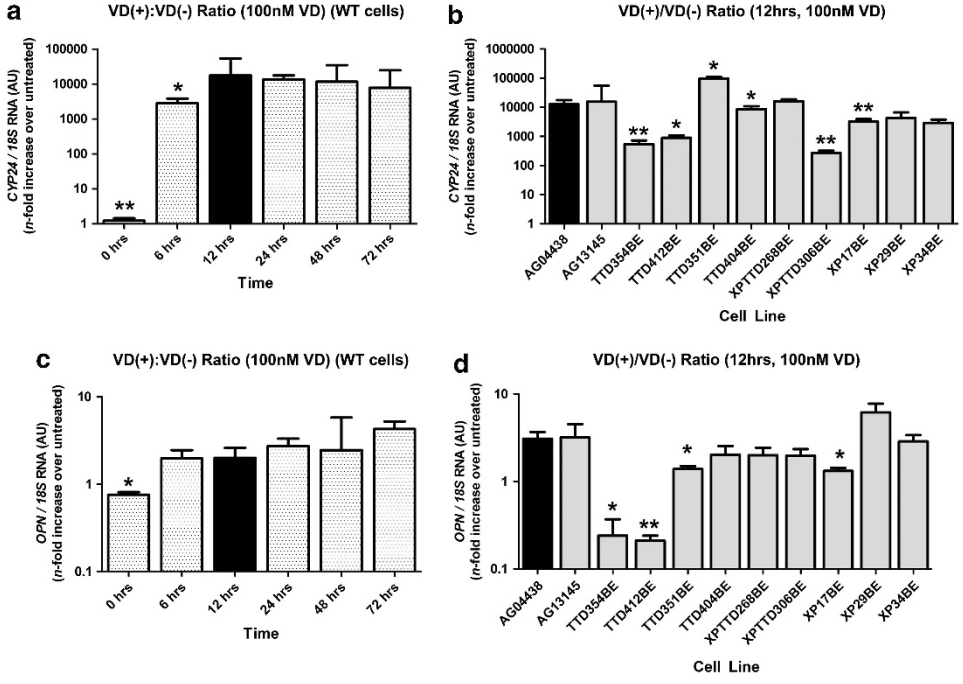

Figure 3 VDR expression of *XPD* mutant fibroblasts in XP, TTD, XP/TTD, and normal cells. Relative mRNA expression of the VDR target genes *CYP24* and *OPN* in normal, TTD, XP/TTD, and XP fibroblasts after treatment with VD was measured using qPCR. The values were normalized relative to 18S RNA expression. Each panel represents the results of two independent experiments performed with duplicate samples (*$P<0.05$; **$P<0.01$, Student's *t*-test compared with normal controls – *black bars*). (**a**) Time response control. *CYP24* expression in normal (AG04438) fibroblasts incubated for different lengths of time with or without vitamin D (100 nM) was measured. (**b**) *CYP24* induction ratio of treated cells relative to untreated cells for the different cell strains. (**c**) Time response control. *OPN* expression in normal (AG04438) fibroblasts incubated for different lengths of time with or without vitamin D (100 nM) was measured. (**d**) *OPN* expression of normal, TTD, XP/TTD, and XP fibroblasts after 12 h of incubation with or without vitamin D (100 nM).

Abnormalities in *OPN* induction in *XPD* mutant fibroblasts further indicate mutation-dependent aberrant transactivation of the VDR

To see whether this effect also involves other VDR target genes, we measured mRNA expression of the *OPN* gene. Osteopontin is implicated in a variety of physiological functions, including bone remodeling,[40] neutrophil chemotaxis,[41] and immune regulation.[42] *OPN* expression in normal cells increased with vitamin D concentration in a time-dependent manner (Figure 3c) and in a dose-dependent manner (Supplementary Figure 3b). Similar to our *CYP24* data (Figure 3b), we found that the greatest reduction in *OPN* induction ratio of treated to untreated cells was in TTD354BE and TTD412BE cells and, to a lesser extent, in XP17BE cells (Figure 3d and Supplementary Figure 3c). Considering these similarities of the abnormalities in *CYP24* and *OPN* expression, the data suggest that VDR transactivation may be less responsive to vitamin D stimulation in some of the *XPD* mutant cells.

We examined *ICAM1*, a gene that is not known to be responsive to VD, as a negative control. There was no significant increase in the ratio of expression of *ICAM1* following VD treatment as compared with untreated cells in normal, or *XPD* mutant TTD or XP cells (Supplementary Figure 4).

***KLF9* expression in *XPD* mutant fibroblasts**

TTD patients often have developmental neurological abnormalities such as microcephaly and brain dysmyelination,[1] whereas XP patients may have progressive neurological degeneration.[4,7,8] The thyroid hormone receptor has been shown to be crucial in normal brain development.[43] We measured the expression of *KLF9* (also known as *BTEB*), a thyroid receptor-responsive gene in the fibroblasts.[25,44] The normal cells had a time- and dose-dependent (Supplementary Figures 5a and b) induction of *KLF9* by treatment with triiodothyronine (T_3). The peak of *KLF9* mRNA induction ratio in normal cells after treatment was reached after about 6 h. However, the relative amount of *KLF9* mRNA expression from cells with or without T_3 treatment increased over time through 72 h. *KLF9* induction ratio of treated cells over untreated cells was slightly reduced in TTD354BE, TTD412BE, XPTTD306BE and XP29BE, when compared with normal cells, but these results were not statistically significant (Supplementary Figure 5c).

DISCUSSION

From the group of patients we examined at NIH,[8] we selected TTD, XP, and XP/TTD patients with *XPD* mutations who had markedly different clinical features (Table 1). These patients all were compound heterozygotes for *XPD* mutations but shared some of the same mutations (Table 1 and Figure 2). TFIIH phosphorylation of nuclear receptors or their co-activated partners (such as Ets1 for VDR) is absent in certain *XPD* mutants (e.g., p.R683W mutants)[24,45] and intracellular TFIIH concentration is reduced in TTD fibroblasts.[10,16,23] XPD is a structural bridge between the CAK complex (consisting of cdk7, cycH, and Mat1) and the TFIIH core, which together regulates RNA polymerase II function by phosphorylating its C-terminal domain. This step controls the transition from transcription initiation to elongation.[24,46,47] Thus, abnormalities in activation of nuclear receptor may be involved in some of the clinical features in these patients.

Ueda *et al*[25] demonstrated that each *XPD* allele contributes to the *CYP24* response. While it is not possible to make unambiguous conclusions from non-isogenic human cell strains, this effect might be mutation dependent and be related to both *XPD* alleles. TTD351BE and XPTTD306BE cells both had p.R722W mutations, but the ratio of vitamin D stimulated to basal *CYP24* expression was elevated in TTD351BE cells, but reduced in XPTTD306BE cells compared with the normal controls (Figure 3b). The second allele in each cell line (p.R378H and p.S51F, respectively) thus might contribute to this differential response. Similar second allele mutation dependency could be evident in the three XP cell lines with p.R683W mutations. This is in agreement with an earlier study[25] that reported separate activities of each allele in XP29BE and XP34BE cells. Interestingly, cells with the p.R683W mutation also had a poor response to retinoid receptor stimulation,[24] and patient XP29BE, with this mutation, had a poor response to oral retinoid therapy for the prevention of skin cancers despite typical retinoid-related toxicities.[35]

As reported previously,[25] there was no significant reduction in the *OPN* induction ratios for the XP29BE and XP34BE cells compared with normal cells. However, XP17BE cells and three of the TTD cell strains (TTD354BE, TTD412BE, and TTD351BE) had reduced *OPN* induction ratios compared with the normal controls (Figure 3d). The results for *KLF9*, the response gene for the thyroid hormone receptor, were similar, although the magnitude of change was much smaller and would require a larger sample size to elucidate.

We did not observe a correlation between the nuclear receptor transactivation abnormalities and the different clinical phenotypes. This may be related to the fact that we were only able to study skin fibroblasts from our patients after birth. These cells may not be the ones that are most severely affected by this transactivation abnormality. For example, more dramatic effects may be seen in osteocytes for VDR transactivation and CNS glial cells for thyroid receptor transactivation as reported in TTD mice,[43] or in fetal cells during embryonic development. Each *XPD* mutation might affect the transactivation process differently.[25] The combination would be expected to lead to variability in nuclear receptor activity among different TTD and XP patients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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