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Analysis of Mutations in the *XPD* Gene in Italian Patients with Trichothiodystrophy: Site of Mutation Correlates with Repair Deficiency, but Gene Dosage Appears to Determine Clinical Severity

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Summary

Xeroderma pigmentosum (XP) complementation group D is a heterogeneous group, containing patients with XP alone, rare cases with both XP and Cockayne syndrome, and patients with trichothiodystrophy (TTD). TTD is a rare autosomal recessive multisystem disorder associated, in many patients, with a defect in nucleotide-excision repair; but in contrast to XP patients, TTD patients are not cancer prone. In most of the repair-deficient TTD patients, the defect has been assigned to the *XPD* gene. The *XPD* gene product is a subunit of transcription factor TFIIH, which is involved in both DNA repair and transcription. We have determined the mutations and the pattern of inheritance of the *XPD* alleles in the 11 cases identified in Italy so far, in which the hair abnormalities diagnostic for TTD are associated with different disease severity but similar cellular photosensitivity. We have identified eight causative mutations, of which four have not been described before, either in TTD or XP cases, supporting the hypothesis that the mutations responsible for TTD are different from those found in other pathological phenotypes. Arg112his was the most common alteration in the Italian patients, of whom five were homozygotes and two were heterozygotes, for this mutation. The presence of a specifically mutated XPD allele, irrespective of its homozygous, hemizygous, or heterozygous condition, was always associated with the same degree of cellular UV hypersensitivity. Surprisingly, however, the severity of the clinical symptoms did not correlate with the magnitude of the DNA-repair defect. The most severe clinical features were found in patients who appear to be functionally hemizygous for the mutated allele.

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Introduction

Trichothiodystrophy (TTD) is a rare autosomal recessive disorder whose diagnostic hallmark is brittle hair. A common feature of affected individuals is a decreased amount of total sulfur content and of cystine and cysteic acid in the hair, together with an abnormal distribution of cystine-rich proteins in the cortex and hair cuticle. Other clinical symptoms associated with TTD are ichthyosis, physical and mental retardation, and abnormal facies (Itin and Pittelkow 1990). Although photosensitivity has been reported in ~20% of the cases and is probably associated with a substantially higher proportion, skin lesions and cutaneous tumors have never been described in association with TTD. DNA-repair investigations have demonstrated that clinical photosensitivity is usually associated with cellular UV hypersensitivity. In these cases, the cellular phenotype is similar to that observed in the nucleotide-excision repair (NER)–defective form of xeroderma pigmentosum (XP), the very well-known autosomal recessive disorder characterized by hypersensitivity to sunlight and by increased incidence of skin cancer. Genetic studies using cell fusion have enabled XP patients defective in NER to be classified into seven different complementation groups, designated "XP-A"–"XP-G" (for a review, see Bootsma et al. 1998).

Despite the remarkable differences at the clinical level, two of the genes with defects identified in XP—namely, the *XPB* and *XPD* genes—have been found associated with TTD (Stefanini et al. 1986, 1992, 1993*a;* Lehmann et al. 1988; Vermeulen et al. 1994). One TTD patient appeared to be defective in a new repair gene that has been designated "*TTDA*" (Stefanini et al. 1993*b*). Whereas XP-B is a very rare group with only three families identified, the XP-D defect has been found in a substantial number of XP patients, in the majority of repair-defective TTD patients, and in rare cases showing the clinical features of Cockayne syndrome (CS) in addition to those of XP (Vermeulen et al. 1991; Broughton et al. 1995). The apparent paradox of the presence of

the same genetic defect associated with clinically distinct disorders has been recently rationalized by the discovery that the XPD protein, an ATP-dependent 5′→3′ helicase (Sung et al. 1993), is a subunit of the transcription factor TFIIH (Drapkin et al. 1994; Schaeffer et al. 1994), involved in both NER and basal transcription. TFIIH is a multifunctional protein complex containing at least nine polypeptides, including the *XPD* and *XPB* gene products (Hoeijmakers et al. 1996). It has been suggested that clinical features diagnostic for XP could result from mutations affecting only the NER function of TFIIH, whereas those typical of TTD and CS could be explained by a subtle impairment of the transcription function (Bootsma and Hoeijmakers 1993; Vermeulen et al. 1994*b*). The mutations found in the patients, which are obviously compatible with cellular viability, do not seriously impede basal transcription. They must impair transcription only under certain conditions or in specific cell compartments affecting, for example, only a limited set of genes that critically demand optimal TFIIH functioning.

Since the XP-D group is large and heterogeneous, the definition of causative mutations in the patients represents a useful means to verify whether distinct pathological phenotypes are caused by different alterations. The *XPD* (originally *ERCC2*) gene was cloned because of its ability to correct the UV sensitivity of a Chinese hamster mutant belonging to rodent complementation group 2 (Weber et al. 1988; Flejter et al. 1992). It is highly conserved and encodes a protein with 50% amino acid identity to *Saccharomyces cerevisiae RAD3* and *Schizosaccharomyces pombe rad15* (Weber et al. 1990; Murray et al. 1992) and with 83%, 98%, and 98% identity to the *Xiphophorus maculatus,* mouse, and Chinese hamster homologues, respectively (Kirchner et al. 1994; Della Coletta et al. 1995; de Boer et al. 1998). To date, analysis of mutations in the *XPD* gene has been performed in 21 XP cases (Frederick et al. 1994; Takayama et al. 1995; Kobayashi et al. 1997; Taylor et al. 1997), eleven TTD patients (Broughton et al. 1994; Takayama et al. 1996, 1997; Taylor et al. 1997) and in 2 XP/CS patients (Broughton et al. 1995; Takayama et al. 1995). These studies have identified several alterations—mainly point mutations resulting in single amino acid substitutions in the carboxy-terminal portion of the protein—most of which are specifically associated with a unique pathological phenotype (XP, TTD, or XP/CS). The recent demonstration that the few mutations observed in both XP and TTD behave as null alleles further supports the hypothesis that the site of mutation determines the clinical features (Taylor et al. 1997). However, despite the significant progress in our understanding of the complex genotype-phenotype relationships associated with the *XPD* gene, several unanswered questions remain, concerning the basis of the cellular heterogeneity of the repair defect and the correlation between clinical features, repair defect, and molecular alteration in TTD.

Since our first report in 1986, we have further extended DNA-repair investigations of TTD, and we thus far have identified 11 cases in Italy in whom photosensitivity results from a defect in the *XPD* gene. The cellular response to UV in all these cases is drastically altered, resulting in a similar degree of UV hypersensitivity; in contrast, striking differences in both the progression and the severity of the disease became evident among the patients over the years.

To clarify the basis of this clinical heterogeneity and of its puzzling relationship with the repair defect, we have performed a molecular analysis in the Italian families with TTD-affected members. We have identified the potentially causative mutations in the *XPD* gene of the patients and have determined the linkage relationship between different mutations, by analysis in the parents. Besides providing additional data on primary alterations responsible for the TTD pathological phenotype, this study sheds new light on the different effect that mutated XPD proteins might have on repair and transcription.

Subjects, Material, and Methods

Case Reports

The study was performed on 11 Italian patients affected by TTD; the diagnosis in all cases was confirmed by analysis of the hair. Pili torti, trichoschisis, and trichorrhexis nodosa were observed. Polarization microscopy revealed the typical appearance of alternating light and dark bands, giving a "tiger tail" pattern. Photosensitivity was reported in all patients, in association with the other symptoms typical of TTD—namely, ichthyosis, delayed physical and mental development, nail dysplasia, a face characterized by a receding chin, small nose, and large ears, and microcephaly. Detailed clinical and DNA-repair data on several of these cases have been published, and related literature references are reported in table 1. The seven patients still alive are ages 4–30 years; the four cases that died during early infancy showed severe physical and mental retardation and frequently suffered from respiratory infections. The oldest patients (TTD1PV, TTD2PV, and TTD3PV) developed freckles during childhood, thus far without any progression to malignancy, and they have short stature (140 cm), began to menstruate at age 18 years, and no longer are prone to infections, although they suffered moderate infections during early childhood. TTD1PV suffers from severe myopia, but she is able to write and to read. Mental development in the sisters TTD2PV and TTD3PV is at the preschool level. TTD6PV died at age 3.5 years, of pneumonia. Her physical and mental de-

Table 1

Italian Patients Affected by TTD

Patient[a] (Sex)	Age at (years)		Impairment		Reference(s)
	Diagnosis	Present[b]	Physical[c]	Mental[d]	
TTD1PV (F)	18	30	Moderate	Moderate	Stefanini et al. (1986, 1992)
TTD2PV (F)	4	20	Moderate	Moderate	Stefanini et al. (1986, 1992)
TTD3PV (F)	3	21	Moderate	Moderate	Stefanini et al. (1986, 1992, 1993*a*)
TTD4PV (F)	1	(4)	Severe	Severe	Stefanini et al. (1986, 1992)
TTD6PV (F)	(At birth)	(3.5)	Severe	Severe	Marinoni et al. (1991), Stefanini et al. (1992, 1993*a*)
TTD7PV (F)	2	(4)	Severe	Severe	Stefanini et al. (1992, 1993*a*)
TTD8PV (M)	1	9	Moderate	Moderate	Stefanini et al. (1992, 1993*a*), Battistella and Peserico (1996)
TTD10PV (F)	12	18	Moderate	Moderate	Peserico et al. (1992), Stefanini et al. (1993*a*)
TTD11PV (M)	4	11	Severe	Severe	Present study
TTD12PV (M)	.75	(2)	Severe	Severe	Present study
TTD15PV (M)	(At birth)	3.5	Severe	Severe	Present study

[a] TTD2PV and TTD3PV are sisters, and TTD12PV and TTD15PV are brothers.

[b] Ages in parentheses are age at death.

[c] Moderate = survival beyond early childhood, delayed puberty, and short stature; Severe = death during childhood and/or failure to thrive/dystrophy.

[d] Moderate = mental development at either preschool level or (in the case of TTD1PV) primary-school level, axial hypotonia, and reduced motor coordination; Severe = very poor mental and motor performances and speech.

velopment was severely compromised. She was unable to stand up and to manipulate.

TTD11PV, born in 1987, shows ichthyosis, peculiar face, brittle hair, and spontaneous nystagmus. At 3 mo of age he experienced sunburn after very short exposure to sunlight. On clinical examination at age 4 years, the skin appeared ichthyotic, with freckles in the photoexposed area; the teeth were dystrophic, and the degree of growth and mental retardation was severe. At age 4.5 years, he developed chronic diarrhea; malabsorption; atrophy of villi, on jejunal biopsy, with multiple food intolerance (but not celiac disease) requiring prolonged parenteral and enteral nutrition; and further regression of the mental and motor capabilities. Subsequently, he experienced repeated and severe infectious illnesses, mainly of the gastrointestinal and respiratory tract. At age 11 years, he is now severely dystrophic (weight 11 kg, height 112 cm) and is unable to stand and speak.

TTD12PV, born in 1992, was the second child of healthy unrelated parents. He was referred to us when he was 9 mo of age; he had brittle hair, ichthyosis, nail dysplasia, peculiar face, and severely retarded physical and mental development. He underwent several episodes of respiratory illness and died at age 2 years.

TTD15PV is a brother of TTD12PV, born in 1994. TTD was diagnosed at birth. He underwent several episodes of respiratory illness and, at age 2 years, he showed severe growth retardation (weight 8,900 g), delayed motor and mental development, neutropenia, and strabismus.

Cells and Culture Conditions

Primary fibroblast cultures were established from the skin of the 11 TTD patients and their parents. Lymphoblastoid cell lines were established by Epstein-Barr–virus transformation of peripheral blood lymphocytes from the parents of TTD4PV, from whom biopsies were not available. Fibroblasts were routinely grown in Ham's F-10 medium (Gibco) supplemented with 5% newborn calf serum (Irvine Scientific) and 10% FCS (Irvine Scientific) and were subcultured by trypsinization. Lymphoblastoid cell lines were cultured in RPMI 1640 medium (Hyclone) supplemented with 15% FCS in a 3% CO_2 atmosphere. Fibroblasts from three healthy donors (C3PV, B119, and CF) and from two XP patients previously assigned to group D were used as reference strains in the study.

DNA-Repair Investigations

The response to UV irradiation was analyzed by measurement of cell survival and by unscheduled DNA synthesis (UDS). Definition of the genetic defect responsible for UV hypersensitivity was accomplished by classic complementation assays. Procedures for cell survival, UDS, and genetic analysis are those routinely used in our laboratory and have been described elsewhere (Stefanini et al. 1992, 1993*a*).

Molecular Analysis of the XPD *Gene*

RNA and genomic DNA were simultaneously extracted, by a cesium chloride–gradient centrifugation procedure, from samples of 2×10^7 fibroblasts or 1×10^8 lymphoblastoid cells (in the case of TTD4PV's parents), and were resuspended in 1 ml of guanidinium thiocyanate buffer (4 M guanidinium thiocyanate, 3 M Na acetate pH 6).

RNA was reverse transcribed into cDNA, with Mo-

Table 2

PCR Amplification Primers for the *XPD* Gene

Amplified Region[a]	Fragment Size[b] (bp)	5′ Primer		3′ Primer	
		Code	Sequence	Code	Sequence
51 to 690	640	AL32	5′-GACCCCGCTGCACAGTCCGG-3′	AL33	5′-CACATTGGCATGCAGGATTG-3′
442 to 1085	644	BB14	5′-ACACCCCTGCGCTTTGGGAAGGAC-3′	BB8	5′-AGCCGCCTCAGGAAGCCCAGGAA-3′
1025 to 1665	641	BB9	5′-AGGAGGCAGTGCCTGGCTCCATCC-3′	E23	5′-AGCGGACATCTCCAGCAGGAGGTTCC-3′
1433 to 2397	965	BB3	5′-AGTCTGTCATCATCACATCTGGGACACTGT-3′	BB16	5′-GTCACCAGGAACCGTTTATGCCCCACCCG-3′
2119 to 2397	279	AL37	5′-GACAAGCGGTTTGCCCGT-3′	BB16	5′-GTCACCAGGAACCGTTTATGCCCCACCCG-3′
−242 to 114	659	U1	5′-GGCAGGCGCAGGAGGACCAA-3′	L2	5′-GAAGTAGACCAGGAGCCCGT-3′
267 to 419	357	U2	5′-TCCGCTGGAGGTGACCAAAC-3′	L5	5′-TCAGGGTGAATACACAAGTT-3′
402 to 610	3767	BB12	5′-TCTGAGCTCCCGCAAAAACTTGTGTATTCA-3′	BB13	5′-CCAGGTTGTAGATGCCAGCGGG-3′
1858 to 2197	855	AL36	5′-GCCATCCTGCTGTCAGTG-3′	BB19	5′-TGAGGTTGGCATCTGTGAG-3′
2128 to 2397	756	BB21	5′-TTTGCCCGTGGGGACAAGCGGGGGA-3′	BB16	5′-GTCACCAGGAACCGTTTATGCCCCACCCG-3′
2128 to +178	941	BB21	5′-TTTGCCCGTGGGGACAAGCGGGGGA-3′	L4	5′-TGGGCTGGTGGGGTGAGAGGGGGTC-3′

[a] Numbers correspond to position on cDNA (Weber et al. 1990). For primers U1 and L4, the numbers −242 and +178 correspond, respectively, to the position on the genomic 5′- and 3′-flanking sequences of the *XPD* transcribed region (GenBank accession L47234).,

[b] The first five fragments (underlined) were amplified from cDNA; the others were amplified from genomic DNA.

loney murine leukemia virus reverse transcriptase (M-MLV RT). In brief, 2 μg of RNA in a total volume of 10 μl were heated to 90°C for 2 min, and then 30 μl of a mix containing 1 × first-strand cDNA buffer (Gibco BRL), 10 mM DTT, 1 mM of each dNTP, 100 ng of oligo(dT)15 (Promega), and 200 U of M-MLV RT (Gibco BRL) were added. After incubation at 37°C for 1 h, the samples were heated to 95°C for 5 min, were increased to a volume of 100 μl by the addition of water, and were stored at −20°C. PCR-amplification conditions were as follows: 10–50 of μl cDNA were used in 100-μl reactions containing 1 × Gene Amp buffer II (Perkin-Elmer), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 100 pmol of each of the required primers (table 2), and 2 U of Ampli*Taq* (Perkin-Elmer). Amplification was performed with 31 cycles each of 94°C for 1 min, 65°C for 2 min, and 70°C for 4 min.

Genomic DNA amplification was performed on 1 μg of DNA samples, with the primers listed in table 2. The PCR conditions for the primer pairs AL36 and BB19, BB21 and BB16, and BB21 and BB16 were as described for cDNA. Amplification with the primer pairs U1 and L2 and U2 and L5 was performed by use of Ampli*Taq* Gold (Perkin-Elmer) and the following cycling parameters: 1 cycle of 95°C for 12 min and then 40 cycles each of 95°C for 1 min and 65°C for 10 min. For the primer pair BB12 and BB13, the XL PCR Kit (Perkin-Elmer) was used; the reaction mix contained 1 × XL buffer, 1 mM $Mg(OAc)_2$, 0.2 mM of each dNTP, 200 pmol of each of the primers, and 6 U of rTth DNA polymerase XL. Amplification was performed by use of 38 cycles each of 94°C for 1 min and 68°C for 10 min.

PCR products were separated and excised from 1.5% low-melting-point agarose gels and then were purified by means of the Wizard PCR Preps Purification System (Promega). Products for the primer pair AL37 and BB16 were separated and excised from 4% NuSieve agarose gels.

The products from the primer pair BB9 and E23 were cloned into the pMOSBlue T-vector by use of the pMOSBlue T-vector kit (Amersham), and double-stranded plasmid DNA was prepared by a conventional mini-alkaline–lysis method.

Purified PCR fragments and plasmid DNA with appropriate inserts were directly sequenced by means of the ThermoSequenase cycle sequencing kit (Amersham), according to the three dNTPs internal-label protocol and the manufacturer's instructions.

Results

DNA-repair investigations in the Italian patients with clinical diagnoses of TTD have allowed us, thus far, to identify 11 cases from nine families in whom the cellular response to UV irradiation is clearly altered. Fibroblast strains from these patients show both a drastic reduction in the capacity to perform DNA-repair synthesis (with UDS levels that are 8%–28% of normal) and an increased sensitivity to the killing effects of UV light (fig. 1). An altered response to UV irradiation, in patients TTD1PV, TTD2PV, TTD3PV, TTD4PV, TTD6PV, TTD7PV, TTD8PV, TTD9PV, and TTD10PV, has been described elsewhere (see refs. cited in table 1), and the values in the present series of experiments are similar to those reported earlier. Genetic analysis performed by complementation studies indicated that the repair alteration in all the patients is due to the presence of a mutation in the *XPD* gene. Despite the similar degree of photosensitivity at the cellular level, the marked differences at the clinical level that were observed among patients over the years (see table 1) prompted us to identify the molecular alterations responsible for the pathological phenotypes. The nucleotide sequence of the coding region of the *XPD* gene was analyzed by reverse-transcription PCR followed by direct sequencing (nucleotides 79–2361 in the report by Weber et al. [1990]). The mutations found in the patients were investigated in the parents by sequencing of the relevant cDNA fragments. The results are described below and are summarized in figure 2.

Patients TTD1PV, TTD2PV, TTD3PV, TTD8PV, and TTD10PV

Complete sequencing of the *XPD* cDNA in the five patients TTD1PV, TTD2PV, TTD3PV, TTD8PV, and TTD10PV (who were from four families,TTD2PV and TTD3PV being sisters) revealed the presence, in the whole of the amplified population, of both a silent A→C transversion at position 546 and a G→A transition at position 413, resulting in an arg112his substitution. All the parents were heterozygous for the G413A transition, confirming that the five patients are homozygotes. All the fathers were heterozygous, and the mothers homozygous, for the silent mutation at position 546. Therefore the A546C change in the fathers is present on the *XPD* allele carrying the G413A mutation, whereas in the mothers the silent mutation is present on both *XPD* alleles.

Patient TTD6PV

Patient TTD6PV showed, in the whole of the amplified cDNA population, both the silent transversion A546C and, at position 2096, an A→G transition resulting in an asp673gly substitution. The patient's father was heterozygous for the asp673gly change (fig. 3*A*). In contrast, no mutation was identified in the cDNA of the

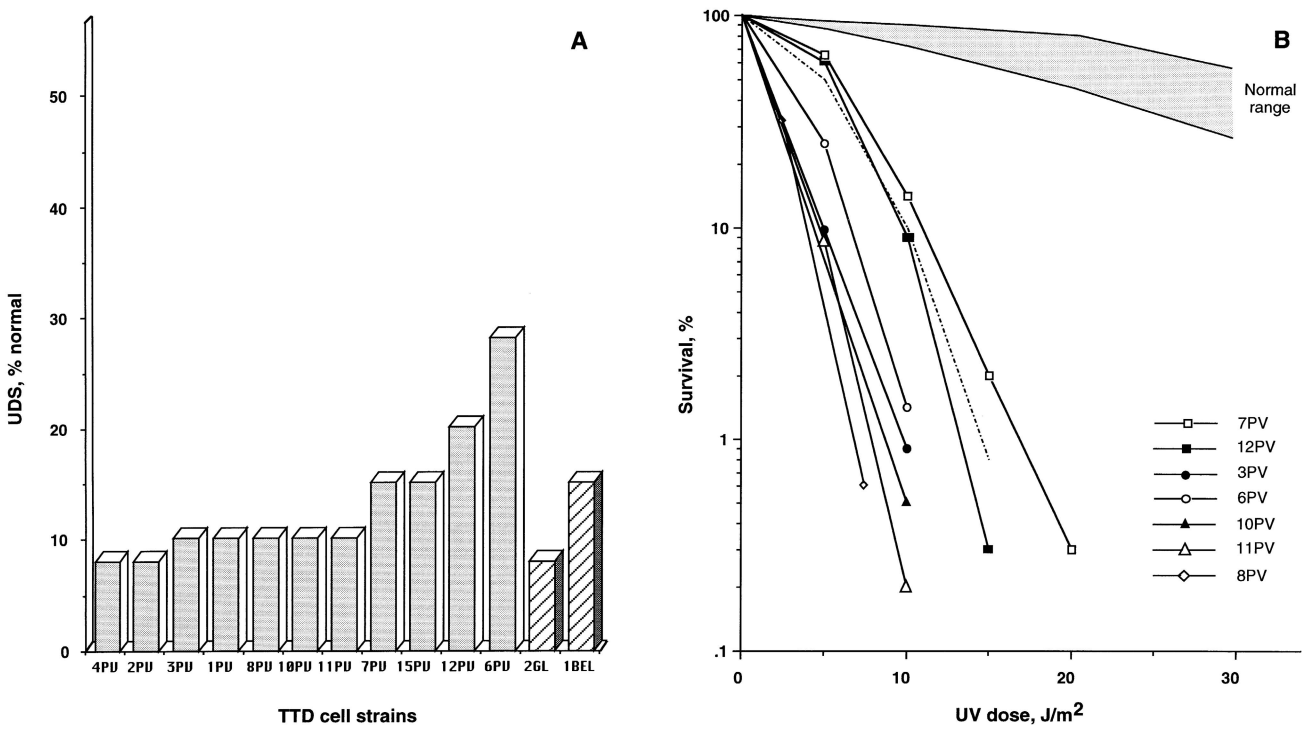

Figure 1 Response to UV irradiation in fibroblast strains from repair-defective TTD patients ("TTD" prefix has been omitted). *A*, Repair synthesis after 20 J/m^2 in all the cases identified in Italy thus far (*gray-shaded cols.*) and in the British patients 2GL and 1BEL (*diagonally hatched cols.*). The mean number of autoradiographic grains per nucleus is expressed as percentage of the level observed in normal cells analyzed in parallel; the reported values are the mean of at least three independent experiments. *B*, Survival, after UV irradiation, of nondividing cells from Italian TTD patients (*symbols*), normal subjects (*gray-shaded area*), and XP patients belonging to the D group (*broken line*). An altered response to UV irradiation in patients TTD1PV–TTD4PV, TTD6PV–TTD10PV, TTD2GL, and TTD1BEL has been described in previous reports (Stefanini et al. 1986, 1992, 1993*a*); the values in the current series of experiments are similar to those reported in those earlier studies.

mother, suggesting the occurrence of an unexpressed allele. The lack of expression of the maternal *XPD* allele was confirmed by the observation that, in the genomic DNA, the patient was heterozygous for the A2096G change. The patient is therefore functionally hemizygous. Previous analysis by Southern blotting did not reveal any large deletion or rearrangement involving the *XPD* gene, either in the patient or in her mother (Mondello et al. 1994). We therefore searched for a causative mutation in the 5′- and 3′-flanking sequences of the unexpressed allele, by amplifying these regions in the genomic DNA of the *XPD* gene in the patient. We used primers starting 242 and 178 nucleotides upstream and downstream, respectively, of the transcribed region. No mutation was revealed by sequencing of either region, indicating that (i) the expression failure is not caused by transcript instability due to alterations in the polyadenylation signal in the genomic 3′-flanking region and (ii) the expression of the *XPD* gene appears to require some transcriptional control elements in addition to those contained in the genomic 5′-flanking region—namely, the classic promoter elements (TATA, CAAT, and GC boxes) and a pyrimidine-rich region postulated to play an important role in the expression of the gene (Weber et al. 1990).

Patients TTD12PV and TTD15PV

The two affected brothers, TTD12PV and TTD15PV, were compound heterozygotes. A G→A transition at position 854, resulting in a cys259tyr substitution, was maternally inherited, and a C→T transition at position 2242, resulting in an arg722trp substitution, was paternally inherited (fig. 3*B*). In addition, the father was heterozygous for a silent C→T transition at 2211. This alteration must be on the normal *XPD* allele of the father, because it was not present in the patients.

Patient TTD4PV

Complete sequencing of the *XPD* cDNA in patient TTD4PV revealed that the patient was heterozygous for three changes: a G→A transition (maternally inherited)

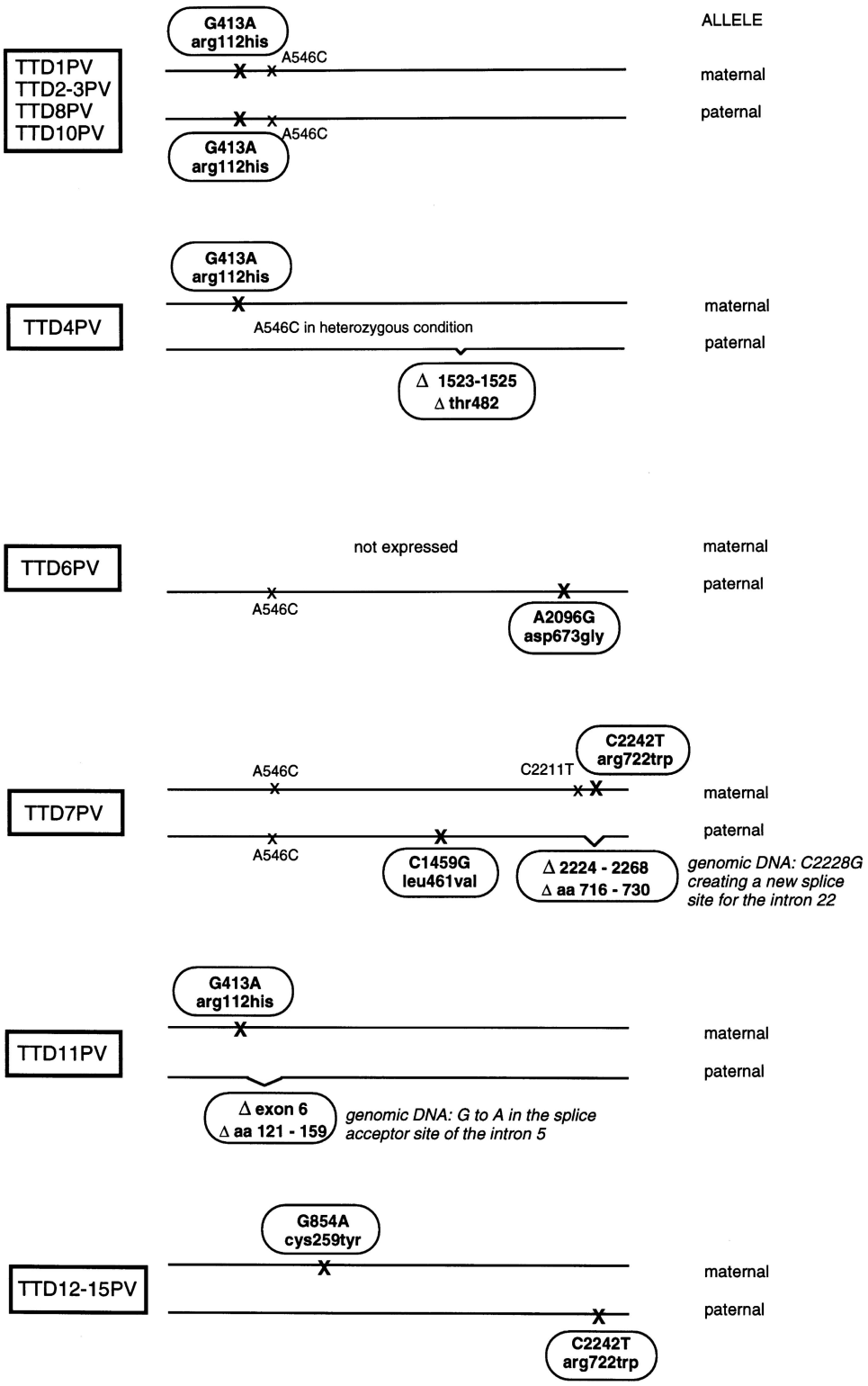

Figure 2 Mutations found in the *XPD* alleles of 11 Italian TTD patients from nine families. Causative mutations detected in *XPD* cDNA, as well as resulting protein alterations, are within the ovals.

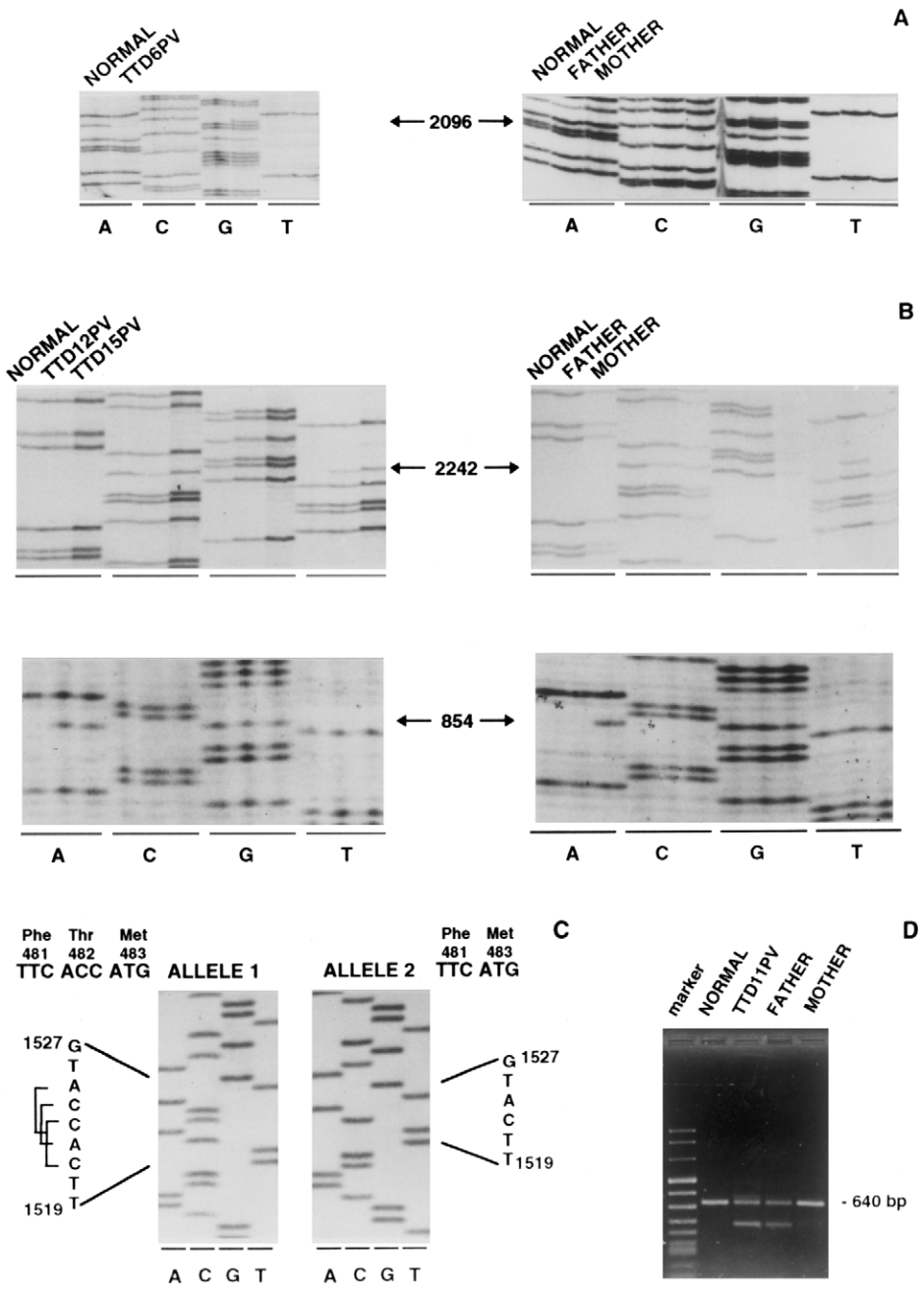

Figure 3 Mutations in the *XPD* cDNA of patients TTD4PV, TTD6PV, TTD11PV, TTD12PV, and TTD15PV. *A–C,* Autoradiographs of sequencing gels, showing A2096G transition in the family of TTD6PV (*A*), G854A and C2242T transitions in the family of the brothers TTD12PV and TTD15PV (*B*), and the three-base deletion in allele 2 of TTD4PV (*C*). *D,* Agarose gel electrophoresis of PCR amplification products of 5′ region of *XPD* cDNA in the family of TTD11PV.

at position 413, resulting in an arg112his substitution; a silent A→C transversion at position 546; and a three-base deletion (inherited fro the father), resulting in loss of thr482. The latter was confirmed by the sequencing of individual clones and could have arisen from the deletion of triplet 1521–1523, triplet 1522–1524, or triplet 1523–1525 (fig. 3*C*).

Patient TTD11PV

Amplification of the 5′ region of the *XPD* cDNA from patient TTD11PV produced both a fragment of normal size and a shorter fragment (fig. 3*D*). Analysis of the normal-size fragment revealed a G→A transition (ma-

ternally inherited) at position 413. Sequencing of the short fragment that was present in both the patient and his father demonstrated that it was missing 117 bases corresponding to exon 6. Analysis of the genomic DNA region including exons 5–7 identified a G→A transition in the last base of intron 5. Both the patient and his father were heterozygous for this mutation, which would destroy the splice acceptor site for intron 5. The loss of 39 amino acids encoded by exon 6 is likely to destroy the activity of the protein completely. (The silent A→C transversion at position 546 was present in both parents, in the heterozygous state, but was not present in the patient. It must therefore be present on the normal *XPD* allele of the parents.)

Patient TTD7PV

Five changes were identified in the *XPD* cDNA of patient TTD7PV (fig. 2). Direct sequencing of the nucleotide fragment 1433–2397 revealed the occurrence of both a paternally inherited heterozygous C→G transversion at position 1459 and a 45-bp deletion of nucleotides 2224–2268. This is consistent with previous reports, in which these two mutations were always found associated on the same allele (Takayama et al. 1995, 1996; Taylor et al. 1997). The fragment containing the deletion could be separated from the other allele by separation of the PCR products amplified from nucleotides 2119–2397, on a 4% NuSieve agarose gel. The normal-size fragment contained a maternally inherited C→T transition at position 2242, resulting in an arg722trp change. Genomic DNA analysis in the members of TTD7PV's family demonstrated that the patient and her father were heterozygous for a C→G transversion corresponding to position 2228 of the cDNA. This mutation creates a new splice-donor site for intron 22, at position 2223/4, resulting in both deletion of the cDNA region 2224–2268 and loss of amino acids 716–730. In addition, two silent changes were observed. The patient and her father were homozygous, and her mother was heterozygous, for the A546C transversion, and the patient and her mother were heterozygous for a C→T transition at position 2211. In conclusion, patient TTD7PV had a maternally inherited C2242T transition associated with the silent mutations A546C and C2211T and had a paternally inherited *XPD* allele carrying both the silent change A546C and the C1459G and C2228G transversions.

Discussion

In Italy, 11 patients from nine families have been identified in whom the hair abnormalities diagnostic for TTD are associated with photosensitivity and the other symptoms typical of the disease. In all these patients, clinical photosensitivity was associated with an altered cellular response to UV light, characterized by low levels of DNA-repair synthesis and survival. As indicated by complementation analysis, this cellular phenotype was due to defect(s) in the *XPD* gene. As summarized in figure 2, in total we detected two silent mutations, an unexpressed allele, and eight potentially causative mutations, four of which have not been described elsewhere. The silent A546C and C2211T changes are polymorphisms frequently observed both in the normal population and in affected individuals (Broughton et al. 1996; present study).

The most frequently observed mutation responsible for the pathological phenotype in the Italian TTD patients was G413A, resulting in arg112his. Five patients (TTD1PV, TTD2PV, TTD3PV, TTD8PV, and TTD10PV) were homozygous, and two patients (TTD4PV and TTD11PV) were heterozygous, for this mutation. All the homozygotes for the G413A mutation were also homozygotes for the A546C silent change. These results strengthen the hypothesis that there was a common ancestor, which was suggested elsewhere by consanguinity studies, of the families of patients TTD1PV, TTD2PV, TTD3PV, and TTD4PV (Nuzzo et al. 1990). The maternal allele of TTD11PV, in which the G413A transition was not linked to the A546C polymorphism, is likely to have a different origin.

New Mutations

We have detected the following several mutations, not described elsewhere, that are expected to compromise the function of the protein severely: (1) Thr482, which is deleted in TTD4PV, is located in a sequence of nine amino acids that are predicted to be in alpha-helical conformation (L. Brocchieri, personal communication) by the PHD secondary-structure–prediction protocol (Rost and Sander 1993, 1994; Rost et al. 1994). (2) In patient TTD11PV, a G→A transition in the last base of intron 5, resulting in the deletion of the whole of exon 6, is likely to abolish function completely. (3) The asp673gly change, which is found in patient TTD6PV, alters a residue that is conserved in five homologues (human, rodent, fish, and the yeasts *Saccharomyces cerevisiae* and *S. pombe*); this mutation must be responsible for the pathological phenotype, because the maternal allele was not expressed in patient TTD6PV's cells. (4) The cys259tyr change, found in patients TTD12PV and TTD15PV, alters a residue that is located in RNA helicase domain V and that is conserved in three of the homologues (human, rodent, and fish) but not in the yeasts *S. cerevisiae* and *S. pombe* (where it is replaced by alanine). This mutation is likely to interfere with the

function of the XPD protein at least as drastically as the arg722trp does. Both the severity of the clinical symptoms and the DNA-repair defect in both brothers are similar to those reported for three other TTD patients (TTD1BEL, TTD1VI, and TTD7PV) in whom the allele carrying this mutation is likely to be the one relevant for the pathological phenotype (Taylor et al. 1997).

Relationship between Mutation and Repair Deficiency

All the mutations found in XP patients in the XP-D group (fig. 4) appear to result in a similar degree of impairment in the cellular response to UV irradiation (Taylor et al. 1997). In contrast, a wide range of sensitivity to the lethal effects of UV light is observed in TTD (Lehmann et al. 1988; Stefanini et al. 1992, 1993*a*). The Italian patients and some of the non-Italian cases (e.g., patients TTD2GL and TTD1BEL in fig. 1) show a drastic reduction in UDS levels, with a cellular sensitivity, to UV, that is similar to or even greater than that in XP-D patients (fig. 1). Other non-Italian TTD patients are characterized by a lower reduction in UDS level, with survival levels significantly affected only at high UV doses (Stefanini et al. 1992, 1993*a*). These different degrees of impairment in the cellular responses to UV in TTD appear to be related to specific mutations. Comparison of figures 1 and 4 shows that a remarkable UV sensitivity is associated with the arg112his substitution, the change most frequently found in TTD, as well as with both the asp673gly change, the only expressed allele in patient TTD6PV, and (somewhat less marked) the arg722trp change, the relevant alterations in patients TTD7PV, TTD1BEL, TTDIVI and, likely, in the brothers TTD12PV and TTD15PV. Mild UV sensitivity is associated with mutations resulting either in a change of arg658 or in the loss of the final portion of the XPD protein. Intriguingly, as already mentioned, the repair

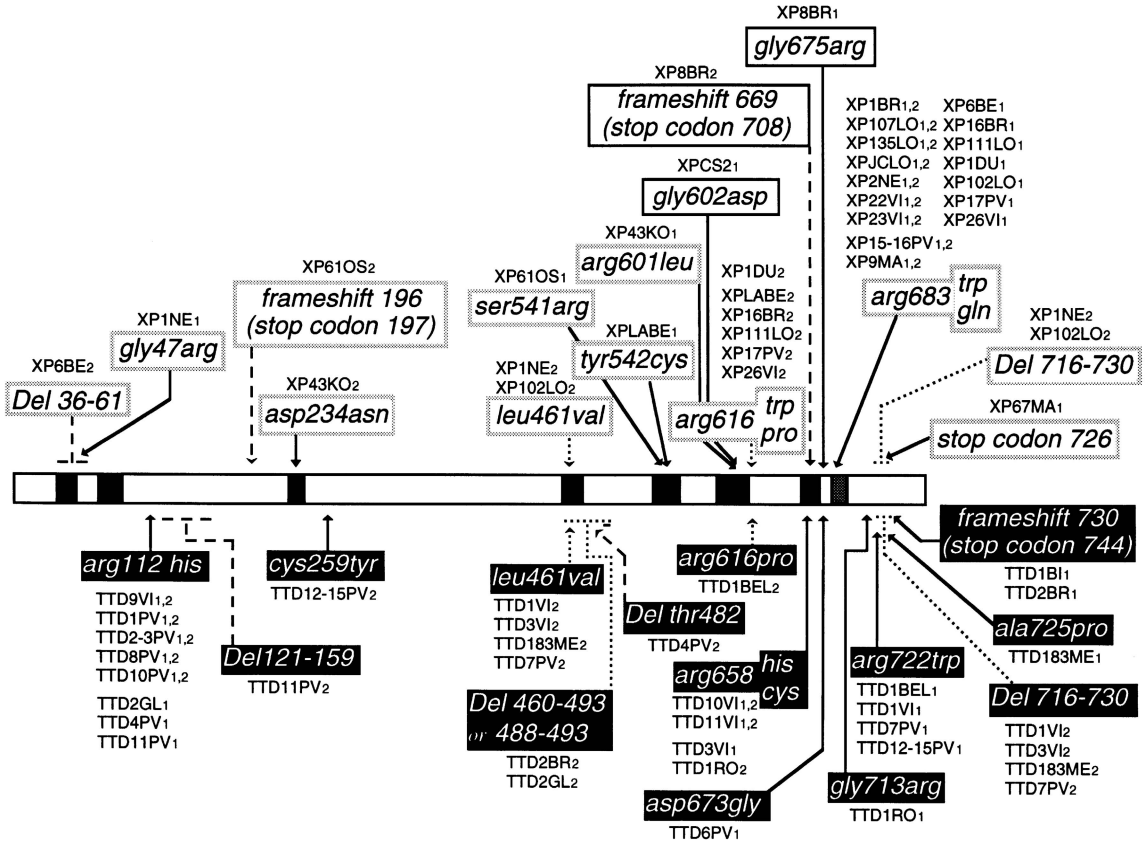

Figure 4 Mutations in the XPD protein in TTD, XP, and XP/CS patients. The diagram shows the XPD protein, with the helicase domains (*blackened boxes*) and the putative nuclear-location signal (*gray-shaded box*). The amino acid changes resulting from the mutations found in the different pathological phenotypes are shown boxed, with the change shown as either white on black (for TTD) or black on white (for XP [*dotted border*] and for XP/CS [*unbroken border*]). The changes responsible for the pathological phenotype, those resulting in deletions likely to affect cellular viability, and mutations described, by Taylor et al. (1997), as being lethal are indicated by unbroken, dashed, and dotted arrows, respectively. Numbers "1" and "2" after the patient code denote the different alleles. The mutation leu461val and the deletion 716–730 have always been found associated in a single haplotype.

defect in TTD never results in an increased incidence of tumors or even in the severe skin alterations typically reported for XP cases. It has been suggested that the clinical features of XP are due to mutations affecting only the repair function of the XPD protein, whereas the TTD phenotype is the result of mutations that also compromise the transcription activity of TFIIH, perhaps interfering with tissue-specific transcription or the transcription of a specific set of genes (Bootsma and Hoeijmakers 1993). Since this model does not obviously explain the lack of cutaneous lesions and skin cancers in TTD, it is necessary to postulate that the transcriptional defect in some way suppresses the development of an NER defect into sunlight-induced skin abnormalities.

Relation between Mutation and Clinical Severity

Perhaps surprisingly, the clinical features of the TTD patients do not correlate with their repair deficiencies. At the cellular level, all the Italian patients have a severe DNA-repair defect, resulting in UV hypersensitivity similar to (in the case of patients TTD7PV, TTD12PV, TTD13PV, TTD14PV, and TTD15PV) or even greater than that in XP-D cells. In contrast, there are substantial clinical differences in the severity of their mental and growth retardation, in their proneness to infections, and in their ages at death (see case reports and table 1). The less-compromised pathological phenotype is present in patients homozygous for the arg112his change, who have the most severe repair defect. They are all alive, at ages 9–30 years, and they show relatively moderate psychomotor retardation and no increased proneness to infections. They are able to stand up, to manipulate things, and to have social relations. If we assume that the features of TTD do indeed result from transcriptional abnormalities (see above), this suggests that, although the arg112his change has the most drastic effect on the repair activity of TFIIH, this is not the case for its transcriptional activity.

In contrast, it is striking that three patients, who are compound heterozygotes for the arg112his change (in the case of TTD4PV and TTD11PV, both of whom are described in the present study) and TTD2GL (described in Lehmann et al. 1988; Tolmie et al. 1994), are more severely affected at the clinical level than are the arg112his homozygotes. Both patient TTD4PV and patient TTD2GL (Tolmie et al. 1994) showed little mental and physical development, had recurrent infections, and died in early infancy. At age 11 years patient TTD11PV is severely dystrophic, unable to speak or stand up, and experiences repeated and severe infectious illnesses. The second allele in these three patients is likely to be totally nonfunctional. We showed this directly for patient TTD2GL (Taylor et al. 1997); in TTD11PV, the paternal allele contains a large deletion; and, in patient TTD4PV, a critical threonine residue is deleted in the paternal allele. These observations allow us to speculate that the main determinant of the severity of the clinical features might be the effective *XPD*-gene dosage. Results of examination of the mutations identified in other severely affected patients (fig. 4) are consistent with this hypothesis. In most of these patients, one of the alleles is completely nonfunctional. In the present study, this is clearly the case for patient TTD6PV (in whom only one allele expressed) and patient TTD7PV (in whom the paternally inherited allele has been shown, by Taylor et al. [1997], to be lethal), as well as for those patients with the arg112his change, discussed above. Likewise, in the severely affected patients TTD1BEL and TTD1VI, described elsewhere (Broughton et al. 1994; Takayama et al. 1996), one allele was shown to be nonfunctional (Taylor et al. 1997). This group of compound heterozygotes is therefore functionally hemizygous. Consequently, not only will their TFIIH contain a partially functionally mutated XPD subunit, but it also will be present at only half the normal amount. This may well result in a more severe impairment of the transcriptional activity of TFIIH. This hypothesis implies that, in tissues critically affected in TTD, the level of XPD and, by implication, of TFIIH is rate limiting for transcription. Although a recent paper by Satoh and Hanawalt (1997) has suggested that this is not the case in cell-free extracts of proliferating human lymphoblastoid cells from XP-D patients with XP or XP/CS, the situation either in TTD or in specialized tissues in vivo may be quite different.

A similar effect of gene dosage on clinical features has recently been reported for two ataxia-telangiectasia families with the same point mutation, 7271G→T, in the *ATM* gene (Stankovic et al. 1998). In one family, the mutation was homozygous, the sensitivity to radiation was greater, but the clinical features were milder, than in the second family, in which the mutation was heterozygous. The mutation in the second allele in the second family resulted in a nonfunctional truncated protein, so that the patient was functionally hemizygous. These findings for the *ATM* gene resemble ours for the *XPD* gene.

In conclusion, our findings demonstrate that in TTD the same mutated allele is associated with a specific UV hypersensitivity, both in the homozygous patients and in the functionally hemizygous patients. In contrast, the severity of the clinical symptoms does not correlate with the extent of the DNA-repair defect, but it appears to be influenced by the dosage of the mutated allele. These findings should stimulate further investigations into the complex genotype-phenotype relationships of the *XPD* gene.

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