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High Prevalence of the Point Mutation in Exon 6 of the Xeroderma Pigmentosum Group A-complementing (XPAC) Gene in Xeroderma Pigmentosum Group A Patients in Tunisia

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Summary

Xeroderma pigmentosum (XP) patients in Tunisia who belong to the genetic complementation group A (XPA) have milder skin symptoms than do Japanese XPA patients. Such difference in the clinical features might be caused by the difference in the site of mutation in the XP A-complementing (XPAC) gene. The purpose of this study is to identify the genetic alterations in the XPAC gene in the Tunisian XPA patients and to investigate the relationship between the clinical symptoms and the genetic alterations. Three sites of mutation in the XPAC gene have been identified in the Japanese XPA patients, and about 85% of them have a G→C point mutation at the splicing acceptor site of intron 3. We found that six (86%) of seven Tunisian XPA patients had a nonsense mutation in codon 228 in exon 6, because of a CGA→TGA point mutation, which can be detected by the *Hph*I RFLP. This type of mutation is the same as those found in two Japanese XPA patients with mild clinical symptoms. Milder skin symptoms in the XPA patients in Tunisia than in those in Japan, despite mostly sunny weather and the unsatisfactory sun protection in Tunisia, should be due to the difference in the mutation site.

Introduction

Xeroderma pigmentosum (XP) is an autosomal recessive hereditary disease characterized by hyperphotosensitivity and development of skin cancers in the sun-exposed area. Seven genetic complementation groups, A-G, of the excision repair-deficient type and one excision repair-proficient variant type have been identified. XP patients belonging to complementation group A (XPA) are more frequent among XP types in Japan than they are in the United States and European countries (Kraemer et al. 1987). In Tunisia, where the frequency of XP patients is higher than it is in Japan, the United States, and Europe, 7 (18%) of 38 patients with XP were assigned to XPA (M. Zghal, unpublished data). Most of the Japanese XPA patients have severe skin symptoms, with early onset (at about 10 years of age) of skin cancers (Takebe et al. 1987), while Tunisian patients develop skin cancers at about 20 years of age, despite unsatisfactory protection from the sunlight (M. Zghal, unpublished data). Recently, a human gene designated the "xeroderma pigmentosum group A-complementing" (XPAC) gene was cloned (Tanaka et al. 1990), and three sites of mutation in the XPAC gene in Japanese XPA patients have been identified (Satokata et al. 1992*a*).

Mutations in the XPAC gene should cause the DNA repair defects in the XPA patients. A point mutation at the splicing junction of intron 3 and exon 4 was found to be predominant in Japanese XPA patients, and two nonsense mutations, one each at codon 116 (exon 3) and codon 228 (exon 6) of the gene (Satokata et al. 1992*a*), were found in a few other patients. The mutation at the splicing junction, by a transversion (G→C) at the 3′ splice acceptor site of intron 3 of the XPAC gene, creates a new cleavage site for the restriction endonuclease *Alw*NI, and this mutation can be detected by the combination of PCR and *Alw*NI RFLP analysis. The

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Table 1

Clinical and Biological Characteristics of XPA Patients in Tunisia

Patient	UDS (% of normal cells)	D_0[a] (J/m^2)	Age at Observation (years)	Age at Onset of Dermatological Symptoms (mo)	Age at First Consultation (years)	Severity of Symptoms		
						Dermatological[b]	Photophobia[c]	Neurological[d]
XP5TU	6	.75	20	12	10	++	++	−
XP8TU	4	.70	35	36	18	++	+	+
XP11TU	1	.70	27	12	6	++	+	++
XP12TU	2	.80	27	6	13	+	+	+++
XP13TU	3	.80	34	2	20	+	+	+++
XP27TU	6	.75	15	24	5	++	+	++
XP33TU	5	.70	9	12	3	++	−	−[e]

[a] UV fluence that gives 37% survival in the colony-formation assay, after exposure to UV (254 nm), in the straight portion of the survival curve.

[b] + = Erythema after exposure to the sun; and ++ = severe erythema or bullae formation after exposure to the sun.

[c] + = Photophobia when patient views the sun; ++ = photophobia when patient is outdoors; +++ = photophobia when patient is near an open window; and − = no symptom of photophobia.

[d] + = Mental retardation only; ++ = mental retardation and reduced neurological reflexes; +++ = inability to walk, because of severe neurological abnormalities such as spasticity or ataxia; and − = no neurological symptoms.

[e] Absence of any neurological abnormalities; however, patient may have been too young for such abnormalities to be positively identified. Her sister, XP11TU, did show such abnormalities.

mutation in exon 3 creates a new cleavage site for the restriction endonuclease *Mse*I, by a transversion (T→A) at codon 116, and the mutation in exon 6 creates an *Hph*I site by a transition (C→T) at codon 228. Satokata et al. (1992*b*) further examined the gene alterations in seven non-Japanese XPA patients and showed that none of them had the same mutation as those found in the Japanese XPA patients. Heterogeneity in clinical characteristics among XPA patients may depend on the site of mutation (Nishigori et al., in press), and this study intends to identify the gene alterations in Tunisian XPA patients and to analyze whether the sites of the genetic alterations in the XPAC gene are related to the clinical features.

Material and Methods

Cell Culture

Fibroblast cells were grown from the skin biopsies of non–sun-exposed areas of seven Tunisian XPA patients and a healthy volunteer, NITU. Clinical characteristics of these patients are listed in table 1. XP11TU and XP33TU are siblings, as are XP12TU and XP13TU. All cultured cells were maintained in Dulbecco's modified minimum essential medium (Nissui Seiyaku, Tokyo) supplemented with 15% FBS (HyClone; Sterile Systems, Logan, UT) in 5% CO_2 atmosphere at 37°C. Cells at fewer than 10 passages were used in all experiments.

UV Irradiation

Germicidal lamps (Toshiba GL10) emitting predominantly 254 nm were used as the source of UV light, and the fluence rate was measured by a UV radiometer, UV-254 (Topcon; Tokyo Kogaku Kikai KK, Tokyo).

Measurement of Unscheduled DNA Synthesis (UDS) after UV Irradiation (254 nm)

UDS after UV irradiation (254 nm) was measured as described by Cleaver and Thomas (1981). Approximately 5×10^4 cells were inoculated onto a glass coverslip in a 35-mm dish and incubated. On the next day, the medium was removed, and the cells were washed with PBS and were irradiated with 20 J of UV/m^2 and then incubated in the medium containing [^{3}H]-thymidine (10 μCi/ml; 25 Ci/mmol; Amersham, Buckinghamshire, UK) for 3 h. After the labeling, cells were fixed and washed with 5% ice-cold trichloroacetic acid. [^{3}H]-thymidine incorporated into nuclear DNA was detected by autoradiography using Kodak nuclear emulsion NTB3 (Kodak, Rochester, NY), with exposure for 1 wk at 4°C. Cells were stained with Giemsa solution, and the number of grains per nucleus was scored for 100 nuclei in each specimen.

Genetic Complementation Test

Cells from a patient and of a strain of a known XP complementation group were seeded together (5×10^5

cells each) in a 35-mm dish. The cells were treated with 46% polyethylene glycol (PEG) (molecular weight 6,000; Nakalai Tesque, Kyoto) for 1 min, to fuse cells, and then the PEG was vigorously washed off. On the next day, the cells were trypsinized and inoculated on coverslips, and UDS measurement was done as described above. The number of grains in the nuclei in the binucleated cells was scored.

Colony Formation after UV (254-nm) Irradiation

Cellular sensitivity to UV was assayed by determining the colony-forming ability after irradiation. Appropriate numbers of cells were seeded into 60-mm dishes and incubated. On the next day, the medium was removed, and the cells were washed with PBS. After cells were irradiated with various fluences of UV, dishes were refilled with the medium and further incubated, with medium changes being done twice a week until colonies appeared. After being fixed and stained, colonies (50 cells or more) were counted.

Extraction of DNA

Genomic DNA was extracted from the cultured fibroblasts as described by Maniatis et al. (1982). Cells in 10-cm dishes were harvested and suspended in the digestion buffer with ribonuclease A for 3 h at room temperature and incubated with 100 μg of proteinase K/ml at 37°C overnight, followed by phenol-chloroform (1:1) extraction three times, and were dialyzed against TE (10 mM Tris-Cl, 1 mM EDTA pH 7.6) buffer.

Amplification of DNA Sequences of the XPAC Gene by PCR

A genomic DNA fragment of the XPAC gene, containing exon 4 with 5′ and 3′ flanking introns, was amplified by PCR using a primer containing an *Eco*RI restriction site at the 5′ end, 5′-GGGAATTCTTGCTGGGCTATTTGCAAAC-3′ (intron 3), and a primer containing a *Bam*HI restriction site at the 5′ end, 5′-GGGGATCCGCCAAACCAATTATGACTAG-3′ (intron 4). A genomic DNA fragment including exon 3 was amplified by PCR using primer 31, 5′-GGGAATTCGAAACTAGAGTTCATTTTCC-3′ (intron 2), and primer 32, 5′-GGGAATTCGTTTTGCCCTAAACCTACAC-3′ (intron 3) (Satokata et al. 1992*a*). A genomic DNA fragment including exon 6 was amplified by PCR using a primer containing an *Eco*RI site at the 5′ end, 5′-GGGAATTCGGATTCACCTGAATAGCACC-3′ (intron 5), and a primer containing a *Bam*HI site at the 5′ end, 5′-GGGGATCCACATTGTGCACACAACCAGG-3′ (intron 6). The primers were synthesized with an Applied Biosystems, 380A DNA synthesizer (Applied Biosystems, Foster City, CA) by the procedures recommended by the supplier. Amplifications with *AmpliTaq* DNA polymerase (Perkin Elmer Cetus Instruments, Norwalk, CT) were performed in an automatic thermocycler (PC-700 Astec, Fukuoka, Japan), as described by Saiki et al. (1988). The amplified fragments were digested with restriction enzymes (New England Biolabs, Beverly, MA)—*Alw*NI for the fragments containing exon 4, *Mse*I for the fragments containing exon 3, and *Hph*I for the fragments containing exon 6, at 37°C for 6 h. The digested DNA was separated by electrophoresis in 5% polyacrylamide gel and was stained with ethidium bromide.

Results

Clinical Characteristics of Tunisian XPA Patients

Skin symptoms were noticed in childhood (2–36 mo) in most of the patients (see age at onset of dermatological symptoms in table 1), according to observation by doctors and the claims by the patients or the parents of the patients at the first consultations. Ages of the first consultations (see table 1) took place later (i.e., at age 3–20 years) than the ages at onset of the skin symptoms, presumably reflecting the mildness and slow progression of the symptoms. Ages at onset of skin cancers in the Tunisian XPA patients and, for comparison, in the Japanese XPA patients with the splicing mutations who developed skin cancers are given in table 2. On average, the onset of skin cancers was later in Tunisian patients, in both histopathological types, basal cell carcinoma and squamous cell carcinoma, than in Japanese patients. No skin cancer developed in XP33TU, 9 years old. Of seven patients, only one, XP5TU, 20 years old, did not show the neurological abnormalities, while skin cancers developed at the age of 11 years (tables 1 and 2).

The rest of the patients developed hearing impairment at about the age of 15 years (M. Zghal, unpublished data), whereas hearing impairment is one of the earliest signs of the neurological abnormalities observed in Japanese XPA patients (Nishigori et al., in press). In two Tunisian patients, 34 and 35 years old, neurological abnormalities are slowly but gradually progressing. None of the Tunisian XPA patients had microcephaly.

DNA Repair Test

UDS in the cells of the patients varied from 1% to 6% of the normal level, as shown in table 1. All patients in

Table 2

Ages at Onset (years) of Skin Cancers in XPA Patients, by Histopathological Type and RFLP Genotype

Patient	Histopathological Type				RFLP Genotype[a]		
	Squamous Cell Carcinoma	Basal Cell Carcinoma	Malignant Melanoma	Kerato-acanthoma	*Hph*I	*Alw*NI	*Mse*I
Tunisian:							
XP5TU	11	15			+/+	+/+	+/+
XP8TU	22				−/−	+/+	+/+
XP11TU	21	15	23		−/−	+/+	+/+
XP12TU	22				−/−	+/+	+/+
XP13TU	20	20			−/−	+/+	+/+
XP27TU	10				−/−	+/+	+/+
XP33TU					−/−	+/+	+/+
Japanese with the homozygous splicing mutation:[b]							
XP1BP	6	6		6	+/+	−/−	+/+
XP15KY		14		14	+/+	−/−	+/+
XP1KN	9				+/+	−/−	+/+
XP1EH	11	7			+/+	−/−	+/+
XP4KG	11	12	14		+/+	−/−	+/+
XP1NI	12				+/+	−/−	+/+
XP11KY		8			+/+	−/−	+/+
XP2KY		9			+/+	−/−	+/+
XP104TO		11			+/+	−/−	+/+

[a] + = Wild type; and − = presence of a mutation detected by each enzymes.
[b] Data taken from our file.

table 1 were assigned to complementation group A. Cellular UV sensitivity was examined by the colony-forming ability and is represented by D_0 values (table 1). Most of the Tunisian XPA cells were slightly less sensitive than those in Japanese XPA patients with the homozygous splicing mutations whose D_0 values are 0.50 J/m^2 or less. (Nishigori et al., in press).

*Hph*I RFLP in Exon 6 of the XPAC Gene in Tunisian XPA Patients

To detect the nonsense mutation in exon 6 of the XPAC gene, we examined the *Hph*I RFLP in the amplified exon 6 DNA fragments. DNA from normal subjects who have a normal XPAC allele gives a 426-bp band. In contrast, DNA from a patient with the homozygous mutation in exon 6 should give two bands—one of 347 bp and the other of 79 bp—because a new cleavage site for *Hph*I is created in the 426-bp fragment by the nonsense mutation, from CGA to TGA, at codon 228 (fig. 1*B*). In six of seven patients, the 347-bp and 79-bp bands appeared (fig. 1*A*), confirming that these six patients belong to XPA and are homozygous for the nonsense mutation at codon 228 in exon 6. The only exceptional case, XP5TU, did not have the *Hph*I site, indicating that the mutation at codon 228 in exon 6 was not involved, though this patient was identified as XPA by the genetic complementation test.

*Alw*NI RFLP in Intron 3 and *Mse*I RFLP in Exon 3

None of the Tunisian XPA patients showed either the *Alw*NI or the *Mse*I restriction site. XP5TU, without the exon 6 mutation, should have a mutation in an unidentified site. The RFLP genotypes are shown in the right-hand columns in table 2.

Discussion

In Japan, XPA is the most frequent type among excision repair–deficient types of XP (Satoh and Nishigori 1988). Most of the XPA patients have severe clinical symptoms such as acute sunburn, early onset of skin cancers in the sun-exposed areas, and neurological abnormalities (Takebe et al. 1987). In Tunisia, about 15% of XP patients are XPA (M. Zghal, unpublished data).

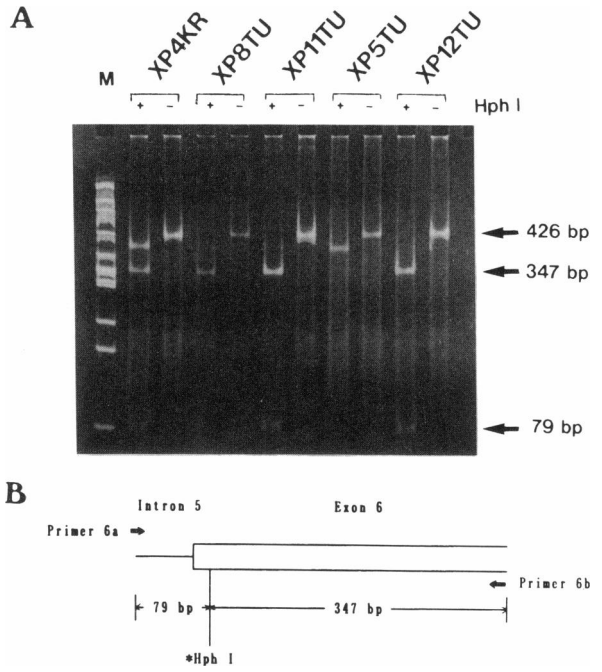

Figure 1 *A*, *Hph*I RFLP analysis of the amplified DNA of exon 6. PCR products were digested with *Hph*I at 37°C for 6 h and were analyzed by 5% acrylamide gel electrophoresis. Lane M shows size markers (*Hinc*II digest of ϕX174 DNA). XP4KR, who is a compound heterozygote for the nonsense mutations in exon 6 and the splicing mutation, was used as a control. *B*, Schematic diagram of the XPAC gene, exon 6. The sites of the primers are shown by arrows. A new cleavage site for *Hph*I is indicated by the asterisk (Satokata et al. 1992*a*).

As shown in table 1, the ages at onset of skin symptoms vary from 2 to 36 mo, whereas most of the Japanese XPA patients reveal their first skin symptoms before the age of 6 mo (Nishigori et al., in press). Tunisian XPA patients in this study developed the first skin cancers at about age 15 years, whereas Japanese XPA patients had their first skin cancers before the age of 10 years (table 2). The latitude of the populated areas in Tunisia is almost the same as that of southern Japan, and the sun protection in Tunisia does not appear to be satisfactory, because most of the patients see doctors only occasionally and cannot buy sunscreens. The weather in Tunisia is far more sunny and clearer than that in Japan, with very few cloudy and rainy days. Considering these environmental conditions, we expected that Tunisian XPA patients should have more severe clinical symptoms than are seen Japanese XPA patients. As shown in tables 1 and 2, this assumption was absolutely wrong, possibly because of the difference in the genetic background, and it prompted us to investigate the sites of the genetic changes in the XPAC gene. We found that the predominant type in Tunisian XPA patients was the homozygous nonsense mutation at exon 6, while nearly 90% (41/46) of Japanese XPA patients have the homozygous splicing mutation at intron 3 (C. Nishigori, unpublished data).

Japanese XPA patients with the splicing mutations in intron 3 usually develop very severe neurological abnormalities. The symptoms start when they are younger than age 10 years, and most of them cannot walk and are on bedrest by their teens. Most of them die at about age 20 years (Nishigori et al., in press). Tunisian XPA patients with the nonsense mutation at codon 228 have neurological abnormalities, but their symptoms are less severe than those in Japanese XPA patients who have the splicing mutations. One of the Tunisian patients is age 34 years, and another is age 35 years, whereas XPA patients older than age 25 years are scarce in Japan.

Satokata et al. (1992*a*) found a Japanese XP patient (XP39OS; Sato et al. 1987) with the homozygous mutation at codon 228 in exon 6, and we found another case of this type, XP1KR (C. Nishigori, unpublished data); both cases had mild dermatological and neurological symptoms. Since XP patients in Japan have received sun protection and skin care intensively in recent years, comparison of the clinical symptoms of XPA patients with the splicing mutation versus XPA patients with the exon 6 nonsense mutation cannot be made precisely, since we had only two Japanese cases who were in the latter category. Additional 6 cases in Tunisia of the same nonsense mutation site, and their milder clinical symptoms despite unsatisfactory care, convinced us to conclude that the clinical symptoms should depend on the site of mutation, even if the mutated gene (i.e., XPAC) is the same. XPAC cDNA encodes a protein of 273 amino acids, and the nonsense mutation is found at position 2 in codon 228. Theoretically, most (84%) of the mRNA of the XPAC gene would be produced with the nonsense mutation in the last exon of the gene, and the mutation site does not appear to be in an important domain such as the zinc-finger motif. The splicing mutation, on the other hand, was found to cause a deletion of most of exon 4. We can speculate that the nonsense mutation in exon 6 does not lose the major function of the XPAC gene, in contrast to the splicing mutation of intron 3, which causes unstableness of the mRNA.

These data suggest that the different sites of gene alteration are reflected in the different clinical features. The difference in the mutation sites appears to lead to a minor difference in the level of DNA repair deficiency, with slightly more UV resistance in Tunisian XP cells

than in Japanese XP cells belonging to the same complementation group.

In Tunisia, the *Hph*I RFLP can be used to screen the XPA patients as well as the heterozygotes, since, in Tunisian XPA, the majority of mutations in XPA may be in codon 228 in exon 6. The incidence of XPA patients in Tunisia may be 10 times greater than that in Japan, probably because of the high frequency of consanguineous marriages. (M. Zghal, unpublished data). Such RFLP analyses require far less time and effort than do the conventional fibroblasts-UDS-DNA repair tests, which take at least a few months. The presence of possible founder effects in the unique mutations in two countries is another point of interest in the distribution of human genotypes and may reflect the stableness of the XPAC gene, which did not yield a variety of mutant genes in one area, either Japan or Tunisia.

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