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Muneo Tanita · Jun Matsunaga · Yoshinori Miyamura
Miwako Dakeishi · Eriko Nakamura · Michihiro Kono
Hiroshi Shimizu · Hachiro Tagami · Yasushi Tomita

Polymorphic sequences of the tyrosinase gene: allele analysis on 16 OCA1 patients in Japan indicate that three polymorphic sequences in the tyrosinase gene promoter could be powerful markers for indirect gene diagnosis

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Abstract Since 1989, a large number of mutations of the tyrosinase gene, which result in oculocutaneous albinism (OCA), have been reported. However, approximately 15% of patients with tyrosinase-related OCA (OCA1) heterozygously carried an uncharacterized mutation, which presumably existed outside of the ordinarily examined area of the tyrosinase gene. In such cases, polymorphic sequence(s) of the tyrosinase gene might be useful to identify the OCA1 allele. In this study, we examined four polymorphic sequences of the tyrosinase gene in 16 patients with OCA1, their relatives, and 108 normally pigmented Japanese individuals. The results showed a complex dinucleotide repeat in the promoter region at –800 to –900 of seven different lengths, and a polythymidine sequence in the 3' end of intron 2 of three different lengths. Polymerase chain reaction-restriction fragment length polymorphism analysis of two polymorphic sequences at –301 (C/T) and –199 (C/A) in the promoter region allows us to classify the tyrosinase gene into three groups. Using these polymorphic sequences, we could identify the OCA1 allele in more than 80% of cases in which the parents' genomic DNA was available. Three polymorphic sequences in the tyrosinase gene promoter are particularly useful for this purpose.

Key words Albinism · Tyrosinase · Mutation · Polymorphism · Gene diagnosis

Introduction

Oculocutaneous albinism (OCA) is an autosomal recessive inherited disorder characterized by the reduced or absent biosynthesis of melanin pigment in melanocytes of the skin, hair follicles, and eyes (King et al. 1995). Until now, three different genes were identified as responsible for OCA1, 2, and 3. Tyrosinase-related OCA (OCA1) results from deficient activity of tyrosinase, which is an essential enzyme of melanin biosynthesis (Lerner et al. 1949). In tyrosinase-negative OCA (OCA1A), tyrosinase activity and melanin biosynthesis are completely absent, and in yellow mutant OCA (OCA1B), tyrosinase activity and melanin biosynthesis are greatly reduced. OCA2 and OCA3 are caused by homozygous gene mutations of P-protein and tyrosinase-related protein 1 (TRP1; DHICA oxidase), respectively (Tomita et al. 2000). Because of no tyrosinase gene mutation, patients with OCA2 and OCA3 have enough tyrosinase activity, and their skin, hair, and eyes are a little pigmented. Therefore they are clinically distinguishable from patients with OCA1, who show no pigment.

The human tyrosinase gene, encoding a 58-kD a glycoprotein composed of 529 amino acids (Shibahara et al. 1988; Takeda et al. 1989), consists of five exons spanning more than 50kb of DNA (Giebel et al. 1991) in chromosome 11q14–q21 (Barton et al. 1988). In 1989, we reported, for the first time, a pathologic mutation of the tyrosinase gene in a patient with OCA1A (Tomita et al. 1989). Since then, a large number of tyrosinase gene mutations associated with OCA1 have been reported (Oetting 2000). In addition to pathological mutations, several polymorphic sequences of the tyrosinase gene were reported (Figure 1). In 1991, Morris et al. showed that a complex dinucleotide (guanine and adenine, GA) repeat located 800–900bp upstream of the protein-coding region of the tyrosinase gene could be classified into five different lengths in 31 unrelated individuals (Morris et al. 1991). Other polymorphic sequences in the promoter were located 301 (cytosine or thymine, C/T) and 199 (adenine or cytosine, A/C) bp, respectively, upstream of the protein-coding region (Oetting and King 1993; Oetting

M. Tanita · J. Matsunaga · H. Tagami
Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan

Y. Miyamura · E. Nakamura · M. Kono · Y. Tomita (✉)
Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel. +81-52-744-2314; Fax +81-52-744-2318
e-mail: tomita@med.nagoya-u.ac.jp

M. Dakeishi
Department of Dermatology, Akita University School of Medicine, Akita, Japan

H. Shimizu
Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Fig. 1. Polymorphic sequences and mutations of the human tyrosinase gene in Japanese patients. The five *boxes* indicate the protein-coding regions of the tyrosinase gene. *Arrows* show the approximate positions of the polymerase chain reaction primers used in this study. Dimensions are not to scale

et al. 1991). In the protein-coding region, two polymorphic sites (codon 192 and 402) have been identified (Giebel and Spritz 1990; Tripathi et al. 1991); however, in our experience, these sites are not polymorphic in the Japanese population.

Sequence analysis of the tyrosinase gene became a routine way to diagnose patients with OCA1 in several laboratories. However, approximately 15% of the patients heterozygously carried an uncharacterized mutation (UCM), which presumably existed outside of the ordinarily examined area of the tyrosinase gene (King et al. 1995). In our laboratory, we usually examined the protein-coding region, exon/intron junctions, and the proximal promoter region (up to 379bp from the coding region) of the tyrosinase gene (Matsunaga et al. 1998). For one patient (Patient 4 in Table 1) with the OCA1A phenotype, who heterozygously carried the R77Q mutation, we further sequenced the patient's tyrosinase gene from the protein-coding region up to position -2114 in the promoter region (Matsunaga et al. 1997). We could not find any pathological mutations, including a tyrosinase distal element (TDE), a strong enhancer for pigment cell-specific expression that exists 1.8kb upstream of the transcription initiation site of the tyrosinase gene (Yasumoto et al. 1994). However, we found a difference between the two OCA1 alleles in the GA repeat region. This finding led us to consider the use of the polymorphic sequences of the tyrosinase gene to identify OCA1 alleles.

To explore the possibility of indirect gene analysis for the diagnosis of OCA1, we examined three polymorphic sequences in the tyrosinase gene promoter and the polythymidine sequence in the 3' end of intron 2 in 16 patients with OCA1, their relatives, and 108 normally pigmented Japanese individuals.

Materials and methods

Patients

Sixteen Japanese patients with OCA1 were included (Table 1). Mutation analysis of the patients 1 (Takeda et al. 1990),

4 (Matsunaga et al. 1997), 5 (Takizawa et al. 2000), 6 (Tomita et al. 1989), 11 and 12 (Matsunaga et al. 1996), 13 (Matsunaga et al. 1998), 14 (Matsunaga et al. 1999), 15 (Nakamura et al. 2001), and 16 (Tomita et al. 2000) have been reported previously. Patients 4 and 5 heterozygously carried a UCM (Table 1). We clinically diagnosed patient 4 as having OCA1A because she had a typical OCA1A phenotype, showing no pigment in the skin, hair, and eyes. Although we could not exclude the possibility that patient 5 had OCA2 or OCA3, we thought that the existence of the R77Q mutation in her tyrosinase gene suggested an association with OCA1 rather than a coincidence with homozygous mutations of P-protein gene or *TRP1* gene.

The patients and all subjects enrolled in this study provided informed consent. This study was approved by the Ethical Committee of Nagoya University Graduate School of Medicine.

Preparation of genomic DNA

Peripheral blood, collected from the patients, their relatives, and phenotypically normal Japanese individuals were used as sources of genomic DNA. We extracted genomic DNA using QIAGEN Blood DNA Kits (QIAGEN, Valencia, CA, USA).

Length analysis for the complex dinucleotide (GA) repeat

The genomic DNA segment containing the region concerned was amplified by polymerase chain reaction (PCR) using ExTaq (TaKaRa, Kyoto, Japan), electrophoresed on an ABI 373A Sequencer (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA), and analyzed for length using ABI GeneScan software. The primers used and the PCR conditions were 5-AGGAAAAACAATATG GCTACAGCATT-3 (TDS4), 5-CCTGATGCATTTTCT GACAACTCC-3 (TDS4R), with an initial heating for 1 min at 94°C, 30 cycles for 20sec at 98°C and 1 min at 65°C, and with a final elongation step for 10 min at 72°C.

Table 1. Mutations and genotypes of the tyrosinase gene in the OCA1 patients and their relatives

Subject	Tyrosinase gene mutation paternal/maternal	Diagnosis	GA repeat bp	−301 C/T	−199 A/C	Intron 2 bp
Patient 1	R77Q/R77Q	OCA1A	359	C	C	209
Patient 2	R77Q/R77Q	OCA1A	359	C	C	209
Patient 3	R77Q/R77Q	OCA1A	359	C	C	209
Patient 4	UCM/R77Q ^a	OCA1A	349/359	C	C	209
Mother			359	C/T	C/A	209
Patient 5	UCM/R77Q ^a	OCA1B	349/359	C	C	209
Father			349	C/T	C	209
Mother			339/359	C/T	C	209
Patient 6	929insC/929insC	OCA1A	357	C	C	209
Father			357	C	C	209
Mother			357	C	C	209
Patient 7	929insC/929insC	OCA1A	357	C	C	209
Patient 8	929insC/929insC	OCA1A	357	C	C	209
Father			357/359	C/T	A/C	209
Mother			357/359	C/T	A/C	209
Patient 9	929insC/929insC	OCA1A	357	C	C	209
Father			349/357	C	C	209
Mother			349/357	C	C	209
Patient 10	929insC/929insC	OCA1A	357	C	C	209
Father			349/357	C	C	209
Mother			339/357	C	C	209
Patient 11	929insC/R278X	OCA1A	357	C/T	C	209
Father			357	C	C	209
Mother			357/370	T	A/C	209
Patient 12	P431L/929insC	OCA1A	357/359	C/T	A/C	209
Father			357/359	T	A/C	209
Mother			349/357	C	C	209
Patient 13	R278X/929insC	OCA1A	357/359	C/T	A/C	209
Father			349/359	C/T	A/C	209
Mother			357/359	C/T	A/C	209
Patient 14	929insC/IVS2-7T>A	OCA1B	357	C	C	207/209
Father			357	C/T	C	209
Mother			357	C/T	C	207/209
Patient 15	R239W/929insC	OCA1A	349/357	C	C	209
Father			349/359	C	C	209
Mother			349/357	C	C	209
Patient 16	D383N/929insC	OCA1A	349/357	C	C	209
Father			349/359	C/T	C	209
Mother			349/357	C/T	C	209

^aUCM means that the tyrosinase gene has no detectable mutation in the examined area

PCR restriction fragment length polymorphism analysis for −301 (C/T) and −199 (C/A) polymorphic sequences

The genomic DNA segment was amplified by PCR using TaKaRa Taq (TaKaRa). The primers used and the PCR conditions were 5-GCTTAGGTCAGGCATTATTATTA CTGA-3 (222P), 5-ATAATACCACTCCCACCTCCA GC-3 (120PR), 30 cycles for 1min at 98°C, 2min at 65°C, and 3min at 72°C. The amplified products were respectively digested with *Mbo* I for −301 (C/T) or *Sfu* I for −199 (C/A), and electrophoresed on a 5% polyacrylamide gel.

Length analysis of the 3' end of intron 2

The genomic DNA segment was amplified by PCR under the same conditions as described for the complex dinucleotide (GA) repeat, except that the primers used were 5-GTAAAGAGTCTCAATACGGAATGAAT-3 (dTTF) and 5-GGCATTGTGCATGCTGCTTTGAG-3 (dTTF4).

Results

Genotype and allele analysis of the tyrosinase gene in the 16 OCA1 patients and their relatives

Table 1 summarizes the results of genotype analysis for the OCA1 patients and their relatives examined in this study. We identified 20 different alleles of the tyrosinase gene, as shown in Table 2. Two polymorphic sequences at −301 (C/T) and −199 (C/A) in the promoter region allowed us to classify the tyrosinase gene into three groups, C-C, T-C, and T-A.

Possibility of indirect gene diagnosis from three polymorphic sequences in the tyrosinase promoter

We tried to examine whether allele analysis of the tyrosinase gene was useful for identifying an OCA1 allele in our

Table 2. Identified alleles in 16 OCA1 patients and their relatives

Haplotype					
GA repeat	−301	−199	Intron 2	OCA1 genotype	Chromosome count
339	C	C	209	Normal	1
349	C	C	209	Normal	6
349	C	C	209	R239W (1A)	2
349	C	C	209	D383N (1A)	2
349	C	C	209	UCM (1A)	1
349	C	C	209	UCM (1B)	2
357	C	C	209	Normal	3
357	C	C	209	929insC (1A)	30
357	C	C	207	IVS2-7T>A (1B)	2
359	C	C	209	Normal	1
359	C	C	209	R77Q (1A)	10
339	T	C	209	Normal	1
349	T	C	209	Normal	2
357	T	C	209	Normal	3
357	T	C	209	R278X (1A)	2
359	T	C	209	Normal	1
359	T	A	209	Normal	4
359	T	A	209	R278X (1A)	2
359	T	A	209	P431L	2
370	T	A	209	Normal	1

cases. Eleven of the 16 cases in which parents' samples were available were subjected to the analysis. As shown in Table 3, in 9 of 11 cases, we were able to diagnose possible sibling haplotypes and various combinations of alleles for those of affected, carrier, or noncarrier status from the allele analysis of the three polymorphic sequences of the patients and their parents.

Polymorphic sequences of the tyrosinase gene in normally pigmented Japanese individuals

The frequency and observed heterozygosity for each polymorphic sequence examined in 108 unrelated normally pigmented Japanese individuals are summarized in Table 4. We found a novel genotype of intron 2, noted as 210 in Table 4. Sequence analysis from 10 individuals showed that this genotype corresponded to an insertion of thymidine into a poly-thymidine sequence (position −21 to −27 from exon 3) without exception (data not shown). Table 5 presents the estimated haplotype frequencies in the Japanese population using the EH program (Terwilliger and Ott 1994; Curtis and Sham 1995).

Discussion

At present, over 90 mutations of the tyrosinase gene associated with OCA1 have been described (Oetting 2000), and common mutations occur by ethnic group. For example, the P81L and T373K were observed in 25%–30% of northern Europeans (Spritz 1993; Spritz et al. 1997), and the R77Q and 929insC (P310insC) are common in Korean (Park et al. 1996, 1997) and Japanese individuals (Tomita et al. 2000). Patients 1 to 3, who were homozygous for the R77Q muta-

tion, showed single haplotype combination 359-C-C-209. Although patients 4 and 5 were heterozygous for the R77Q mutation, genotype data of the parents indicated that the R77Q mutation and the 359-C-C-209 moved together. Therefore, we concluded that R77Q mutation was associated with haplotype 359-C-C-209 in 5 cases (8 chromosomes). In the same way, the data on five homozygous cases (patients 6 to 10) and 5 heterozygous cases (patients 11, 12, 14, 15, and 16) for the 929insC mutation in Table 1 suggested that the 929insC mutation was primarily associated with haplotype 357-C-C-209 in 10 cases (15 chromosomes). Therefore, we presumed that the R77Q and 929insC mutations derived from a common origin (founder), respectively. On the assumption that associations of the 359-C-C-209-R77Q and 357-C-C-209-929insC had no exception, we identified 20 alleles of the tyrosinase gene, as shown in Table 2. Interestingly, the R278X mutation was associated with two haplotypes, 357-T-C-209 and 359-T-A-209. This fact suggested that the R278X mutation had two different origins, or occurred in a very ancient era. The R278X mutation was observed in various ethnic groups other than the Japanese population, such as Guayanan (Tripathi et al. 1993), Moroccan Jew (Gershoni-Baruch et al. 1994), and Indo-Pakistani (paper in preparation).

Allele frequencies of the polymorphic sequence at −199 (C for 0.898 and A for 0.102, as shown in Table 4) in Japanese individuals were virtually the same as those in Caucasians (C for 0.893 and A for 0.107) and Orientals (C for 0.887 and A for 0.113) (Oetting et al. 1991). Therefore, a base substitution of A for C at −199 presumably occurred long before humans divided into Caucasians and Orientals. We speculate that C at −301 and C at −199 (C-C) is a probable candidate for the ancestral haplotype of the human tyrosinase gene. During evolution of the human tyrosinase gene, two mutations occurred. The first appeared in a C-C allele, where a base substitution of T for C at −301

Table 3. Tentative indirect gene diagnosis using three polymorphic sequences in the tyrosinase gene promoter

Patients and parents		Siblings	
Subject	Estimated allele combination	Possible haplotype	Diagnosis from allele analysis
Patient 5	349-C-C and 359-C-C	349/359-C-C	Affected
Father	349-C-C and 349-T-C	339/349-C/T-C	Carrier
Mother	339-T-C and 359-C-C	349/359-C/T-C	Carrier
		339/349-T-C	Noncarrier
Patient 6	357-C-C and 357-C-C	357-C-C	Impossible
Father	357-C-C and 357-C-C		
Mother	357-C-C and 357-C-C		
Patient 8	357-C-C and 357-C-C	357-C-C	Affected
Father	357-C-C and 359-T-A	357/359-C/T-A/C	Carrier
Mother	357-C-C and 359-T-A	359-T-A	Noncarrier
Patient 9	357-C-C and 357-C-C	357-C-C	Affected
Father	349-C-C and 357-C-C	349/357-C-C	Carrier
Mother	349-C-C and 357-C-C	349-C-C	Noncarrier
Patient 10	357-C-C and 357-C-C	357-C-C	Affected
Father	349-C-C and 357-C-C	339/357-C-C	Carrier
Mother	339-C-C and 357-C-C	349/357-C-C	Carrier
		339/349-C-C	Noncarrier
Patient 11	357-C-C and 357-T-C	357-C/T-C	Affected or Carrier
Father	357-C-C and 357-C-C	357/370-C/T-A/C	Carrier or Noncarrier
Mother	357-T-C and 370-T-A		
Patient 12	357-C-C and 359-T-A	357/359-C/T-A/C	Affected
Father	357-T-C and 359-T-A	349/359-C/T-A/C	Carrier
Mother	349-C-C and 357-C-C	357-C/T-C	Carrier
		349/357-C/T-C	Noncarrier
Patient 13	357-?-? and 359-?-?	357/359-?-?	Affected
Father	349-?-? and 359-?-?	349/357-?-?	Carrier
Mother	357-?-? and 359-?-?	359-?-?	Carrier
		349/359-?-?	Noncarrier
Patient 14	357-C-C and 357-C-C	357-C-C	Affected
Father	357-C-C and 357-T-C	357-C/T-C	Carrier
Mother	357-C-C and 357-T-C	357-T-C	Noncarrier
Patient 15	349-C-C and 357-C-C	349/357-C-C	Affected
Father	349-C-C and 359-C-C	349-C-C	Carrier
Mother	349-C-C and 357-C-C	357/359-C-C	Carrier
		349/359-C-C	Noncarrier
Patient 16	349-C-C and 357-C-C	349/357-C-C	Affected
Father	349-C-C and 357-T-C	349/359-C/T-C	Carrier
Mother	357-C-C and 359-T-C	357-C/T-C	Carrier
		357/359-T-C	Noncarrier

Table 4. Estimated genotype frequencies of the tyrosinase gene in normally pigmented Japanese individuals

Polymorphic sequences	Length or nucleotide	Frequency
GA repeat (0.72) ^a	318	0.005
	339	0.056
	349	0.394
	357	0.329
	359	0.213
	370	0.000
	380	0.005
-301 (0.49) ^a	C	0.671
	T	0.329
-199 (0.20) ^a	A	0.102
	C	0.898
Intron 2 (0.16) ^a	207	0.000
	209	0.921
	210	0.079

^aObserved heterozygosity; the length of 207 corresponds to a deletion of two thymidines associated with the IVS2-7T>A mutation

Table 5. Estimated haplotype frequencies of the tyrosinase gene in normally pigmented Japanese individuals

Haplotype				
GA repeat	-301	-199	Intron 2	Frequency
318	C	C	209	0.005
339	C	C	209	0.056
349	C	C	209	0.394
357	C	C	209	0.107
357	T	C	209	0.143
357	T	C	210	0.079
359	C	C	209	0.111
359	T	A	209	0.097
359	T	C	209	0.005
380	T	A	209	0.005

Because there was overwhelming evidence for association between markers ($\chi^2 = 200.38$, $P < 0.0001$), haplotype frequencies under the hypothesis of allelic association were shown

resulted in a T-C allele. The second mutation probably took place in a T-C allele. The estimated allele frequencies in the Japanese population were 0.671 for C-C, 0.227 for T-C, and 0.102 for T-A.

Recently, there has been increased awareness of and attention paid to genetic diseases. Identification of prevalent and/or novel mutations of the tyrosinase gene will facilitate the DNA-based diagnosis for OCA1. Haplotype analysis is a classical way to identify pathological alleles, although allele analysis of the tyrosinase gene is still useful under certain situations, and tells us the origin of the mutated allele.

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