Genet. Res., Camb. (2014), vol. 96, e14. © Cambridge University Press 2014
doi:10.1017/S0016672314000160

Tyrosinase gene mutations in the Chinese Han population with OCA1

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(*Received 4 March 2014; revised 25 August 2014*)

Summary

Oculocutaneous albinism (OCA) is a heterogeneous autosomal recessive genetic disorder that affects melanin synthesis. OCA results in reduced or absent pigmentation in the hair, skin and eyes. Type 1 OCA (OCA1) is the result of tyrosinase (*TYR*) gene mutations and is a severe disease type. This study investigated *TYR* mutations in a Chinese cohort with OCA1. This study included two parts: patient genetic study and prenatal genetic diagnosis. A total of 30 OCA1 patients were subjected to *TYR* gene mutation analysis. Ten pedigrees were included for prenatal genetic diagnosis. A total of 100 unrelated healthy Chinese individuals were genotyped for controls. The coding sequence and the intron/exon junctions of *TYR* were analysed by bidirectional DNA sequencing. In this study, 20 mutations were identified, four of which were novel. Of these 30 OCA1 patients, 25 patients were *TYR* compound heterozygous; two patients carried homozygous *TYR* mutations; and three were heterozygous. Among the ten prenatally genotyped fetuses, three fetuses carried compound heterozygous mutations and seven carried no mutation or only one mutant allele of *TYR* and appeared normal at birth. In conclusion, we identified four novel *TYR* mutations and showed that molecular-based prenatal screening to detect *TYR* mutations in a fetus at risk for OCA1 provided essential information for genetic counselling of couples at risk.

Introduction

Oculocutaneous albinism (OCA) affects melanin synthesis resulting in reduced or absent pigmentation in the hair, skin and eyes, with severe visual defects being a major effect of this disease (Ray *et al.*, 2007; Montoliu *et al.*, 2014). Although patients with OCA have similar phenotypes the disease actually is due to autosomal recessive mutations in different genes including *TYR, OCA2, TRYP1* and *SLC45A2*. OCA1A is the most common and severe form of albinism in most populations (Oetting & King, 1999; King *et al.*, 2003; Li *et al.*, 2006 *a*; Wei *et al.*, 2010; Chaki *et al.*, 2011; Preising *et al.*, 2011). One in every 40 000 individuals has OCA1 (type I) albinism (Ray *et al.*, 2007), and OCA1 is the most common type of albinism in Japanese (Suzuki & Tomita, 2008), non-Hispanic Caucasians (Hutton & Spritz, 2008), Danes (Grønskov *et al.*, 2009), a mixed population of Europeans, Asians and Africans (Rooryck *et al.*, 2008), and Chinese (Wei *et al.*, 2010). Other subtypes include OCA type II, which is caused by a mutation in the pink-eyed dilution gene (*OCA2*; MIM 203200) (Rinchik *et al.*, 1993); OCA2 is the most common type in Africans and African–Americans (Durham-Pierre *et al.*, 1994). OCA type III is caused by a mutation in the tyrosinase-related protein (*TYRP1*; MIM 203290) (Boissy *et al.*, 1996), and OCA type IV is caused by a mutation in the membrane-associated transport protein (*SLC45A2*; MIM 606574) (Spritz, 1993; Fernandez *et al.*, 2008; Hutton & Spritz, 2008), and is the only gene affected in OCA4 patients (Ko *et al.*, 2012). Although OCA4 is rare worldwide, OCA4 is the second most common type in Japanese (Suzuki & Tomita, 2008).

TYR encodes a 58 kD (529 amino acids) bifunctional type-1 integral membrane protein that is required for melanin biosynthesis in the melanocytes of hair follicles, skin and eyes (Shibahara *et al.*,

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1988; Takeda *et al.*, 1989; Hearing *et al.*, 1992). The enzyme catalyses the conversion of tyrosine to l-dihydroxy-phenylalanine (DOPA) and subsequently oxidizes DOPA to dopaquinone in melanocytes (Hearing *et al.*, 1992). The TYR protein also has 5,6-dihydroxyindole oxidase activity (Hearing *et al.*, 1992). The tyrosinase activity is more stable in the presence of two other factors, tyrosinase-related protein (TRP)-1 and TRP-2 (Hearing *et al.*, 1992). Human *TYR* is located at 11q14-q21 and contains five exons (Barton *et al.*, 1988; Giebel *et al.*, 1991).

OCA1 can be classified into two subtypes: OCA1A in which there is a complete lack of enzyme activity, and OCA1B in which there remains residual enzyme activity (Tomita *et al.*, 1989; Ray *et al.*, 2007; Hutton & Spritz, 2008; Montoliu *et al.*, 2014). OCA1A is the most severe form of OCA1. OCA1B patients typically have little or no pigmentation at birth but progressive melanization can occur over time. The range of pigmentation can vary from little pigmentation to almost normal pigmentation, and the degree of pigmentation is dependent upon family pigment patterns and ethnicity (Oetting & King, 1999; King *et al.*, 2003; Li *et al.*, 2006 *a*; Ray *et al.*, 2007; Chaki *et al.*, 2011; Preising *et al.*, 2011). The prevalence of OCA subtypes differs widely among different populations.

Over 273 mutations in *TYR* have been identified in different ethnic groups (Li *et al.*, 2006 *b*; Simeonov *et al.*, 2013). Although a distribution of *TYR* mutations has previously been described in Chinese patients (Wang *et al.*, 2009; Wei *et al.*, 2010), there is still a paucity of information regarding the types, frequency and distribution of mutations. In addition, diagnosis of OCA subtype of a patient based purely on clinical features is challenging. Further characterizing OCA1 mutations may help in the development of molecular tools that could be used for prenatal diagnosis of the disease. Prenatal screening for albinism by molecular analysis or high performance liquid chromatography and sequencing has been performed in Israeli families (Rosenmann *et al.*, 2009) and Taiwanese families (Lin *et al.*, 2006), respectively. There is a need for a comprehensive genetic analysis in a large sample to better characterize mutations in *TYR* that may cause OCA1 in mainland China. In this study, we characterized *TYR* mutations associated with OCA1 in 30 affected Chinese patients, and identified four novel mutations that had not previously been described.

Methods

Subjects and methods

This study was performed at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) and was approved by the hospital's ethics committee and performed according to the principles of the Declaration of Helsinki. All patients gave their written informed consent.

Study subjects

All subjects analysed in this study were recruited from the Genetic Counseling clinic of the Prenatal Diagnosis Center of the hospital and had albinism type I, type II or type IV. A clinical information form recorded the hair, skin and eye colour at birth, the age of sample submission and fundus examination by an ophthalmologist on the most affected family members. This study focused on OCA1 patients only. Inclusion criteria were symptoms of the eyes (severely poor eyesight, refractive error and photophobia) and skin (white or light yellow skin or hair that did not change with age). Normal pigmentation in a Chinese population is black hair, black eye colour and yellow skin colour. A total of 39 families were screened, including 35 patients with albinism, family members of three patients with albinism who had died and a person receiving genetic counselling. Patients were screened using DNA sequencing of the *TYR, OCA2* and *SCL45A2* genes. We did not assess patients for type III albinism as this is only found in black patients. We found 30 patients had type 1 albinism due to mutation in the *TYR* gene, three patients had type II albinism with mutations in the *OCA2* gene and two patients had type IV albinism resulting from mutations in the *SCL45A2* gene. The parents of the three deceased patients were heterozygous for *TYR* mutations. The couple receiving genetic counselling were heterozygous for *TYR* mutations. Only patients who carried the *TYR* mutation were included in the study. Patients with albinism but without *TYR* mutation were excluded. In addition, exclusion criteria were abnormalities in the immune system, symptoms in other organs (syndromic albinism) or solely ocular symptoms (ocular albinism). After birth, all surviving babies had a full OCA1 examination including eye examinations.

Collection of samples and genomic DNA extraction

Peripheral blood samples were collected for DNA analysis from study subjects. Genomic DNA was isolated using a TIANamp DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions.

PCR and DNA sequencing

All exons and exon/intron junctions of the *TYR* gene were amplified by PCR. Specific primers were designed according to the UCSC Genome

https://doi.org/10.1017/S0016672314000160 Published online by Cambridge University Press

Bioinformatics database (see Supplementary Table 1 for primer sequences; available online). PCR was performed using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction included 20–50 ng of genomic DNA, 1 μl of each primer and 13 μl 2 × Taq PCR MasterMix (containing a cocktail of dNTP, Tris-HCl, taq polymerase, KCl and $MgCl_2$) and ddH_2O to a final volume of 25 μl. The PCR amplification was as follows: 96 °C for 5 min, followed by 35 cycles of 96 °C for 30 s, then 58 to 64 °C (depending on the primer) for 40 s, followed by 72 °C for 1 min. Following the last cycle, the reaction products were further extended at 72 °C for 7 min. PCR products were visualized by electrophoresing in 1·5% agarose gel and ethidium bromide staining. Recovered PCR amplicons (50 ng) were bidirectionally sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carslbad, CA, USA).

Novel mutation naming and verification

Novel mutations were identified using PubMed and OCA databases (http://www.hgmd.cf.ac.uk/ac/; http://www.ncbi.nlm.nih.gov/SNP/; and http://www.ifpcs.org/albinism/index.html). To confirm that the mutation was new and not a single nucleotide polymorphism, the relevant region of the gene was sequenced using DNA isolated from 100 unrelated healthy individuals and was analysed with PROVEN and Polyphen-2. Novel mutations were named according to Mutalyzer (https://mutalyzer.nl/check)

Prenatal testing

Fetal tissue was obtained from transabdominal chorionic villi isolated during the first trimester or amniocentesis performed during the second trimester. Fetal DNA was also isolated using the TIANamp DNA Kit. Maternal contamination was ruled out from fetal samples and paternity was confirmed using PowerPlex 16 HS System Kit (Promega Corporation, Madison, WI, USA) and GeneMapper ID v3·2 Soft.

Results

A total of 98 subjects from 34 families were subject to *TYR* gene mutation analysis (Table 1). Among them, subjects 1–30 were living OCA1 patients and these probands plus both parents were genotyped (n = 90). Subjects 31, 33 and 34 were deceased albinism probands and their genotypes were deduced from the genotypes of their asymptomatic parents (n = 6); the parents each carried heterozygous *TYR* mutations. Subject 32 was the fetus from a single couple who had poor development of optic disc, displayed pathological photophobia and had normal eyesight without nystagmus; this couple were identified as asymptomatic carriers of OCA1 mutations after genotyping. The parents of OCA1 subjects were asymptomatic and not in consanguineous marriages. A total of 100 unrelated healthy Chinese individuals were genotyped as controls. Most subjects had white hair and skin colour, and were positive for nystagmus (Table 1). Interestingly, Chinese children with typical oculocutaneous albinism usually presented with red irises, rarely blue irises, at birth (Fig. 1).

TYR gene mutation analysis

DNA analysis revealed 20 different *TYR* alleles among which 16 had previously been reported (Tables 1 and 2). The four novel alleles that were not present in healthy controls were an insertion in the coding region (c.560_561ins25 bp), a nucleotide change in the second nucleotide of the third intron (IVS3 + 2 T > C), a deletion in the first nucleotide of the fourth intervening sequence (IVS4 + 1delG) and a non-conservative missense mutation (p.G446 V).

Seven *TYR* alleles accounted for 74·6% of the alleles with mutations detected in this study. The most common allele was p.R299H (12/68 mutant alleles analysed) (exon 2), followed by c.232_233insGGG (7/68) (exon 1), p.R116X (7/68) (exon 1), p.R278X (6/68) (exon 2), c.929_930insC (6/68) (exon 2), IVS2-10delTT-7 T > A (5/68) (intron 2) and p.W400L (4/68) (exon 4) (Table 2). All other identified alleles occurred in only one or two of the subjects.

A total of 29 OCA1 subjects were compound heterozygous for mutant alleles of the *TYR* gene. Two subjects were homozygous (Subject 3 and 13), and three subjects were single heterozygous for mutant *TYR* alleles((Subjects 2, 14 and 25) (Table 1).

The novel missense mutation p.G446 V changes a glycine at the copper-binding site of tyrosinase, to valine, which likely impacts copper binding and enzyme activity. We tested for pathogenicity of this mutation using PROVEN and Polyphen-2, which indicated the mutation likely affects the structure and function of the protein.

The mutation c.560_561ins25 bp, was a frameshift mutation that caused an abnormality in the amino acid sequence downstream of the mutation (Leu-Leu-Cys-Val-Lys-Leu-Ser-Pro-Thr-Ala-Trp-Gly-Ile-STOP).

Two novel mutations, IVS3 + 2 T > C and IVS4 + 1delG were located in the recognition sequence of the donor splice site of the third and fourth introns, respectively. These nucleotide changes are expected to alter the RNA splicing pattern and frequency, mRNA sequence, levels of mRNA and, subsequently, protein levels and function.

https://doi.org/10.1017/S0016672314000160 Published online by Cambridge University Press

Table 1. *Clinical characteristics and identified mutations in 34 OCA1 subjects*

Subject	Sex	Age (years)	Hair colour at birth	Hair colour at analysis	Skin colour	Iris colour	Nystagmus	OCA1 subtype	Subject genotype	
									Paternal allele	Maternal allele
1	M	0·5	White	White	White	Red–brown	Positive	1A	c.929_930insC	p.R299H
2	M	7	White	White	White	Red–brown	Positive	1A	c.929_930insC	—
3	F	0·06	White	White	White	Amber	Positive	1A	p.R299C	p.R299C
4	M	23	White	Yellow	White	Grey	Negative	1B	p.R299H	p.R116X
5	M	2·5	White	White	White	Red–brown	Positive	1A	p.R77Q	p.R278X
6	F	6	White	White	White	Red–brown	Positive	1A	c.232_233insGGG	p.W400L
7	F	22	White	White	White	Red–brown	Positive	1A	c.929_930insC	p.R116X
8	M	1	White	Yellow	White	Black	Positive	1B	IVS2-10delTT-7 T > A	p.E219 K
9	F	50	Unknown	Brown	White	Grey	Negative	1B	c.232_233insGGG	IVS2-10delTT-7 T > A
10	F	0·07	White	White	White	Red–brown	Positive	1A	c.560_561ins25 bp[a]	c.929_930insC
11	M	2	White	White	White	Red–brown	Positive	1A	IVS2-10delTT-7 T > A	c.929_930insC
12	F	0·83	White	White	White	Red–brown	Positive	1A	c.232_233insGGG	p.M1 V
13	F	13	White	White	White	Red–brown	Positive	1A	p.E294 K	p.E294 K
14	M	24	White	White	White	Red–brown	Positive	1A	p.R299H	—
15	M	5·5	White	Brown	White	Grey	Positive	1B	p.R77Q	p.R299H
16	F	0·58	White	White	White	Red–brown	Positive	1A	p.G446V[a]	p.R299H
17	F	32	White	White	White	Red–brown	Positive	1A	p.G253E	p.R299H
18	M	30	White	White	White	Red–brown	Positive	1A	c.929_930insC	p.R299H
19	F	0·02	White	White	White	Red–brown	Positive	1A	p.W400L	p.R77Q
20	M	48	White	White	White	Red–brown	Positive	1A	p.R116X	IVS4 + 1delG[a]
21	F	0·17	White	White	White	Red–brown	Positive	1A	IVS3 + 2 T > C[a]	p.R116X
22	F	5	White	White	White	Red–brown	Positive	1A	p.R278X	p.R299H
23	F	4	White	White	White	Red–brown	Positive	1A	p.C24Y	c.929_930insC
24	F	7	White	Yellow	White	Black	Positive	1B	c.232_233insGGG	IVS2-10delTT-7 T > A
25	F	21	White	White	White	Red–brown	Positive	1A	—	p.R299H
26	M	0·25	White	White	White	Red–brown	Negative	1A	c.232_233insGGG	IVS2-10delTT-7 T > A
27	F	3	White	White	White	Red–brown	Positive	1A	p.K142M	p.R278X
28	M	1·4	White	White	White	Red–brown	Positive	1A	p.W400L	p.G253E
29	M	1·2	White	White	White	Red–brown	Positive	1A	p.R299H	p.R116X
30	F	7	White	White	White	Red–brown	Positive	1A	p.R299H	p.Q399X
31	F	/	White	White	White	Blue	/	/	p.R299H	c.232_233insGGG
32									p.R299H	p.R278X
33	M	/	White	White	White	Blue	/	/	p.R278X	p.R116X
34	M	/	White	White	White	Red–brown	Positive	/	p.W400L	p.R116X

[a] Previously unknown alleles.
A dash (—) in the genotype column denotes a putative uncharacterized allelic mutation that may not be in another portion of *TYR* gene and was not identify by the PCR primers used.
/, no correlated information; F, female; M, male.

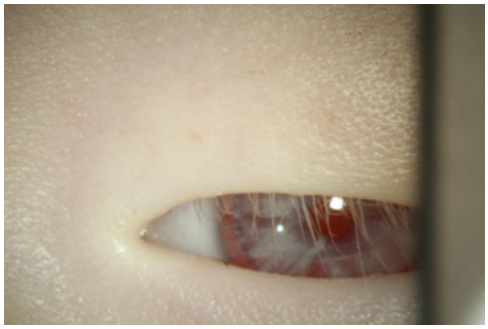

Fig. 1. Image of iris of Chinese child aged 1 year and 9 months showing the red iris often seen in Chinese children with typical oculocutaneous albinism.

Table 2. *Mutation frequency of* TYR *gene in Chinese OCA1 patients*

Mutation type	Allele	Location	Mutation frequency
Missense	p.M1 V	Exon 1	1/68
	p.C24Y	Exon 1	1/68
	p.R77Q	Exon 1	3/68
	p.K142M	Exon 1	1/68
	p.E219 K	Exon 1	1/68
	p.G253E	Exon 1	2/68
	p.E294 K	Exon 2	1/68
	p.R299H	Exon 2	12/68
	p.R299C	Exon 2	1/68
	p.Q399X	Exon 4	1/68
	p.W400L	Exon 4	4/68
	p.G446 V	Exon 4	1/68
Nonsense	p.R116X	Exon 1	7/68
	p.R278X	Exon 2	6/68
Insertion	c.232_233insGGG	Exon 1	7/68
	c.560_561ins25 bp	Exon 1	1/68
	c.929_930insC	Exon 2	6/68
Splice	IVS2-10delTT-7 T > A	Intron 2	5/68
	IVS3 + 2 T > C	Intron 3	1/68
Deletion	IVS4 + 1delG	Intron 4	1/68

Prenatal genetic diagnoses

Parental genotypes for ten high-risk fetuses suggested that these fetuses had a 25% risk of carrying two mutant alleles of *TYR*. Prenatal DNA analysis of high-risk fetuses (n = 10) identified three fetuses (Subjects 12, 32 and 34) that carried compound heterozygous alleles (Table 3). Following genetic counselling, the parents of these fetuses chose to terminate the pregnancy. Analysis of the aborted tissue confirmed that these fetuses carried two mutant *TYR* alleles. Five fetuses (Subjects 5, 6, 8, 11 and 33) were heterozygous carrying one mutant allele and two fetuses (Subjects 30 and 31) did not carry any detectable mutant allele (Table 3). After birth, none of these seven subjects showed symptoms of OCA1. DNA analysis supported the paternity of all fetuses analysed.

Discussion

In this study, we assayed *TYR* for mutations in 30 OCA1 individuals and ten fetuses at risk for the disease. We identified four novel mutations. Two of the novel mutations (p.G446 V and c.560_561ins25 bp) altered the coding sequence and the other two (IVS4 + 1delG and IVS3 + 2 T > C) affected donor splice site sequences. All four mutations were associated with the OCA1A clinical diagnosis, suggesting they are likely null or strong loss-of-function alleles and that the companion mutation on the other chromosome also produced little active enzyme. It is possible that IVS4 + 1delG and IVS3 + 2 T > C disrupt gene function by altering splicing to produce a non-functional protein product or an unstable mRNA. Further studies are required to elucidate how these mutations alter gene function and to determine if these four novel mutations are specific to Han Chinese or are present in other populations.

The most common mutation in our study was p.R299H; 12/68 analysed chromosomes carried this mutation. Codon 299 is highly conserved and is located close to the two copper binding sites. Disrupting copper binding may inhibit enzyme activity. Prior works also found mutations at position 299 (R299H, R299S and R299C) accounted for almost 60% of missense mutant alleles in Chinese OCA1 patients and represent almost 35% of all OCA1 mutations in Chinese (Tsai *et al.*, 1999; Hsieh *et al.*, 2001; Lin *et al.*, 2006). By contrast, mutations at 299 account for <1·0% in white populations (Spritz *et al.*, 1997; Opitz *et al.*, 2004) possibly indicating the difference in founder mutations between populations. The p.W400L, c.232_233insGGG and p.C24Y mutations have only been reported in Chinese OCA patients to date (Wang *et al.*, 2009; Wei *et al.*, 2010). The c929_930insC mutation appears to be common in east Asian populations as it is prevalent in Chinese, Japanese and Korean OCA1 patients (Goto *et al.*, 2004; Suzuki & Tomita, 2008; Wang *et al.*, 2009; Wei *et al.*, 2010; Ko *et al.*, 2012; Park *et al.*, 2012). The other alleles we found at a high frequency (i.e. pR116X, p.R278X and IVS-10delTT-7 T > A) have also been shown to be common in Chinese in previous studies (Wei *et al.*, 2010). On the other hand, p.R278X is the third most common mutation in Japanese and Chinese patients (11·6 and 11·8%, respectively) (Ko *et al.*, 2012).

In these 34 OCA1 patients, 29 patients were confirmed to carry compound heterozygous mutations in the two *TYR* alleles and two were homozygous for a *TYR* mutation. For the other three of the patients, only one mutation-carrying allele was detected although we sequenced all five exons and all intron/exon boundaries of the other allele. This may indicate that another mutation(s) outside the region we

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Table 3. *Prenatal genetic diagnosis of the ten high-risk fetuses*

Pedigree	Sex	Age years)	Hair colour (at birth/at analysis)	Skin colour	Iris colour	Nystagmus	OCA1 subtype	Proband genotype		Fetus genotype	Fetus outcome	Baby phenotype
								Paternal allele	Maternal allele			
5	M	2·5	White/white	White	Red–brown	Positive	1A	p.R278X	p.R77Q	p.R278X	Normal birth	Normal
6	F	6	White/white	White	Red–brown	Positive	1A	c.232_233insGGG	p.W400L	p.W400L	Normal birth	Normal
8	M	1	White/yellow	White	Black	Positive	1B	IVS2-10delTT-7 T > A	p.E219 K	p.E219 K	Normal birth	Normal
11	M	2	White/white	White	Red–brown	Positive	1A	IVS2-10delTT-7 T > A	c.929_930insC	IVS2-10delTT-7 T > A	Normal birth	Normal
12	F	0·83	White/white	White	Red–brown	Positive	1A	c.232_233insGGG	p.M1 V	p.M1 V c.232_233insGGG	Odinopoeia	/
30	F	7	White/white	White	Red–brown	Positive	1A	p.R299H	p.Q399X	No mutation	Normal birth	Normal
31	F	/	White/white	White	Red–brown	/	/	Deceased		No mutation	Normal birth	Normal
32	/	/	/	/	/	/	/	No proband		p.R278X p.R299H	Odinopoeia	/
33	M	/	White/white	White	Red–brown	/	/	Deceased		p.R116X	Normal birth	Normal
34	M	/	White/White	White	Red–brown	Positive	/	Deceased		p.R116X p.W400L	Odinopoeia	/

/, no correlated information; F, female; M, male.

sequenced, such as in the regulatory regions (both upstream and downstream) or in other intronic sequences, are present that may alter or regulate splicing efficiency and/or accuracy. It is also possible that a mutation in other genes either directly or indirectly affect *TYR* gene expression or activity. A prior study that genetically analysed OCA1 in Caucasian patients with albinism found 26% of patients with OCA1 did not have two mutations in *TYR* (Simeonov *et al.*, 2013). They further analysed the *TYR* gene as well as other genes involved in OCA and found possible reasons for this phenotype in two cases: in one case the patient carried a sequence variant in the *SLC24A5* gene and in another case the patient was hemizygote for OCA1 (Simeonov *et al.*, 2013). *SLC24A5* protein is required for proper routing of tyrosinase and mutations in this gene result in a similar phenotype as OCA2 mutations. Further studies are required to identify additional mutations within this Han Chinese population and to elucidate the mechanism resulting in the OCA1 phenotype.

We genotyped ten high-risk fetuses for mutations in *TYR*, and found three fetuses carried mutations on both alleles and were highly likely to have the disease. Five fetuses carried only one mutant allele and the other two carried no *TYR* mutations, which is similar frequencies to a previous report (Rosenmann *et al.*, 2009). The parents of the three affected fetuses chose to terminate the pregnancy. After birth, the seven other babies showed no signs of OCA1. These data confirm that molecular-based prenatal screening is possible for this disease.

It would be of interest to understand how the different *TYR* mutations affect gene expression and function. This information may give insight into disease severity and possibly facilitate the development of treatment for the disease. In conclusion, we identified four novel *TYR* mutations and 16 known *TYR* mutations in a Chinese OCA1 population. This molecular-based prenatal screening to detect *TYR* mutations in a fetus at risk for OCA1 provided essential information for genetic counselling of "at risk" couples.

Supported by Youth Innovation Fund of First Affiliated Hospital of Zhengzhou University. We are grateful to the albino patients and their family members, the volunteers for donating bloodsamples, and all participants in the study for their kind cooperation.

Declaration of interest

None.

Supplementary material

The online supplementary material can be found available at http://journals.cambridge.org/GRH

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