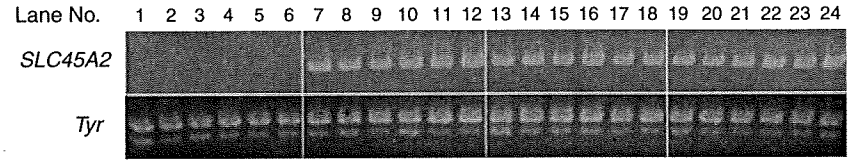

Figure 1. Expression of *SLC45A2* mRNA in each of the clones. RNA was extracted from stable transformants with pIREShyg3 for mock-transfected cells (lane 1–6), pIREShyg3-SLC45A2mutant-p.D157N (lane 7–12), pIREShyg3-SLC45A2mutant-p.G188V (lane 13–18), and pIREShyg3-SLC45A2 wild-type (lane 19–24). Then the RNAs were amplified by RT-PCR method with primer sets for human *SLC45A2* gene (upper) and mouse tyrosinase gene (*Tyr*) as a control (bottom). The primer sequences were following, human *SLC45A2*-F, GTGGCCATGGGTAGCAACAGTG; -R, GAATGCCCTTTGCAACCTCTG; mouse *Tyr*-F, CGACCTCTTTGTATGGATGC; -R, GCTTGTTCCAAGTAAGGCTC. Expressions of *SLC45A2* mRNA were confirmed in all clones excepted mock-transfected cells.

converted to melanin content via a standard curve using sepia melanin (Sigma, Poole, UK) (Sviderskaya et al., 1997). The melanin content was normalized to protein content, determined using a Protein Assay Kit from Bio-Rad (Hercules, CA, USA).

As shown in Figure 2A, the mock-transfected cells and cells transfected with p.D157N-mutant and p.G188V-mutant cDNA were very similar in appearance, and all had a low level of visible pigmentation. In contrast, cells transfected with wild-type *SLC45A2* cDNA were darkly pigmented.

These subjective assessments were substantiated by a measurement of melanin content in the cells (Figure 2B). Mock, p.D157N-mutant and p.G188V-mutant cDNA transfections produced cells with approximately 50 μg melanin/mg of protein, whereas cells transfected with wild-type cDNA contained a substantial amount of melanin (339.6 μg melanin/mg of protein). These results indicated that human SLC45A2 protein can compensate for the mouse Slc45a2 protein in *uw* cells, and that the SLC45A2 molecule is critical for melanogenesis. Furthermore, the p.D157N, and p.G188V-mutant proteins were functionally incapable of melanin synthesis and should be pathologic. Transfection experiments performed at 31°C yielded similar results, indicating that neither of the p.D157N or p.G188V substitutions produces a temperature-sensitive SLC45A2 protein (data not shown).

We have developed an in vitro system to study the function of the SLC45A2 protein, to assess the relationship between specific mutations of the *SLC45A2* gene and the consequent phenotypes. The two substitutions

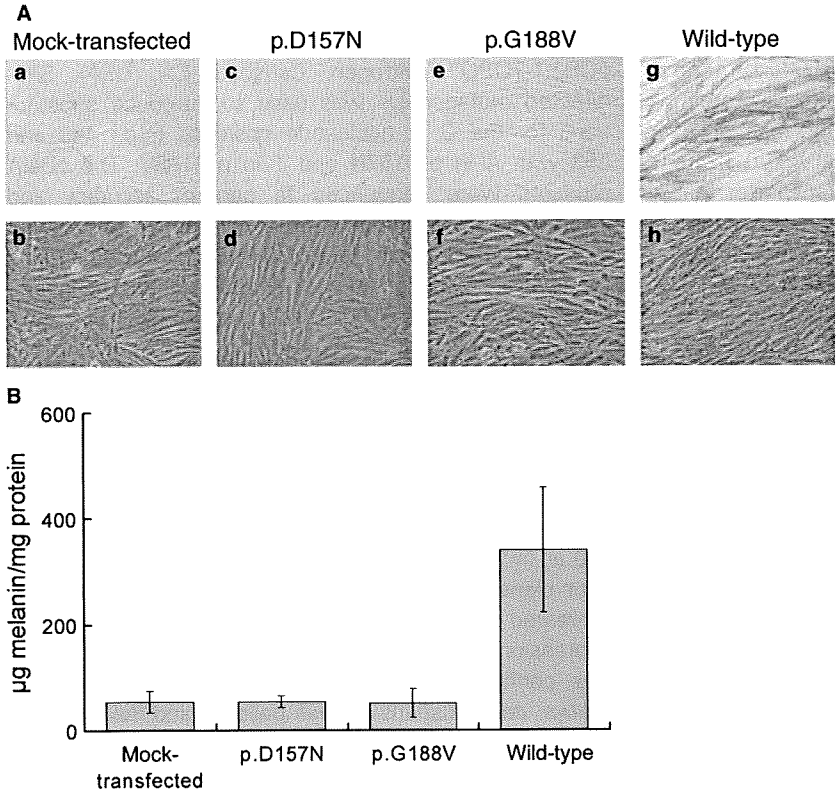

Figure 2. (A) Differential complementation of hypopigmentation of *uw* cells after transfection with normal and mutant human *SLC45A2* cDNA constructs. Bright-field (upper) microscopy shows melanin, and phase-contrast optics (lower) shows cells. a, b. mock-transfected with pIREShyg3; c, d. p.D157N mutant; e, f. p.G188V mutant; g, h. wild-type human *SLC45A2* cDNA. Cells transfected with wild-type cDNA showed some visible pigmentation. In contrast, cells with mutant (p.D157N or p.G188V) cDNA had very little melanin. (B) Differing melanin content of *uw* cells transfected with different cDNA constructs. Means ± SEM were calculated from six independent clones for each treatment. Transfected cells were compared with mock-transfected cells. A significant increase in melanin content was observed only after transfection with the wild-type sequence ($P < 0.01$).

tested here, p.D157N and p.G188V, have been associated with phenotypes of different clinical severities (Inagaki et al., 2004). However, the in vitro system used in this study could not detect the differences in melanin production between cells transfected with p.D157N-mutant or p.G188V-mutant cDNA, suggesting that both mutant alleles might have very low functional activity in melanogenesis, and that the differences in melanin synthesis could be minimal. However, these minimal differences might give rise to different clinical severities in vivo.

Acknowledgements

The authors are grateful to Dr VJ Hearing (National Cancer Institute, NIH, Bethesda, MD, USA) for kindly providing *uw* mutant melanocytes. This work was supported by grants 18390312 to TS and 19390294 to YT from the Ministry of Education, Science and Culture of Japan, and partially by the Cosmetology Research Foundation.

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Gene Corner

A Japanese piebald patient with auburn hair colour associated with a novel mutation p.P832L in the *KIT* gene and a homozygous variant p.I120T in the *MC1R* gene

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Piebaldism (MIM 172800) is an inherited disorder characterized by congenital poliosis and leucoderma on the frontal scalp, forehead, ventral trunk and extremities. Some patients with piebaldism have café-au-lait spots. Piebaldism is caused by a mutation of the KIT protooncogene encoding c-kit, and shows autosomal dominant inheritance.[1–3]

Red hair colour (RHC) can be observed in as many as 6% or more Icelanders,[4] although it is apparently rare in Asians. RHC is associated with inactive variants of the melanocortin-1 receptor (MC1R) gene and is believed to be recessive.[5] MC1R is a seven transmembrane G-protein-coupled receptor. Certain variants are related to the RHC phenotype of fair skin, poor tanning ability and elevated risk of freckles, solar lentigines, malignant melanoma, basal cell carcinoma and squamous cell carcinoma.[6]

Case and methods

A 1-year-old Japanese girl presented with aberrant hair and skin colour. Physical examination revealed: (i) poliosis on the frontal region of the scalp; (ii) auburn hair colour on the other regions of the scalp; (iii) leucoderma on the forehead, the ventral trunk, knees and elbows; (iv) fair skin on the face except for the forehead, and on the dorsal trunk, upper arms and thighs; and (v) multiple café-au-lait spots on regions of both leucoderma and fair skin (Fig. 1a, b). Her irides were symmetrically brown. There was no family history of the disease or parental consanguinity.

We performed polymerase chain reaction (PCR) and direct sequencing using genomic DNA on the coding regions of KIT and MC1R as described.[2,3,7] The parents provided written informed consent and the study was approved by the Genetic Ethics Committee of Kinki University. Genomic DNA was prepared from peripheral blood leucocytes. The identified mutation in KIT and variants in MC1R were reconfirmed by three independent sequencings. PCR products of exon 18 in KIT from family members and healthy volunteers were studied by single-strand conformational polymorphism analysis as described.[8]

Results and discussion

Sequence analysis of KIT from the proband revealed a novel missense substitution in exon 18. The C-to-T transition at nucleotide position 2495 resulted in a shift from a proline residue to a leucine at amino acid position 832. The substitution was present neither in the proband's parents nor in any of the 123 controls, suggesting that the substitution is not a common polymorphism but a novel pathological mutation. The *de novo* mutation was located in the highly conserved enzymatic site of the tyrosine kinase domain.[2,3] Missense mutations in the domain have a dominant-negative effect, reducing c-kit function by ~75%, resulting in a characteristically severe phenotype.[2,3] This novel p.P832L mutation is highly likely to be pathological.

Sequence analysis of MC1R from the proband showed two homozygous missense polymorphisms. The T-to-C transition at nucleotide position 359 resulted in a shift from an isoleucine residue to a threonine residue at amino acid position 120. The absence of G-to-A transition at nucleotide position 488 suggested the presence of an ancestral arginine residue at amino acid position 163, but not the glutamine residue of the dominant variant in the Japanese population.[7,9] Both parents were heterozygous at codon 120 (359T/C) and codon 163 (488G/A). Our previous study with 238 normal Japanese volunteers indicated that the frequency of 359T/C was 0·979/0·021 and that of 488G/A was 0·229/0·771.[7] Nakayama *et al.*[9] showed that the p.I120T substitution occurred on the p.R163 background haplotype and reported a frequency of 0·04 in 25 Japanese individuals. The homozygosity of both p.T120 and p.R163 in the proband was consistent with the reported haplotype. They studied the function of the p.I120T allele in a pharmacological cyclic adenosine monophosphate assay.[9] They concluded that the variant p.T120 showed a significantly decreased responsiveness of intracellular output signals at low concentrations of an MC1R agonist, Nle4-D-Phe7-alpha-MSH.[9] The assay showed no significant difference in the variant p.R163Q.[9]

A unique point in our patient was the auburn hair. A person having the homozygous MC1R p.T120 variant has not been identified in caucasian or Japanese populations.[7,10] The possibility of the presence of the homozygous variant p.T120 was 0·000441 (one person per 2268 Japanese people).[7] It is statistically apparent that the presence only of the homozygous variant cannot cause the auburn hair colour, because red or auburn hair is extremely rare in the Japanese population.

The white forelock, complete leucoderma on the ventral trunk and the café-au-lait spots appeared to be piebaldism. However, very strangely for piebaldism, the patient's back was

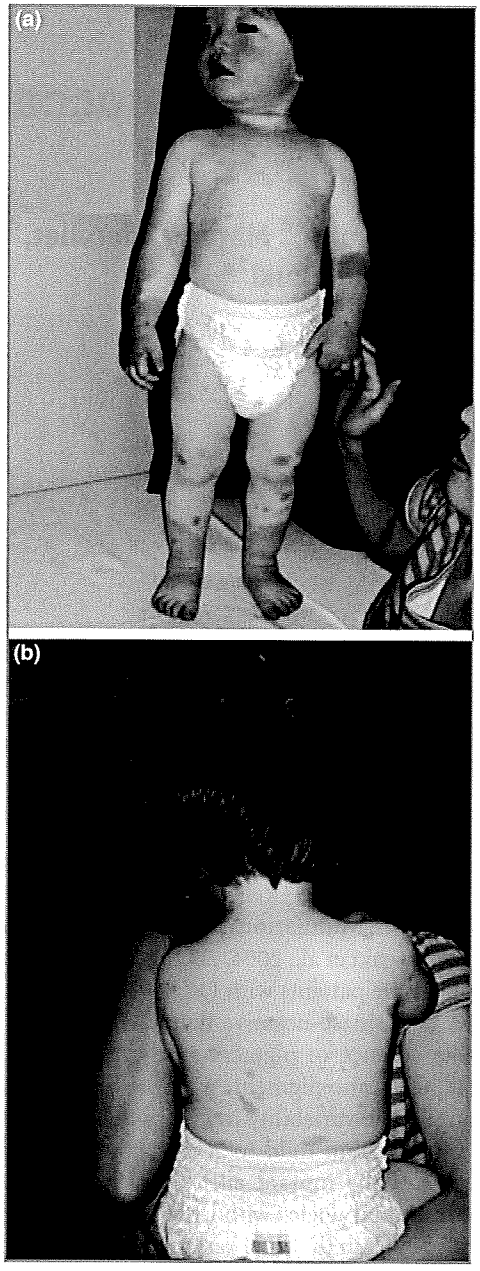

Fig 1. Clinical assessment of the child with piebaldism. Leucoderma was present on the forehead, ventral trunk and extremities (a, b); auburn hair colour on the rest of the scalp (b); fair skin on the face except for the forehead, and on the back, upper arms and thighs (a, b) and multiple café-au-lait spots on regions of both leucoderma and fair skin (a,b).

white. It is hard to explain why the back is white in piebaldism, but it might be possible to speculate that c-kit and MC1R acted in synergy in the development and migration of the melanoblasts.

We report a peculiar pigmentary anomaly caused by a novel KIT gene mutation, p.P832L, and a homozygous MC1R gene variant, p.I120T. The presence of the mutation of KIT and the inactive homozygous variant of MC1R together might cause the unique pigmentary changes. The clinical features indicated the possible interaction between c-kit and MC1R in determining hair colour.

Department of Dermatology, N. Oiso
Kinki University School of Medicine, K. Kishida*
377-2 Ohno-Higashi, K. Fukai†
Osaka-Sayama, Osaka 589-8511, Japan T. Motokawa‡
*Department of Dermatology, N. Hosomi§
Tokyo Medical University, Tokyo, Japan T. Suzuki¶
†Department of Dermatology, Y. Mitsuhashi*
Osaka City University Graduate School of Medicine, R. Tsuboi*
Osaka, Japan A. Kawada
‡Cutaneous Drug Research Department,
POLA Chemical Industries, Inc., Yokohama, Japan
§Division of Dermatology,
Kashiwara Municipal Hospital,
Kashiwara, Japan
¶Department of Dermatology,
Yamagata University School of Medicine,
Yamagata, Japan
E-mail: naoiso@med.kindai.ac.jp

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Key words: *auburn hair colour*, KIT, MC1R, *piebaldism*

Conflicts of interest: none declared.

CLINICAL REPORT

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Oculocutaneous Albinism Type IV: A Boy of Moroccan Descent With a Novel Mutation in *SLC45A2*

Takayuki Konno,[1] Yuko Abe,[1] Masakazu Kawaguchi,[1] Katrien Storm,[2] Martine Biervliet,[2] Winnie Courtens,[3] Michihiro Kono,[4] Yasushi Tomita,[4] and Tamio Suzuki[1]*

[1]Department of Dermatology, Yamagata University School of Medicine, Yamagata, Japan

[2]Department of Medical Genetics, University and University Hospital of Antwerp, Antwerp, Belgium

[3]Center of Human Genetics UCL, Cliniques Universitaires St-Luc, Brussels, Belgium

[4]Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan

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Oculocutaneous albinism type IV (OCA4 [MIM606574]) caused by mutations of the *SLC45A2* gene is an autosomal recessive disorder of pigmentation characterized by reduced biosynthesis of melanin pigment in the skin, hair, and eye. We had the opportunity to examine a Belgian boy of Moroccan descent with clinically severe OCA and screened the mutation in his *SLC45A2* gene. Sequencing of exon 1, of which the PCR product showed aberrant patterns in the SSCP gel, revealed that the patient was a homozygote for p.H38R mutation. We demonstrated that the p.H38R-mutant protein was functionally incapable of melanin synthesis using melanocyte cultures (*under white* cells; *uw*) established from a mouse model of OCA4. This is the second report of the occurrence of OCA4 in a member of an African ethnic group.

Key words: melanin; albinism; melanogenesis; Moroccan

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Am J Med Genet Part A 149A:1773–1776.

INTRODUCTION

Oculocutaneous albinism type IV (OCA4) [OMIM606574] is an autosomal recessive hypopigmentary disorder caused by mutations in the Solute Carrier Family 45, member 2 gene (*SLC45A2*), formerly called the Membrane-Associated Transporter Protein gene, *MATP*. In 2001, Newton et al. [2001] reported a Turkish OCA patient with a homozygous G to A transition in the splice acceptor sequence of exon 2 in the *SLC45A2* gene, thus identifying *SLC45A2* as the fourth gene capable of causing OCA. The *SLC45A2* gene spans 40 kb at chromosome 5p13.3, encodes a 530-amino acid polypeptide with 12 putative transmembrane domains, and is only expressed in melanocytes. Tyrosinase processing and trafficking is disrupted before delivery to early melanosomes in melanocyte cultures (*under white* cells; *uw*) established from a mouse model of OCA4 [Costin et al., 2003].

More than 30 different mutations have so far been reported in Turkish [Newton et al., 2001], European Caucasians [Rundshagen et al., 2004; Rooryck et al., 2008], Moroccan [Rooryck et al., 2008], Korean [Suzuki et al., 2005], Japanese [Inagaki et al., 2004, 2006], Brazilian [Lezirovitz et al., 2006], and Indian [Sengupta et al., 2007] OCA4 patients. Multifarious phenotypes have been found in OCA4 patients [Inagaki et al., 2004, 2006; Suzuki and Tomita, 2008]. For example, some patients with OCA4 were initially thought to have OCA1A (tyrosinase-negative type) (OMIM 203100) because of a complete absence of pigment in the hair, eyes, and skin. Other patients had some pigment, with brown hair and irides without nystagmus. The variability in clinical expression in OCA4 phenotypes varying from mild to severe albinism might be due to the genotypes of the mutant alleles. Furthermore, combinations of different mutant alleles with different activities of melanogenesis could also explain the wide variety of clinical phenotypes observed in these OCA4 patients. Type IV appears to be a rare form of OCA worldwide, except in Japan where type IV has been found in 27% of OCA patients [Suzuki and Tomita, 2008]. Only one OCA4 patient of Turkish descent was detected among 102 albinic individuals (or <1%) from diverse North American, Asian, European and African populations [Newton et al., 2001], and only five individuals were

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*Correspondence to:
Tamio Suzuki, M.D., Ph.D., Professor and Chairman, Department of Dermatology Yamagata University Faculty of Medicine, 2-2-2, Iida-Nishi, Yamagata 990-9585, Japan.
E-mail: tamsuz@med.id.yamagata-u.ac.jp
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identified among 176 (or 2.8%) German patients [Rundshagen et al., 2004]. Rooryck et al. [2008] recently reported that *SLC45A2* mutations appear to be more prevalent (17%) than previously described in Caucasians. So far only one mutation, p.K247X, has been reported in African populations, indicating that OCA4 might be very rare in African ethnic groups. Here, we describe a boy of Moroccan origin with relatively severe OCA4.

CLINICAL REPORT

The patient was a 5-year-old boy of Moroccan ethnicity. The pregnancy was uneventful, he was born at term and presented with white hair, blue irides, nystagmus, and white skin. He had a relatively large birth weight (4,200 g) and length (52 cm). We saw him at the age of 5 years with his parents for genetic counseling. The boy was in good health, had a normal psychomotor development and had never been hospitalized. Physical examination showed a boy with white hair, white eyebrows and eyelashes, blue irides, persistent nystagmus, and white skin with no apparent tanning ability (Fig. 1). No naevi were present. He had a normal weight (50th centile) and head circumference (75th centile) with a length at the 97th centile. It was a very active boy presenting multiple ecchymoses especially on the lower limbs. Complementary investigations could exclude a Hermansky–Pudlak syndrome, a form of OCA with bleeding diathesis since his hematological examination and platelet aggregation studies were normal. His karyotype was also normal 46,XY. Family history was unremarkable; he was the only person with clinical signs of albinism in the family. His parents were first cousins. His 1-year-old sib was unaffected. A recurrence risk for albinism in this couple was explained to be 25% affecting both boys and girls, as could be confirmed by the molecular studies.

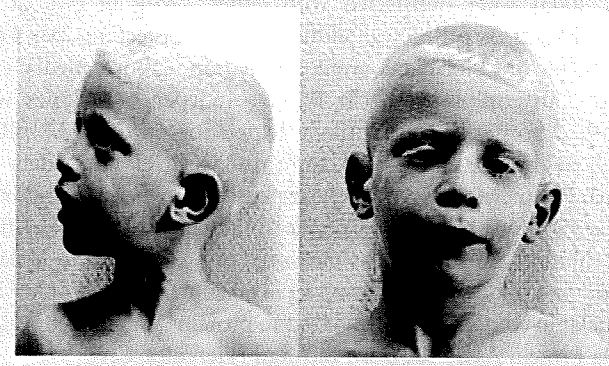

FIG. 1. Clinical features of the patient at 5 years of age. He had blue irides with nystagmus, virtually white skin with no apparent tanning ability and white hair.

METHODS

Mutational analysis of the *SLC45A2* gene was performed as previously described [Inagaki et al., 2004]. Briefly, genomic DNA was extracted from peripheral blood and used as a template for mutational screening using PCR-based single strand conformation polymorphism/heteroduplex (SSCP/HD) analysis. PCR products showing aberrant patterns on SSCP/HD gels were reamplified and sequenced to identify the mutation.

Functional analysis was performed as previously described [Konno et al., 2009]. Briefly, we constructed wild-type and mutant *SLC45A2* p.H38R cDNA. The cDNAs were inserted into the mammalian expression plasmid pIREShyg3 (BD Biosciences, San Jose, CA), creating pIREShyg3-*SLC45A2* wild and pIREShyg3-*SLC45A2* mutant

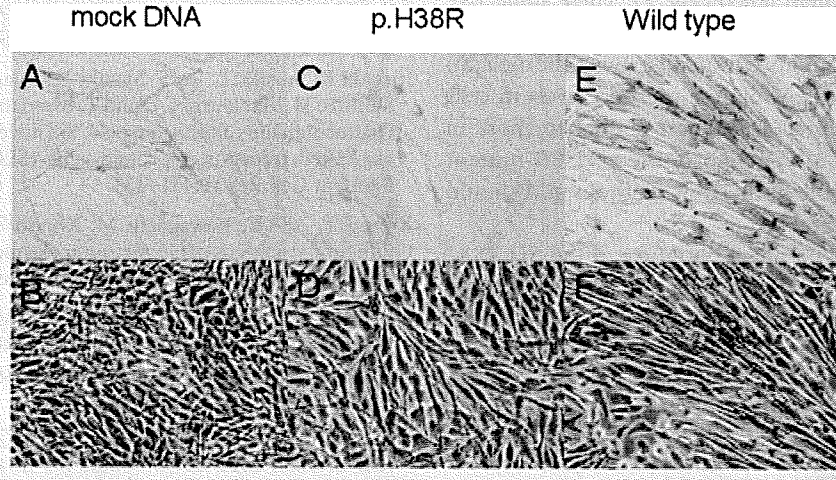

FIG. 2. Differential complementation of hypopigmentation of *uw* cells after transfection with normal and mutant human *SLC45A2* cDNAs. Melanin was observed using bright-field microscopy (upper) and phase-contrast (lower) was used to examine cells. (A,B) Mock-transfection without added DNA; (C,D) p.H38R mutant; (E,F) wild-type human *SLC45A2* cDNA. The transfected cells with the wild-type cDNA showed some visible pigmentation. In contrast, the cells with the p.H38R mutant cDNA had very little melanin.

-p.H38R. Following an initial 24 hr-culture period, *uw* cells [Costin et al., 2003] were transfected with either 1.6 μg of one of the two constructs or pIREShyg3 alone (mock transfection) per 4 cm^2 flask. Stable transformants were selected in culture media containing 500 μg/ml hygromycin B. Five independent clones were established from each transformant. Expression of *SLC45A2* mRNA in each of the five clones was confirmed with RT-PCR (data not shown). For melanin assays, each suspension was pelleted and incubated at 95°C for 1 hr after resuspension in 100 μl of 1 N NaOH. After a 100 × dilution, the OD_{475} was measured and converted to melanin content via a standard curve using sepia melanin (Sigma, Poole, UK). The melanin content was normalized to protein content, determined using a Protein Assay Kit from Bio-Rad (Hercules, CA).

This study was approved by the Ethics Committees of Nagoya University School of Medicine and Yamagata University School of Medicine. Informed consent was obtained from the patient's parents.

RESULTS AND DISCUSSION

The mutation screen for the genes responsible for OCA1–4 revealed a homozygous mutation at NM_016180.3: c.113A > G in the *SLC45A2* gene causing an amino acid substitution, p.H38R. And, his parents turned out to be heterozygous for the mutation. This missense mutation involves a conserved amino acid residue since it is known to be present among all species carrying an *SLC45A2* ortholog, including the pig, horse, dog, mouse, cow, chicken, medaka, *Xenopus*, *Tetraodon*, and zebrafish. The substitution was not detected in any of the 27 individuals of Moroccan origin who were used as controls. These data indicated that residue H38 is functionally important and that p.H38R is not a common non-pathological polymorphism.

To experimentally assess the function of the p.H38R-mutant SLC45A2 protein, we tested the ability of the wild-type versus the mutant polypeptide to produce melanin in melanocytes (*uw* cells) obtained from the OCA4 mouse. Over-expression of normal human SLC45A2 protein in *uw* cells restored melanin production, while the p.H38R-mutant protein failed (Fig. 2). These subjective assessments were substantiated by melanin content. Cells transfected with wild-type cDNA contained more than twice the melanin found in mock-transfected cells, while the melanin levels in cells transfected with p.H38R-mutant cDNA were similar to those in mock-transfected cells (Fig. 3). Therefore, the p.H38R-mutant protein is functionally incapable of melanin synthesis and should be pathologic thus causing albinism.

In conclusion, we identified a novel mutation of the *SLC45A2* gene, p.H38R, in a Belgian son of Moroccan consanguineous parents. This study confirms that both parents were carrier of the mutation, that it is an autosomal recessive inheritance and that the recurrence rate for this couple to have a child with albinism (OCA4) is 25%. This is the second report of the occurrence of OCA4 in a member of an African ethnic group, suggesting that OCA4 might be present in many ethnic groups.

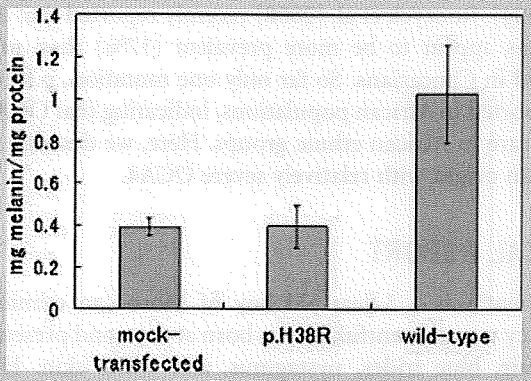

FIG. 3. Melanin content of *uw* cells transfected with different cDNAs. Means ± SEM were calculated using five independent clones from each treatment. Transfected cells were compared to mock-transfected cells. A significant increase in melanin content was observed only after transfection with the wild-type sequence ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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