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Gap junction development in the human fetal hair follicle and bulge region

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Summary

Background Gap junctions, composed of connexin (Cx) subunits, are channels that allow intercellular communication between adjacent cells and are thought to play a key role in the regulation of cell proliferation and differentiation. The Cx expression pattern and formation of gap junctions in human fetal hair follicles has yet to be clarified, including the prominent follicular bulge region that is believed to be a site rich in stem cells.

Objectives To study the expression of two major Cxs, Cx26 and Cx43, in developing hair follicles in skin samples from a series of human fetuses of estimated gestational age (EGA) 88–163 days, and to determine quantitatively the presence of gap junctions.

Methods We used immunofluorescence labelling to investigate the sequential expression pattern of Cx26 and Cx43 in developing human hair follicles. Gap junction formation was observed by electron microscopy and the numbers of gap junctions were analysed quantitatively.

Results Both Cx26 and Cx43 expression were observed at 88 days' EGA in the inner part of the hair peg. At 135 days' EGA, Cx26 was expressed in the outer root sheath (ORS) and the inner root sheath (IRS), while Cx43 was expressed chiefly in the IRS, hair matrix and sebaceous glands. At 163 days' EGA, Cx26 expression was most intense in the outermost layer of the ORS, in contrast to Cx43 expression which was in the inner part of the ORS. In the bulge region, only Cx43 was expressed in a subset of cells in the bulge. Ultrastructurally, gap junctions were observed at 102 days' EGA in the hair peg, and the number of gap junctions increased as the hair follicle matured. Gap junctions were also observed between the bulge cells in considerable numbers.

Conclusions The changing expression patterns of Cx26 and Cx43 and the increasing gap junction numbers suggest a close association of Cx expression and gap junction formation with hair follicle morphogenesis. In addition, the present ultrastructural observations demonstrate that considerable numbers of the bulge cells, a putative site rich in hair follicle stem cells, form gap junctions during human hair follicle development.

Key words: bulge, connexin 26, connexin 43, electron microscopy, morphogenesis, stem cell

Gap junctions are intercellular channels that are involved in cell–cell communication.[1] Small molecules can pass through the gap junction channels, such as ions and second messengers with molecular weights less than 1 kDa.[2] Gap junctions are considered to be important for maintaining tissue homeostasis and for communicating certain intracellular signals. Disturbance of gap junction intercellular communication is observed in malignant transformation[3] and in many cases of congenital hearing impairment.[4] These findings suggest a significant role of gap junction communication in normal cell proliferation, differentiation and intercellular signalling.

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Gap junctions are composed of connexin (Cx) molecules, of which there are about 20 subtypes. More than 10 Cx subtypes have been identified in human skin.[5] However, the major Cx subtypes in human skin are Cx26 and Cx43. Distribution of these Cxs in human adult skin has been studied in detail.[6] Cx43 is expressed in suprabasal keratinocytes in the epidermis, and also in the hair follicle including the sebaceous gland, and the eccrine sweat gland. Cx26 is expressed in the hair follicle and the eccrine sweat gland, but not in the normal human adult epidermis nor the sebaceous gland.

Cx expression patterns are abnormal in skin tumours[7] and hyperproliferative disorders of the epidermis,[8,9] and therefore Cxs are thought to be important in keratinocyte proliferation and differentiation. Recently, Cx26 gene mutations have been reported in palmoplantar keratoderma with sensorineural deafness (Vohwinkel's syndrome)[10] and keratitis–ichthyosis–deafness syndrome.[11] These also suggest a role of gap junction intercellular communication in normal keratinization. Fetal skin exhibits a changing pattern of proliferation and differentiation. For this reason the expression of Cxs in fetal skin is of considerable interest. In developing rodent skin, changing Cx expression patterns have been reported, including within the hair follicle.[12–14] In humans, we have recently demonstrated both the changing expression patterns of Cx26 and Cx43 and morphological gap junction formation in developing fetal epidermis.[15] However, there have been no reports of the expression patterns of Cx26 and Cx43 and gap junction formation in developing human fetal hair follicles, as far as we know. Here, we report Cx26 and Cx43 expression patterns in human fetal hair follicles and gap junction formation at different developmental stages. In addition, we have demonstrated the presence of gap junctions in the bulge region, which is prominent during the fetal period and is thought to be enriched with stem cells.[16]

Materials and methods

Tissue

Human embryonic and fetal skin specimens were obtained from several institutes with the approval of the appropriate ethical boards and in accordance with their policies. The fetuses were confirmed not to have any obvious skin abnormalities or family history of skin disorders. The ages and the autopsy sites of fetuses included in the present study are summarized in Table 1. Estimated gestational age (EGA) was determined from maternal histories, fetal measurements (crown, rump and foot length) and comparative histological appearance of the epidermis.[17–20] For comparison, one skin specimen from adult scalp was used.

Antibodies

The antibodies used in the present study were rabbit polyclonal anti-Cx26 antibody (Zymed Laboratories, San Francisco, CA, U.S.A.) and mouse anti-Cx43 monoclonal antibody, clone 4E6.2 (Chemicon International, Temecula, CA, U.S.A.).

Immunofluorescent labelling

Fetal skin was snap-frozen in isopentane and 6-μm thick sections were cut using a cryostat. The sections were washed with 0·01 mol L^{-1} phosphate-buffered saline (PBS) for 10 min and then incubated in a primary antibody solution for 1 h at 37 °C. Antibody dilutions were 1/10 for the anti-Cx26 antibody and 1/100 for the anti-Cx43 antibody. The sections were then incubated in fluorescein isothiocyanate (FITC)-conjugated goat antimouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) solution or in FITC-conjugated goat antirabbit immunoglobulin (Jackson Immunoresearch Laboratories) solution for 30 min at room temperature, followed by

Table 1. Samples used in this study

Estimated gestational age (days)	88	96	102	108	120	135	152	163
Site	Scalp	Trunk	Trunk	Scalp	Scalp	Scalp	Scalp	Scalp
Observation	IF	IF	EM	IF, EM	EM	IF	EM	IF
Differentiation state	Hair peg	Hair peg	Hair peg	Hair peg	Hair peg	Bulbous hair peg	Lanugo hair	Lanugo hair

IF, immunofluorescence; EM, electron microscopy. $n = 1$ for all samples except 120 days' EM samples ($n = 2$).

10 μg mL^{-1} propidium iodide solution as a nuclear counterstain (Sigma Chemical Co., St Louis, MO, U.S.A.) for 10 s (the nuclear staining was not performed in the adult specimen). The sections were extensively washed with 0·01 mol L^{-1} PBS between incubations. The stained sections were mounted using a glycerol-based mounting medium (Permafluor; Shandon, Pittsburgh, PA, U.S.A.) and stored in the refrigerator in the dark. Immunostaining was detected as green (FITC) and nuclear staining was observed as red (propidium iodide). Overlap of both FITC and propidium iodide was demonstrated as a yellowish colour. Fluorescence images were observed using an Olympus IX70 confocal laser scanning microscope. Image collection was performed by software Fluoview version 2·0 (Olympus America Inc., Melville, NY, U.S.A.). As negative control, nonimmunized normal rabbit serum or mouse myeloma supernatant was used instead of primary antibodies.

Electron microscopy

Fetal skin was fixed in one-half strength Karnovsky's fixative or 2% glutaraldehyde solution, postfixed in 1% OsO_4, dehydrated, and embedded in Epon 812.[21] All the samples were ultrathin sectioned at a thickness of 70 nm, and stained with uranyl acetate and lead citrate.[22] Photographs were taken using a Hitachi H-7100 or H-800 transmission electron microscope.

For statistical analysis, we chose several developing hair follicles: the numbers of gap junctions were counted at 102, 120 and 152 days' EGA, and the numbers of gap junctions were divided by the numbers of cells observed.

Results

Connexin 26 and connexin 43 are differentially expressed in developing human fetal hair follicles

Induction of hair follicle formation was observed from 88 days' EGA and onwards. The expression of Cx26 and Cx43 was observed from this period. Both Cxs were expressed in the inner part of hair pegs (Fig. 1a,f). By 135 days' EGA, the hair follicle structure had matured and formed a bulbous hair peg. Cx26 expression was observed in the outer root sheath (ORS) and the inner root sheath (IRS), whereas it was not expressed in hair matrix or sebaceous gland tissue (Fig. 1b,c). Conversely, Cx43 expression was mainly in the IRS, sebaceous gland and hair matrix (Fig. 1g,h), although weak expression of Cx43 was also observed in the ORS (Fig. 1h). By 163 days' EGA, hair follicles had differentiated to lanugo hair follicles, and Cx26 was expressed in the ORS and IRS, with the most intense staining in the outermost layer of the ORS (Fig. 1d). Conversely, Cx43 expression was more prominent in the IRS (Fig. 1i). Similar distributions of Cx26 and Cx43 in the IRS and ORS were observed in adult hair follicles (Fig. 1e,j). Sebaceous glands were also Cx26 negative and Cx43 positive in adult tissue (data not shown). The immunofluorescence results are summarized in Table 2. The 96 days' EGA specimen was from the trunk, but the staining pattern of Cxs was similar to those from scalp tissue (data not shown).

In fetal hair follicles, the bulge region was easily identified as a prominent protrusion of the ORS below the sebaceous gland (Fig. 1c,h). Cx26 expression was not observed in the bulge region (Fig. 1c). Cx43 expression was negative in most cells of the bulge, but a few cells stained weakly (Fig. 1h).

Gap junction formation was confirmed in developing hair follicles, including bulge cells, by electron microscopy

Typical gap junction structures were observed from 102 days' EGA in the hair peg, in small numbers. Later, more gap junctions were observed in the IRS and ORS of fetal hair follicles (Fig. 2a–c). The numbers of cells observed were 193 at 102 days' EGA, 228 at 120 days' EGA, and 132 at 152 days' EGA. (The bulge cells were not included in the ORS cell group.) The numbers of observed gap junctions were 6 at 102 days' EGA, 11 at 120 days' EGA, and 15 at 152 days' EGA. The mean number of gap junction per one ORS or IRS cell was 0·031, 0·048 and 0·114, respectively (Fig. 2g). Gap junction numbers per cell increased with time.

Bulge regions were present from 120 days' EGA. Gap junctions were also observed between cells in the bulge areas (Fig. 2d–f). The number of cells studied was 90 at 120 days' EGA, and 223 at 152 days' EGA. The numbers of observed gap junctions were 5 at 120 days' EGA and 17 at 152 days' EGA. The mean number of gap junctions per bulge cell was 0·056 and 0·076, respectively (Fig. 2g). The cells in the bulge region had a high nuclear/cytoplasmic ratio and scant intracellular organelles, which is suggestive of an undifferentiated stem cell-like phenotype (Fig. 2d). The cells with gap junctions and those without them were morphologically indistinguishable.

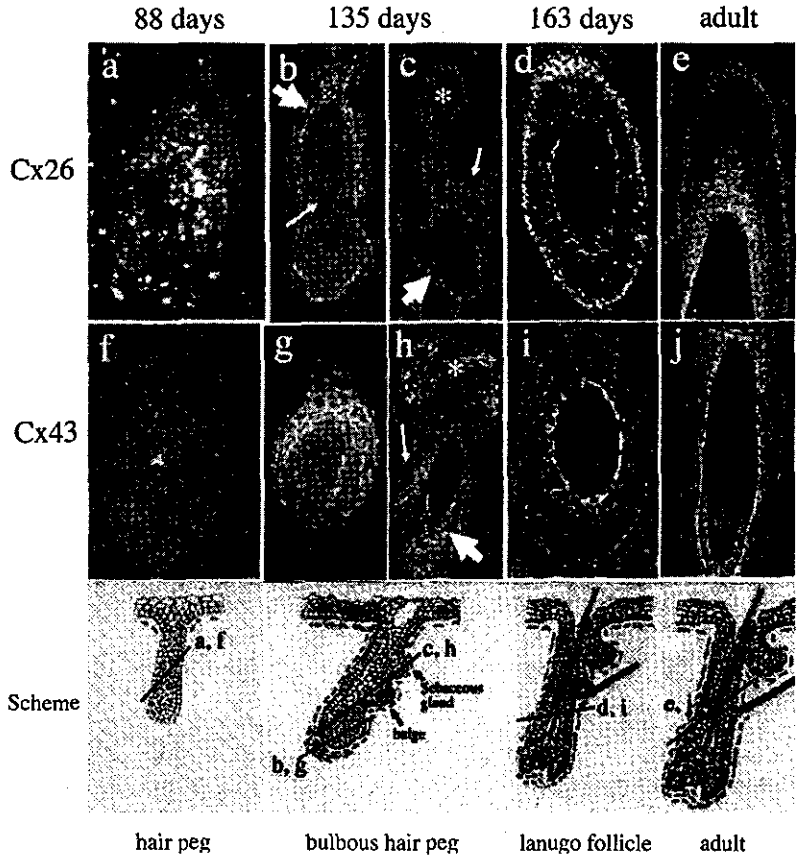


Figure 1. Connexin (Cx) 26 and Cx43 expression in developing fetal hair follicles. Induction of hair follicle formation was observed from 88 days' estimated gestational age (EGA) (a,f). Cx26 was expressed in the hair peg at 88 days' EGA (a). At 135 days' EGA, the hair follicle developed to form a bulbous hair peg and Cx26 expression was observed in the outer root sheath (ORS) (b: large arrow) and the inner root sheath (IRS) (c: large arrow). Cx26 expression was not observed in the hair matrix (b: small arrow) and sebaceous gland (c: asterisk). In the bulge region there was no obvious Cx26 expression (c: small arrow). At 163 days' EGA, the hair follicle developed into a lanugo hair follicle and the expression of Cx26 in the ORS was more intense within the basal layer (d). In the adult follicle, Cx26 expression in the basal layer of the ORS was more prominent (e). Cx43 expression was observed in the central portion of the hair pegs from 88 days' EGA (f). At 135 days' EGA, the expression of Cx43 was prominent within the IRS (h: large arrow) and sebaceous glands (h: asterisk), together with a high expression level in the hair matrix (g), although relatively weak expression was observed in the ORS (h). In the bulge region, most cells were negative, but a few cells were weakly positive (h: small arrow). At 163 days' EGA, Cx43 expression was observed in the IRS and the inner part of the ORS (i) and this expression pattern was also seen in the adult hair follicle (j). Green, fluorescein isothiocyanate staining of Cx26; red, nuclear propidium iodide staining. Original magnification ×400. A schematic representation of human fetal hair follicles at each stage of development is shown in the bottom row.

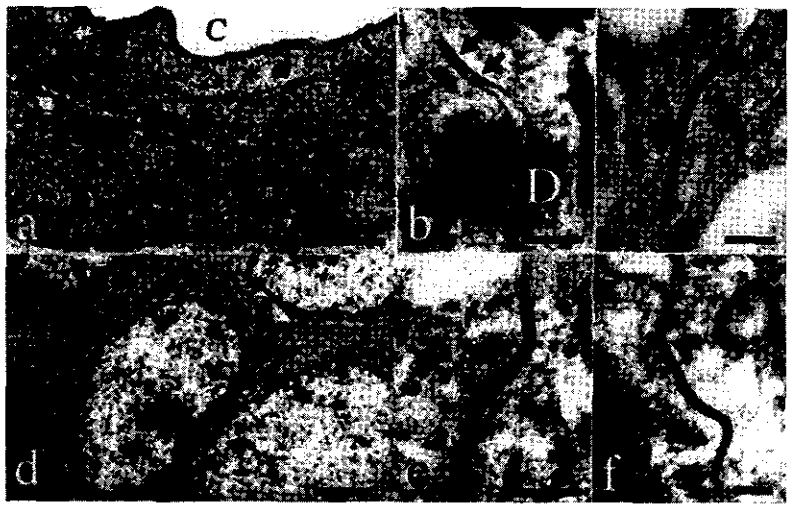
Table 2. Summary of immunofluorescence results in each developing stage of fetal hair follicle

	Hair peg	Bulbous hair peg					Lanugo hair follicle				
		ORS	IRS	Matrix	SG	Bulge	ORS	IRS	Matrix	SG	Bulge
Cx26	+	+	+	−	−	−	+ +[b]	+ +	−	−	−
Cx43	+	+	+ +	+ +	+ +	+[a]	+ +[c]	+ +	+ +	+ +	+[a]

Cx, connexin; ORS, outer root sheath; IRS, inner root sheath; SG, sebaceous gland. [a]A few cells were positive; [b]stronger in the outermost layer; [c]stronger in the inner layer.

Discussion

Our results have demonstrated changing patterns of Cx expression and gap junction formation during human hair follicle development. Cx26 was expressed at the initial stage of hair morphogenesis, as seen in Figure 1(a). During the same period, Cx26 was not expressed in the interfollicular epidermis except



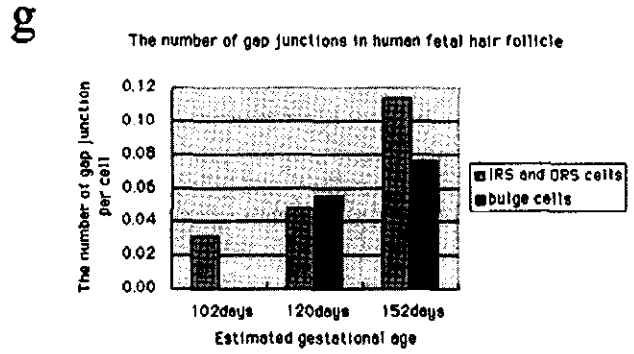


Figure 2. *Ultrastructural features of gap* junctions and quantification of gap junctions in developing fetal hair follicles. The typical structure of gap junctions was observed in the hair follicles from 102 days' estimated gestational age (EGA). (a) Outer root sheath (ORS) and inner root sheath (IRS) cells at 120 days' EGA. The IRS cells (asterisk), which contained large trichohyaline granules, were undergoing keratinization and the hair canal (C) was being formed. (b) A gap junction observed between the ORS cell at 120 days' EGA (arrows). D, desmosome. (c) A gap junction between the IRS cells at 152 days' EGA. The typical pentalaminar structure was observed more clearly than at 120 days' EGA. Gap junctions were also observed in the bulge regions at 120 and 152 days' EGA. (d) Bulge cells at 120 days' EGA. The cells in the bulge region were poorly differentiated and densely packed. (e) A gap junction between bulge cells at 120 days' EGA. (f) A gap junction between bulge cells at 152 days' EGA. Bars: (a) 5 μm; (b) 100 nm; (c) 100 nm; (d) 2 μm; (e) 100 nm; (f) 100 nm. (g) Gap junction numbers were counted and divided by the total number of cells observed. The number of gap junctions per cell increased with fetal age. The bulge regions were observed at 120 and 152 days' EGA.

between periderm cells.[15] Therefore, Cx26 expression is a characteristic feature of the hair follicle from a very early period of hair morphogenesis. Cx26 and Cx43 were coexpressed in the early hair peg, but as the hair follicle formed individual compartments such as the ORS and IRS, a distinct/characteristic distribution of each Cx emerged. The relatively strong expression of Cx26 between the basal cells of the ORS may suggest an increased requirement to proliferate during the hair follicle development in this period, in agreement with previous reports suggesting that Cx26 might reflect a hyperproliferative state.[9]

Ultrastructurally, gap junction numbers per follicular epithelial cell in the ORS and IRS increased with fetal age. In the developing human fetal epidermis, the number of gap junctions increased as the epidermis developed, similar to this study.[15] These data suggest a close association between gap junction formation and maturation of fetal skin, including hair follicle compartment formation and morphogenesis. The numbers of gap junctions per cell counted using electron microscopy were very small, at up to only 0·114 per cell. This seems too small because the immunofluorescence showed clear staining of Cxs in the hair follicle. The thickness of ultrathin sections was about 100 nm and the total diameter of the keratinocyte was estimated at 10 μm, and therefore the exact number of gap junctions per 'whole' cell may be 100-fold more than the results observed using this method of electron microscopy. Furthermore, Cx molecules not yet assembled into gap junctions might be present on the plasma membrane together with the more visible classical gap junction structure. The small number of visible gap junctions may suggest that not all Cx proteins are assembled into the gap junctions but that some Cx proteins (possibly the majority of these proteins) are expressed freely on the plasma membrane.

Using electron microscopy, gap junctions were also observed in the bulge region. Recently, an absence of Cx43 expression and a lack of gap junction intercellular communication in epidermal stem cells were reported using immunofluorescent and microinjection

techniques,[23] although an ultrastructural study was not performed in this report. Our electron microscopic observations revealed the presence of gap junctions in the bulge cells. The mean gap junction number per bulge cell was similar to that of IRS and ORS cells at 120 days' EGA (0·056 vs. 0·048), although it was about two-thirds of that of IRS and ORS cells at 152 days' EGA (0·076 vs. 0·114). The number of gap junctions in the bulge region was unexpectedly high for a putative stem cell-rich population,[16,24–26] especially at 120 days' EGA.

The cells forming gap junctions may be relatively differentiated cells, such as transit amplifying cells. However, in the prominent bulge region of human fetal follicles, most cells are thought to be stem cells.[24] We could not find any difference between gap junction positive cells and negative cells because both cells exhibited a uniform, undifferentiated morphology in this region. From this viewpoint, the considerable number of gap junctions in the bulge region suggests the presence of stem cells forming gap junctions. Further investigation of specific markers of stem cells may clarify this point.

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Tyrosinase gene analysis in Japanese patients with oculocutaneous albinism

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Summary

Background: Oculocutaneous albinism (OCA) is a heterogeneous congenital disorder. Tyrosinase is a key enzyme in melanin biosynthesis, and tyrosinase gene mutations cause the OCA1 subtype.
Objective: This study was intended evaluate the frequency and details of tyrosinase gene mutations in Japanese OCA patients.
Patients and methods: We examined nine non-consanguineous OCA families, sequenced the tyrosinase gene of the patients and also confirmed a splicing site mutation using exon trapping system.
Results: Tyrosinase gene mutations were identified in five out of nine OCA families (55%). IVS2-10deltt-7t-a was present in 3 out of 18 alleles in three families (16%), P310insC was present in three alleles in three families (16%) and R278X was found in three alleles (16%), including those in one heterozygous and one compound homozygous patient. G97V (290 G-T) was found in 1 out of 18 alleles, and we could not find G97V in the mutation database. We have added this mutation as 9th mutation of Japanese OCA1 patients. In 8 of 18 alleles, four families, no tyrosinase mutations were identified. They were presumed not to be OCA1, but other subtypes of OCA. Exon trapping system demonstrated IVS2-10deltt-7t-a mutation generated the abnormal splicing site, and inserted the codon 4 bases in mRNA level resulting in premature termination codon downstream.
Conclusion: This study provided new information about OCA1 mutations, and highlights the requirement of broader detailed search to make precise diagnosis of OCA.

1. Introduction

Oculocutaneous albinism (OCA) is a heterogeneous hereditary disorder that can be caused by 14 dif-

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ferent genes. Oculocutaneous albinism type 1 (OCA1) that results from mutations in the tyrosinase gene is the best-understood and most frequent type of OCA in Japan [1]. OCA1A presents with a life-long absence of melanin pigment after birth, whereas OCA1B develops minimal to moderate cutaneous and ocular pigment. More than a hundred of different mutations in the tyrosinase gene, which results in OCA, have been reported in the literature [2] and database [3–5]. However, genetic analysis of Japanese OCA1 has yet not been completed. In spite of the variety of the tyrosinase mutations in the world, only eight tyrosinase mutations have been found in Japanese OCA1 patients [6]. Among them, mutations in codons 77 (R77Q) and 310 (P310insC) are the major ones [6]. In the present study, we have analyzed and examined nine OCA patient kindred's who were referred to Hokkaido University. We compared the clinical features and tyrosinase mutations to verify the reports of common mutations in Japanese patients. Also, because IVS2-10deltt-7t-a mutation has not been reported how the abnormal splicing occurred, we have identified the aberrant splicing site by exon trapping system.

2. Patients and methods

2.1. Patients

Nine OCA patients were diagnosed in Hokkaido University Hospital. Consequently, 18 alleles from nine OCA patients of non-consanguineous OCA families, whose subtypes were previously clinically unknown, were examined. Three of them exhibited a complete absence of pigmentation and rest of them showed minimal to moderate pigmentation gradually accumulating with age (Table 1). Five out of nine patients were born on the islands of Hokkaido, Northern Japan. This study was evaluated and approved by the Ethical Committee of Hokkaido University Graduate School of Medicine.

2.2. Identification of mutations in the tyrosinase gene

Genomic DNA was isolated from peripheral blood mononuclear cells of the patients and their parents. In search for mutations, all five exons were amplified separately by PCR using seven sets of primers as described previously [7]. Specifically, for amplification of the 397 bp DNA fragment comprising exon 1 containing G97V (290 G-T), the following primers were used: forward 5′-ACTCCAATTAGCCAGTTCCT-3′ and reverse 5′-AGTCGTCTCTCTGTGCAGTT-3′. There was no proper restriction enzyme to verify the G97V mutation. PCR amplification was carried out using the following PCR primers: 5′-CTGATGGAGAAG-GAATGCTG-3′ and 5′-CTTGCAGTTTCCACAGTTGG-3′ for generation of a 225 bp product. We changed the last base of the latter primer (underlined) from the original sequence, so that, the combination of this change and the upstream sequence created a new BamHI site in the PCR product. Since the G97V mutation was also located just one base upstream from that primer, G97V abolished this BamHI site. Therefore, BamHI digestion of the 225 bp PCR product with G97V produced 203 bp and 22 bp bands, whereas that without G97V did 225 bp band. For amplification of the 337 bp DNA fragment comprising exon 2 containing R278X and P310insC mutations, the following primers were used: forward 5′-TCCTACT-GACTGGTGGTGAC-3′ and reverse 5′-AGGACTTTGGA-TAAGAGACTG-3′. For the amplification of a 256 bp DNA fragment comprising exon 3 containing IVS2-10deltt-7t-a mutations, the following primers were used: forward 5′-TCCTACTGACTGGTGGTGAC-3′ and reverse 5′-AGGACTTTGGATAAGAGACTG-3′. For PCR amplification, 250 ng of genomic DNA was used as the template in an amplification buffer containing 20

Table 1 Clinical features and mutations in nine families of Oculocutaneous albinism

Patient	Sex	Age	Clinical features (hair/skin)	Hometown	Tyrosinase gene mutation		Diagnosis
					Paternal	Maternal	
1	M	0	Blond/white	Hokkaido	G97V	IVS2-10deltt-7t-a	OCA1B
2	F	0	Blond/white	Kanagawa	P310incC	IVS2-10deltt-7t-a	OCA1B
3	F	6	Blond/white	Tokyo	IVS2-10deltt-7t-a	P310incC	OCA1B
4	F	1	White/white	Hokkaido	P310incC	R278X	OCA1A
5	M	1	White/white	Hokkaido	R278X	R278X	OCA1A
6	M	0	Blond/white	Aomori		–	OCA
7	F	0	White/white	Hokkaido		–	OCA
8	M	0	Blond/white	Tokyo		–	OCA
9	F	17	Blond/white	Hokkaido		–	Hermansky–Pudlak syndrome

Patient 1 and 2 are twins. (–) No mutation in tyrosinase gene.

pmol of each primer, 1.5 mM $MgCl_2$, 200 μmol of each nucleotide and 0.5 unit of taq polymerase (Amplitaq, Perkin-Elmer Life Sciences, San Francisco, CA, USA) in a total volume of 50 μl. The amplification conditions were 94 °C for 5 min, followed by 40 cycles of reaction at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, followed by incubation at 72 °C for 7 min. The PCR products were directly sequenced by di-deoxy dye-terminator method using an automated sequencer (ABI Prism Genetic Analyzer 3100, PE biosystems, Foster city, CA, USA).

2.3. Identification the splicing site of IVS2-10deltt-7t-a mutation

Exon trapping system (Invitrogen, Carlsbad, CA) is an approach used for the direct isolation of transcribed sequences from genomic DNA [8]. To a genomic TYR fragment containing exon 3 and the acceptor site for the splicing, we synthesized two primers, 5′-AAACTCGAGTCTCAATACGGAATGAA-3′ and 5′-AAAGGATCCTGCCTAATCCACCTTC-3′, based on the sequence of tyrosinase gene (Gene Bank/EMBL Data Bank accession number, M63239). The primers contained restriction enzyme sites at the 5′ and 3′ ends for sub cloning. PCR was performed using normal human genomic DNA and patient genomic DNA with IVS2-10deltt-7t-a mutation as a template. After sequence analysis, the DNA fragment was digested with XhoI and BamHI and sub cloned into the multicloning site of a pSPL3 expression vector (Invitrogen), which contained a portion of the HIV-1 tat gene, an intron, splice donor and acceptor sites, and some flanking exon sequences. The recombinant plasmid was transfected into A375 cells using *N*-[1-(2,3-dioleoyloxy)proryl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche Molecular Biochemicals). Total RNA was extracted from the culture cells and RT-PCR was performed using the trapping vector-specific oligonucleotide primers (Invitrogen). The PCR products were sequenced to know abnormal splicing.

3. Results and discussion

Patients 1, 2 and 3 presented with creamy white skin, blond or light brown hair, and pale brown iris pigmentation (Fig. 1a and b). They lacked pigment at birth but developed yellow hair pigmentation as they grew older and accumulated pigment in their hair, eyes, and skin with time. Patient 1 was heterozygote for G97V (290 G-T) and IVS2-10deltt-7t-a. IVS2-10deltt-7t-a has been reported as an OCA1B allele [9,10]. As far as we know, G97V was a novel mutation. His mother and normal individual did not show this mutation. Although we suspected his father was heterozygous for this mutation, genomic DNA of his father was not available (Fig. 2). This mutation was confirmed by restriction endonuclease analysis. This substitution was not found in 100 normal alleles (data not shown). Patients 2 and 3

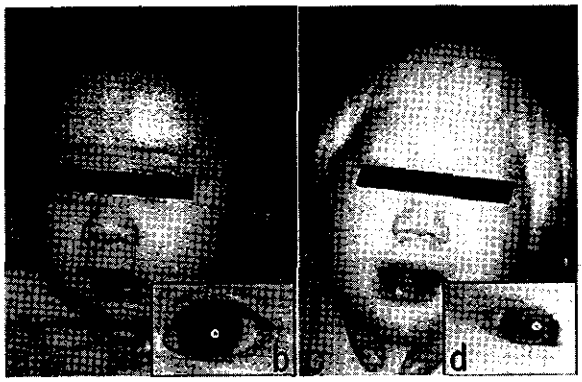

Fig. 1 Clinical features of OCA cases. Case 1 OCA1B (left); compound heterozygote of IVS2-10deltt-7t-a and P310insC. The skin had minimal pigmentation. The hair was blond (a). The iris developed brown pigmentation as she grew older (b). Case 2 OCA1A (right); compound heterozygote of R278X and P310insC. The hair and skin was white (c). The irises were pink (d). The patient had severe nystagmus and photophobia.

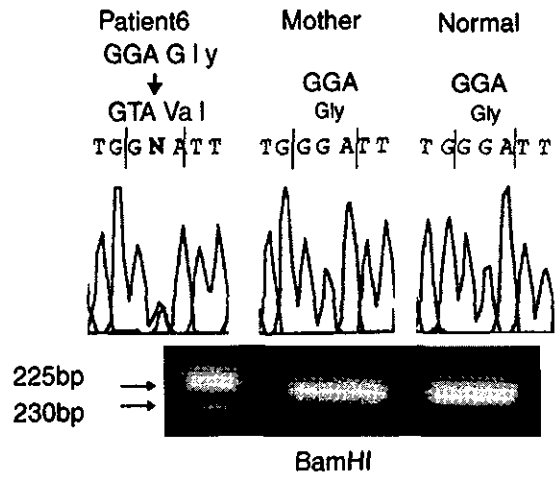

Fig. 2 Detection of a novel mutation G97V. Patient 6 has a heterozygous 290 G-T substitution (exon 1) in codon 97, replacing glutamine with valine. Since no proper restriction enzyme site was found around the G97V mutation, we changed one base of the PCR primer from the original sequence to create a site for BamHI (see Section 2). The digestion of 225 bp PCR product with G97V produced 203 bp band. His mother and normal human did not show this mutation. Although we suspected his father was heterozygous for this mutation, genomic DNA of his father was not available.

were heterozygote for IVS2-10deltt-7t-a and P310insC (Table 1).

Patients 4 and 5 were revealed complete loss of pigmentation of whole body; pink skin, white hair, and white eyelash. Their irises were pink (Fig. 1c and d). They had severe nystagmus and photophobia. They had no Mongolian spots as babies. Genetic examination revealed that they were OCA1A. Patient 4 was compound heterozygotes for P310insC and R278X while patient 5 was homozygote of R278X (Table 1).

Patients 6, 7 and 8 presented with blond or white hair and white or creamy white skin. The patients suffered to various extents from nystagmus. They did not harbor any mutations in the tyrosinase gene in spite of complete tyrosinase gene sequencing. Therefore, these four patients rather had cases of OCA that were not associated with tyrosinase deficiency. Detailed clinical examination revealed that patient 9 most likely had Hermansky–Pudlak syndrome, because she had a bleeding diathesis with defects in the ability to aggregate platelets.

The genotype analysis of the tyrosinase gene in these patients is summarized in Table 1. We examined 18 alleles of the entire tyrosinase gene and identified mutations in 10 alleles (55%). The four kinds of mutation were identified: G97V in one allele, IVS2-10deltt-7t-a in three alleles (16%), 310insC in three alleles (16%) and R278X in three alleles (16%). We could not identify any tyrosinase gene mutations in eight alleles (44%). This study has detected G97V mutation in the OCA1B patient. Only eight pathological mutations have detected in Japanese patients (Fig. 3) so far, whereas worldwide database of tyrosinase gene mutation has collected more than 100 different mutations. Since we could not find G97V in the database and in 100 normal alleles, we have added this mutation as 9th mutation of Japanese OCA1 patient.

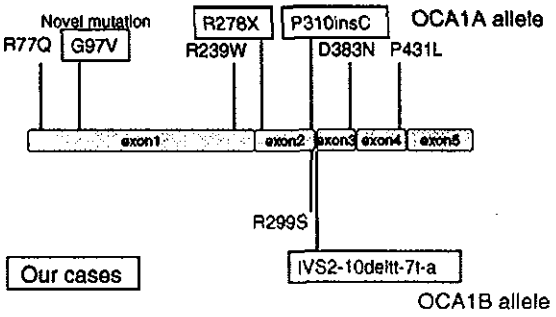

Fig. 3 A summary of tyrosinase gene mutations reported in Japanese OCA1patients. The mutations identified in OCA1A (black letters) are described in the upper half of the schema. The mutations identified in OCA1B (blue letters) were described in the lower half of the schema. The mutations identified in the present study are marked by a red square.

According to the previous reports, 310insC and R77Q were common among eight pathological OCA1 mutations in Japan [6,11] (Fig. 3). In our examination, 310insC was also a frequent allele, although we could not find any R77Q defects. Nevertheless, each OCA family tends to have their own particular combination of tyrosinase mutations that is rarely duplicated despite a global search (Fig. 4). R278X is an exceptional and frequent mutation in several races and in the different places [12–15]. According to haplotype analysis, R278X had at least two different origins, which was thought to have occurred a long time in the past [16]. Our study is unique because we have found the R278X mutation in our patients from Hokkaido, whose inhabitants had moved from various districts of Japan in the 19th century. The result of tyrosinase analysis in Hokkaido may reflect the result found in various districts of Japan.

IVS2-10deltt-7t->a was another frequent mutation in our study. This mutation was identified in Korean and Japanese OCA1B patients [9,10]. Because we found IVS2-10deltt-7t->a in different unrelated three kindred of Japanese OCA1B patients, we thought that IVS2-10deltt-7t->a might be a common OCA1B mutation in Japan, which is geographically close to Korea. However, no report has shown mechanism whereby this mutation induces abnormal mRNA. In this study, we detected the abnormal splicing site by exon trapping system, which revealed insertion of 4 bp (ACAG) upstream from the common acceptor site of exon 3. Consequently, insertion of the additional four bases resulted in premature termination codon downstream (Fig. 5). The PCR products were sequenced by TA cloning. The abnormal sequenced is found in approximately 20% of all the sequenced. Relatively low expression of aberrant mRNA implied that this mutation generated mild phenotype OCA1B. We confirmed for the first time that IVS2-10deltt-7t-a resulted in pathological splicing site.

The number of uninvestigated OCA1B patients might be unexpectedly large, since adult Japanese OCA1B patients cannot be recognized as OCA patients. We found three OCA1B unrelated patients out of nine OCA families. The number of OCA1B patient might become large if more broad detailed examination is performed. Owing to the systematic genetic allele examination, information of OCA that has been caused by something other than tyrosinase gene defects has been known. Recent genetic analysis of Japanese OCA showed that OCA2 is the major types but less than OCA1 [17] and OCA4 is one of the common types of albinism in Japan [18]. We should also pay attention to the non-OCA1A type OCA to

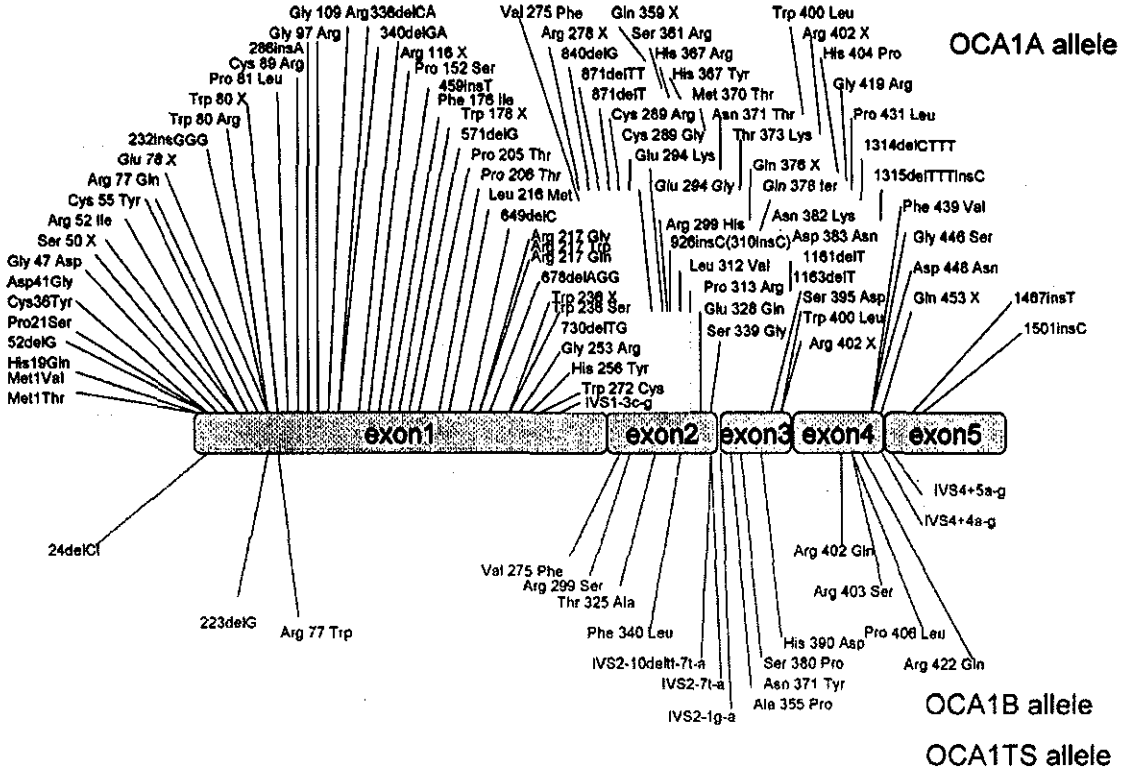

Fig. 4 Database of tyrosinase gene mutations. The mutations identified in OCA1A (black letters) are described in the upper half of the schema. The mutations identified in OCA1B (blue letters) and OCATS (Temperature sensitive variant of OCA) (red letters) are described in the lower half of the schema.

make precise diagnosis. This study has suggested additional possible hot spots mutation areas within the tyrosinase gene in the Japanese population and points towards a possible hidden higher frequency of non-OCA1A patients in Japan. Systematic genetic analysis all over the country is necessary to know the precise distribution and allele frequency of tyrosinase mutations in the Japanese population [19].

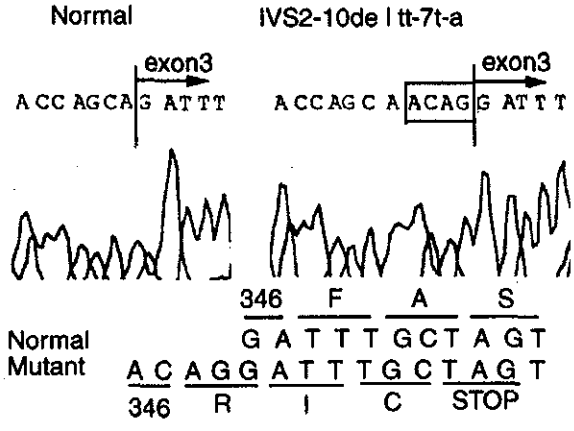

Fig. 5 Generation of aberrant mRNA from IVS2-10deltt-7t-a splicing site mutation. This mutation inserted 4 bp (ACAG) upstream from the common acceptor site of exon 3. Consequently, insertion of the additional four bases resulted in premature termination codon downstream.

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Oculocutaneous Albinism Type 4 Is One of the Most Common Types of Albinism in Japan

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Oculocutaneous albinism (OCA) is a complex genetic disease with great clinical heterogeneity. Four different types of OCA have been reported to date (OCA1, OCA2, OCA3, and OCA4). *MATP* was recently reported in a single Turkish OCA patient as the fourth pathological gene, but no other patients with OCA4 have been reported. Here, we report the mutational profile of OCA4, determined by genetic analysis of the *MATP* gene in a large Japanese population with OCA. Of 75 unrelated patients that were screened, 18 individuals (24%) were identified as having OCA4; they harbored seven novel mutations, including four missense mutations (P58S, D157N, G188V, and V507L) and three frameshift mutations (S90CGGCCA→GC, V144insAAGT, and V469delG), showing that *MATP* is the most frequent locus for tyrosinase-positive OCA in Japanese patients. We discuss the functional melanogenic activity of each mutant allele, judging from the relationship between the phenotypes and genotypes of the patients. This is the first report on a large group of patients with OCA4.

Introduction

Oculocutaneous albinism (OCA) is a group of autosomal recessive disorders caused by mutations of melanogenic genes, including *tyrosinase* (for OCA1 [MIM 203100]) (Tomita et al. 1989), P protein (for OCA2 [MIM 203200]) (Rinchik et al. 1993), and tyrosinase-related protein 1 (*TYRP1*) (for OCA3 [MIM 203290]) (Boissy et al. 1996). Individuals with OCA1A are born with a complete absence of pigment in the hair, eyes, and skin. In patients with OCA1B and OCA2, the phenotypes are typically somewhat less severe than in those associated with OCA1A, and the two disorders display considerable clinical overlap (Lee et al. 1994). The OCA3 phenotype is characterized by reddish skin and hair color in African blacks and is caused by a mutation in *TYRP1*. Rufous OCA is now classified as OCA3 (Manga et al. 1997). Recently, a novel melanogenic gene located in chromosome segment 5p and named "*MATP*" (*membrane-associated transporter protein*) or "*AIM-1*" (*antigen in melanoma-1*) was identified as the fourth pathological OCA gene. The human *MATP* gene encodes a 530–amino-acid polypeptide that contains 12 putative transmembrane domains, exhibits structural homology to plant sucrose-proton symporters, and is expressed in a high percentage of melanoma cell lines (Harada et al. 2001). Its homolog in medaka fish, *b*, encodes a transporter that mediates melanin synthesis (Fukamachi et al. 2001). In a single Turkish patient with OCA, a homozygous G-to-A transition in the splice-acceptor sequence of exon 2 of the *MATP* gene was identified, and that type of OCA was termed "OCA4" (MIM 606574) (Newton et al. 2001). No other patient with OCA4 was subsequently reported, suggesting that OCA4 might be a very minor type of OCA in the worldwide OCA population. Little is known about the clinical phenotype of OCA4, although the Turkish patient with generalized hypopigmentation and ocular abnormalities was reported to be within the phenotypic range commonly associated with OCA2 (Newton et al. 2001).

We examined 75 Japanese patients with OCA, in which 35 (47%) and 6 (8%) were classified as having OCA1 and OCA2, respectively, according to the sequence analyses of the tyrosinase gene and the *P* gene (Tomita et al. 2000; Suzuki et al. 2003). The remaining

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34 patients were further examined to see whether any of them had *MATP* mutations. In this study, we report 18 individuals who have been identified with OCA4.

Subjects and Methods

Patients

A total of 34 Japanese patients with OCA (20 females and 14 males), who were unrelated and in whom no mutation in either the tyrosinase gene or the *P* gene was detected, were included in this study. The degree of hypopigmentation in each patient varied from mild to severe, similar to tyrosinase-related OCA (OCA1A and OCA1B). No patient had a family history of inbreeding.

This study was approved by the ethics committee of the Nagoya University School of Medicine. Informed consent was obtained from each patient, or from the patient's parents, in the case of children.

Identification of the Genomic Organization and Mutation Screening of the MATP *Gene*

The GenBank database was screened with the *MATP* complementary DNA sequence (AF172849). From one human genomic contig (NT_023085), the intron/exon boundaries of the *MATP* gene were analyzed, and primer sequences were designed for the mutation screening of the *MATP* gene (table 1). Genomic DNA was isolated from the peripheral blood of each patient by use of a genomic DNA purification kit (Qiagen). The human *MATP* gene spans 7 exons. In exon 1, three primer sets were designed—for the 5′ side, the middle part, and the 3′ side—because DNA fragments >350 bp were not suitable to detect mutations precisely in our system. In exon 3, two primer sets were designed, for the same reason as in exon 1. The amplified fragments were then screened for mutations by simultaneous analyses of SSCPs and the heteroduplex method (Spritz et al. 1992). Three kinds of SSCP gels, with glycerol concentrations of 0%, 7%, and 10%, were used to elevate the sensitivity of our mutation screening system. Standard PCR amplification procedures were employed, with an annealing temperature of 58°C for all primers, except the primer sets for exons 3 and 5, which were done at 61°C. PCR products showing aberrant patterns were reamplified and sequenced directly. In patients with one or no mutations detected by the SSCP method, all of their PCR products were directly sequenced to identify any mutations.

Results and Discussion

Mutations of the MATP *Gene*

Among the 75 Japanese patients with OCA in our study, 34 patients lacked any mutation in the coding or adjacent noncoding sequences of the tyrosinase gene or the *P* gene. The diagnoses of OCA1 or OCA2 were therefore essentially eliminated. We then screened for mutations in the *MATP* gene of those 34 patients with OCA. PCR-SSCP/heteroduplex screening and direct sequencing of the *MATP* gene finally detected seven novel mutations in 18 individuals (3 males and 15 females) (table 2). These novel mutations included four missense substitutions (P58S, D157N, G188V, and V507L), two deletion mutations (S90CGGCCA→GC and V469delG), and one insertion mutation (V144insAAGT). All of the four missense mutations were found at amino acid residues conserved among medaka fish, mouse, and human.

We examined the frequency of the seven mutant alleles in the *MATP* gene of 104 unrelated normally pigmented Japanese subjects (208 alleles), and no mutant allele was detected (table 3). This indicates that those seven alleles might be very rare in the general Japanese population and could be defined statistically as pathological alleles.

In six patients (4, 11, 15, 19, 20, and 25 [table 2]), only one heterozygous mutation was found, although we directly sequenced all PCR products amplified from the DNA of these six patients to find a second mutation.

Table 1

Primer Pairs Used to Amplify the *MATP* Exon Segments

Exon No.	PCR Primer		PCR Product Size (bp)
	Forward	Reverse	
1 (5′ side)	5′-AGGCTCCACGTCAAATCCAG-3′	5′-GGTCACATACGCTGCCTCCA-3′	260
1 (middle)	5′-CAGACTCATCATGCACAGCA-3′	5′-ATGCCCACGAGCATCATGAC-3′	252
1 (3′ side)	5′-CAGCATTGTGTGGTTCCTCA-3′	5′-GGTCAAACACATGAACATCCTC-3′	261
2	5′-AACGTGGATGATTCTAAAACAGGA-3′	5′-CTCATTGTCTGGGGAGCTGA-3′	280
3 (5′ side)	5′-GGGAGTGTCTATGCATGAGG-3′	5′-GATAGAACCATACTCGTACATTCC-3′	324
3 (3′ side)	5′-GCCCCACTTACAGAGGTTGC-3′	5′-CAACAAAGAGCAAGAATATTTTCCCTTG-3′	224
4	5′-AGCTGGCTGAGTTTCTGCAG-3′	5′-CCTCAACAGGTGTTAATGGAGG-3′	265
5	5′-AGAGGTGGAGAAGCAGAGTG-3′	5′-GAAGACATCCTTAGGAGAGAG-3′	236
6	5′-ATGAGGCACTGCCAGCTGTA-3′	5′-CCCAAGGCAGAGGTTCAATG-3′	286
7	5′-GCCCTAAATGACAGTTCCTTG-3′	5′-TGTGCTTCACTGTCTCTGAG-3′	326

Table 2

Mutations of the *MATP* Gene in 18 Japanese Patients with OCA4

			Mutation		Clinical Phenotype			Parents	
Patient	Age	Sex	1st	2nd	Hair Color	Iris Color	Nystagmus	Mother	Father
3	23 years	F	D157N	G188V	Light yellow	Blue	Positive	G188V	D157N
4	1 year	M	G188V	NI	Brown	Red-brown	Negative	NM	G188V
6	13 years	F	D157N	V469delG	Light yellow	Blue	Positive	V469delG	D157N
8	71 years	F	D157N	D157N	White	Blue	Positive	ND	ND
9	25 years	F	S90CGGCCA→GC	V144insAAGT	White	Blue	Positive	ND	ND
10	1 year	F	G188V	S90CGGCCA→GC	Blond	Gray-blue	Negative	G188V	S90CGGCCA→GC
11	25 years	F	D157N	NI	Blond	Brown	Negative	ND	NM
12	5 years	M	G188V	G188V	Pale blond	Red-brown	Negative	G188V	G188V
15	11 mo	F	G188V	NI	Light yellow	Brown	Negative	G188V	NM
17	1 year	F	D157N	G188V	Yellow	Blue	Positive	G188V	D157N
19	10 years	F	D157N	NI	Brown-black	Brown	Negative	ND	ND
20	2 mo	F	D157N	NI	Light yellow	Gray-blue	Negative	D157N	NM
25	6 years	F	D157N	NI	Blond	Gray	Positive	D157N	NM
36	1 year	F	D157N	V507L	Blond	Gray-blue	Negative	V507L	D157N
38	1 mo	M	D157N	V144insAAGT	Light yellow	Gray	Positive	D157N	V144insAAGT
42	6 mo	F	P58S	S90CGGCCA→GC	Light yellow	Blue	Negative	P58S	S90CGGCCA→GC
50	24 years	F	D157N	D157N	Light yellow	Blue	Positive	D157N	ND
51	1 year	F	D157N	S90CGGCCA→GC	Light yellow	Blue	Positive	D157N	S90CGGCCA→GC

Note.—ND = an examination was not done; NM = no mutation was found in our examination; NI = mutation not identified.

It seems likely that the other *MATP* allele contains an occult mutation that either was not detected by our SSCP/heteroduplex screening procedure or occurs in a gene region not sampled in the PCR products.

The frequency of OCA4 in the Japanese albino population was 24% (18/75), indicating that mutations in the *MATP* gene are one of the most common causes of tyrosinase-positive OCA in Japan. Although all PCR products amplified from their DNA were directly sequenced to find any mutations in the *MATP* gene, 16 patients still remained as an unclassified type of OCA.

Of the four missense mutations identified, three were within transmembrane domains and one was the first amino acid in the second cytoplasmic loop (table 3). This is in complete contrast to mutations of the P protein associated with OCA2, because most missense substitutions in patients with OCA2 occurred within the loops between the transmembrane domains (Spritz 1994). Both the MATP and the P proteins have 12 transmembrane domains arranged similarly to various transporters and appear to be integral membrane proteins of melanosomes.

Identification of Polymorphism

SSCP revealed several polymorphisms in the exonic or the nearby intronic sequences in the *MATP* gene, and the frequencies were determined for the 34 patients with OCA and the 104 unrelated normally pigmented Japanese subjects (table 4). Two of the exonic polymorphisms resulted in amino acid changes (E272K and T500P), whereas the remaining ones were silent. E272K is a common polymorphism (0.37), whereas T500P is rare (0.01) in the Japanese population. Three of the polymorphisms (IVS3+14A→G [MATP1-1; dbSNP accession number ss16339967], IVS4-6T→C [MATP1-2; dbSNP accession number ss16339968], and A1498C [MATP1-3; dbSNP accession number ss16339969]) were novel.

The Relationship between the Genotype and the Phenotype in Patients with OCA4

The Pro(CCA)58Ser(TCA) mutation in exon 1 occurred within the first transmembrane domain (table 3) and was identified only in patient 42. The other mutation in that patient was a S90CGGCCA→GC frameshift mutation, which creates a stop codon at 111 and thus produces no functional protein. Patient 42 was a 6-mo-old girl with light yellow hair and blue irides.

The Asp(GAC)157Asn(AAC) mutation in exon 2 causes a substitution from an acidic to a neutral amino acid at the first residue in the second cytoplasmic loop. This mutant allele was found in 12 patients, and 2 of them (patients 8 and 50) were homozygous for this allele. The allele frequency of D157N in all patients with OCA4 was 0.39 (14/36), indicating that D157N is the most common mutant allele in Japanese patients with OCA4. Patient 8 was 71 years old with nystagmus and had no pigmentation in her hair or eyes. Patient 50, who was 24 years old, also showed light yellow hair and blue irides with nystagmus. Patients 6, 38, and 51 (fig. 1*B*) had the D157N allele plus another mutant allele—V469delG, V144insAAGT, or S90CGGCCA→GC, respectively—which may have no functional activity in melanogenesis because the mutations caused frameshifts. All of those patients presented with light yellow hair,

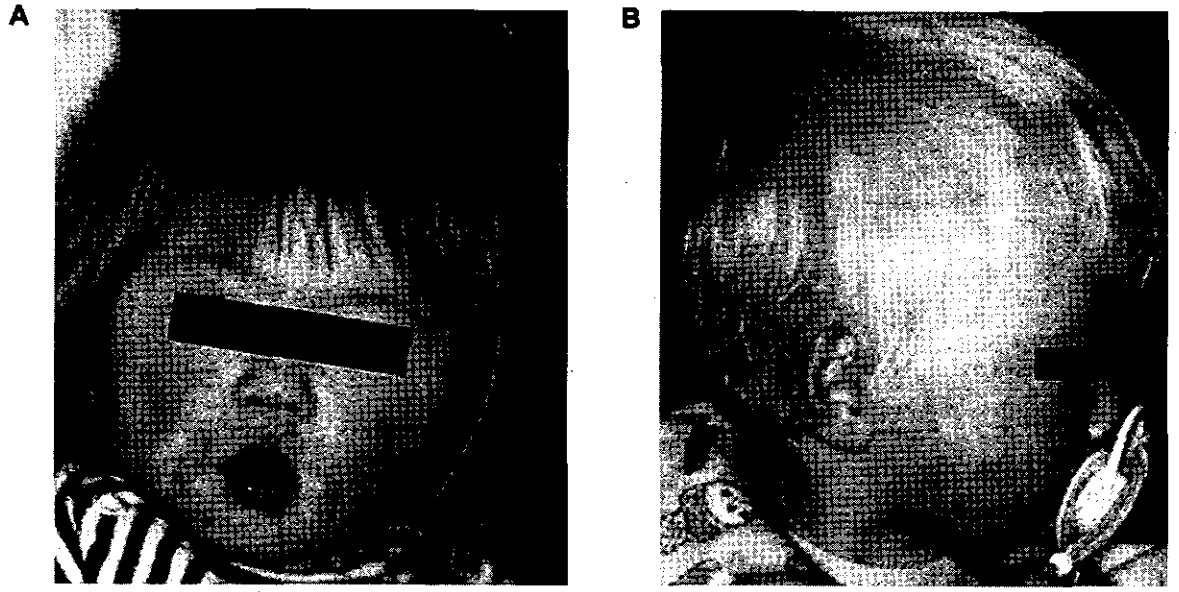

Figure 1 The diverse clinical phenotypes of patients with OCA4. Both patients are 1-year-old Japanese girls. Patient 36, with D157N and V507L mutations, has blond hair and gray-blue irides (*A*). Patient 51, with D157N and S90CGGCCA→GC mutations, has light yellow hair and blue irides (*B*). Note the milder hypopigmentation of patient 36 compared with that of patient 51.

blue eyes, and nystagmus, as did patients 8 and 50. These results indicate that the D157N mutant allele might have a very low functional activity in melanogenesis.

The Gly(GGT)188Val(GTT) mutation resulted from a single-nucleotide mutation from G to T at the first nucleotide of exon 3, which caused an amino acid substitution within the fifth transmembrane domain (table 3). This mutant allele was found in six patients, including one homozygote for this allele (patient 12). The allele frequency was 0.19 (7/36). The clinical phenotype of patient 12 presented with some pigmentation—that is, the hair was pale blond, and the irides were red-brown without nystagmus. Patient 10, who had the G188V allele and the S90CGGCCA→GC allele, presented with blond hair and gray-blue eyes. All other patients who had the G188V allele also presented with some generalized pigmentation (patients 3, 4, 15, and 17), although the degree of hypopigmentation in each patient varied. These results indicate that the G188V allele might have some functional activity in melanogenesis.

The Val(GTG)507Leu(CTG) mutation in exon 7 occurred within the 12th transmembrane domain, which is nearest to the C-terminal of the MATP protein. A single individual (patient 36 [fig. 1*A*]) had this mutant allele and presented with blond hair and gray-blue irides. Her other mutant allele was D157N, which appears to have a very low functional activity in melanogenesis, as mentioned above.

Three different frameshift mutations were identified in this study. The first one was found in four patients (9, 10, 42, and 51) who exhibited clear heteroduplexes on SSCP gels with the primer set for exon 1; the consequent sequencing revealed a two-plus-two nucleotide deletion (CGGCCA→GC) from Ser90 to Ser92. This deletion results in a change after amino acid 90 with a consequent truncation of the distal MATP nonsense polypeptide, so that 21 new amino acids were translated before a premature stop occurred at codon 111. The second frameshift mutation was observed in two patients (9 and 38) who have a 4-bp insertion in exon 2 (V144insAAGT), which results in a 10–amino-acid frameshift followed by a premature stop at codon 154. A single nucleotide deletion of G in exon 7 (V469delG) was identified as the third frameshift mutation in one individual (patient 6). This deletion results in a frameshift with a truncation of the MATP nonsense polypeptide at codon 469 that lacks the C-terminal 2 transmembrane domains of the predicted protein. Patient 9 had two different frameshift mutation alleles, S90CGGCCA→GC and V144insAAGT, which are both predicted to have no functional activity in melanogenesis. This patient was 25 years old and had no generalized pigmentation, a phenotype similar to OCA1A (tyrosinase-negative OCA). Therefore, it was impossible to distinguish her or patient 8 from patients with OCA1A on the basis of their clinical phenotypes. However, we could have identified her as non-OCA1A with a positive

Table 3

Positions of *MATP* Gene Mutations Identified in This Study and Its Frequencies in Japanese Patients with OCA4 and Normally Pigmented Japanese Subjects

Pathogenic Variant	Position in the MATP Protein	No. of Alleles Identified in: Japanese Patients with OCA4 (n = 36)	No. of Alleles Identified in: Normally Pigmented Japanese Subjects (n = 208)
P58S	1st transmembrane domain	1 (.03)	0
D157N	2nd cytoplasmic loop	14 (.39)	0
G188V	5th transmembrane domain	7 (.19)	0
V507L	12th transmembrane domain	1 (.03)	0
S90CGGCCA→GC	Frameshift in 1st cytoplasmic loop	4 (.11)	0
V144insAAGT	Frameshift in 4th transmembrane domain	2 (.06)	0
V469delG	Frameshift in 5th cytoplasmic loop	1 (.03)	0

Note.—n = number of alleles.

DOPA test by use of electron microscopy, because her specimen showed stage IV melanosomes when treated with DOPA, indicating that the tyrosinase activity in her melanosomes was fairly high (data not shown).

Finally, we speculate that the D157N allele might suppress the functional activity of the G188V allele on melanogenesis, similar to the amino acid substitution of D153N in the mouse *underwhite* gene of Uw^{dbr}, which is known as a dominant allele that reduces melanogenesis when heterozygous (Sweet et al. 1998; Newton et al. 2001). Patient 10, whose clinical phenotype included blond hair and gray-blue irides without nystagmus, had both the G188V and the S90CGGCCA→GC mutant alleles. On the other hand, patients 3 and 17, who had the D157N and the G188V mutation alleles, respectively, presented with yellow hair and blue irides with nystagmus, showing somewhat more severe hypopigmentation than patient 10. This may suggest that D157N might have a dominant negative effect, because the MATP mutant protein derived from the G188V mutant allele appeared to have some functional activity in melanogenesis by itself, as mentioned above. More advanced approaches for functional assays will be required to confirm the dominant negative effect of the D157N substitution on melanogenesis, because other differing background genes in these patients may contribute to their more severe hypopigmentation.

In conclusion, this study demonstrates that seven novel mutations in the *MATP* gene were found in 18 Japanese patients with OCA, indicating that OCA4 is one of the major types in Japan. The study also establishes that the clinical phenotype of the patients varied, depending on their mutant genotypes. Further accumulation of data correlating phenotypes with mutant genotypes is expected to give new insights into investigations on melanogenesis.

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Table 4

Polymorphisms Detected in the *MATP* Gene and Its Frequencies in Japanese Patients with OCA and Normally Pigmented Japanese Subjects

Nucleotide Change[a]	Amino Acid Change	Exon	Allele Frequency in: Japanese Patients with OCA	Allele Frequency in: Normally Pigmented Japanese Subjects
G814A	E272K	EX3	30/68 (.44)	76/208 (.37)
IVS3+14A→G	None	IVS3	1/68 (.01)	0/208 (.00)
G987A	T329T	EX4	18/68 (.26)	57/208 (.27)
IVS4-6T→C	None	IVS4	1/68 (.01)	0/208 (.00)
IVS4-44A→C	None	IVS4	34/68 (.50)	129/208 (.62)
A1498C	T500P	EX7	0/68 (.00)	2/208 (.01)
G1594A	None	EX7	0/68 (.00)	1/208 (.005)

[a] Nucleotide 1 begins at the first nucleotide of codon 1.

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for MATP1-1 [accession number ss16339967], MATP1-2 [accession number ss16339968], and MATP1-3 [accession number ss16339969])

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *MATP* genomic DNA [accession number NT 023085], *MATP* cDNA [accession number AF172849])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for OCA1, OCA2, OCA3, and OCA4)

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ORIGINAL PAPER

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Differences in recurrent COL7A1 mutations in dystrophic epidermolysis bullosa: ethnic-specific and worldwide recurrent mutations

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Abstract Dystrophic epidermolysis bullosa (DEB) is caused by mutations in the gene encoding type VII collagen (COL7A1). Although most COL7A1 mutations are unique to individual families, small numbers of mutations are recurrent. The recurrent mutations R578X, 7786delG, and R2814X seem to be exclusive to a specific ethnic group, the British population. The mutations 5818delC, 6573+1G→C, and E2857X are present only in individuals of Japanese ethnic origin. On the other hand, the mutations 425A→G and G2043R have been found in several different ethnic groups. The purpose of this study was to clarify whether these recurrent mutations are also found in patients of other ethnic groups with DEB, mainly Asian patients. We demonstrated the absence of the recurrent mutations R578X, 7786delG, and R2814X in 42 non-British patients with DEB and detected the mutations 425A→G in a French patient and G2043R in Japanese and Chinese patients with DEB. The mutations 5818delC, 6573+1G→C, and E2857X were detected in 11 Japanese patients (13 alleles) with DEB. Our results confirm that R578X, 7786delG, and R2814X mutations are specifically limited to British patients, and the mutations 5818delC, 6573+1G→C, and E2857X are frequent in Japanese patients. On the other hand, the mutations 425A→G and G2043R can be found in different ethnic groups. In conclusion, our results further support the notion that recurrent mutations can be classified into two types, ethnic-specific mutation and worldwide mutation.

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Introduction

Epidermolysis bullosa (EB) is a group of inherited blistering diseases classified into three major subgroups on the basis of the level of cleavage within the skin [9]. Dystrophic EB (DEB), characterized by blister formation beneath the lamina densa within the papillary dermis and abnormality of anchoring fibrils, is further divided into two forms based on the inheritance pattern, autosomal dominant and recessive forms. Both dominant and recessive forms of DEB are caused by mutations in the gene encoding type VII collagen (COL7A1) [2, 13, 21]. It is known that the COL7A1 consists of 118 exons, the highest number of exons in any gene [3]. In recent studies, small numbers of recurrent mutations have been identified. The mutations R578X, 7786delG, and R2814X are frequently observed in COL7A1 exclusive to British patients with RDEB [14, 16], although R578X was first described in Italians [8]. These three recurrent mutations have been observed in 25% of British patients with DEB [16]. The mutations 5818delC, 6573+1G→C, and E2857X are present only in Japanese patients with recessive DEB (RDEB) [18]. The mutation 425A→G, splice site mutation leading to premature termination codon, was first described with the localisata variant of RDEB in an Italian patient [10]. Another three cases of the mutation 425A→G have since been reported: a Hungarian patient [7] and an Italian patient [11, 12] with RDEB. In Italian patients with DEB, six recurrent mutations have been found: 7344G→A, 425A→G, 8441-14del21, 4783-1G→A, 497insA, and G1664A. In particular, the last three mutations are found only in Italian patients [12]. The mutation G2043R has been reported to be a recurrent mutation in dominant DEB (DDEB) [15]. This mutation is known to be a worldwide cause of DEB, and has been reported in Hungarian, Italian, Hispanic-Mexican, Scottish, Austrian, North American (Caucasian), Norwegian, Spanish and white British patients [4, 6, 15,

17, 20, 22]. Therefore, it seems that recurrent mutations can be classified into two types, one limited to a specific ethnic group and the other found in various ethnic groups.

The ethnic-specific recurrent mutations have been studied in British and Japanese patients and in corresponding ethnic patients with DEB, but not in patients of other ethnic groups. The purpose of this study was to analyze the frequency of these recurrent mutations in 42 patients with DEB, mainly Asian, to further clarify the specificity of ethnic-specific recurrent mutations.

Materials and methods

Patients

Mutation analysis was performed in 42 patients with DEB, including 39 Japanese, 1 Korean, 1 Chinese, and 1 French (Table 1). The diagnosis was based on a combination of clinical observations, electron microscopic study, and immunofluorescence staining with a range of antibodies directed to the epidermal basement membrane zone. The subtypes of DEB among the 42 patients are shown in Table 1, including RDEB (n=24), DDEB (n=12), and unclassified DEB (n=6). The patients analyzed in this study were not related.

Table 1 Results of the mutation analysis in 42 patients with DEB

Patient	Diagnosis	Ethnic group	R578X Exon 13	7786delG Exon 104	R2814X Exon 114	425A→G Exon 3	G2043R Exon 73	5818delC Exon 70	6573+1G→C Exon 81/intron 81	E2857X Exon 116
1:ST	DDEB	Japanese	–	–	–	–	–	–	–	–
2:OT	DDEB	Japanese	–	–	–	–	–	–	–	–
3:HM	DDEB	Japanese	–	–	–	–	–	–	–	–
4:TM	DDEB	Japanese	–	–	–	–	–	–	–	–
5:HH	DDEB	Japanese	–	–	–	–	–	–	–	–
6:MM	DDEB	Japanese	–	–	–	–	–	–	–	–
7:MN	DDEB	Japanese	–	–	–	–	–	–	–	–
8:AM	DDEB	Japanese	–	–	–	–	–	–	–	–
9:SN	DDEB	Japanese	–	–	–	–	–	–	–	–
10:SY	DDEB	Japanese	–	–	–	–	–	–	–	–
11:TN	DDEB	Japanese	–	–	–	–	+	–	–	–
12:SH	DDEB	Chinese	–	–	–	–	+	–	–	–
13:HA	RDEB	Japanese	–	–	–	–	–	–	–	+
14:OM	RDEB	Japanese	–	–	–	–	–	–	–	–
15:YA	RDEB	Japanese	–	–	–	–	–	+	–	–
16:HY	RDEB	Japanese	–	–	–	–	–	–	–	–
17:KD	RDEB	Japanese	–	–	–	–	–	–	–	–
18:KY	RDEB	Japanese	–	–	–	–	–	–	+	–
19:HS	RDEB	Japanese	–	–	–	–	–	+	–	–
20:MY	RDEB	Japanese	–	–	–	–	–	–	–	–
21:MK	RDEB	Japanese	–	–	–	–	–	–	–	–
22:KS	RDEB	Japanese	–	–	–	–	–	+	–	–
23:KG	RDEB	Japanese	–	–	–	–	–	–	–	–
24:KM	RDEB	Japanese	–	–	–	–	–	–	–	–
25:OY	RDEB	Japanese	–	–	–	–	–	–	–	–
26:YM	RDEB	Japanese	–	–	–	–	–	–	–	–
27:TK	RDEB	Japanese	–	–	–	–	–	–	–	–
28:OA	RDEB	Japanese	–	–	–	–	–	–	–	+
29:KR	RDEB	Japanese	–	–	–	–	–	+	–	+
30:YK	RDEB	Japanese	–	–	–	–	–	–	+	+
31:TR	RDEB	Japanese	–	–	–	–	–	–	+	–
32:AM	RDEB	Japanese	–	–	–	–	–	–	–	–
33:KR	RDEB	Japanese	–	–	–	–	–	–	+	–
34:HY	RDEB	Japanese	–	–	–	–	–	+	–	–
35:CS	RDEB	Korean	–	–	–	–	–	–	–	–
36:RF	RDEB	French	–	–	–	+	–	–	–	–
37:SA	DEB	Japanese	–	–	–	–	–	–	–	–
38:HN	DEB	Japanese	–	–	–	–	–	–	–	–
39:TY	DEB	Japanese	–	–	–	–	–	–	–	–
40:TN	DEB	Japanese	–	–	–	–	–	–	–	–
41:TY	DEB	Japanese	–	–	–	–	–	–	–	–
42:IY	DEB	Japanese	–	–	–	–	–	–	–	–