Correspondence

Possible modifier effects of keratin 17 gene mutation on keratitis–ichthyosis–deafness syndrome

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MADAM, Keratitis–ichthyosis–deafness (KID) syndrome (OMIM 148210, 242150) is a rare type of ectodermal dysplasia caused by mutations in the gap junction protein beta-2 gene (*GJB2*)[1] or beta-6 gene (*GJB6*).[2] On the other hand, mutations in genes encoding keratin 6a, 6b, 16 and 17 (*KRT6A*, *KRT6B*, *KRT16* and *KRT17*) are known to cause pachyonychia congenita (PC; OMIM 16720, 17210). PC and KID syndrome share similar symptoms, such as palmoplantar hyperkeratosis and onychodystrophy. This study reports a Japanese patient with atypical KID syndrome with the combined heterozygous mutations of a recurrent mutation in *GJB2* and a novel mutation in the V1 region of *KRT17*.

The proband was a 40-year-old Japanese woman. She was the child of healthy, nonconsanguineous parents. From childhood, she had shown diffuse mutilating palmoplantar hyperkeratosis (Fig. 1a), nail dystrophy (Fig. 1b), hypotrichosis, sensorineural hearing loss, and vascularized keratitis. Periorificial hyperkeratosis was not seen. From these findings, the diagnosis of KID syndrome was made. She had had recurrent bacterial and fungal skin infections. In her twenties, painful tumours appeared on her lower limbs. In her thirties, tumours on both buttocks developed to take on a papilloma-like appearance (Fig. 1c). Etretinate with topical or systemic antibiotics and antifungal agents did not alleviate her symptoms. Skin abrasion was repeatedly conducted on the tumours. Histopathology of the lesions revealed epidermal pseudocarcinomatous hyperplasia with dilation of vessels in papillary and reticular dermis accompanied by mixed immune cell infiltrates, excluding the involvement of squamous cell carcinoma (Fig. 1d). Vacuolated keratinocytes, suggesting human papillomavirus infection, were not detected.

Genomic DNA extracted from peripheral blood was used as a template for polymerase chain reaction (PCR) amplification. Direct sequencing of *GJB2*, *GJB6*, *KRT6A*, *KRT6B*, *KRT16* and *KRT17* was performed as described elsewhere.[3–5] The medical ethical committee of Hokkaido University approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles. The proband gave her written informed consent.

Mutation analysis of the proband's genomic DNA revealed a c.148G>A transition (p.Asp50Asn) in *GJB2* (Fig. 2a), which is the most prevalent mutation in patients with KID syndrome.[1] Furthermore, the proband was found to be heterozygous for a c.177C>A transversion (p.Ser59Arg) in *KRT17* (Fig. 2b). Restriction enzyme digestion of the PCR products by PvuII was carried out to confirm the c.177C>A in *KRT17* (Fig. 2c). The c.177C>A in *KRT17* was novel and was not detected in 200 alleles from 100 normal Japanese individuals. Mutation screening on the proband's parents could not be performed because the father was not alive and the mother did not consent. Keratin 17 (K17) immunohistochemistry on skin samples from several different sites revealed K17 expression in whole epidermis although its expression level did not vary between nonlesional and lesional skin specimens (data not shown).

As the clinical manifestations of the proband were atypical and more severe than those of other patients with KID syndrome – as evidenced, for example, by diffuse mutilating palmoplantar hyperkeratosis and recurrent granulation tissue formation on the buttock – we hypothesized that mutations in other genes might have affected the proband's phenotype through modifier effects. Modifier genes are defined as genes that affect the phenotypic expression of another gene, and several studies have demonstrated that modifier genes are involved in manifestations of inherited disorders.[6] *KRT6A*, *KRT6B*, *KRT16* and *KRT17*, the causative genes of PC, which affects the nails and the palmoplantar area, were selected as candidates for modifier gene investigation in our case, although we cannot exclude the possibility that there are some other genes which modify KID syndrome phenotype.

Most of the keratin mutations are within the helix boundary motifs, which are crucial for keratin monomers to form dimers and subsequent keratin networks.[7] The *KRT17* mutation found in the proband was located not within the helix boundary motifs but in the V1 region of K17 (Fig. 2d). In other keratin genes, such as *KRT5* and *KRT16*, some mutations have been reported within the V1 region, and the phenotypes resulting from these mutations are milder than those resulting from the mutations within the helix boundary motifs.[7] The V1 regions of keratin intermediate filament have glycine loops[8] and it has been suggested that these structures modulate flexibility and other unknown physical attributes of keratin filaments by interacting with similar structures in loricrin.[9] Ser^{59} is located within a highly conserved segment composed of the glycine loop in K17 (Fig. 2e). p.Ser59Arg in K17 is predicted to be probably damaging by PolyPhen-2, with a score of 0·893.[10]

Based on these findings, it is conceivable that the p.Ser59-Arg variant in K17 has a modifying effect on the pathogenic

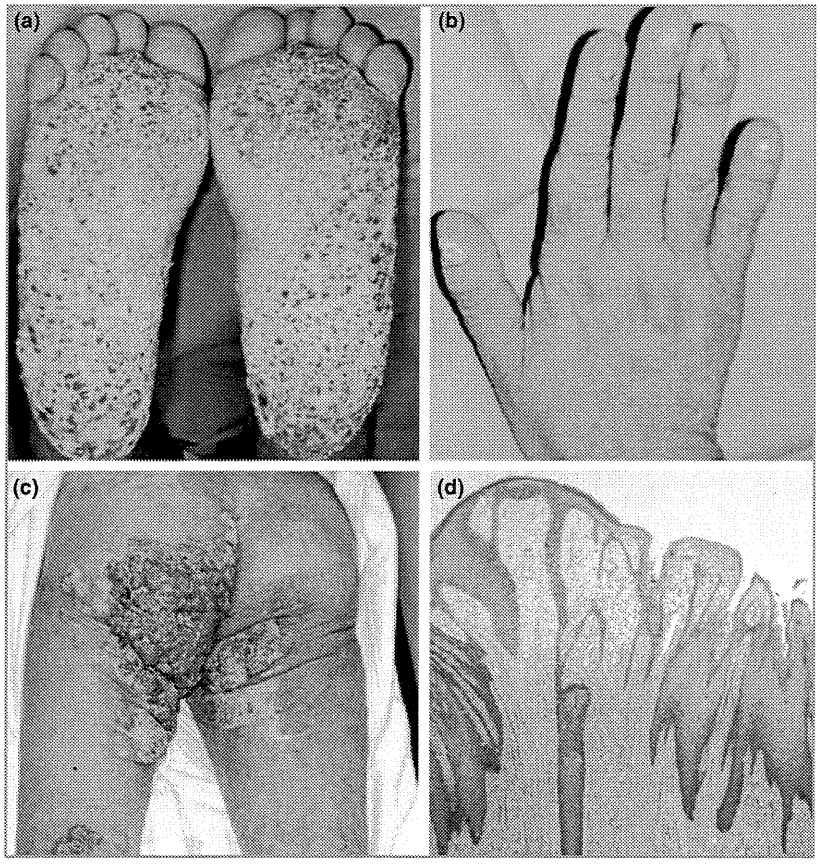

Fig 1. Clinical features of the proband. (a) Numerous erosive papules are coalesced into a hyperkeratotic plaque on the proband's soles. (b) Nail dystrophy is seen in the fingers. (c) A tumour is observed on the left buttock. Scars after skin abrasion are seen on the dorsal aspects of the thigh and on the right buttock. (d) Specimens from the tumour show pseudocarcinomatous hyperplasia of the epidermis. Dilated vessels with monocytic infiltrates are seen in the dermis (haematoxylin and eosin; original magnification × 100).

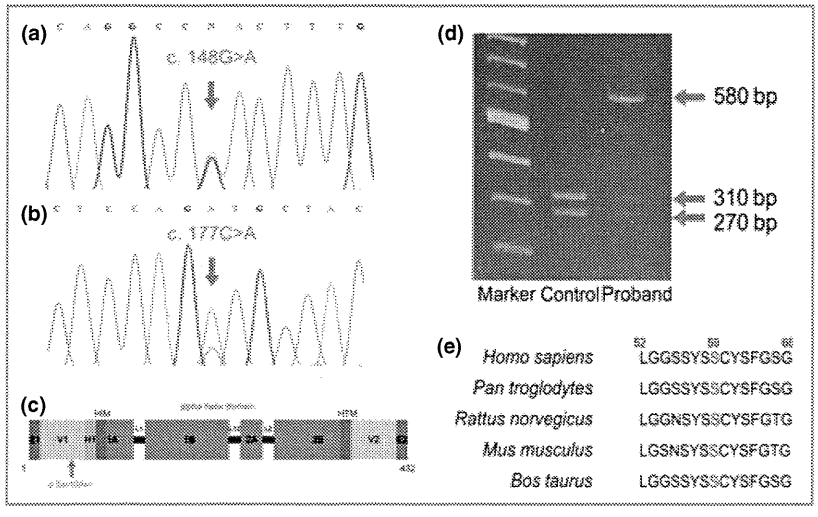

Fig 2. Mutation analysis. (a) The proband was heterozygous for a c.148G>A transition (p.Asp50Asn) mutation in *GJB2* (arrow). (b) c.177C>A (p.Ser59Arg) in *KRT17* was detected in the proband's genomic DNA (arrow). (c) PvuII restriction enzyme digestion of the polymerase chain reaction (PCR) products from genomic DNA of the proband and a normal control. c.177C>A resulted in the loss of a site for PvuII. PvuII restriction enzyme digestion of the PCR products from a normal controls reveals 270- and 310-bp bands. In contrast, 270-, 310- and 580-bp bands were detected in the proband, suggesting that she was heterozygous for c.177C>A. (d) A schematic of the structure of keratin 17. Note that Ser^{59} is located at the V1 region of the keratin molecule (arrow). HIM, helix initiation motif; HTM, helix termination motif. (e) Keratin 17 amino acid sequence alignment shows the level of conservation in diverse species of the amino acid Ser59 (red characters).

GJB2 mutation p.Asp50Asn and may contribute the proband's phenotype. Nevertheless, the limited scope of this study (single case report) does not allow us to determine the clinical significance of p.Ser59Arg in K17, and the influence of other genetic and epigenetic factors cannot be excluded.

*Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
†Department of Dermatology, University of Miyazaki Faculty of Medicine, Miyazaki, Japan
‡Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan
E-mail: natsuga@med.hokudai.ac.jp

K. NATSUGA*
S. SHINKUMA*
M. KANDA*
Y. SUZUKI*
N. CHOSA†
Y. NARITA†
M. SETOYAMA†
W. NISHIE*
M. AKIYAMA*‡
H. SHIMIZU*

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Novel adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*) mutations associated with congenital ichthyosiform erythroderma

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MADAM, Autosomal recessive congenital ichthyosis (ARCI) is a keratinization disorder, characterized by general desquamation. ARCI is a heterogeneous entity, including harlequin ichthyosis (HI, MIM 242500), lamellar ichthyosis type 2 (LI2, MIM 601277) and congenital ichthyosiform erythroderma (CIE, MIM 242100). The reported mutations in CIE include adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*),[1] transglutaminase 1 (*TGM1*),[2] lipoxygenase-3, 12(R)-lipoxygenase,[3] *NIPAL4*[4] and *CYP4F22*.[5] Mutations in *ABCA12* also result in LI2 and HI.[6,7] We report *ABCA12* mutations in four unrelated Japanese patients with CIE and identified five unreported and two recurrent mutations.

Patient 1 is a 3-year-old girl with generalized scales on erythroderma, ectropion, eclabium, severely deformed ears and alopecia (Fig. 1a–c). Her elder sister displayed similar symptoms and died after dehydration and infection. Patient 2 is a 9-year-old girl with generalized scales on an erythrodermic skin, mild ectropion, alopecia of the forehead and mild auricular malformation. Her younger sister died after severe skin symptoms and subsequent complications. Patient 3 is a 4-month-old boy, born as a collodion baby, with systemic whitish scales and generalized erythrodermic skin. There is no family history. Patient 4 is a 3-month-old boy, born as a collodion baby, with generalized whitish scales on a mild erythrodermic skin (Fig. 1d,e). Ectropion, eclabium and auricular malformation were not seen. There is no family history. Pathological findings of all patients revealed hyperkeratosis, mild acanthosis and perivascular lymphocytic infiltration.

We initially examined for *ABCA12* mutation, because *ABCA12* mutations have been found frequently in Japanese patients with CIE. For analysis of the *ABCA12* gene, polymerase chain reaction (PCR) fragments were amplified with 53 primer pairs, as previously reported.[6] We identified five unreported and two recurrent mutations (Table 1). Patient 1 had compound heterozygosity of missense/small deletion mutations [(p.Thr1575Pro)+(c.6031delG)]. Patients 2 and 3 had compound heterozygosity of missense/splice-site mutations [(p.Arg986Trp)+(c.5940–1G>C), (p.Asn1380Ser)+(c.5128+3A>G), respectively]. Patient 4 had compound heterozygosity

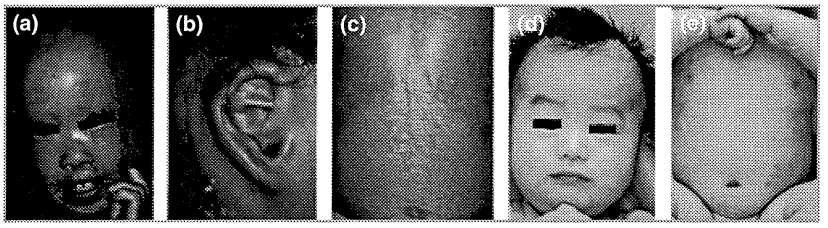

Fig 1. (a–c) Clinical features of patient 1. The whole body was covered with whitish scales on the erythrodermic skin. Ectropion, eclabium and alopecia of the forehead were seen. (d,e) Clinical features of patient 4. Whitish scales and generalized erythrodermic skin were seen.

Table 1 Summary of mutation analysis of *ABCA12* in the present study

Patient	Age, sex	Mutation	Maternal	Paternal
1	3 years, girl	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	c.6031delG
2	9 years, girl	Compound heterozygous	p.Arg986Trp (c.2956C>T)	c.5940–1G>C
3	4 months, boy	Compound heterozygous	p.Asn1380Ser (c.4139A>G)	c.5128+3A>G
4	3 months, boy	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	p.Gly1651Ser (c.4951G>A)

of missense mutations [(p.Thr1575Pro)+(p.Gly1651Ser)]. Each of the parents was a heterozygous carrier. Five mutations (p.Thr1575Pro, c.6031delG, p.Arg986Trp, c.5940–1G>C and c.5128+3A>G) have not been reported previously. Two recurrent mutations (p.Asn1380Ser and p.Gly1651Ser) have been reported previously in LI2.[6] These mutations were not found in 200 normal, unrelated Japanese alleles.

In cDNA from the skin of patient 2, reverse transcriptase-PCR (RT-PCR) across the c.5940–1G>C mutation site showed a single band of 526 bp. Subcloning and direct sequencing

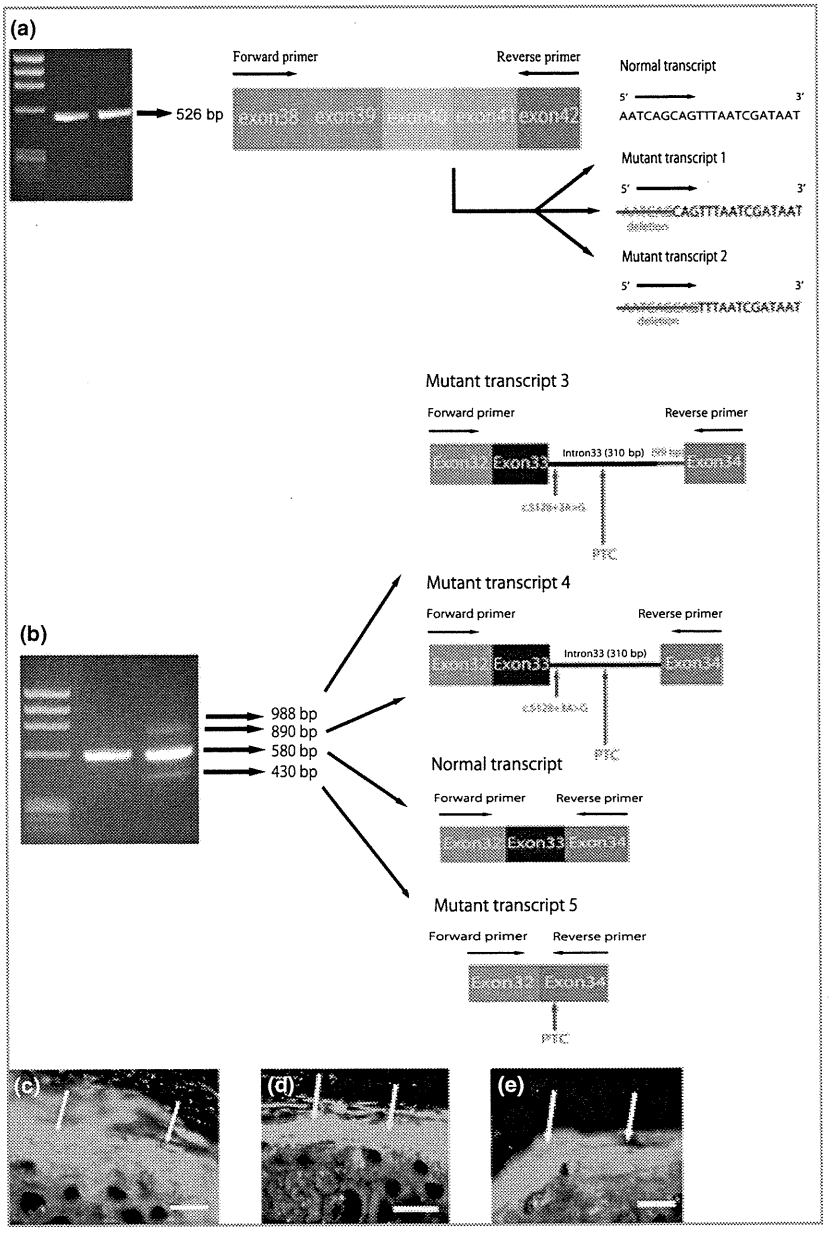

Fig 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA fragments around the splice-site mutations and immunofluorescent analysis. (a) In patient 2, RT-PCR, subcloning and direct sequencing through the exon 40–41 boundary revealed two mutant transcripts as well as a normal transcript. Mutant transcript 1 had lost 6-bp nucleotides from exon 41, which resulted in a 2-amino acid deletion (Ile1981_Ser1982del). Mutant transcript 2 had lost 9-bp nucleotides from exon 41, which resulted in a 3-amino acid deletion (Ile1981_Ser1983del). Both mutant transcripts were within-frame deletions. (b) In patient 3, three aberrant mutant transcripts, all of which led to a premature termination codon, were identified by RT-PCR, subcloning and direct sequencing through the exon 33–34 boundary. Mutant transcript 3 was 988 bp in length with the inclusion of 310 bp and another 99 bp of intron 33. Mutant transcript 4 was 890 bp in length with the inclusion of 310 bp of intron 33. Mutant transcript 5 had exon 33 skipping. (c–e) Immunofluorescent labelling of ABCA12 in the skin. (c,d) A dot-like pattern of ABCA12 staining was seen in the cytoplasm of keratinocytes in the upper epidermis in patient 1 (c) and patient 2 (d). (e) In the normal control epidermis, ABCA12 staining was relatively strong in the granular layers and seemed to be dominant at the cell periphery. Bar = 5 μm.

revealed two mutant transcripts with in-frame deletions (Fig. 2a). In cDNA from the skin of patient 3, RT-PCR across the c.5128+3A>G mutation site identified four bands of 988, 890, 580 and 430 bp, with a single 580-bp band in the control sample (Fig. 2b). Subcloning and direct sequencing revealed three aberrant mutant transcripts, all of which led to premature termination codons. Immunofluorescence using anti-ABCA12 antibody revealed a diffuse staining of ABCA12 in the granular layers of control skin (Fig. 2e) and of the non-ABCA12 form (TGM1) from patient CIE (data not shown), while a dot-like staining in the cytoplasm was observed in patients 1 and 2 (Fig. 2c,d).

ABCA12 is a membrane lipid transporter that functions in the lipid transport from the trans-Golgi network to lamellar granules.[8] *ABCA12* mutations result in heterogeneity, including LI2, HI and CIE.[1,6,7] LI2 is characterized by generalized scales without serious erythroderma, and caused by either homozygote or compound heterozygote for missense mutations within the first nucleotide-binding folds of *ABCA12*.[6] HI is the severest form of ARCI, characterized by generalized large, plate-like scales with ectropion, eclabium and flattened ears.[7] HI is usually caused by homozygous or compound heterozygous truncation mutations in *ABCA12*.[7] In contrast, CIE with *ABCA12* mutation clinically shows milder manifestations.[1] Thus far, 17 different mutations in *ABCA12* have been reported in 12 cases of CIE. Eleven of 12 cases have at least one missense mutation. Only three of 17 mutations (p.Asn1380Ser, p.Ile1494Thr and p.Arg1514His) were located in the first nucleotide-binding folds. Other mutations were located outside ABCA12 active transporter sites: two nucleotide-binding folds and 12 transmembrane domains. The mutation p.Thr1575Pro was identified in two unrelated patients with different clinical severity. Patient 1 with severer features had a heterozygous truncation mutation (c.6031delG) on another allele, while patient 4, with a milder phenotype, had another heterozygous missense mutation (p.Gly1651Ser). We suggest that the phenotypic variability in these two patients was caused by different mutations.

We identified two *ABCA12* splice-site mutations, which were not reported in CIE: c.5128+3A>G and c.5940–1G>C. RT-PCR analysis across the site of the c.5940–1G>C mutation in patient 2 revealed two mutant transcripts. These findings demonstrate expression of the in-frame shorter transcript lacking two or three amino acids due to this splice-site mutation, which may account for the mild phenotype. In contrast, RT-PCR analysis across the site of the c.5128+3A>G mutation in patient 3 revealed three aberrant mutant transcripts, all of which led to premature termination codons. Therefore, patient 3 had a compound heterozygosity for missense/truncated combinations of mutations.

Using high-throughput sequencing analyses, screening of all ARCI-related genes is currently possible, but the cost is still expensive.[9] Once this is overcome, the elucidation of the pathogenesis of ARCI will greatly progress in the near future.

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Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan — S. FUKUDA, T. HAMADA, N. ISHII, S. SAKAGUCHI

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan — K. SAKAI*, M. AKIYAMA†, H. SHIMIZU*

†*Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, 466-8550, Japan* — K. MASUDA‡

‡*Department of Dermatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, 62-8566, Japan* — K. IZU§, K. TEYE, D. TSURUTA, T. KARASHIMA

§*Department of Dermatology, Kyushu Kosei-Nenkin Hospital, Kitakyushu, 806-8501, Japan* — T. NAKAMA, S. YASUMOTO

Correspondence: Takashi Hashimoto. — T. HASHIMOTO

E-mail: hashimot@med.kurume-u.ac.jp

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Morikazu Medical Science Promotion Foundation, the Japan Lydia O'Leary Memorial Foundation, the Cosmetology Research Foundation, the Japanese Dermatological Association (Shiseido Award), the Fukuoka Foundation for Sound Health, and Galderma K.K. (Galderma Award).

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Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite

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MADAM, Androgenetic alopecia (AGA) is a term that describes the androgen-dependent and genetically determined nature of the disease.[1] However, although it is known that androgen replacement therapy can induce AGA, no report has previously been issued regarding the development of iatrogenic AGA in a hermaphrodite undergoing androgen therapy. Herein, we describe a unique case of a castrated male phenotype 46XX true hermaphrodite receiving exogenous androgen supplementation who developed male-type hair loss.

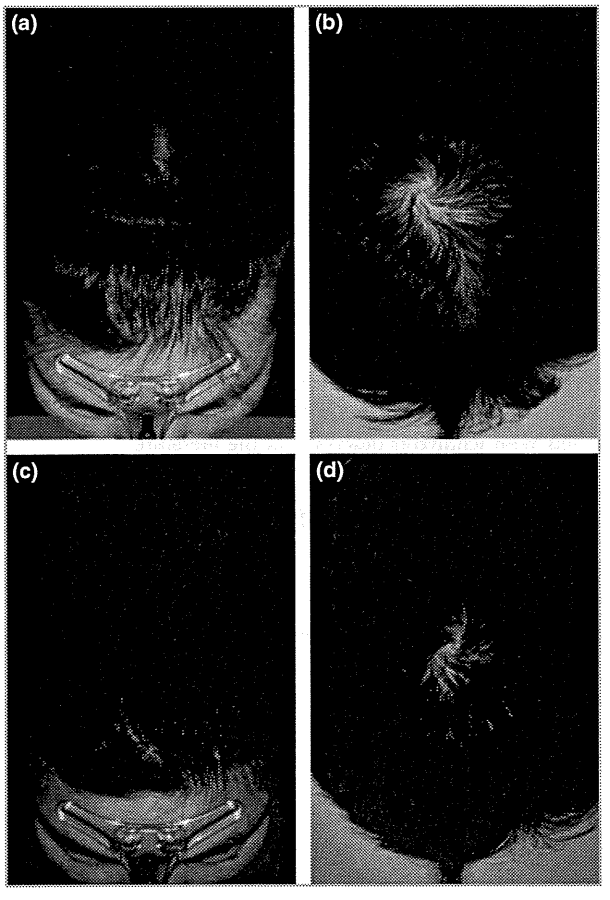

Fig 1. Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite showed a great improvement compared with baseline (a, b) after 4 months of finasteride treatment (c, d).

A 21-year-old male phenotype 46XX true hermaphrodite presented with a 3-year history of progressive hair loss. At the age of 16 years he was diagnosed as a 46XX true hermaphrodite with bilateral ovotestis, and subsequently underwent bilateral orchiectomy and testis prosthesis insertion. In addition, he was then given testosterone replacement therapy (testosterone enanthate, Jenasteron®; Jenapharm, Jena, Germany) for surgically induced andropausal status, which halted the development of secondary sexual characteristics. After 3 years of androgen therapy, progressive hair thinning developed on the scalp. Hair examination revealed nonscarring Norwood–Hamilton type III vertex alopecia with frontotemporal recession or BASP classification M1V2 alopecia (Fig. 1a, b).[2] Digital microscopy (Folliscope®; LeadM Corporation, Seoul, Korea) showed miniaturized hair shafts, and hair shaft size variation over the vertex scalp (Fig. 2). Serum testosterone, at the time, was 4·1 ng mL^{-1} (normal 2·7–10·7) and serum dehydroepiandrosterone sulphate was 1845 ng mL^{-1} (normal 800–5600). Under a diagnosis of iatrogenic androgen-induced alopecia, finasteride (1 mg daily) therapy was started. After 4 months of treatment, the hair loss stabilized and scalp hair regrowth was observed, despite the continuance of testosterone replacement therapy (Fig. 1c, d).

True hermaphroditism is an extremely rare disorder, which is defined as the coexistence of testicular and ovarian tissue in the same subject. The most frequent karyotype of true hermaphrodites is 46XX.[3] Gender assignments for hermaphrodites are made according to genetic, gonadal, social and psychologically determined sex, and the requests of patients and their relatives.[4] To be reared as male or female, surgical correction of ambiguous external genitalia, surgical removal of dysgenetic gonads, and sex hormone replacement for the surgically induced andropausal or menopausal state are required. The unwanted dermatological side-effects of testosterone replacement therapy include acne, excessive hair growth and male pattern baldness. As in our case, to be reared

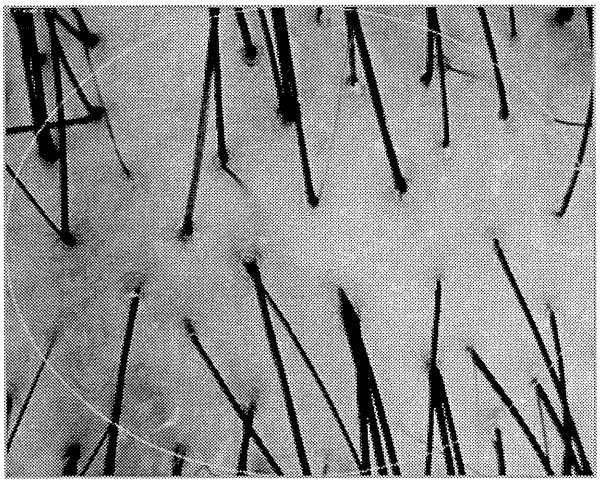

Fig 2. Photomicrograph showing miniaturized hair shafts, and variations in hair shaft size over the vertex scalp (original magnification ×50).

REVIEW ARTICLE

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Malignant skin tumours in patients with inherited ichthyosis

K. Natsuga, M. Akiyama* and H. Shimizu

Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
**Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

Summary

Inherited ichthyoses are rare genodermatoses caused by mutations in the genes involved in epidermal development. Although there have been case reports on patients with ichthyosis who developed skin malignancies, it is still unknown whether or not patients with ichthyosis have an increased risk of skin malignancies. Here, we review case series of skin malignancies in patients with ichthyosis and show biological findings which might lead to cancer susceptibility. A survey of the literature revealed 28 cases of inherited ichthyoses with skin malignancy, including 12 cases of keratitis–ichthyosis–deafness (KID) syndrome, seven of autosomal recessive congenital ichthyosis, three of Netherton syndrome and six of miscellaneous ichthyosis. Twenty-four of the 28 cases developed single or multiple squamous cell carcinomas (SCCs). The age at diagnosis of the first skin malignancy ranged from 15 to 54 years. As patients with these particular subtypes of ichthyosis seem to be prone to skin malignancies, including SCC, at an unusually young age, routine cancer surveillance of these patients is strongly recommended.

Correspondence
Ken Natsuga.
E-mail: natsuga@med.hokudai.ac.jp

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Conflicts of interest
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Skin cancer poses a serious problem in patients with inherited disorders, such as Gorlin syndrome, Cowden syndrome, xeroderma pigmentosum and epidermolysis bullosa. The prognosis for these patients is greatly influenced by skin malignancies, which develop at an unusually early age.

Ichthyoses are disorders characterized by skin dryness. Congenital ichthyoses are caused by mutations in the genes organizing keratinocyte differentiation and skin barrier function, although some of the causative genes are still undetermined.[1] There have been sporadic case reports of skin malignancies in patients with congenital ichthyosis. However, the epidemiology among these patients remains unknown because of the limited number of cases.

This review article summarizes skin malignancies in congenital ichthyoses described in the English language literature and discusses the biological background underlying skin barrier defects and carcinogenesis.

Skin malitgnancies in each ichthyosis subtype

Twenty-eight cases of skin malignancy in congenital ichthyoses were found in the literature: 12 cases of keratitis–ichthyosis–deafness (KID) syndrome, seven of autosomal recessive congenital ichthyosis (ARCI), three of Netherton syndrome (NS) and six of miscellaneous ichthyosis. The first malignancies were diagnosed at the ages of 15–54 years. Reported skin malignancies include squamous cell carcinoma (SCC), basal cell carcinoma (BCC), malignant proliferating trichilemmal tumour (MPTT), malignant melanoma (MM), malignant fibrous histiocytoma and cutaneous lymphoma, although single or multiple SCC was the malignancy in most of the cases (24 out of 28). Table 1 summarizes the skin malignancies in patients with ichthyosis described in the literature.

Keratitis–ichthyosis–deafness syndrome

Keratitis–ichthyosis–deafness syndrome (KID) syndrome is an autosomal dominant disease characterized by congenital erythrokeratoderma as well as sensorineural deafness and eye involvement.[2,3] Heterozygous mutations in *GJB2*, which encodes connexin 26 (Cx26), are responsible for the disease.[4,5] Mutations in *GJB6*, the gene encoding connexin 30 (Cx30), are causal in some cases which overlap with Clouston syndrome.[6,7]

There are 12 reports of patients with sporadic KID syndrome in the literature who developed skin malignancies, including SCC and MPTT (Table 1).[5,8–15] The age of onset for SCC in KID syndrome is 15–43 years, which is earlier than that for SCC in the normal population (around the age of 70 years).[16,17] p.Asp50Asn in Cx26, the most prevalent muta-

Table 1 Skin malignancies in patients with ichthyosis

Ichthyosis subtype	Age at the diagnosis of first skin malignancy (years)	Skin malignancy	Causative gene	Reference
KID	35	SCC	NE	8
KID	28	Multiple SCC	NE	9
KID	43	SCC	NE	10
KID	38	SCC	*GJB2*	5
KID	31	Multiple SCC	*GJB2*	12
KID	31	Multiple MPTT	NE	11
KID	15	SCC	NE	13
KID	28	Multiple SCC/MPTT	*GJB2*	15
KID	24	Multiple MPTT	ND	15
KID	30	Multiple SCC	*GJB2*	14
KID	38	SCC	*GJB2*	14
KID	40	SCC	*GJB2*	14
CIE	44	SCC, MM	*ABCA12*	23, 32
CIE	37	MM, cutaneous lymphoma	*ABCA12*	23
CIE	43	Multiple SCC/BCC	NE	31
CIE	51	Multiple SCC/BCC	NE	31
CIE	25	SCC, MFH	NE	29
LI	27	Multiple SCC/BCC	NE	33
LI	33	Multiple BCC	NE	30
NS	23	Multiple SCC/BCC	NE	52
NS	29	Multiple SCC	NE	53
NS	29	Multiple SCC/BCC	NE	54
ICM	54	Multiple SCC	NE	63
ICM	40	Multiple SCC	NE	62
MAUIE	21?	SCC	NE	33
MAUIE	26	Multiple SCC	NE	64
EI	49?	Multiple SCC/BCC	NE	68
CHILD	29	SCC	NE	71

KID, keratitis–ichthyosis–deafness syndrome; CIE, congenital ichthyosiform erythroderma; LI, lamellar ichthyosis; NS, Netherton syndrome; ICM, ichthyosis Curth–Macklin; MAUIE, micropinnae, alopecia universalis, congenital ichthyosis and ectropion; EI, epidermolytic ichthyosis; CHILD, congenital hemidysplasia with ichthyosiform erythroderma and limb defects; SCC, squamous cell carcinoma; MPTT, malignant proliferating trichilemmal tumour; MM, malignant melanoma; MFH, malignant fibrous histiocytoma; BCC, basal cell carcinoma; NE, not examined; ND, not detected.

tion in KID syndrome, was found in six patients who developed SCC or MPTT.[5,12,14,15] SCC was reported in roughly 10% of patients with KID syndrome and has been proposed as a distinguishing manifestation of the disease.[5] In a recent case series, three out of 14 (21%) patients with KID syndrome developed SCC.[14] Recurrent and chronic infection of the skin in KID syndrome has been suggested to be partly responsible for the increased risk of SCC[8,13] or to be one of the many factors involved in multiple-step carcinogenesis.[15] Also, alteration of E-cadherin expression due to dysfunctional Cx26 is hypothesized to lead to cancer susceptibility.[5] Mutated Cx26 might lead to tumorigenesis through a decrease in gap junction communication, a possibility that is supported by a mouse carcinogenesis model.[18] Overexpression of Cx26 has been shown to suppress tumour growth and induce apoptosis in prostate cancer cells through Bcl-2 downregulation.[19]

In a mouse model for KID syndrome in which Cx26 harbouring the p.Ser17Phe mutation was introduced as a heterozygous mutation under control of the endogenous Cx26 promoter, the basal layer showed increased cell proliferation.[20] However, progressive skin growth and increased susceptibility to SCC were not observed.[20]

Autosomal recessive congenital ichthyosis

Congenital ichthyosiform erythroderma (CIE) and lamellar ichthyosis (LI) are two major types of ARCI.[1] CIE is characterized by fine, white scaling with erythroderma. In contrast, the typical manifestation of LI is coarse brown/dark scaling. Their causative genes are *ALOXE3*,[21] *ALOX12B*,[21] *ABCA12*,[22,23] *CYP4F22*,[24] *NIPAL4*[25] and *TGM1*.[26,27] CIE and LI have been proposed as representing variations of a single group of disorders, although the typical cases of each type have distinct clinical features.[28]

In the literature, five patients have been reported with CIE and two with LI who developed skin malignancies (Table 1).[23,29–33] They began to suffer from SCC between the ages of 25 and 51 years.[29,31–33] There is the possibility that chronic inflammation due to skin barrier defects is associated with skin carcinogenesis in CIE/LI patients,[23] as discussed in the section on KID syndrome. Scarring from chronic inflammation was suggested to underlie SCC in one CIE case,[32] although scar formation was not histologically evident in SCC specimens from two other patients with CIE.[31] The increased proliferation observed in CIE keratinocytes *in vitro*[34] might account for the early onset of SCC. It is notable that the long-term administration of systemic retinoids did not prevent SCC development in some patients with CIE,[31,32] although the retinoids might have reduced the number or severity of the SCCs.

Genetic analysis was performed on only two of the patients with CIE, both of whom had missense mutations in *ABCA12*.[23] The patients developed MM at the ages of 47 and 37, respectively. It is unclear why the ABCA12-deficient patients with CIE developed skin malignancies at those early ages. ABCA12 is an ATP-binding cassette (ABC) transporter that is thought to play a pivotal role in keratinocyte lipid transport.[35,36] ABCA12 is expressed mainly in keratinocytes, and not in melanocytes or lymphocytes.[35,37,38] A recent study also confirmed that ABCA12 is only weakly expressed in normal melanocytes and is largely absent in melanoma cells.[39] ABCA transporters are involved in regulating lipid transport and metabolism, and cholesterol levels may be a limiting factor in membrane maintenance in rapidly dividing cancer cells.[40] From these facts, it is unlikely that ABCA12 deficiency directly promotes skin tumorigenesis including that of MM. Other ABCA members that compensate for ABCA12 dysfunction might be related to tumorigenesis in patients with CIE.

Abca12-deficient mouse models have been developed, all of which showed neonatal lethality,[41–44] and these models

reproduce the severest subtype of ARCI: harlequin ichthyosis.[35,45] In one mouse model (*Abca12*-null mice), epidermal proliferation was not altered at E18.5 compared with wild-type mice.[43] From this finding, it is unlikely that loss of ABCA12 function directly causes proliferation of keratinocytes and leads to SCC development.

No patients with CIE or LI who developed skin malignancies have been reported to have mutations in *TGM1*, although *TGM1* is thought to be the most prevalent causative gene for CIE/LI.[46,47] *TGM1* encodes tranglutaminase-1, which forms the cornified envelope (CE) in the cornified layer through cross-linking of CE precursor proteins.[47] Increased proliferation in the epidermis of the *Tgm1*-null neonate skin grafted onto athymic nude mice was observed,[48] which might imply that patients with CIE/LI with *TGM1* mutations might be susceptible to skin SCC.

Netherton syndrome

Netherton syndrome (NS) is an autosomal recessive disorder characterized by trichorrhexis invaginata (bamboo hair), congenital ichthyosis and atopic diathesis.[49,50] NS is caused by mutations in *SPINK5*, which encodes the serine protease inhibitor LEKTI.[51]

Three NS cases have been reported who developed skin malignancies (Table 1).[52–54] Surprisingly, multiple SCCs (or multiple BCCs) were observed for these patients in their twenties. In one patient, epidermodysplasia verruciformis-associated human papillomavirus (HPV) DNA (HPV-19, -23, -38 and HPV-RTRX9) was preferentially detected in malignant lesions.[52] The authors speculated that impaired epidermal defence mechanisms could have promoted latent HPV DNA persistence in the patient's skin.[52] However, polymerase chain reaction amplification using HPV universal primers failed to detect HPV DNA in tumour specimens of another patient.[54] This shows that HPV infection is not always responsible for skin carcinogenesis in patients with NS at an early age. Patients with NS show recurrent infections other than HPV.[55] From the findings that several immunological abnormalities including those of memory B cells and natural killer cells are common in NS and that the patients respond well to intravenous immunoglobulin therapy,[55] it is possible to conclude that cognate and innate immunodeficiency might be associated with skin carcinogenesis in NS.

Although other serine protease inhibitors are implicated in skin carcinogenesis,[56,57] the role of LEKTI in skin cancers is unclear. NS mouse models in which LEKTI is deficient have been reported.[58–60] In one model, increased proliferation of the epidermis was observed,[59] which might underlie a susceptibility to SCC.

Miscellaneous

In each other form of ichthyosis, only a few cases have been described as having skin cancers. Ichthyosis Curth–Macklin (ICM) is a very rare form of keratinopathic ichthyosis that is characterized by massive spiky hyperkeratosis.[1,61] Mutations in the V2 domain of keratin 1 have been reported in patients with ICM. Two patients developed multiple SCC at the ages of 54 and 40 years, respectively (Table 1).[62,63] However, one patient had a history of whole-skin X-ray therapy, which might have led to the multiple skin cancers.[63]

Micropinnae, alopecia universalis, congenital ichthyosis and ectropion (MAUIE) syndrome is a syndromic form of ichthyosis that was not included in the revised nomenclature and classification of inherited ichthyoses.[1] Causative genes of MAUIE syndrome have not been reported. Two patients with MAUIE syndrome were found to have developed SCC in their twenties (Table 1).[33,64]

Epidermolytic ichthyosis (EI), formerly called bullous CIE, is a major subtype of keratinopathic ichthyosis[1] that is caused by mutations in the genes encoding keratin 1 or keratin 10 (*KRT1* or *KRT10*, respectively).[65–67] One patient with EI was reported to have multiple SCC/BCC (Table 1), although the patient had a history of whole-skin X-ray therapy.[68]

Congenital hemidysplasia with ichthyosiform erythroderma and limb defects (CHILD) syndrome is a rare X-linked dominant disorder[69] that is caused by mutations in *NSDHL*.[70] One patient with CHILD syndrome developed SCC in the affected skin.[71]

Ichthyosis vulgaris, the most prevalent type of inherited ichthyosis, is caused by mutations in *FLG*, the gene encoding filaggrin.[72] To our knowledge, there have been no reports on the incidence of skin malignancies in ichthyosis vulgaris. Several cohort studies have reported cancer incidence in patients with atopic dermatitis (AD), in which loss-of-function mutations in *FLG* are a major predisposing factor.[73] Although many studies have confirmed that AD is associated with an increased risk of lymphoma, the estimated risk of nonmelanoma skin cancer (NMSC) in patients with AD differs among studies. Some studies reported an increased risk of NMSC in patients with AD,[74,75] whereas others demonstrated no association between NMSC and AD.[76,77] Further studies are needed to evaluate precisely the cancer risk in patients with ichthyosis vulgaris.

Future directions

Because of the limited number of patients with inherited ichthyoses, it is still almost impossible to calculate accurately the incidence of skin malignancies in these patients. However, our review of the literature shows that patients with ichthyosis can develop skin malignancies, mostly SCC, at an early age, although the literature may be biased in favour of describing only 'interesting' cases.

Generally, impaired barrier function in patients with ichthyosis might permit breech of the stratum corneum by contact chemical carcinogens. However, epithelial desquamation has been suggested as protecting against natural chemicals.[78,79] If this is true, one might guess that more rapid epidermal turnover in ichthyosis skin would be protective against, rather than contributory to, skin carcinogenesis. There are common

types of ichthyosis, such as ichthyosis vulgaris and recessive X-linked ichthyosis, which do not seem to be associated with skin cancer at a young age. On the other hand, patients with KID syndrome, ARCI and NS have been reported to develop SCC at an early age. These differences might be explained by causative genetic defects in each ichthyosis subtype.

Recent developments in bioengineering techniques have resulted in many animal models of inherited ichthyosis.[80] Experiments on ichthyosis skin carcinogenesis, including two-stage carcinogenesis assay, might provide clues to understanding the pathomechanisms underlying skin cancer in inherited ichthyosis, although neonatal lethality will prevent these experiments in several mouse models.

In the future, a worldwide registry on ichthyoses with follow-up information would be desirable towards enabling a full evaluation of skin malignancies in patients with ichthyosis. At present, routine surveillance for skin malignant changes is strongly recommended for patients with KID syndrome and inflammatory types of congenital ichthyosis such as CIE/LI and NS, even if the patients are taking systemic retinoids.

What's already known about this topic?

- There have been sporadic case reports of malignant skin tumours in patients with congenital ichthyosis.
- The frequency of skin malignancies in patients with ichthyosis is unknown.

What does this study add?

- Patients with congenital ichthyosis, especially those with KID syndrome, congenital ichthyosiform erythroderma, lamellar ichthyosis and Netherton syndrome, can develop cutaneous squamous cell carcinoma at unusually young ages.

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Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia

*Takamasa Katagiri,[1,2] *Aiko Sato-Otsubo,[3] Koichi Kashiwase,[4,5] Satoko Morishima,[6] Yusuke Sato,[3] Yuka Mori,[3] Motohiro Kato,[3] Masashi Sanada,[3] Yasuo Morishima,[7] Kohei Hosokawa,[2] Yumi Sasaki,[2] Shigeki Ohtake,[1] †Seishi Ogawa,[3,5] and †Shinji Nakao,[2] on behalf of the Japan Marrow Donor Program

[1]Clinical Laboratory Science, Division of Health Sciences, and [2]Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan; [3]Cancer Genomics Project, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [4]Tokyo Metropolitan Red Cross Blood Center, Tokyo, Japan; [5]Core Research for Evolutional Science and Technology, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama, Japan; [6]Department of Hematology, Fujita Health University, Aichi, Japan; and [7]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan

Idiopathic aplastic anemia (AA) is a common cause of acquired BM failure. Although autoimmunity to hematopoietic progenitors is thought to be responsible for its pathogenesis, little is known about the molecular basis of this autoimmunity. Here we show that a substantial proportion of AA patients harbor clonal hematopoiesis characterized by the presence of acquired copy number-neutral loss of heterozygosity (CNN-LOH) of the 6p arms (6pLOH). The 6pLOH commonly involved the HLA locus, leading to loss of one HLA haplotype. Loss of HLA-A expression from multiple lineages of leukocytes was confirmed by flow cytometry in all 6pLOH(+) cases. Surprisingly, the missing HLA-alleles in 6pLOH(+) clones were conspicuously biased to particular alleles, including HLA-A*02:01, A*02:06, A*31:01, and B*40:02. A large-scale epidemiologic study on the HLA alleles of patients with various hematologic diseases revealed that the 4 HLA alleles were over-represented in the germline of AA patients. These findings indicate that the 6pLOH(+) hematopoiesis found in AA represents "escapes" hematopoiesis from the autoimmunity, which is mediated by cytotoxic T cells that target the relevant autoantigens presented on hematopoietic progenitors through these class I HLAs. Our results provide a novel insight into the genetic basis of the pathogenesis of AA. (*Blood.* 2011;118(25):6601-6609)

Introduction

Acquired aplastic anemia (AA) is a rare condition associated with BM failure and pancytopenia.[1] A series of classic observations and experiments have unequivocally supported that the autoimmunity to hematopoietic stem/progenitor cells (HSPCs) critically underlies the pathogenesis of the BM failure in the majority of AA cases. According to the widely accepted model of immune-mediated BM failure, activated cytotoxic T cells (CTLs) that recognize an auto-antigen(s) presented on HSPCs through their class I HLA molecules have a major role in initiating the autoimmune reactions.[2-4] However, no definitive evidence exists that supports this model or the presence of such CTL repertoires. Moreover, little information is available about their target antigens or about the way by which they are recognized by effector T cells.

Another long-standing issue on AA is its close relationship with clonal hematopoiesis.[5,6] It was first suspected from an apparent overlap between AA and paroxysmal nocturnal hemoglobinuria (PNH)[7,8] and was also implicated by the frequent development of late clonal disorders in AA, such as myelodysplastic syndromes, PNH, or even acute myeloid leukemia (AML).[9-11] Clonal hematopoiesis can be explicitly demonstrated by conventional clonality assays at presentation in a substantial proportion of newly diagnosed typical AA cases.[12] Although it has been expected that the inciting autoimmune insult somehow confers selective pressures on the evolution of clonal hematopoiesis,[5] the exact mechanism for such immunologic selection or escape is still unclear.

The objectives of this study, therefore, were to characterize the clonal nature of the hematopoiesis that is maintained even under the severe autoimmune insult in AA, and to explore the genetic/immunologic mechanism that could underlie the pathogenesis of AA. To achieve these aims, we performed single nucleotide polymorphism (SNP) array-based analysis of genomic copy numbers and/or allelic imbalances in peripheral blood (PB) specimens obtained from 306 patients with AA. Initially, we found that AA patients frequently showed clonal/oligoclonal hematopoiesis that lost specific HLA alleles as a result of copy number-neutral loss of heterozygosity (CNN-LOH) of the 6p arms, which led us to further analyses of the contribution of 6pLOH(+) clones to residual hematopoiesis and a large-scale epidemiologic study on the HLA alleles that are over-represented in AA, involving a total of 6,613 transplants registered in the Japan Marrow Donor Program (JMDP).

Methods

Subjects

PB specimens from a total of 306 patients with AA were analyzed for the presence of genetic alterations using SNP arrays (see Figure 1). The clinical

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*T.K. and A.S.-O. contributed equally to this study.

†S. Ogawa and S.N. contributed equally to this study.

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Table 1. Patient characteristics

	Newly diagnosed (n = 107)	Previously treated (n = 199)
Median age at diagnosis, mo (range)	64 (9-88)	24 (2-80)
Sex, male/female, no.	58/49	110/89
Severity of AA at onset, no. (%) of patients		
Severe	79 (74)	185 (93)
Nonsevere	28 (26)	14 (7)
History, mo, median (range)	19 (0.1-251)	51 (0.1-372)
Past treatment, no. (%) of patients		
ATG + CsA	—	39 (20)
CsA alone	—	51 (26)
Anabolic steroid alone	—	13 (7)
Unknown*	—	96 (48)

ATG indicates antithymocyte globulin; CsA, cyclosporine A; and —, not applicable.

*Information regarding previous therapies of 96 cases (from Japan Marrow Donor Program) was unavailable.

characteristics of these patients are summarized in Table 1 and supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Among the 306 patients, 107 were newly diagnosed and 199 were previously treated. Ninety-six patients received allogeneic BM transplantation from unrelated donors through the JMDP, and their HLA information was available from the JMDP. The other 210 were newly genotyped for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles as described elsewhere.[13] A total of 103 patients had been treated with anti–thymocyte globulin plus cyclosporine, cyclosporine alone, or anabolic steroids at the time of sampling. All patients and healthy persons provided their informed consent before sampling in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University and also by that of the Graduate School of Medicine, University of Tokyo.

Analysis of genomic copy numbers and detection of 6pLOH

Genomic copy numbers, as well as allele-specific copy numbers, were analyzed by using GeneChip 500K arrays (Affymetrix) as previously described.[14,15] Briefly, genomic DNA from AA patients and normal controls were analyzed on GeneChip 500K arrays separately. After adjusting several biases introduced during experiments, signal ratios of the corresponding probes between test (patient) and controls were calculated across the genome to obtain genome-wide copy numbers. Genetic lesions, including copy number gains and losses, as well as CNN-LOHs, were first detected using a hidden Markov model-based algorithm implemented in the CNAG software.[14,15] Known copy number variations were carefully excluded by referring to the Database of Genomic Variants (www.projects.tcag.ca/variation). CNN-LOH in 6p involving the HLA locus was more specifically and sensitively detected by statistically evaluating the mean differences in allele-specific copy numbers between heterozygous SNPs on 6p (N = ~ 1400) that were telomeric from the 5′-end of the HLA-A locus (rs1655927) and all non-6p heterozygous SNPs (N = ~ 105 000) using the Mann-Whitney *U* test with the R package (www.r-project.org). Possible false-positive findings arising from multiple testing involving the 306 samples were evaluated by maintaining the false discovery rate under 0.01 as previously described,[16] where the microarray data of 1000 JMDP donor specimens obtained from an ongoing whole genome association study (unpublished data) were used to calculate an empiric null distribution.[17,18]

Determination of the missing HLA alleles in 6pLOH(+) clones in patients with AA

The 500K SNP data of the 1800 JMDP donor-recipient pairs (JMDP dataset), together with their HLA genotyping information, was used to generate an HLA SNP haplotype table on the GeneChip 500K platform, which contains the consensus SNPs of the 3 major haplotypes (P1, P2, and P3) in Japanese subjects[18] and the SNP sequences of all observed HLA haplotypes complementary to P1 to P3 within the JMDP set (N = 1576; data not shown). To determine the missing HLA haplotype in each 6pLOH(+) patient, those "HLA" haplotypes were first selected from the aforementioned HLA haplotype table that were compatible with the observed HLA genotypes of that patient. Among these, a candidate haplotype was selected such that it contained the minimum number of SNPs that were incompatible with the patient's genotype. For each candidate haplotype, genomic copy numbers were inferred at the heterozygous SNPs along that haplotype using the circular binary segmentation algorithm,[19,20] which divided the haplotype into one or more discrete segments with different mean copy numbers. Finally, each copy number segment was thought to be "missing," when the alternative hypothesis (Ha: $S_i \neq \overline{S_i}$, for $^{\forall}i$) was supported against the null hypothesis (H_0: $S_i = \overline{S_i}$, for $^{\forall}i$) using the Wilcoxon signed rank test with a significance level of .05, where S_i represents the allele-specific copy number at the ith heterozygous SNP site within the segment of the candidate haplotype with $\overline{S_i}$ being the corresponding value for the complementary haplotype (supplemental Figure 1). Finally, for those HLA types that appeared more than 8 times among 6pLOH(+) cases, their contribution to the observed allelic loss of HLA haplotypes was evaluated by multivariate logistic regression analysis with stepwise backward selection

Flow cytometry

Heparinized PB and BM were collected from the patients at diagnosis and/or after treatment. HLA-A expression on granulocytes, monocytes, B and T cells, and BM $CD34^+$ cells was analyzed by flow cytometry using a FACSCanto II instrument (BD Biosciences) with the FlowJo 7.6.1 program (TreeStar). The monoclonal antibodies used for this study are provided in supplemental Table 2.

Human androgen receptor assay

The human androgen receptor gene was amplified from genomic DNA of 23 female patients, including 3 6pLOH(+) patients, as described by Ishiyama et al[21] with some modifications. Clonality was assessed using an "S value" as a marker of skewing in granulocytes and T lymphocytes.

Association of HLA types with AA

A total of 6613 patients who had received allogeneic BM transplantation through the JMDP between 1992 and 2008 were investigated to see whether the HLA alleles frequently missing in CNN-LOH in 6p with the development of AA could represent risk alleles for the development of AA. Thus, the frequencies of patients with each of the candidate risk alleles (HLA-A*31:01, B*40:02, A*02:01, and A*02:06) and those having none of these alleles were compared between 407 patients with AA and those with other hematopoietic disorders (1827 with AML, 1606 with acute lymphocytic leukemia, 1014 with chronic myeloid leukemia, 825 with myelodysplastic syndrome, 566 with non-Hodgkin lymphoma, and 368 with other hematopoietic neoplasms; supplemental Table 3) by calculating the Fisher *P* values in the corresponding 2 × 2 contingency tables.

Results

Genetic lesions in AA detected by SNP array analysis

After excluding known or suspected copy number variations, a total of 50 genetic lesions were identified in 46 of the 306 (15%) PB specimens of our AA case series (Table 1; Figure 1). Among these by far, the most conspicuous was the recurrent CNN-LOH involving the 6p arm, which was detected in 28 cases as a significant dissociation of allele-specific copy number graphs in 6p regions using a hidden Markov model–based algorithm implemented in the CNAG software[2,14,15] (Figure 2A-2B). Of particular interest was that all CNN-LOH in 6p commonly affected the HLA locus, causing a haploid loss of HLA alleles and uniparental HLA expression. In some cases, the breakpoint of the 6pLOH was

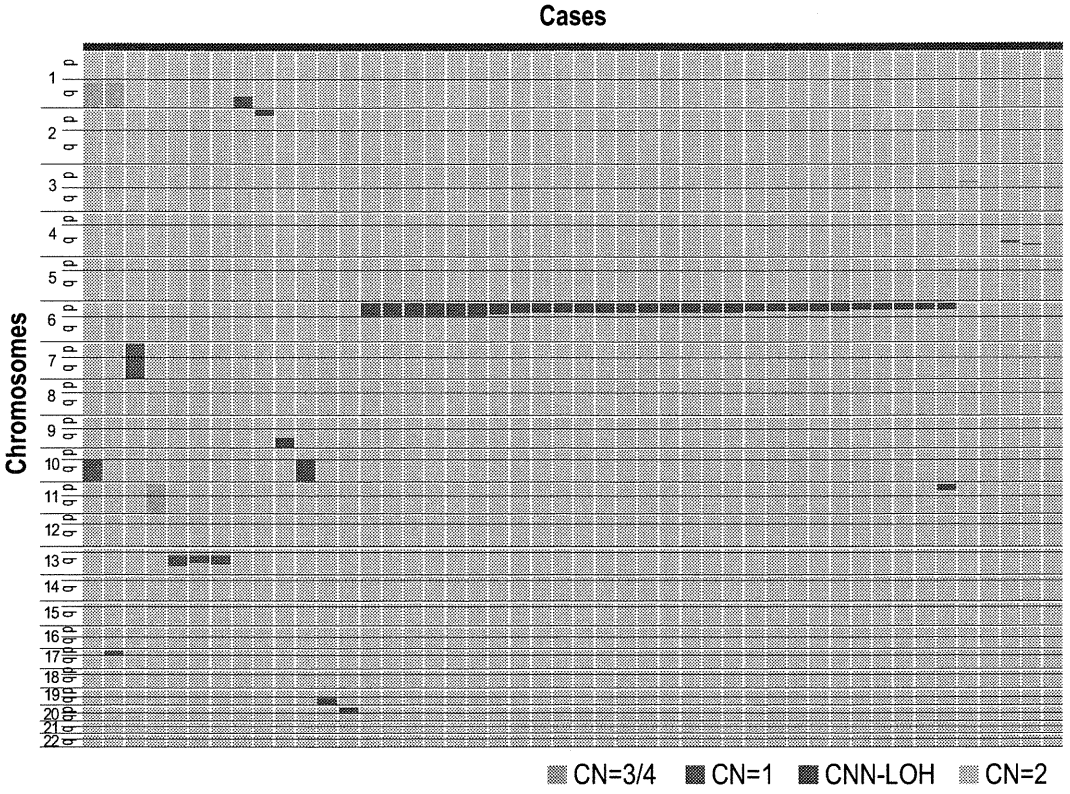

Figure 1. Copy number changes and allelic imbalances in 46 of the 306 AA cases. The copy number changes and allelic imbalances (or CNN-LOHs) in each case are summarized in the chromosomal order vertically for 46 AA cases with copy number abnormalities. Gains and losses, as well as CNN-LOHs, are shown in the indicated colors.

predicted to fall within the HLA locus (Figure 2B). These findings strongly indicated that the HLA locus was the genetic target of these 6pLOHs. Also supporting this was the finding that, in half of the cases, the dissociations in the allele-specific copy number graphs were gradually attenuated to the baseline over several mega base pair regions rather than showing a discrete breakpoint, indicating the presence of multiple 6pLOH(+) clones within a single case that had different breakpoints but still shared the same missing HLA alleles (Figure 2C). Moreover, the 6pUPDs existing only in a minor population were more sensitively detected by statistically evaluating the size of dissociation of allele-specific copy numbers in the 6p arm. With this improved statistical test, CNN-LOH in 6p was found in a total of 40 cases (13%; Figure 2D; supplemental Figure 2), where the false discovery rate was maintained at 0.01 to avoid too many false positive findings. In all 6pLOH(+) cases, substantial numbers of heterozygous SNP calls were retained within the affected regions, thus indicating that the CNN-LOHs in 6p were not constitutional but represented acquired genetic events only found in the affected subclones (Figure 1). Indeed, all 6pLOH(+) cases were shown to have "heterozygous" HLA alleles in high-resolution HLA typing of their PB (Table 2). Moreover, 6pLOH was not detected in the CD3-positive T cells in selected cases (cases 25 and 26, supplemental Figure 3). By quantitatively comparing the observed differences in allele-specific copy numbers in the 6pLOH segments with what were expected assuming 100% LOH(+) components, the 6pLOH(+) clones were estimated to account for 0.2% to 53.9% of the PB leukocytes (Table 2). The trend of the lower percentages of the 6pLOH(+) fraction in newly diagnosed patients compared with those in patients at remission was thought to reflect the fact that the former patients tended to have lower counts of granulocytes and monocytes, which were the predominant targets of 6pLOH (see supplemental Table 1).

The disease status of the 40 patients at the sampling was before treatment in 16 cases, during remission for 1 to 16 years after therapies in 15, and before BM transplantation for refractory disease in 9. All evaluable 6pLOH(+) AA cases responded to immunosuppressive therapy (IST) (23 of 23), whereas 101 of 126 evaluable cases with 6pLOH(−) responded ($P = .014$; Table 3).

Uniparental expression of HLA-A in multilineage hematopoietic cells

The genetic loss of one HLA haplotype in SNP array analysis was further confirmed by expression analysis of HLA-A in PB leukocytes using flow cytometry in 19 eligible cases with 6pLOH(+), in which the HLA-A alleles were heterozygous and fresh PB samples were available. Loss of expression of one HLA-A antigen was confirmed in all 19 6pLOH(+) cases (Figure 3A; supplemental Figure 4). The HLA-A missing cells in the PB were shown to have appeared shortly after the onset or before the initiation of treatments in 2 cases, and were confirmed to persist for 1 to 16 months (median, 6 months) in 14 patients (supplemental Table 1; supplemental Figure 5). The percentage of granulocytes lacking HLA-A antigens in the 2 patients who were responsive to IST remained almost the same during the convalescent period of 2 to 3 months (supplemental Figure 6). Importantly, uniparental expression of HLA-A alleles was detected in multiple cell lineages, including granulocytes, monocytes, B cells, and, to a lesser extent, in T cells. Moreover, uniparental HLA-A expression was demonstrated in BM $CD34^+$ cells in 5 patients whose BM samples were available for flow cytometry. All 5 patients possessed various proportions of BM $CD34^+$ cells (49.7%-71.3%), which had lost the expression of one

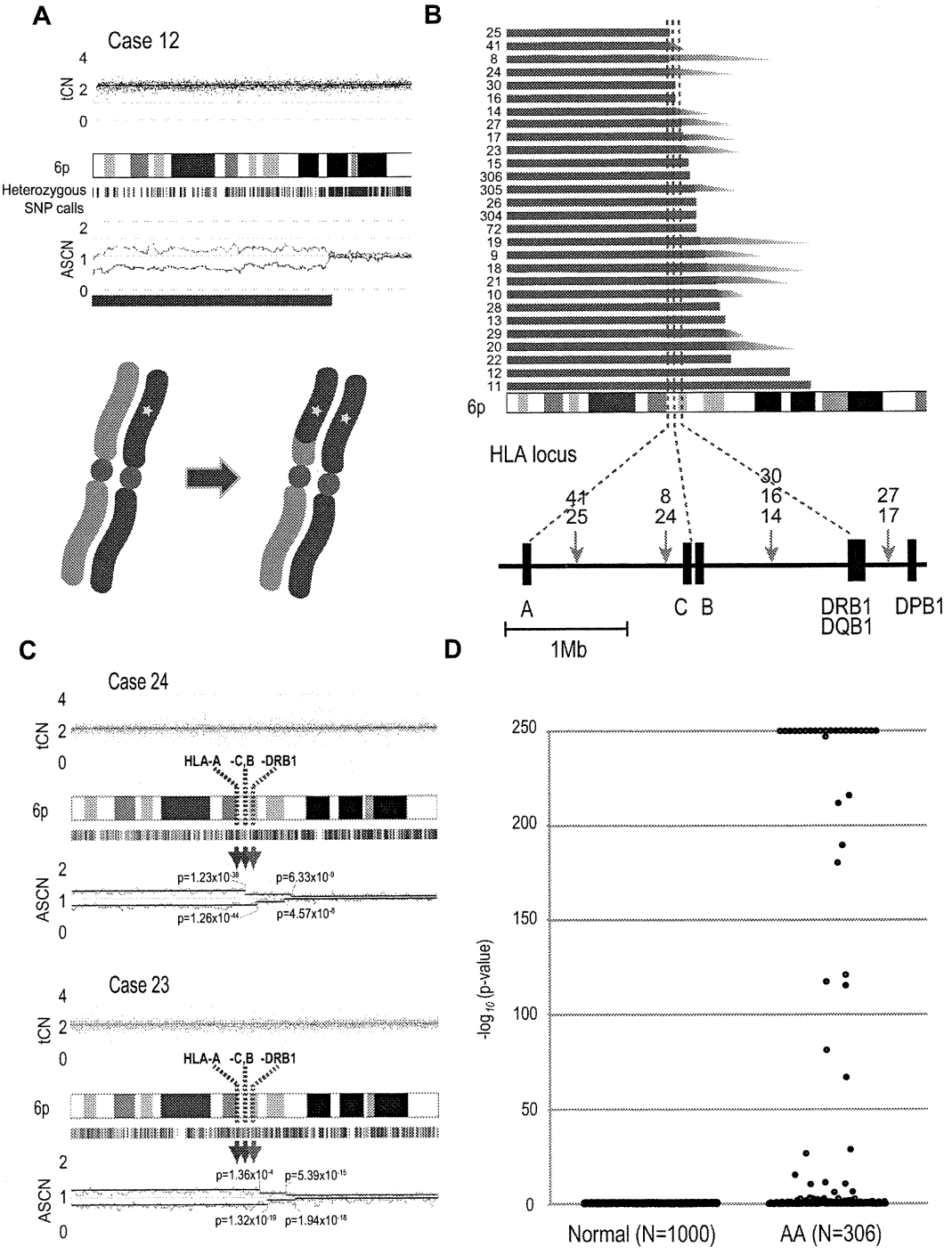

Figure 2. Acquired 6pLOHs in AA patients that target the HLA locus. (A) Typical CNAG outputs in SNP array analysis showing CNN-LOH (purple line) that appears as significant dissociation in allele-specific copy number graphs (red and green lines) from the baseline with normal total copy numbers (tCN; top panel). As a result of an allelic conversion, the affected segment causes LOH (* indicates 1; bottom panel). The "acquired" origin of these lesions is indicated by the retention of substantial numbers of heterozygous SNP calls (green bars below the chromatogram) that would otherwise mostly disappear. (B) The breakpoints of 6pLOHs found in a total of 28 AA cases, all involving the HLA locus in common. In more than half of cases (indicated by arrowheads in panel B), the exact location of the breakpoint was difficult to uniquely determine, where dissociation of the allele-specific copy number graphs continuously tapered along the 6p arm, indicating the presence of multiple 6pLOH(+) clones with common missing alleles (C). Indeed, the breakpoint containing regions are separated into multiple segments having significantly different copy numbers in the circular binary segmentation model, as indicated by solid lines with *P* values. Note that the most telomeric breakpoint is located within (case 24) or centromeric to (case 23) the HLA locus in each case. (D) A skewed distribution of the logarithm of *P* values in AA cases compared with normal persons. The *P* values were calculated in the Mann-Whitney *U* test, with which the difference in the mean allele-specific copy numbers between 6p and other chromosomal regions was evaluated (see "Analysis of genomic copy numbers and detection of 6pLOH"). A total of > 250 values are plotted as 250.

HLA-A antigen; and in each case, the missing HLA-A allele was identical to that in the PB leukocytes (Figure 3B). The uniparental expression of HLA-A in case 13 was also observed in the $CD34^+$ compartment of the archived BM specimen obtained 2 years before analysis (supplemental Figure 7). Together, these findings suggested that the 6pLOH involved early HSPCs and that the 6pLOH occurred at the level of long-term repopulating stem cells.

Table 2. 6pLOH(+) AA cases and imputed allelic status of HLA alleles

UID	6pUPD(+) fraction,* %	Missing alleles						Retained alleles					
		A	B	C	DRB1	DQB1	DPB1	A	B	C	DRB1	DQB1	DPB1
19	53.9	31:01†‡	40:02†	03:04†	12:01	03:01	05:01	24:02	52:01	12:02	15:02	06:01	05:01
12	51.8	02:01†‡	40:02†	03:03	15:01	06:02	05:01	26:02	40:06	08:01	09:01	03:03	05:01
17	51.6	24:02	13:01	03:04†	12:02	03:01	04:02	24:02	52:01	12:02	15:02	06:01	09:01
304	49.3	31:01†‡	55:02	01:02	12:02	03:01	41:01	24:02	07:02	07:02	01:01	05:01	04:02
11	48.0	02:06†‡	40:02†	03:04†	15:01	06:02	ND‖	11:01	67:01	07:02	16:02	05:02	ND‖
21	46.2	31:01‡§	51:01	14:02	14:05	05:03	03:01	24:02	07:02	07:02	01:01	05:01	04:02
24	44.9	31:01†	40:02†	03:04†	11:01	03:01	02:01	24:02	40:06	08:01	09:01	03:03	05:01
26	44.3	31:01†‡§	40:01	03:04†	04:05	04:01	03:01	26:03	52:01	12:02	15:02	06:01	09:01
27	43.5	02:06†	40:02†	03:04†	04:10	04:02	02:01	11:01	52:01	12:02	15:02	06:01	09:01
10	42.1	31:01†	40:02†	03:04†	08:03	06:01	02:01	24:02	51:01	14:02	09:01	03:03	02:01
8	40.8	02:06†‡	40:02†	03:03	12:01	03:01	05:01	24:02	52:01	12:02	15:02	06:01	04:02
23	35.2	02:01†	40:02†	03:04†	09:01	03:03	02:01	24:02	54:01	01:02	04:05	04:01	04:02
25	32.1	02:06†‡			No LOH			01:01			No LOH		
9	23.5	02:06†‡	39:01	07:02	08:02	04:02	02:01	24:02	15:18	07:04	04:01	03:01	14:01
20	21.7	26:01‡	40:02†	03:03	15:01	06:02	05:01	02:18	46:01	01:02	08:03	06:01	05:01
14	21.7	31:01†‡	51:01	14:02	09:01	03:03	05:01	24:02	52:01	12:02	15:02	06:01	09:01
22	20.6	02:01†	39:01	07:02	08:03	06:01	05:01	24:02	52:01	12:02	15:02	06:01	09:01
18	17.6	02:01†‡	40:06	08:01	09:01	03:03	02:01	24:02	35:01	03:03	15:01	06:02	04:02
15	17.4	02:06†	40:06	08:01	09:01	03:03	02:01	24:02	07:02	07:02	01:01	05:01	02:01
41	15.2†	31:01†‡	35:01	03:03	09:01	03:03	03:01	26:01	39:01	07:02	08:03	06:01	05:01
28	12.8	24:02	54:01	01:02	01:01	05:01	04:02	24:02	52:01	12:02	15:02	06:01	09:01
29	11.7	31:01†	40:02†	03:04†	15:01	06:02	02:01	24:02	54:01	01:02	04:05	04:01	05:01
305	10.3	02:06†‡	40:02†	15:02	15:02	06:01	04:01	24:02	51:01	14:02	09:01	03:03	02:01
13	9.6	24:02‡	40:02†	03:04†	15:01	06:02	02:01	02:01‡	35:01	08:01	09:01	03:03	02:01
306	8.5	24:02‡	40:02†	03:04†	09:01	03:03	02:01	26:02	40:06	08:01	09:01	03:03	02:01
16	8.1	11:01	40:06	08:01		No LOH		24:02	46:01	01:02		No LOH	
30	8.0	02:06†	39:01	07:02		No LOH		24:02	40:06	08:01		No LOH	
72	5.6	02:01†	40:02†	03:04†	09:01	03:03	05:01	02:07	46:01	01:02	08:03	06:01	02:02
36	4.0	02:01†‡	ND¶	ND#	15:02	06:01	09:01	24:02	ND¶	ND#	15:02	06:01	09:01
124	3.5	24:02	40:02†	03:04†	12:01	03:01	02:01	24:02	52:01	12:02	15:02	06:01	09:01
223	2.8	31:01†‡	48:01	03:04†	09:01	03:03	05:01	02:06†	39:01	07:02	15:01	06:02	02:01
215	2.8	31:01†	51:01	14:02	08:02	04:02	04:02	03:01	44:02	05:01	13:01	06:03	05:01
181	1.3	02:06†	13:01	03:04†	12:02	03:01	05:01	24:02	52:01	12:02	15:02	06:01	09:01
97	1.0	24:02	07:02	07:02	01:01	05:01	05:01	02:01†	39:01	07:02	15:01	06:02	02:01
252	0.9	ND**	40:02†	03:04†	09:01	03:03	05:01	ND**	46:01	01:02	04:05	04:01	05:01
118	0.9	02:06‡§	40:02†	03:04†	08:02	03:02	05:01	24:02	52:01	12:02	15:02	06:01	09:01
298	0.8	24:02	40:02†	03:04†	15:01	06:02	05:01	24:02	52:01	12:02	15:02	06:01	09:01
188	0.7	24:02	52:01	12:02	15:02	06:01	09:01	02:01†	52:01	12:02	11:01	03:01	05:01
291	0.7	31:01†	51:01	14:02	15:01	06:02	02:01	24:02	40:01	03:04†	11:01	03:01	05:01
196	0.2	ND†† (A*02:06/24:02, B*35:01/51:01, C*03:03/15:02, DRB1*04:03/15:01, DQB1*03:02/06:02, DPB1*0:201/02:01)											

UID indicates unique ID.

*The percentage of 6pUPD(+) fraction is derived from total peripheral blood leukocytes that include lymphoid as well as myeloid element.

†HLA types significantly deviated to missing alleles.

‡The allelic loss was confirmed by flow cytometry.

§The missing haplotype was determined by flow cytometry.

‖DPB1*04:02/05:01.

¶B*15:18/52:01.

#C*08:01/12:02.

**A*02:01/02:07.

††Missing allele was not determined because copy number changes in these segments were not statistically significant.

Clonality of the HLA-missing granulocytes

The human androgen receptor-based clonality assays in granulocytes were performed in 3 6pLOH(+) and 20 6pLOH(−) patients, in which all 3 6pLOH(+) and 4 (20%) of the 6pLOH(−) patients showed evidence of clonality in granulocyte populations (supplemental Figure 8).

Missing HLA alleles in 6pLOH

Given that the HLA is the genetic target of 6pLOH in AA, the missing HLA alleles in 6pLOH are of particular interest because in this context they are thought to be directly involved in the presentation of the target auto-antigens to CTLs and, therefore, to be critically important in the pathogenesis of AA. We determined the missing HLA alleles in each 6pLOH(+) AA patient by the haplotype imputation of HLA alleles based on the large data of HLA haplotypes observed in the JMDP set, followed by statistical evaluation of allele-specific copy numbers along the imputed haplotypes (Figure 4). The imputed haplotypes were confirmed in 4 cases by the family studies on the HLA. The allelic status was imputed at least partially in 39 of the 40 6pLOH(+) cases. The imputed results were consistent with the patterns of uniparental expression of HLA-A in flow cytometry in 18 cases with 6pLOH (Table 2; Figure 4), except for those in case 26, in which no valid SNP haplotype

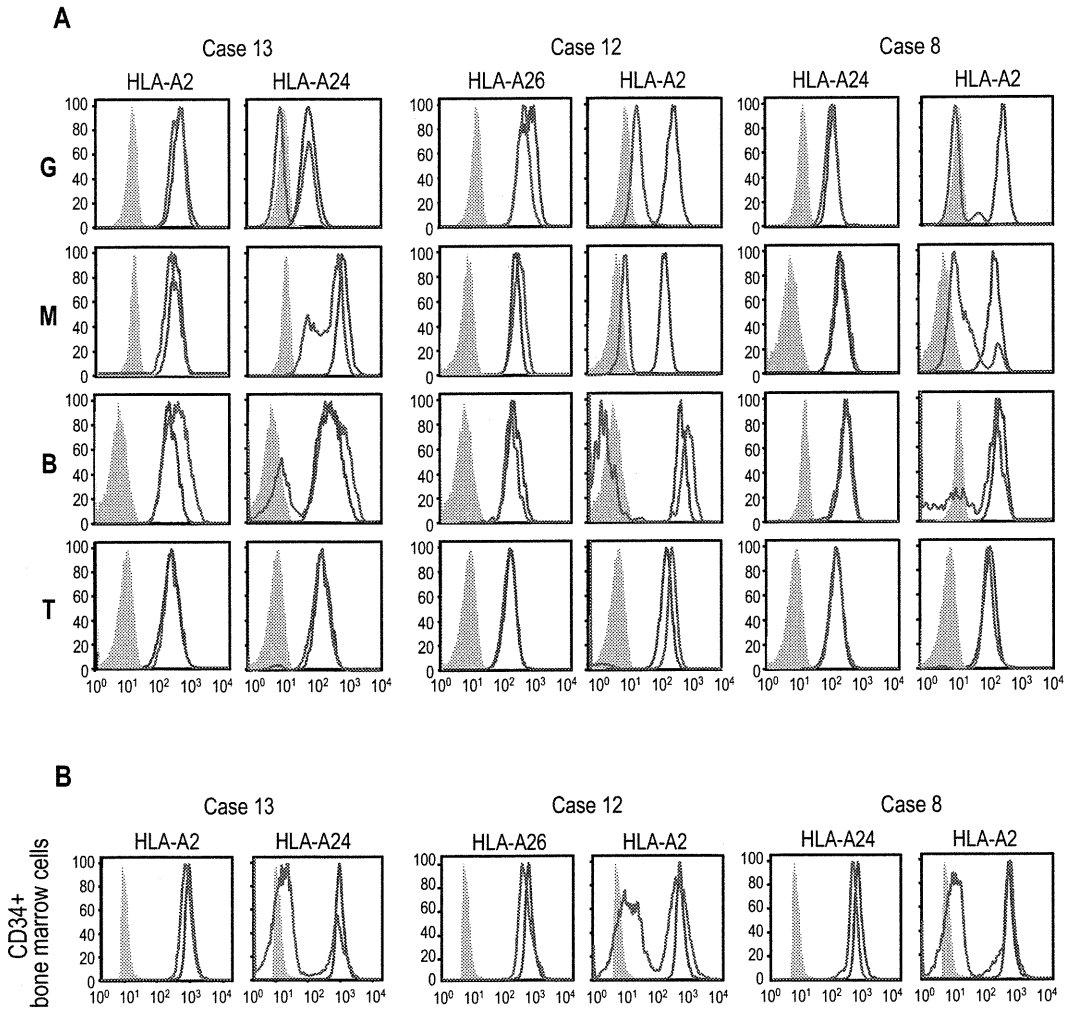

Figure 3. Uniparental expression of HLA in AA cases with CNN-LOH in 6p. Allele-specific expression of HLA-A antigens in AA specimens was examined by flow cytometry using monoclonal antibodies that specifically recognize the indicated HLA types (red lines), where leukocytes from healthy persons were used as a control (blue lines). (A-B) The uniparental expression of HLA-A antigens in PB leukocytes and BM CD34$^+$ cells obtained from 3 AA cases with CNN-LOH in 6p. Different leukocyte compartments were separately examined, including granulocytes (G), monocytes (M), B-lymphocytes (B), and T-lymphocytes (T).

around the HLA-A locus was identified and the status of HLA-A was determined by flow cytometry. The missing HLA alleles in 6pLOH(+) AA showed a conspicuous deviation to some selected HLA alleles, including HLA-A*31:01, B*40:02, C*03:04, and, to a lesser extent, HLA-A*02:01 and A*02:06. After the effects of linkage disequilibrium between individual HLA alleles were taken into consideration by multivariate analysis, 4 HLA alleles were shown to remain as the principal determinants of the missing haplotypes, HLA-A*31:01, B*40:02, A*02:01, and A*02:06 (supplemental Table 4).

Over-representation of frequently missing HLAs in AA populations

Because these missing HLA alleles in 6pLOH could be involved in the pathogenesis of AA, we next tested whether these relevant HLA alleles are associated with the risk of the development of AA among the 6,613 JMDP registrants. As shown in Table 4, the 4 major missing HLA alleles, HLA-A*31:01, B*40:02, A*02:01, and A*02:06, were more frequently observed in AA cases compared with nonsignificant HLA alleles (ie, all HLA alleles other

Table 3. Response rate (CR + PR) according to the Camitta criteria

	Newly diagnosed (n = 107)		Previously treated (n = 103)	
	6pLOH(−) (n = 91), no. (%)	6pLOH(+) (n = 16), no. (%)	6pLOH(−) (n = 88), no. (%)	6pLOH(+) (n = 15), no. (%)
Immunosuppressive therapies (all)	36/49 (73)	11/11 (100)	65/77 (84)	12/12 (100)
ATG + CsA	14/19 (74)	7/7 (100)	27/33 (82)	5/5 (100)
CsA alone	22/30 (73)	4/4 (100)	38/44 (86)	7/7 (100)
Anabolic steroid alone	0/0 (0)	0/0 (0)	7/11 (64)	2/2 (100)
Unknown/not evaluable	42	5	0	1

CR indicates complete remission; PR, partial remission; ATG, antithymocyte globulin; and CsA, cyclosporine A.

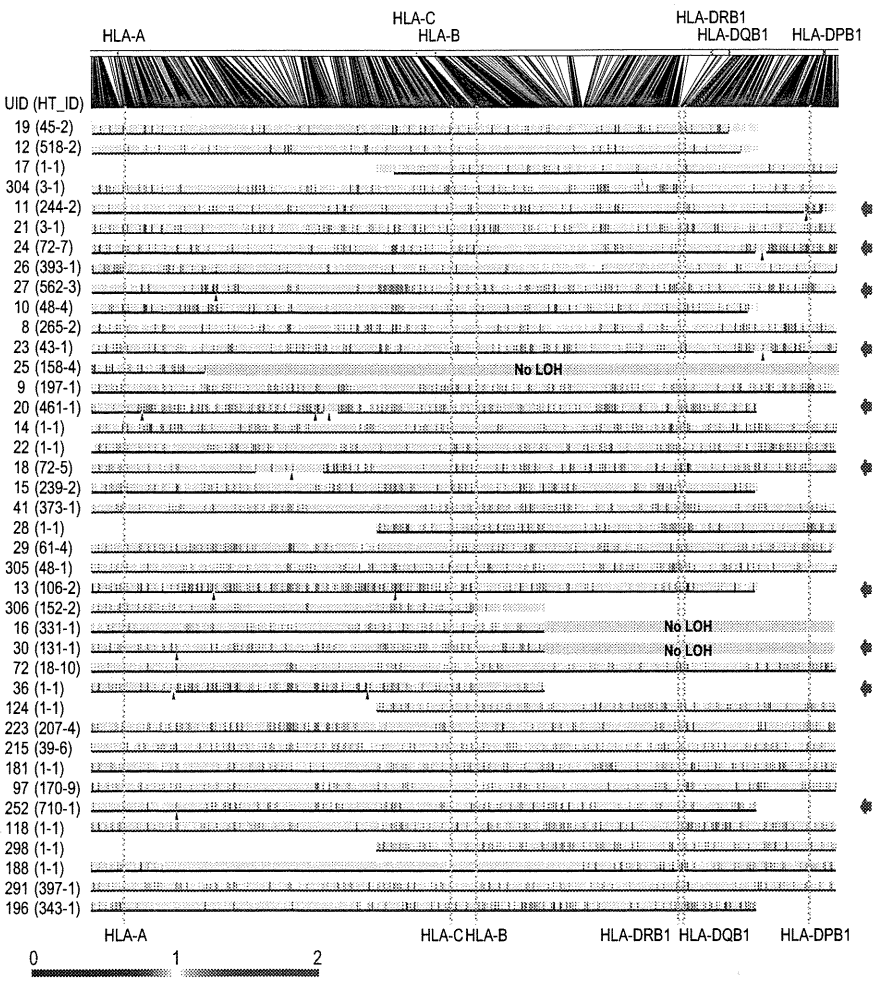

Figure 4. Imputation of missing HLA haplotypes. The observed allelic copy numbers at heterozygous SNP sites along each candidate SNP haplotype are color-coded as indicated at the bottom. Green bars showed the SNPs that are incompatible with the patient's genotype. Case IDs and haplotype ID (HT_ID) are indicated on the left. The locations of the 500K SNPs and HLA-A, C, B, DRB1, DQB1, and DPB1 are indicated in the figure. For each allele, genomic copy numbers were imputed using the circular binary segmentation algorithm. This divided each haplotype into one or more segments having discrete mean allelic copy numbers (blue arrows on the right). The positions of breakpoints are indicated by arrowheads. Finally, the mean allelic copy number of each segment was statistically compared with that of the corresponding segment on the other haplotype using the Wilcoxon signed rank test. Missing HLA haplotypes were determined based on the result of the statistic tests. Purple and blue lines indicated the retained and missing segments, respectively, whereas the allelic status was not determined statistically for those segments shown by green lines.

than these 4 alleles), where the odds ratios for the risk of the development of AA between each of these alleles and nonsignificant alleles were 1.87 (95% confidence interval [CI], 1.43-2.43) for A*02:01, 2.22 (95% CI, 1.70-2.90) for A*02:06, 1.37 (95% CI, 1.00-1.88) for A*31:01, and 1.95 (1.48-2.58) for B*40:02 (Table 4). The combined relative risk for all these alleles was 1.75 (1.42-2.17; $P = 1.3 \times 10^{-7}$).

Discussion

The origin of clonal hematopoiesis in AA is a focus of long-standing disputes, in which a profoundly reduced hematopoietic stem cell pool and/or escape from the autoimmune insults have been implicated in the evolution of the clonal hematopoiesis in AA.[5,22,23] Our findings on 6pLOH in AA provide an intriguing insight not only into the underlying mechanism of the clonal hematopoiesis in AA but also into the origin of the autoimmunity that is responsible for the pathogenesis of AA. A recent study from the United States also reported 3 cases with 6pLOH.[24] With a sensitive detection algorithm, the presence of the 6pLOH(+) components was demonstrated in as many as 13% of typical cases with AA, and the evidence from the subsequent studies strongly indicated that the HLA genes are the genetic targets of 6pLOH in AA patients. First, the HLA locus was commonly and critically involved in all 6pLOHs found in AA. Second, some AA patients carried multiple 6pLOH(+) subclones with different breakpoints, but in all cases, the 6pLOH involved the HLA locus and occurred in a manner that targeted the same parental HLA allele. Moreover, particular class I HLA alleles were over-represented among 6pLOH(+) cases and consistently found in the missing haplotypes. Finally, many of these HLA alleles were shown to be tightly

Table 4. Association of missing HLA alleles with AA in Japanese patients

Risk allele	AA (N = 407)	Other diseases (N = 6206)	Total (N = 6613)	P (χ^2 test)	Odds ratio (95% CI) (vs no risk alleles)
A*02:01	103	1173	1276	2.5×10^{-6}	1.87 (1.43–2.43)
A*02:06	100	957	1057	$< 1.0 \times 10^{-7}$	2.22 (1.70–2.90)
A*31:01	58	899	957	0.048	1.37 (1.00–1.88)
B*40:02	86	938	1024	1.8×10^{-6}	1.95 (1.48–2.58)
All risk alleles	268	3250	3518	1.3×10^{-7}	1.75 (1.42–2.17)
No risk alleles	139	2956	3095	—	—

— indicates not applicable.