A novel *IGnT* allele responsible for the adult i phenotype

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BACKGROUND: The adult i phenotype has been characterized as the presence of a very low level of I antigen but a high quantity of I antigen on red blood cells (RBCs). It has been noted that this rare phenotype is partially associated with congenital cataracts. It has been demonstrated that the human *I* locus expresses three *IGnT* forms, *IGnTA*, *IGnTB*, and *IGnTC*, and that the *IGnTC* gene is responsible for the I antigen expression on RBCs. This report describes molecular genetic analysis of a Taiwanese person with the adult i phenotype but without congenital cataracts.

STUDY DESIGN AND METHODS: The five exon regions of the *IGnT* gene of the adult i individual were amplified by polymerase chain reaction (PCR) and cloned, and the sequences were determined. The activity of the IGnT enzyme expressed from the mutant *IGnTC* gene identified in this i adult was analyzed.

RESULTS: The presented adult i individual possesses wild-type *IGnTA* and *IGnTB* genes but a mutant *IGnTC* gene with a 243T>A nucleotide substitution, which predicts an amino acid alteration of Asn81Lys. PCR-restriction fragment length polymorphism analysis has been used to show that this *IGnTC*243A* allele is uncommon in the general Taiwanese population. The activity of the IGnT enzyme expressed from the mutant *IGnTC*243A* gene was significantly reduced when compared with that expressed from the wild-type *IGnTC* gene.

CONCLUSION: A novel *IGnTC* allele with a 243T>A missense mutation was demonstrated in our adult i Taiwanese without congenital cataracts. The molecular basis revealed for this adult i case agrees with the proposed molecular genetic mechanism, accounting for the partial association of the adult i phenotype with congenital cataracts.

The blood group I and i antigens were first detected on human red blood cells (RBCs) by cold agglutinating autoantibodies.[1-8] Their expressions were found to have a reciprocal relationship and to be developmentally regulated. Adult human RBCs fully express I antigen, with only a few i antigens, whereas the i antigen is predominately present on fetal and neonatal RBCs. After birth, the quantity of I antigen gradually increases as the level of i antigen decreases, until the normal Ii status of adult RBCs is reached after about 18 months of life.[5,9] Like ABH antigens, the Ii are also referred to as histo-blood group antigens[10] because they are not just detected on RBCs, but are known to also be present on the surface of most human cells and on soluble glycoproteins in various body fluids, including milk,[11] plasma,[12] saliva,[12] amniotic fluid, ovarian cyst fluid, and urine.[7,8] The i and I antigens have been characterized as linear and branched repeats of the *N*-acetyllactosamine carbohydrate structures, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-R and Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-R, respectively, carried on glycolipids and glycoproteins.[13-15] The *N*-acetyllactosamine repeats are synthesized by the sequential action of β-1,3-*N*-acetylglucosaminyltransferase and β-1,4-galactosyltransferase. Conversion of i into an I antigenic structure requires the enzyme activity of I-branching β-1,6-*N*-acetylglucosaminyltransferase (I β6GlcNAcT).[14,16,17]

ABBREVIATION: I β6GlcNAcT = I-branching β-1,6-*N*-acetylglucosaminyltransferase.

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Most of the adult RBCs abundantly express I antigen; however, in a small percentage of individuals, the RBCs are rich in the i antigen and very low level in I.[4,5,9] This phenotype is called the adult i phenotype and is believed to result from lack of I-branching transferase activity.[15,16] The frequency of the adult i phenotype is very low, with only a few occurrences in thousands or tens of thousands.[7] In spite of its rareness, however, the adult i phenotype has attracted considerable attention because it appears to be partially associated with congenital cataracts. This association was first demonstrated in Japanese[18-20] and later also in three Taiwanese pedigrees.[21,22] The association of the two traits appears strong in these two populations, as 29 of the 31 Japanese with the adult i phenotype and all 5 adult i members in the 3 Taiwanese families have congenital cataracts. The association, however, does not seem to be as pronounced in white populations, with only a few cases formally reported as having both the adult i phenotype and congenital cataracts.[23-25]

In 1993, Bierhuizen and colleagues[26] first identified the cDNA encoding the I β6GlcNAcT through an expression cloning strategy. The gene, located on chromosome 6p24, is designated *IGnT* (recognized by the HUGO Gene Nomenclature Committee as *GCNT2*).[27,28] In 2003, both our research team[29] and Inaba and coworkers[30] expanded the complexity of the human *IGnT* locus and demonstrated that three *IGnT* forms, which have different exon 1, but identical exon 2 and exon 3, coding regions, are expressed from the *IGnT* locus through utilization of alternative promoters (Fig. 1). The three *IGnT* transcripts, designated *IGnTA*, *IGnTB*, and *IGnTC*, encode three functional I β6GlcNAcT enzymes sharing 66 percent amino acid sequence identities and were found to have different expression profiles in different human tissues. Further, molecular genetic analysis of the two adult i groups, with or without congenital cataracts, was accomplished in our laboratory,[29] with the results demonstrating that the *IGnTC* gene form is responsible for the blood group I antigen expression on RBCs. The most significant conclusion drawn in that article is of a molecular genetic mechanism accounting for the partial association of the adult i phenotype with congenital cataracts. It is proposed that the mutation event that occurs in the exon 1C region of the *IGnTC* gene may lead to the trait of adult i phenotype but not that of congenital cataracts, whereas the mutation that occurs in the common exon 2 to 3 region, and that consequently results in elimination of the activities of all three of the IGnT enzymes, may lead to both the studied traits.

To date, seven different mutations, including six nonsynonymous point mutations and one deletion, have been identified in the *IGnT* alleles of adult i individuals.[22,29-31] Without exception, i adults without congenital cataracts have mutations resulting in the *IGnTC* defect only, whereas those with congenital cataracts have mutations in the common exon 2 to 3 region, which lead to defects in all three *IGnT* genes. It has been noted that the association between the adult i phenotype and congenital cataracts is strong in Asian populations. Three Taiwanese pedigrees and one Japanese analog with both the adult i phenotype and congenital cataracts have been analyzed.[22,29,30] Although adult i cases without cataracts have been identified in Japanese persons,[19,20] molecular genetic analysis has never been performed for these individuals. In this study, a Taiwanese person with the adult i phenotype but without congenital cataracts was identified, and a novel *IGnTC* allele with a missense mutation of 243T>A is demonstrated.

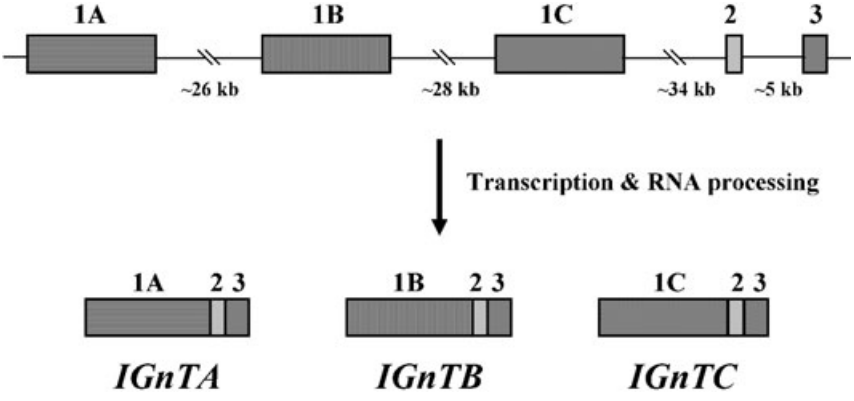

Fig. 1. Schematic representation of organization of the human *I* locus and the structures of the expressed *IGnT* genes. Three *IGnT* transcripts, *IGnTA*, *IGnTB*, and *IGnTC*, which have different exon 1, but identical exon 2 and exon 3, coding regions, are expressed from the human *I* locus (adapted from Yu et al.[29]). The coding nucleotides of exons 1A, 1B, and 1C have 925, 919, and 925 bp, respectively, and the common exon 2 and exon 3 have respective coding nucleotides of 93 and 191 bp. It has been demonstrated that the *IGnTC* gene is responsible for the I antigen expression on RBCs.

MATERIALS AND METHODS

Samples

An individual with the adult i phenotype was identified with a standard hemagglutination test with the monoclonal anti-I antibody, which was a gift from Red Cross Blood Center of Osaka, Japan. This individual's daughter was typed as common I phenotype, and neither of the two family members have cataracts. Both the family members and the 58 randomly selected individuals analyzed in this study are Taiwanese, and informed consent was obtained from all participants before participation. Genomic DNA samples were prepared from their peripheral blood cells with a DNA blood mini kit (QIAamp, Qiagen

GmbH, Hilden, Germany). Our use of human subjects was conducted under the tenets of the Helsinki protocol, and the program was approved by the Institutional Review Board at Mackay Memorial Hospital.

Molecular cloning and sequence analysis for the *IGnT* gene

The five exon regions of the *IGnT* gene (exons 1A, 1B, 1C, 2, and 3) were polymerase chain reaction (PCR)-amplified with the primer pairs as described previously.[29] Fifty nanograms of genomic DNA and 5 pmol of each forward and reverse primer were combined in 12.5 µL of PCR buffer containing 0.2 mmol per L of dNTP and 0.625 U of *PfuUltra* hotstart DNA polymerase (Stratagene, La Jolla, CA). The PCR products were cloned into the pCR4Blunt-TOPO vectors with a PCR cloning kit (Zero Blunt TOPO PCR cloning kit, Invitrogen, Carlsbad, CA). DNA sequences were determined with a cycle sequencing kit (BigDye Terminator, Applied Biosystems, Foster City, CA). Multiple clones from two batches of PCR products were sequenced to identify PCR errors from actual sequence polymorphisms.

PCR-restriction fragment length polymorphism analysis

The 243T>A nucleotide substitution identified in the exon 1C region of the *IGnT* gene of the adult i case destroys a *Tfi*I recognition sequence (GAWTC), and thus a PCR-based restriction fragment length polymorphism (RFLP) analysis was developed to demonstrate the mutation in the *IGnTC* gene. Fifty nanograms of genomic DNA and 5 pmol of each forward (ICF2, GCAAATTCAACCTCT CACACCGATC, 51 nucleotides upstream of the translational start ATG of *IGnTC*) and reverse (ICR6, TGAGTCAGTTCTCTAGGCGAGCAG, antisense sequence, 49 nucleotides downstream of exon 1C) primer were combined in 12.5 µL of PCR buffer containing 0.2 mmol per L dNTP and 0.25 U of *Taq* polymerase (Promega, Madison, WI). The PCR program included 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 1 minute at 72°C. The 1073-bp PCR products were subjected to digestion by *Tfi*I restriction endonuclease and then analyzed by 1.5 percent agarose gel electrophoresis.

Functional analysis of the enzyme encoded from the *IGnTC* cDNA

Total RNA samples from the adult i case and a common I individual were prepared from their peripheral blood cells with the QIAamp RNA blood mini kit (Qiagen). The first-strand cDNAs were primed with oligo(dT) primer and synthesized by reverse transcriptase (SuperScrip III, Invitrogen). The cDNA fragment encompassing the region of nucleotides 76 to 1206, which encodes the amino acid residues 26 to 402, of the *IGnTC* gene was amplified by PCR as described previously[29] and inserted into the mammalian expression vector pSecTaq2A (Invitrogen), which is designed for the secretion of the expressed protein into cell culture medium. The expression vectors bearing wild-type *IGnTC* and mutant *IGnTC*243 A* cDNAs were selected. The constructed and mock pSecTaq2A plasmids were prepared with a plasmid kit (EndoFree, Qiagen) for transfection. Expression of the constructed and mock plasmids in COS-7 cells, and the subsequent GlcNAcT assay were performed as has been described previously.[22]

RESULTS

Missense mutation of 243T>A identified in *IGnTC* gene of the adult i individual

The coding regions of the five exons of the *IGnT* gene of the adult i individual were PCR-amplified and cloned, and the sequences were determined. Multiple clones from the exon 1A, 1B, 2, and 3 regions were analyzed, and all were found to have wild-type sequences; however, all five clones from the exon 1C region were found to possess a T-to-A nucleotide substitution at position 243. The wild-type sequences were also demonstrated from direct sequencing of the PCR products encompassing the respective exon 1A, 1B, 2, and 3 regions. Direct sequencing of the PCR product encompassing the exon 1C region demonstrated the homozygous state of the 243T>A substitution (Fig. 2). Taken together, these results suggest that the adult i individual is most likely to have wild-type *IGnTA* and *IGnTB* genes and be homozygous for the 243T>A mutation in the *IGnTC* gene. The possibility that the individual possesses only one chromosome with the mutant *IGnTC*243A* allele and another with the *IGnT* locus deleted, however, cannot be totally excluded. The 243T>A nucleotide change predicts an amino acid

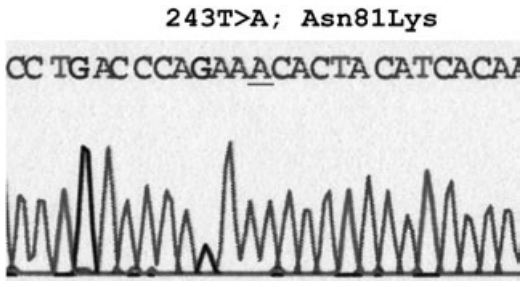

Fig. 2. Sequencing result of the exon 1C region of our adult i Taiwanese person. The exon 1C region of the *IGnT* gene of the adult i individual was amplified by PCR and the sequence was analyzed by direct sequencing. The homozygous state of the T-to-A nucleotide change (underlined) at position 243 is demonstrated. The 243T>A substitution predicts the amino acid alteration of Asn81Lys in the encoded IGnTC enzyme.

alteration of Asn to Lys at residue 81 in the encoded IGnTC enzyme.

The exon 1C region of the i adult's daughter, who was typed as the common I phenotype, was analyzed by direct sequencing after PCR amplification. The result showed heterozygous status of T and A nucleotides at position 243 (data not shown), suggesting that she is a heterozygote bearing the wild-type *IGnTC* and mutant *IGnTC***243A* alleles.

The 243T>A mutation in the *IGnTC* gene is uncommon in the general population

The 243T>A mutation identified in the *IGnTC* gene of the adult i case destroys a *Tfi*I site and, thus, a PCR-RFLP analysis was developed to detect the mutation. The 1073-bp PCR product, which encompasses the exon 1C region and was amplified from the wild-type *IGnTC* allele, was cleaved into 585-, 316-, and 172-bp fragments by *Tfi*I digestion, while that amplified from the mutant *IGnTC* allele with the 243T>A mutation was cleaved into 585- and 488-bp fragments. The PCR-RFLP method was employed to inspect the incidence of the mutation in the general Taiwanese population. As shown in Fig. 3, the adult i case was homozygous for the 243T>A mutation (Fig. 3, Lane 1) from PCR-RFLP analysis, whereas her daughter was a heterozygote with one *IGnTC***243A* allele (Fig. 3, Lane 2). None of the *IGnTC* alleles of the 58 randomly selected individuals demonstrated the mutation (one of the results is shown in Fig. 3, Lane 3). This demonstrates that the *IGnTC* allele with the 243T>A mutation is infrequent in the general population and agrees with the observed rarity of the adult i phenotype.

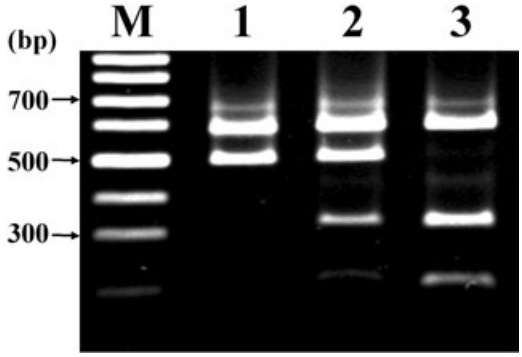

Fig. 3. PCR-RFLP analysis to demonstrate the *IGnTC*243A* allele. The 243T>A mutation identified in the *IGnTC* gene of the adult i individual destroys a *Tfi*I recognition sequence. The 1073-bp PCR product amplified from the exon 1C region with wild-type 243T nucleotide was cleaved into 585-, 316-, and 172-bp fragments by *Tfi*I digestion, whereas that amplified from the mutant *IGnTC* allele with the 243T>A mutation was cleaved into 585- and 488-bp fragments. The *Tfi*I-cleaved products were analyzed by 1.5 percent agarose gel electrophoresis. Samples from the adult i individual (Lane 1) and her daughter (Lane 2), together with samples from 58 randomly selected individuals were subjected to PCR-RFLP analysis. One of the results obtained from the random individuals is shown (Lane 3). Lane M = molecular mass standards of the 100-bp ladder.

Enzyme activity of the I β6GlcNAcTs encoded from the IGnTC*243 A cDNA

The effect of the Asn81Lys change, resulting from the 243T>A mutation, on the enzyme activity of I β6GlcNAcT was tested. Table 1 lists the amounts of GlcNAc transferred to the acceptor substrate, LS-tetrasaccharide c (NeuNAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc), from the donor substrate UDP-GlcNAc by the medium concentrates harvested from the cells transfected with the respective expression vectors. The enzyme expressed from the wild-type *IGnTC* cDNA displayed GlcNAc-transferring activity. In contrast, only residual activity was observed in the enzyme encoded from the mutant *IGnTC*243A* cDNA. This result indicates that the Asn81Lys alteration in IGnTC β6GlcNAcT significantly reduces the original GlcNAcT activity.

DISCUSSION

In this present study, a novel *IGnTC* gene with a 243T>A missense mutation was identified in a Taiwanese person with the adult i phenotype but without congenital cataracts. The mutation predicts the amino acid alteration of Asn81Lys in the encoded IGnTC enzyme. Molecular genetic analyses demonstrated that this i adult possesses wild-type *IGnTA* and *IGnTB* genes and is homozygous for the *IGnTC***243A* mutant allele. The population screening and enzyme function assay strongly suggest that this *IGnTC***243A* mutant allele is responsible for formation of the adult i phenotype.

It has been demonstrated that the human *I* locus expresses three *IGnT* forms, *IGnTA*, *IGnTB*, and *IGnTC*, which have different exon 1, but identical exon 2 and exon 3, coding regions.[29,30] Based on the observed presence of normal quantities of I antigen in the milk, plasma,

TABLE 1. The GlcNAcT activities of the enzymes encoded from the wild-type *IGnTC* and mutant *IGnTC* 243A* cDNAs*

	Vector	*IGnTC*	*IGnTC *243A*
GlcNAc transferred (pmol)	19.0 ± 4.7	76.4 ± 22.6	24.3 ± 7.9

* The results of the mean and standard deviation of four tests are shown. Endogenous transfer of GlcNAc in the absence of acceptor substrate was corrected for each test. The amounts of the transferred GlcNAc in the vector control indicate the background levels of the assay.

and saliva of i adults,[32,33] it has been proposed that different I-branching enzymes may be responsible for the I-antigen synthesis in different tissues. The molecular genetics of the human *I* locus and the differential expression profiles for the tissue-specific *IGnT* gene forms support this proposition and provide a new view of the formation and expression of the I antigen. Our previous investigation of the two adult i groups (with and without cataracts) has shown that mutation in the *IGnTC* gene, leading to a defective IGnTC enzyme, is associated with the adult i phenotype, and it was demonstrated that *IGnTC* is the only one of the three *IGnT* genes expressed in the reticulocytes.[29] Further, it was demonstrated that *IGnTB* is the only one of the three *IGnT* genes expressed in the human lens-epithelium cells. These results demonstrate that the exact gene form responsible for the expression of the blood group I antigen on RBCs is the *IGnTC*. More interestingly, the results obtained from our molecular analysis of the two adult i groups suggest a molecular genetic mechanism that accounts for the partial association between the adult i phenotype and congenital cataracts. A defect in *IGnTC* gene function leads to the adult i phenotype, whereas congenital cataracts occur in those i adults who have mutations in the common exon 2 to 3 region, which lead to defects in all three IGnT enzyme functions, but not in analogs who only have defects in the *IGnTC* form. This proposed molecular genetic mechanism suggests that a defect in the *I* locus may lead directly to the development of congenital cataracts.

To date, seven different mutations, including six nonsynonymous point mutations and one deletion, have been identified in the *IGnT* alleles of individuals with the adult i phenotype. The six point mutations include 505G>A and 683G>A (locating exon 1C, identified in white persons without cataracts),[29] 983G>A (locating exon 2, identified in Arab persons with cataracts),[31] 1006G>A (locating exon 2, identified in Japanese persons with cataracts),[30] 1049G>A (locating exon 3, identified in Japanese and Taiwanese persons with cataracts),[22,29,30] and 1154G>A (locating exon 3, identified in Taiwanese persons with cataracts).[22,29] The deletion is a loss of a large chromosomal segment encompassing the region from exon 1B through exon 3 (identified in Taiwanese persons with cataracts).[22,29] These analyzed adult i individuals belong to four different populations, with the revealed molecular genetic bases agreeing with the proposed molecular genetic mechanism accounting for the partial association between the adult i phenotype and congenital cataracts. To date, seven cases with the adult i phenotype but without congenital cataracts have been analyzed (six reported in our previous article[29] and one analyzed in our laboratory subsequently), with all seven cases belonging to the white population. Carriage of the wild-type *IGnTA* and *IGnTB* genes but double-dose mutation of 505G>A or 683G>A in the *IGnTC* gene was demonstrated in these individuals. The molecular genetic analysis of our adult i Taiwanese person reveals that a novel *IGnTC* mutant allele is responsible for the adult i phenotype and adds one more example of i adults without congenital cataracts possessing wild-type *IGnTA* and *IGnTB* genes and a defective *IGnTC* gene only.

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