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ORIGINAL ARTICLE

Analysis of the *CYP21A2* Gene with Intergenic Recombination and Multiple Gene Deletions in the RCCX Module

Shwu-Fen Chang[1] and Hsien-Hsiung Lee[2]

The most frequent bimodular RCCX module of the *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB* gene sequence is located on chromosome 6p21.3. To determine RCCX alterations, we used the polymerase chain reaction (PCR) product containing the tenascin B (*TNXB*) and *CYP21A2* genes with *Taq*I digestion and Southern blot analysis with *Ase*I and *Nde*I endonuclease digestion of genomic DNA from congenital adrenal hyperplasia patients with common mutations resulting from an intergenic conversion of *CYP21A1P*, such as an I2 splice, I172N, V281L, F306-L307insT, Q318X, and R356W, and dual mutations of I236N/V237E in the *CYP21A2* gene. The results showed that a 3.7-kb fragment of the *CYP21A2* gene was detected in each case, and 21.6- and 11.3-kb DNA fragments were found in the RCCX region by a Southern blot analysis with these corresponding mutations. However, the IVS2-12A/C->G (I2 splice) haplotype in combination with the 707–714delGAGACTAC (without the P30L mutation) mutation produced a 3.2-kb *Taq*I fragment in the PCR product analysis and a specific 9.3-kb fragment by the Southern blot method. Therefore, we concluded that the rearrangement in the RCCX region resulting from processing of either an intergenic recombination or multiple gene deletions can be identified by the PCR analysis and Southern blot method based on a fragment-distinguishing configuration without a family study.

Introduction

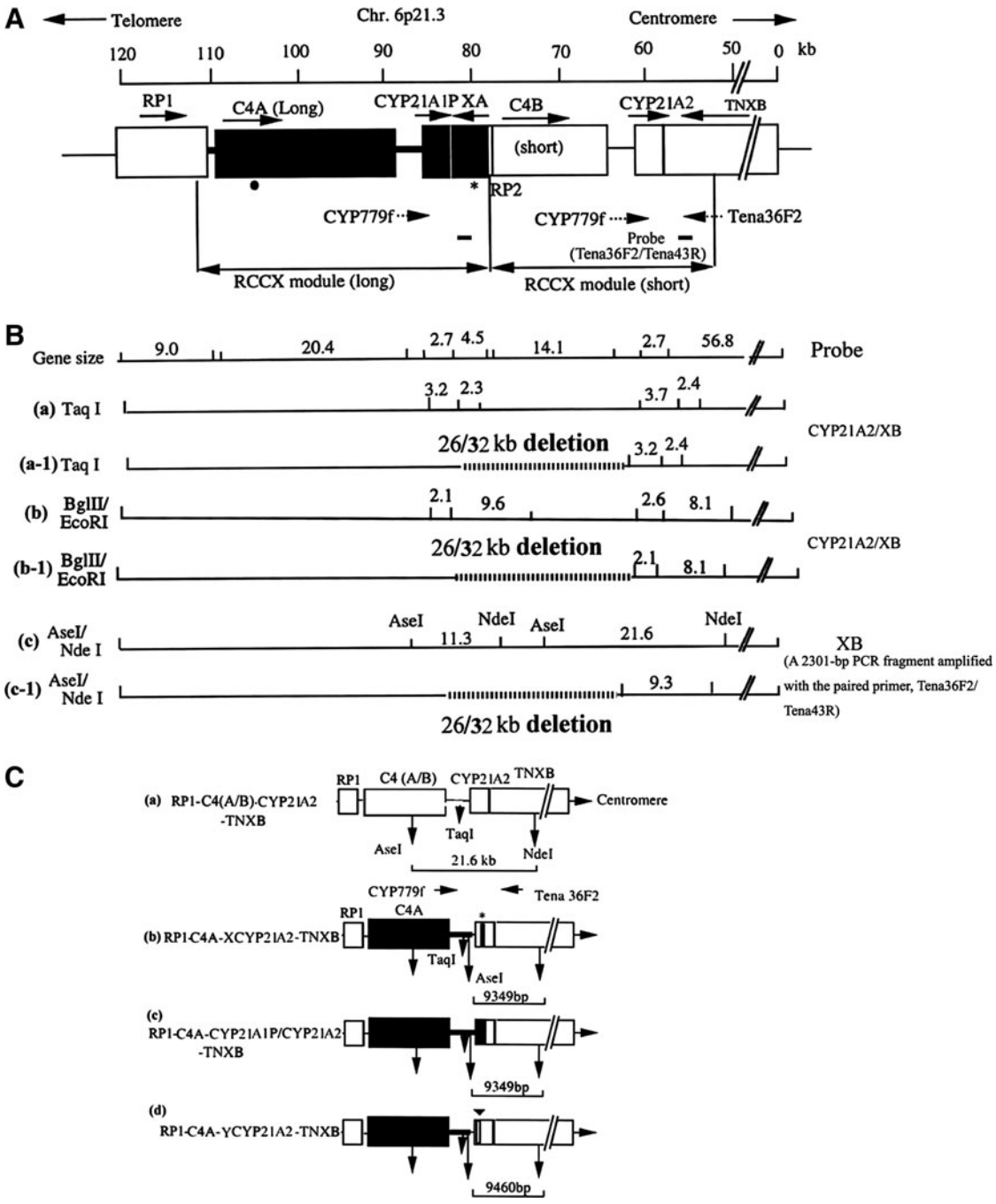
More than 90% of congenital adrenal hyperplasia (CAH) cases are caused by mutations of the *CYP21A2* gene. To date, 133 different *CYP21A2* mutations have been reported (Human Gene Mutation Database, 2010). Among them, 15 mutations (Lee, 2001) identified so far are considered to be a result of the processing of an intergenic recombination of DNA sequences from the neighboring duplicate *CYP21A1P* gene. The other 92 mutations are believed to be spontaneous mutations. The *CYP21A2* and *CYP21A1P* genes are located on chromosome 6p21.3 and are adjacent to and alternate with the *C4A* and *C4B* genes that encode the fourth component of serum complement (White *et al.*, 1984). This region is composed of a portion of the *RP* gene (serine/threonine nuclear protein kinase) (Shen *et al.*, 1994), a full-complement *C4* gene (Shen *et al.*, 1994), a full *CYP21A2* (*CYP21A1P*) gene, and a portion of the *TNX* gene (Gitelman *et al.*, 1992; Bristow *et al.*, 1993). The C4 protein is coded by two genes, *C4A* and *C4B*. The occurrence of a long (20.4 kb) or a short *C4* gene (14.1 kb) is due to the presence of an endogenous retroviral sequence (of 6.7 kb), named HERV-K (C4), in intron 9 (Yu, 1991). The *TNX* gene contains *XA* (*TNXA*) and *TNXB*. *TNXB*, located downstream of the *CYP21A2* gene, is partially duplicated in the downstream *CYP21A1P* gene, where a truncated gene exists, termed *TNXA*. Both *TNXA* and *TNXB* are transcribed on the opposite strand. There are two *RP* genes, *RP*1 and *RP*2. The *RP*2 gene is truncated and corresponds to *RP*1 adjacent to *TNXA*. These genes are arranged in the most frequent (~69%) bimodular RCCX module of the *RP1-C4-CYP21A1P-XA-RP2-C4-CYP21A2-TNXB* gene sequence in Caucasians (Blanchong *et al.*, 2001) (Fig. 1A). Therefore, the bimodule may consist of a long module including part of *RP1*, *C4* (long), *CYP21A1P*, and *TNXA* and a short one containing *RP2*, *C4* (short), *CYP21A2*, and part of the *TNXB* gene (Koppens *et al.*, 2002) (Fig. 1A). In ethnic Chinese (i.e., Taiwanese), 47% of *C4* genes adjacent to the *RP2* gene are the short gene and 53% are the long gene (Lee *et al.*, 2006a). All of the *C4* genes adjacent to the *RP1* gene examined were the long gene (Lee *et al.*, 2006a). In addition, 87% and 85% of the *C4* genes adjacent to the *CYP21A1P* and *CYP21A2* genes, respectively, carried the *C4A* and *C4B* genes (Lee *et al.*, 2006d). The RCCX module has three possible forms: monomodular, bimodular, and trimodular. Chromosomes with four RCCX modules are very rare (Blanchong *et al.*, 2000).

Examining gene deletions and conversions in the RCCX module has traditionally used the gold standard of the Southern blot method with a single restriction digestion of

[1]Graduate Institute of Cell and Molecular Biology, Taipei Medical University, Taipei, Taiwan.
[2]School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan.

*Taq*I [Fig. 1B(a)] or double digestions of *Bgl*II/*Eco*RI [Fig. 1B(b)]. However, those analyses are based on the absence or presence of fragments seen with hybridization using a probe (or probes) (Fig. 1B), leading to two problems. First, a heterozygously deleted *XA-RP2-C4B* allele [Fig. 1B(a-1)] cannot be detected in the presence of a normal allele (Morel and Miller, 1991). Second, it is possible that an allele with the trimodular *RP1-C4-CYP21A1P-XA-RP2-C4-CYP21A1P-XA-RP2-C4-CYP21A2-TNXB* sequence can interfere with the identification of a bimodule with deletion of *XA-RP2-C4B* (Morel and Miller, 1991). Therefore, a family study should be used in these two cases to deduce the lack of an unusual configuration of the RCCX module (Gitelman *et al.*, 1992; Bristow *et al.*, 1993; Shen *et al.*, 1994; Yang *et al.*, 1999), because a healthy carrier cannot independently be distinguished from a normal individual. To overcome these disadvantages, we developed a Southern blot method based on a "fragment-distinguishing" configuration (Lee *et al.*, 2003a) using *Ase*I and *Nde*I restriction endonuclease digestions, which produced two fragments of 21.6 and 11.3 kb from the genome of normal individuals. This analysis can examine the interchange region between *RP1-CYP21A1P-XA-RP2-C4-CYP21A2-TNXB* gene loci and is free from the aforementioned interference.

In this study, we analyzed the *CYP21A2* allele with a single mutation from *CYP21A1P* sequences (containing 11 common mutations), such as an I2 splice (nt 655 or IVS2-12A/C > G),



I172N, V281L, F306-L307insT, R316X (Lee *et al.*, 1998), Q318X, and R356W, or dual mutations of I236N and V237E (Lee *et al.*, 2006b) and others in Taiwanese CAH patients, using a 6.2-kb polymerase chain reaction (PCR) product containing the partial *TNXB* and entire *CYP21A2* genes (Lee *et al.*, 2003c, 2006c) and a fragment-distinguishing Southern blot analysis (Lee *et al.*, 2003a) to examine whether these mutated alleles influenced the arrangement of the RCCX region.

Materials and Methods

We collected data on families with CAH patients who were discovered during a concerted search among hospitals throughout Taiwan (Lee *et al.*, 2008). All families requested an extensive molecular diagnosis and provided informed consent. The study strictly followed the guidelines of the institutional review boards of the hospitals involved.

To examine the status of the *CYP21A2* gene, a 6.2-kb genomic fragment in which the *CYP21A2* and *TNXB* genes were juxtaposed was amplified with the primers, CYP779f and Tena36F2 (Lee *et al.*, 2003c, 2006c), in a PCR (Fig. 1A). Primer CYP779f (5′-AGGTGGGCTGTTTTCCTTTCA-3′) is located at the 5′ end of the *CYP21A1P* and *CYP21A2* genes (nt 87,443–87,463; GenBank accession no. AL049547), and the Tena36F2 (5′-AGGCGCTCGCTATGAGGTGAC-3′) primer is located in exon 36 of the *TNXB* gene (nt 81,255–81,275; GenBank accession no. AL049547), which contains a 121-bp deletion of the *tenascin* A gene (*TNXA*) (Gitelman *et al.*, 1992). For mutational detection of the *CYP21A2* gene, the 6.2-kb PCR product was used as a template for the secondary PCR amplification in the amplification-created restriction site (ACRS) primer analysis (Lee *et al.*, 1996). The ACRS primer pairs and reaction conditions, including P30L, the I2 splice (IVS2-12A/C > G), I172N, the E6 cluster, V281L, F306-307insT, R316X (Lee *et al.* 1998), Q318X, and R356W, were described previously (Lee *et al.*, 1996). The R483P mutation was determined by DNA sequencing using the PCR product amplified with the S10 primer pair as described previously (Lee *et al.*, 1998). To examine the status of the *CYP21A2* gene, the 6.2-kb PCR products were digested with 10 units of *Taq*I enzyme in a 10 μL volume at 65°C for 2 h (Lee *et al.*, 2003c) and analyzed by electrophoresis on 0.65% agarose gels.

For the fragment-distinguishing Southern blot analysis, genomic DNA was digested with *Ase*I and *Nde*I restriction endonucleases and then resolved on a 0.65% agarose gel. The protocols used for Southern blotting and the subsequent hybridization process were all as described in a previous study (Lee *et al.*, 2003a). A 2301-bp fragment (nt 81,255–83,526; GenBank accession no. AL049547) was amplified with the paired primers, Tena36F2/Tena43R. Tena43R (5′-CTCCCTTC CTGACCCTCCGCT-3′) is located in the *TNXB* and *TNXA* genes (nt 83,506–83,526; GenBank accession no. AL049547) (Fig. 1A). The fragment covering the *TNXB* or *TNXA* gene was labeled with ^{32}P and used as a probe for the Southern hybridization analysis.

Results

To investigate intergenic recombinations and multiple gene deletions that occurred within the RCCX module in CAH patients, a 6.2-kb PCR fragment amplified with the allele-specific paired primers of CYP779f and Tena36F2 (Fig. 1A) was obtained from all CAH patients including normal individuals ($n > 200$ chromosomes) (only one sample is shown here) (Fig. 2A, lane UC). After *Taq*I [T/CGA] digestion (2 *Taq*I sites at nt 87,404 and 83,662; GenBank accession no. AL049547) (Lee *et al.*, 2006c), the 6.2-kb PCR product produced three fragments of 3740, 2410, and 60 bp (the last one of which ran out of the gel) from one of the normal individuals (Fig. 2A, lane N). The 2410-bp fragment was derived from sequences containing the *TNXB* gene (nt 83,662–81,225; GenBank accession no. AL049547), whereas the 3740-bp fragment represented the *CYP21A2* gene. In an analysis of a CAH patient with *CYP21A2* mutations of an I2 splice in one chromosome (identified by the ACRS method; data not shown) and R483P (identified by sequencing; data not shown) in the other, the 6.2-kb PCR product produced three fragments of 3740, 2410, and 60 bp (the last one of which ran out of

FIG. 1. **(A)** Bimodular form (*RP1-C4-CYP21A1P-XA-RP2-C4-CYP21A2-TNXB*) of the RCCX region of chromosome 6p21.3. The white box represents the structure of the wild-type gene, whereas the black box represents a nonfunctional gene (*CYP21A1P*, *XA*, and *RP2* genes) or a different functional gene (*C4A*). Sizes of the genes (in **B**) from the ATG start codon to the TGA stop codon, including *RP1*, *C4A*, *CYP21A1P*, *XA*, *RP2*, *C4B*, *CYP21A2*, and *TNXB*, in the RCCX module of the figure are based on the sequences in GenBank with accession nos. AL049547 and AF019413. The presence of *C4A* (the long gene of 20.4 kb) or *C4B* (the short gene of 14.1 kb) depends on the presence of the endogenous 6.7-kb retroviral sequence, HERV-K (C4), in intron 9 (indicated by ●). The bimodular form in the *C4-CYP21* area, consisting of long and short RCCX modules, is also shown. Solid arrows indicate the orientation of transcription, and dashed arrows show the locations of the primers (Tena36F2/CYP779f) for the 6.2-kb PCR amplification. A 121-bp deletion in exon 36 of the *XA* gene is marked with an asterisk (*). Top: Scale in kb, with the *TNXB* gene starting at 0. **(B)** Strategies for identification of the *CYP21A2* gene by the gold standard of the Southern blot method digested with *Taq*I (a), double digestions of *Bgl*II/*Eco*RI (b), and the fragment-distinguishing Southern blot method using *Ase*I/*Nde*I double digestions (c) probed with different gene sequences. Representations of the RCCX region with deletion of *XA-RP2-C4B* loci analyzed by these two Southern methods are shown in (a-1), (b-1), and (c-1), respectively. The dashed line (26/32 kb long) indicates the area of *XA-RP2-C4B* in the RCCX region. The Southern blot analysis based on a fragment-distinguishing configuration using *Ase*I and *Nde*I digestion of genomic DNA indicates two fragments of 11.3 and 21.6 kb for normal individuals and a 9.3-kb fragment produced by a deletion of *XA-RP2-C4B* loci in the RCCX region. Hybridization used the probe consisting of the 2301-bp PCR product derived from the paired primers, Tena36F2/Tena43R. The probe is indicated by solid bars in **A**. **(C)** Representation of four sequence organizations of the RCCX module with multiple gene deletions: (a) *RP1-C4(A/B)-CYP21A2-TNXB*, (b) *RP1-C4A-XCYP21A2-TNXB*, (c) *RP1-C4A-CYP21A1P/CYP21A2-TNXB*; (d) *RP1-C4A-YCYP21A2-TNXB*. Mutation of the IVS-12A/C > G in combination with 707–714delGAGACTAC (without the P30L mutation) in the *CYP21A2* gene (*XCYP21A2*) is indicated by an asterisk (*). The chimeric *CYP21A1P/CYP21A2* gene is indicated by a half-white, half-black box. Duplication of the 111-bp fragment (W21-P57dup) in exon 1 of the *CYP21A2* gene is indicated by a black inverted triangle (▼). PCR, polymerase chain reaction.

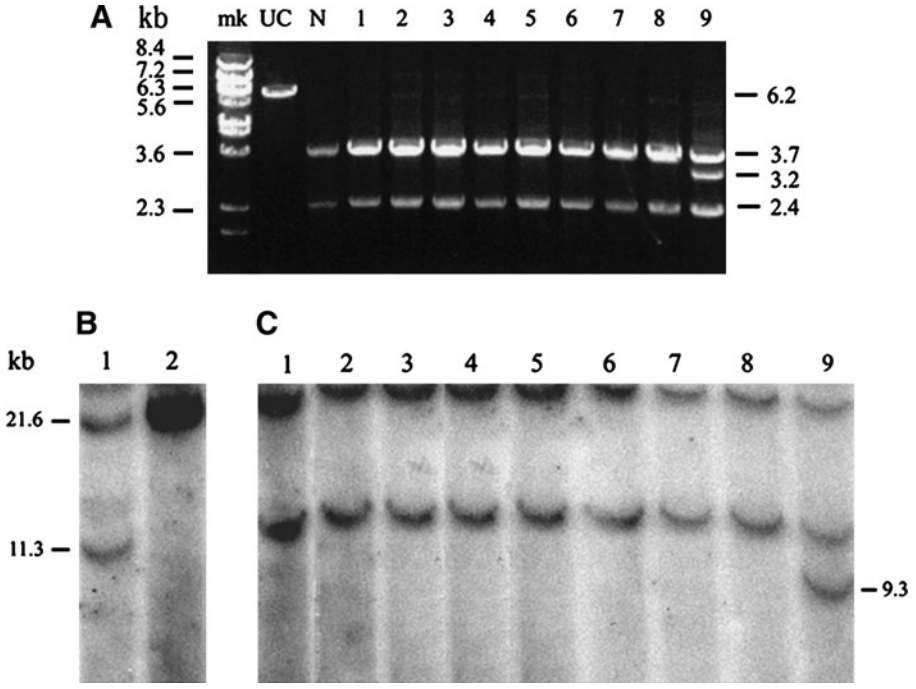


FIG. 2. **(A)** Analysis of the 6.2-kb PCR product by *Taq*I digestion on a 0.65% agarose gel. Lane UC is the 6.2-kb PCR product amplified with the paired primers, Tena36F2/CYP779f, from a normal individual. Lane N is the 6.2-kb PCR product digested by *Taq*I from a normal individual, and lanes 1–5 are from CAH patients with mutations of IVS2-12A/C > G/R483P, I172N/R316X, I172N/Q318X, R356W/F306-307insT, and I172N/I236N and V237E, respectively. Lanes 6–8 are from CAH carriers with V281L, H392C-V397ins, and S329-R333Cdel in one chromosome, respectively. Lane 9 is from a CAH carrier with dual mutations of IVS2-12A/C- > G in combination with 707–711del-GAGACTAC (without the P30L mutation). "mk" is a Lambda DNA-*Bst*EII-digested marker (New England BioLabs). **(B)** Southern blot analysis of *Ase*I and *Nde*I digestion of genomic DNA. Hybridization used the probe consisting of the 2301-bp PCR product derived from the paired primers, Tena36F2/Tena43R. Lane 1 shows the genome of a normal individual who had two fragments of 11.3 and 21.6 kb, and lane 2 shows the genome of a normal individual who had one fragment of 21.6 kb. **(C)** Southern blot analysis of genomes of CAH patients with the mutations of IVS2-12A/C > G/R483P in lane 1, I172N/R316X in lane 2, I172N/Q318 X in lane 3, R356W/F306-307insT in lane 4, and I172N/I236N and V237E in lane 5. Lanes 6–8 are genomes from CAH carriers with mutations of V281L, H392C-V397ins, and S330-R333Cdel in one chromosome, respectively. Lane 9 is genome from a CAH carrier with the dual mutations of IVS2-12A/C- > G in combination with 707–711delGAGACTAC (without the P30L mutation). CAH, congenital adrenal hyperplasia.

the gel) (Fig. 2A, lane 1). Further, the 6.2-kb PCR fragment amplified from CAH patients with compound heterozygosity of I172N/R316X (identified by the ACRS method; data not shown) (Fig. 2A, lane 2), I172N/Q318X (identified by the ACRS method; data not shown) (Fig. 2A, lane 3), R356W/F306-307insT (identified by the ACRS method; data not shown) (Fig. 2A, lane 4), and I172N/I236N and V237E mutations (identified by the ACRS method; data not shown) (Lee *et al.*, 2006b) (Fig. 2A, lane 5) also generated three fragments of 3740, 2410, and 60 bp (the last one of which ran out of the gel) from two defective *CYP21A2* alleles. In addition, the 6.2-kb PCR fragment amplified from CAH carriers with a mutation at V281L in one chromosome (identified by the ACRS method; data not shown) (Fig. 2A, lane 6) or the mutations of H392C-V397ins (Lee *et al.*, 1998) (Fig. 2A, lane 7) and S329-R333Cdel (Lee *et al.* 1998) (Fig. 2A, lane 8) produced three fragments of 3740, 2410, and 60 bp (the last one of which ran out of the gel) from one mutated *CYP21A2* allele. However, the *CYP21A2* gene in a CAH carrier with dual mutations of IVS2-12A/C- > G in combination with 707–711delGAGACTAC (without the P30L mutation) in one chromosome (Lee *et al.*, 2003c) generated two extra fragments of 3207 and 533 bp (*Taq*I site at nt 86,870; GenBank accession no. AL049547) (Lee *et al.*, 2006c), in addition to the 3740-, 2410-, and 60-bp DNA fragments (Fig. 2A, lanes 9) (both the 533- and 60-bp fragments ran out of the gel). Accordingly, the appearance of the 3740-bp fragments allele(s) either in the normal individual (Fig. 1A, lane N) or in CAH patients (Fig. 1A, lanes 1–8) indicates that the juxtaposed sequence of the *CYP21A2* and *TNXB* genes within the RCCX module is not impaired and maintains the order of *RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB* (Fig. 1A) (Table 1), whereas a 3207-bp fragment of the *CYP21A1P*-like gene detected within the *CYP21A2* region implies that the defective allele with dual mutations of IVS2-12A/C- > G in combination with 707–711delGAGACTAC (without the P30L mutation) exists in the *XCYP21A2* structure, and genes within this module were organized in the order of *RP1-C4A-XCY-P21A2-TNXB*, Fig. 1B(a-1)], which may have resulted from multiple gene deletions of the *XA-RP2-C4* sequence [a 26/32-kb deletion; Fig. 1B(a-1)] (Lee *et al.*, 2003c). Therefore, the appearance of the 3740-bp fragment presenting the intact RCCX and the 3207-bp fragment presenting an impaired RCCX by the PCR method may be used as an indicator to examine the configuration of the rearrangement in the RCCX region. We also used the PCR amplification product to analyze Taiwanese CAH patients ($n = 396$ chromosomes; Table 2).

To further validate the observed genomic arrangement that occurred within the RCCX module in CAH patients as analyzed by the PCR method, a fragment-distinguishing Southern blot analysis was used to investigate the alternative genomic organization. According to the genome sequences among *CYP21A1P*, *TNXA*, *RP2*, *C4B*, *CYP21A2*, and *TNXB* (Fig. 1A) from GenBank (accession nos. AF019413 and AL049547) (Fig. 1A), two DNA fragments of 21.6 and 11.3 kb [Fig. 1B(c)] were generated from the genome of normal indi-

Table 1. Status of the Proposed RCCX Module in *CYP21A2* Deficiency Caused by Intergenic Recombination and Multiple Gene Deletions

Mutation locus	TaqI *fragment (kb)*	AseI/NdeI *fragmen t (kb)*	*Sequence of the RCCX module*
Normal individual A	3.7	21.6 + 11.3	*RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB*
Normal individual B	3.7	21.6	*RP1-C4A/B-CYP21-TNXB*
Intergenic recombination	3.7	21.6 + 11.3	*RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB*
I2 splice			
I172N			
I236N,V237E[a]			
V281L			
Q318X			
R356W			
F306-L307insT			
Spontaneous mutation	3.7	21.6 + 11.3	*RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB*
H62L			
IVS2 + 1G > A[b]			
R316R[b]			
S329-R333delC[b]			
H392C-V397ins[b]			
Multiple gene deletions			
Chimera *CYP21A1P/CYP21A2*[c]	3.2	9.3	*RP1-C4A-CYP21A1P/CYP21A2-TNXB*
IVS2–12A/C- > G combined with 707-714delGAGACTAC (without the P30Lmutation)[d]	3.2	9.3	*RP1-C4A-XCYP21A2-TNXB*
W21-P57 duplication[e]	3.3	9.4	*RP1-C4A-YCYP21A2-TNXB*

[a]Based on Lee *et al.* (2006b).
[b]Based on Lee *et al.* (1998).
[c]Based on Lee (2004).
[d]Based on Lee *et al.* (2003c).
[e]Based on Lee *et al.* (2003b).

viduals after cleavage with the *Ase*I [AT/TAAT] and *Nde*I [CA/TATG] restriction endonucleases detected by the Southern blot analysis when probed with a fragment of the *TNXA* and *TNNB* sequences. The 21.6-kb fragment contained sequences spanning exon 16 of the *C4B* gene (*Ase*I, nt 99,188; GenBank accession no. AL049547), the intergenic sequences between the *C4B* and *CYP21A2* genes, the entire *CYP21A2* gene, and the end of the *TNXB* gene at intron 31 (*Nde*I, nt 77,510; GenBank accession no. AL049547). The 11.3-kb fragment was composed of sequences upstream of the *CYP21A1P* gene (*Ase*I, nt 86,855; GenBank accession no. AL049547) to exon 8 of the *C4B* genes (*Nde*I, nt 101,868; GenBank accession no. AL049547), which includes a truncated *TNXA* gene (4.5 kb) and the *RP2* gene (913 bp) [Fig. 1B(c)]. From the Southern blot analysis, these two fragments were detected in most samples from healthy individuals (Fig. 2B, lane 1) ($n > 50$ chromosomes; data not shown) (only one sample is shown here). However, the genome from another healthy individual showed only the 21.6-kb fragment without the 11.3-kb one in two alleles (Fig. 2B, lane 2). Genomes of CAH patients with mutations of an I2 splice/R483P (Fig. 2C, lane 1), I172N/R316X (Fig. 2C, lane 2), I172N/Q318X (Fig. 2C, lane 3), R356W/F306-307insT (Fig. 2C, lane 4), I172N/I236N, and V237E (Fig. 2C, lane 5) were detected as having both 21.6- and 11.3-kb fragments. In addition, genomes of CAH carriers with a mutation in one chromosome of V281L (Fig. 2C, lane 6), H392C-V397ins (Fig. 2C, lane 7), and S329-R333Cdel (Fig. 2C, lane 8) also displayed 21.6- and 11.3-kb fragments, whereas an additional 9.3-kb fragment (Fig. 2C, lanes 9) was detected

Table 2. *CYP21A2* Analysis of Taiwanese Congenital Adrenal Hyperplasia Patients Using the 6.2-kb Polymerase Chain Reaction Product with *Taq*I Digestion

Mutation locus	*Chromosome*
(a) Intergenic recombination	
I2 splice	136
I172N	94
707-714del	1
I236N and V237E	3
I236N,V237E and M239k	1
F306-L307insT	8
Q318X	25
R356W	47
(b) Spontaneous mutation	
H62L	7
IVS2 + 1G > A	5
W21-P57dup	5
C201W	1
V304M	1
R316R	3
S329-R333delC	1
H392C-V397ins	1
(c) Multiple gene deletions (or unequal cross-over recombination)	
IVS2-12A/C > G in combination with 707-714del (without the P30L mutation)	26
Chimeric *CYP21A1P/CY21A2 (CH-1)*[a]	1
Chimeric *CYP21A1P/CY21A2 (CH-2)*[a]	2
Chimeric *CYP21A1P/CY21A2 (CH-3)*[a]	1
Total	369

[a]Based on Lee (2005).

from a CAH carrier in one of the chromosomes with dual mutations of IVS2-12A/C->G in combination with 707–711delGAGACTAC (without the P30L mutation). The above data indicate that gene arrangements in the RCCX module may be diverse in normal individuals in either the *RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB* sequence of the bimodule (Fig. 1A) or the *RP1-C4A/B-CYP21A2-TNXB* sequence of the monomodule [Fig. 1C(a)]. The fragment of 21.6 kb carrying the *CYP21A2* gene is an essential genetic indicator of healthy individuals. In addition, the defective *CYP21A2* allele(s) with mutations including I2 splice, I172N, V281L, Q318X, R356W, F306-307insT, and E6 cluster (I236N and V237E) or the spontaneous mutations of R316X, H392C-V397ins, and S329-R333Cdel showing the 21.6-kb fragments in the Southern blot analysis indicate a lack of disruption of the *RP1-C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB* sequence as in most of the healthy individual alleles. The appearance of the 9.3-kb fragment, a defective *CYP21A2* allele with a 26/32-kb sequence deletion (a short C4 gene or a long C4 gene) [Fig. 1B(c-1)], resulted from disruption of the RCCX region by multiple gene deletions to become the proposed *RP1-C4A-XCYP21A2-TNXB* genomic arrangement [Fig. 1C(b)] as in the case of IVS2-12A/C->G in combination with the 707–711delGAGACTAC mutation (without the P30L mutation).

Discussion

The most frequent mutations of *CYP21A2* deficiency in ethnic Chinese (i.e., Taiwanese) are the I2 splice (nt 655, IV2-12A/C>G), I172N, and R356W (Table 2), which show high similar incidences worldwide in different races (White and Speiser, 2000; Dain *et al.*, 2002; Stikkelbroeck *et al.*, 2003). The spontaneous mutations of IVS2+1G>A, R316X, S329-R333delC, and H392C-V397ins (Table 1) were unique in our CAH population (Human Gene Mutation Database). The H62L mutation producing a nonclassical form (Speiser, 2008) was also present in Brazilian and Scandinavian patients (Soardi *et al.*, 2008), and the V281L mutation, the most common nonclassical disease appearing in high frequencies in patients in France, Austria, Italy, Spain, Turkey, Argentina, and Portugal (Dain *et al.*, 2002; Stikkelbroeck *et al.*, 2003; Friaes *et al.*, 2006), was not found in Japanese (Koyama *et al.*, 2002), Taiwanese (Tsai *et al.*, 2009), or Tunisian patients (Kharrat *et al.*, 2004). At present, six types of chimeric *CYP21A1P/CYP21A2* genes exist in different populations (Concolino *et al.*, 2009). Interestingly, the haplotype of IVS2-12A/C>G in combination with the 707–714del mutation (without the P30L mutation) is most prevalent in ethnic Chinese CAH patients (Lee *et al.*, 2004).

Several possibilities for the mechanism of microconversions were proposed, such as the existence of a chi-like sequence (GCTGGTGG) (Smith *et al.*, 1981) and a minisatellite consensus (Jeffreys *et al.*, 1985). From our examination (Table 3), three chi-like sequences upstream of intron 2 might explain how the highest frequency of the I2 splice mutation occurred in CAH patients with a 21-hydroxylase deficiency (White and Speiser, 2000). In addition, two chi-like sequences upstream of intron 4 might also account for the formation of the Q318X and R356W mutations for the greatest population next to the I2 splice mutation (White and Speiser, 2000). However, a high degree of sequence homology and the tandem repeating order of these genes seem the most likely causes for misalignment to occur at meiosis, which would generate illegitimate genetic recombinations or unequal crossovers (Koppens *et al.*, 2003).

Table 3. Distribution of the Chi-Like Sequence in *CYP21A2* Genes *CYP21A1P* and *CYP21A2*

	CYP21A1P *and* CYP21A2	
Initiator for the chimera	*Location*	*Sequence (nt)*[a]
Chi sequence: GCTGGTGG		
Chi-like sequences		
GCTGGGGC	5′ end	−54 to −48
GCTGGGGG	IVS2	393 to 400
GCTGGTCT	IVS2	461 to 468
GCTGGAGG	IVS2	649 to 642
GCTGGGCT	IVS3	828 to 835
GCTGGGGG	IVS4	1062 to 1055
GCTGGGGT	IVS5	1264 to 1271
GCTGTGGG	IVS6	1449 to 1441
GCTGGAGG	IVS7	1921 to 1928
GCTGCTGG	IVS7	1943 to 1950
GCTGGGTG	3′ end	2830 to 2838

[a]Based on Higashi *et al.* (1986).

Both the PCR product and fragment-distinguishing Southern blot method (Fig. 2A, B) reveal that the defective *CYP21A2* allele, when processing a micro-interchange of sequences from the *CYP21A1P* gene, or dual mutations of I236N/V237E, and either a 10-base deletion (S329-R333delC) or a 16-base duplication (H392C-V397ins), showed a 3.7-kb *Taq*I fragment as the functional *CYP21A2* gene and a 21.6-kb DNA fragment of the gene array (Table 1) in the RCCX region. This indicates that the intact gene arrangement remains in the order of *C4A-CYP21A1P-XA-RP2-C4B-CYP21A2-TNXB* (Fig. 1A). However, the case of 111-bp duplication of W21-P57 (Table 1) presenting a 3.3-kb *Taq*I-PCR fragment (data not shown) and a 9.4-kb genomic fragment [Fig. 1C(d)] in our previous study (Lee *et al.*, 2003b) imply that the genomic RCCX organization was altered to the *RP1-C4A-YCYP21A2-TNXB* sequence (*YCYP21A2* indicating the mutated *CYP21A2* haplotype with a W21-P57 duplication) [Fig. 1C(d)], which was caused by multiple gene deletions [Fig. 1C(d)]. The *CYP21A2* haplotype with dual mutations of IVS2-12A/C->G in combination with 707–711del GAGACTAC (without the P30L mutation) (Fig. 2A, lanes 9) and the chimeric *CYP21A1P/CYP21A2* genes (data not shown) (see Lee, 2004) appearing with the 3.2-kb *Taq*I-fragment and 9.3-kb DNA fragment resulted from multiple gene deletions [Fig. 1B(a-1)]. This led us to conclude that a 26/32-kb deletion of *XA-RP2-C4B* (Fig. 1A) generated the particular gene sequences in the order of *RP1-C4A-XCYP21-TNXB* [Fig. 1C(b)], *RP1-C4A-YCYP21A2-TNXB* [Fig. 1C(d)], and *RP1-C4A-CYP21A1P/CYP21A2-TNXB* [Fig. 1C(c)]. It is worth noting that genomes from healthy individuals without the 11.3-kb fragment (Fig. 2B, lane 2) carrying the *CYP21A1P* pseudogene do not influence the physiological activity of 21-hydroxylase, because the *CYP21A2* gene exists and is functional.

As described above, we can use the PCR product and the Southern blot method based on a fragment-distinguishing configuration to propose the gene order in the RCCX module

after intergenic recombination and multiple gene deletions without a family study.

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Disclosure Statements

No competing financial interests exist.

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Address correspondence to:
Hsien-Hsiung Lee, B.S.
School of Chinese Medicine
College of Chinese Medicine
China Medical University
91 Hsueh-Shih Road
Taichung 404
Taiwan

E-mail: hhlee@mail.cmu.edu.tw; leehsienhsiung@gmail.com