Duplication and deletion of 21 hydroxylase gene among the normal Korean subjects and adrenogenital syndrome patients

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Steroid 21 hydroxylase deficiency is a major cause of congenital adrenal hyperplasia (CAH) and is caused by genetic impairment of the gene (*CYP21B*). In the human genome, *CYP21B* is located within the MHC class III region on the short arm of chromosome 6. Most of the genes in this region are highly polymorphic and crowded. Also the *CYP21B* gene is accompanied by its pseudogene (*CYP21A*) and tandemly arranged with two genes of the fourth component of complement. A highly complex gene cluster in this area may predispose genetic instability of *CYP21*, i.e. mutations. In this study, we tried to investigate the frequency of duplication and deletion of *CYP21* and patterns of the genetic alterations of these genes. We also compared the genetic alterations in normal subjects with those of the CAH patients. The results showed that 15% of the normal Korean population have duplication or deletion of *CYP21*. There was one normal subject heterozygous for the deletion of *CYP21B*. Of the 5 CAH patients examined, 2 were found to show abnormal patterns. One was a large-scale gene conversion and the other a gene conversion associated with deletion involving both *CYP21B* and C4 locus II gene. Through this study, we came to the conclusion that the duplication or even deletion of *CYP21* and C4 might be quite a common event in the Korean population and these rearrangements must be regarded as polymorphisms. It could contribute to a high incidence of CAH by providing a genetic pool of instable *CYP21*.

Keywords: CAH, *CYP21*, deletion, duplication, gene conversion, polymorphism

INTRODUCTION

Congenital adrenogenital hyperplasia (CAH) refers to a group of steroidogenic disorders in which an enzymatic defect results in impaired synthesis of cortisol. The most common classic CAH is caused by 21-hydroxylase (21OH) deficiency, and *CYP21B* is the gene responsible for this enzymatic defect (Miller and Levine, 1987; White *et al.*, 1987). This disorder is known to be one of the most common forms of inborn error of metabolism. While the most extreme case of CAH, i.e. salt loser, is reported to be 1 in 14,500 live births (Pang *et al.*, 1988), which gives a computed heterozygote frequency of 1 in 61 persons worldwide, the non-classic form is not a rare disease. According to recent reports, the frequency of non-classic CAH is ethnic-specific (Speiser *et al.*, 1985): an incidence in Ashkenazic Jews being 1/27, in Hispanics 1/53, in Yugoslavs 1/63 in a heterogenous New York population being 1/100 (Speiser *et al.*, 1985; Dumic *et al.*, 1990).

The clinical symptoms of classic salt loser are evident in the neonatal period, manifested by ambiguous external genitalia, and sometimes at risk of a hypovolemic hypoglycemic adrenal crisis. In contrast to the classic types, non-classic types represent premature development of pubic hair, male type baldness in female, secondary amenorrhea or oligomenorrhea and eventually short stature (Lobo and Goebelsmann, 1980).

In the human genome, there are usually two 21OH genes (*CYP21B* and its pseudogene *CYP21A*), which are tandemly arranged with two genes of the fourth component of complement (*C4* locus I and *C4* locus II) (Carroll *et al.*, 1985), within the class III region of the major histocompatibility complex (MHC) on the short arm of chromosome 6 in the order as 5' *C4* locus I (*C4A*)-*CYP21A* and *C4* locus II (*C4B*)-*CYP21B* in most individuals (White *et al.*,1985)

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CYP21B and *CYP21A* share 98% of sequence homology.

The fourth component of complement genes (*C4*) are important in CAH genetics, because each *C4* gene is 1.5 kb separated by each *CYP21*. In the Caucasian population, deletion or duplication of *C4* and *CYP21* often occur, and this genetic alteration predisposes to immunologic abnormalities or CAH (Jin *et al.*, 1992; Tom and Farid, 1981). However, in the Korean population, there has not been a study on the frequency of the duplication or deletion polymorphisms.

The aim of this study is to survey the frequency and patterns of the duplication or deletion polymorphisms involving *CYP21* and *C4* in the Korean population. We also tried to compare the gene structures of CAH patients to recombination events in the general population.

MATERIALS AND METHODS

Study population

DNA samples collected from peripheral blood leukocytes of Korean blood donors were used for the evaluation of *CYP21* and *C4*. A total of 145 individuals were enrolled in this study and were regarded as normal control subjects. None of the control individuals showed any evidence of CAH, and their 17OH progesterone levels were within the normal range. In one control subject who was later proved to have a deletion of *CYP21B*, ACTH stimulation test was performed to rule out the non-classic form of CAH. Blood was withdrawn before and 60 min following intravenous injection of 0.25 mg bolus of Cortrosyn™, but the increase of 17OH progesterone was not distinguishable from the level in other normal control individuals. This individual, therefore, was included in the normal control group. For a comparision of *CYP21* abnormalities in CAH patients, the DNAs of 5 CAH patients were investigated and two showed abnormal findings in Southern blot analysis. All of the enrolled CAH patients were salt losers detected in infancy.

Genomic DNA extraction and Southern hybridization

Genomic DNA was prepared from peripheral blood leukocytes according to the method previously described (Gross-Bellard *et al.*, 1973). For Southern blotting, four micrograms of each DNA samples were digested with restriction enzymes, *Taq*I, *BamH*I, *EcoR*I or *Hind* III according to the manufacturer's instructions. Restriction fragments of genomic DNA were separated by electrophoresis on 0.7% agarose gel and blotted onto the nylon membrane. Probes were labelled with [α-P^{32}]dATP by nick translation or the random primer method. The blot was fixed with ultraviolet light and was hybridized with radioactive probes at 65°C for 12-18 h in a mixture containing 6x SSC, 10mM EDTA, 0.1% SDS, 5x Denhart's solution and 0.1 mg/ml salmon sperm DNA. The hybridized blots were washed twice at room temperature for 5 min in 2X SSC, 0.1% SDS and once at 65°C for 120 min in 0.1x SSC and 0.1% SDS. Hybridization patterns on the blots were visualized by autoradiography.

Probes

We used *CYP214* cDNA, which was kindly provided by Fujii-Kuriyama (Cancer Institute, Tokyo, Japan) (Higashi *et al.*, 1986). The complement 4 gene cDNA (pAT-A) was a kind gift from Dr. M.C.Caroll (Harvard Medical School, Boston, USA) (Belt *et al.*, 1984). From the large-scale prepared plasmids, a 500-bp *BamH*I/ *Kpn*I fragment from the 5' end of the full length *C4* -cDNA, a 500-bp *EcoR*I fragment from a 21OHase genomic clone, and C21A-1 were electroeluted and used as probes.

Interpretation of RFLP pattern

The intensity of hybridization bands for *CYP21* and *C4* was evaluated by scanning densitometry within the same digest on the blot. Relative intensities of the *C4* locus II genes / *C4* locus I genes and *CYP21A*/*CYP21B* were calculated and classified as deletion or duplication if the relative intensities were 0, 5 or less and 1.5.or more, respectively (Jin *et al.*, 1992). In all patients classified as deletion/duplication, segragation analysis in their families were performed to confirm the assignments. Type I deletion was defined as heterozygous deletion of *CYP21A* and type II deletion as the *C4* locus II gene.

RESULTS

Among several RFLPs detected by different restriction enzymes, *Taq*I RFLP was most informative and we used it as a standard. *Hind* III RFLP was useful for the evaluation of partial deletion of *C4* locus I.

Fig. 1 shows several *Taq*I-polymorphic alleles of the *CYP21* and *C4*. There are basically 3 fragments for the *C4* genes. The intact *C4* locus I gene (previous *C4A*) represents a 7.0 kb fragment, while the *C4* locus II gene appears as either 6.0 kb or 5.4 kb fragment according to the presence or absence of intron. Thus the 7.0 kb fragment is a constant band, while the 6.0 kb and/or 5.4 kb fragments are polymoric. *CYP21B* and *CYP21A* represent 3.7 kb and 3.2 kb bands, respectively. Patterns for the *C4* genes most commonly observed were 2 copies each of the *C4* locus I and *C4* locus II genes. (lanes 1 and 3 of the Fig.1).

Duplication of *CYP21* and *C4* was observed in 12% of the normal control subjects analyzed. This RFLP pattern was quite characteristic. The duplicated *C4* bands were always

derived from the *C4* locus II gene (either 6.0 kb or 5.4 kb) and always accompanied by *CYP21A* duplication. We found another pattern of duplication in a control person. This person had triplication of *CYP21* and *C4*, i.e. 2 copies of the *C4* locus I gene and 4 copies each of the *C4* locus II, *CYP21B* and *CYP21A* genes.

There were 2 different patterns of *C4* deletion (Fig. 2). For example, there were 4 bands and a second upper band was 6.4 kb (lane 2, Fig. 2). This 6.4 kb band represents a *C4* locus I deletion and was observed in one control person among the subjects examined. The type I deletion (*C4* locus I-deletion) was not accompanied by the *CYP* gene deletion. In lane 5, a density of the locus II-band was around half of that of the locus I-band and an intensity ratio of 0.5 was also true for the *CYP21B*/*CYP21A* bands. This pattern was quite consistent and was also found in 4 other normal control subjects. We defined this as type II deletion.

Thus, Fig. 1 shows homozygous deletion of *CYP21A* with the *C4* locus II gene deletion and hemizygous deletion in lanes 3, 4, and 5. In the Fig. 1, lane 3 represents triplication involving *CYP21A* and the *C4* locus II gene.

Table 1 summarizes frequencies of deletion or duplication of the *CYP21* gene in normal subjects and CAH patients. Deletion of *CYP21A* gene was observed in 5/145, duplication in 17/145 and triplication in 1/145 of normal subjects. This implicates that 23/145 normal subjects do have 5 or more copies of the *CYP21* gene or have 3 copies instead of 4 copies of the gene.

Of the patients examined, 2 showed abnormal Southern findings. Lane 1 shows a decreased intensity of *CYP21B* compared to *CYP21A*. Densitometry shows that the intensity of *CYP21B* is one third of that of *CYP21A*. We interprete this as large-scale gene conversion (from *CYP21B* to *CYP21A*). In the remaining patients there was no signal for *CYP21B* at all, although a signal representing the *C4* locus II gene appeared and its density of Fig 3 is half of that of the locus I gene. It is likely that this finding is due to a gene conversion of *CYP21B* to *CYP21A* and a hemizygous, type II *C4* deletion and *CYP21B*, because the *CYP21B* deletion is always accompanied by that of the *C4* locus II gene as indicated previously (Carroll *et al.*, 1985; White *et al.*, 1985).

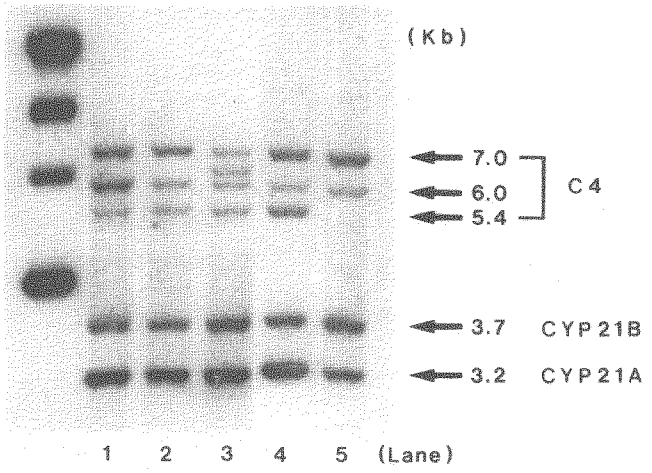

Fig. 1. Several *Taq* RFLP patterns of the *CYP21* and *C4* genes Lane 2 shows 2 copies of each of the *C4* locus I gene (7.0kb), *C4* locus II (6.0kb and 5.4kb), *CYP21B* (3.7kb) and *CYP21A* (3.2kb) genes. Lane 1 and 4, and lane 5 depict a duplication and a deletion of *C4* locus II and *CYP21A* genes, respectively, while lane 3 shows a deletion of the *C4* locus I gene.

DISCUSSION

In the present study, we estimated the frequency of *CYP21* gene duplications and deletions among normal Korean individuals, being as high as 16%. One of the individuals examined had 4 copies of the *CYP21* genes instead of usual copies. It is likely that relatively high alterations of the genes reflect a high frequency of CAH.

In our CAH patients, we observed a large-scale gene conversion and deletion of *CYP21B* and the *C4* locus II gene. These patients manifested with salt losing since the age of 1 month. In Caucasian data, the gene conversion or deletion accounts for 20% of CAH patients, and the remaining 80% of patients have a small deletion or point mutation, leading to frame shift or premature termination (Owerbach *et al.*, 1992; Tajima *et al.*, 1993).

Gene conversion is a fundamental mechanism of CAH. It is known that the region around the *CYP21* gene is frequently involved in gene conversion and unequal crossing-overs (Collier *et al.*, 1989, 1993), probably because of the high homology and tandem-repeat organization of the *CYP21* and *C4* genes. *CYP21A* gene is a pseudogene which has 99% homology to the *CYP21B* gene.

Table 1. Frequencies of deletion or duplication of 21 hydroxylase genes in normal subjects and patients

Gene alterations	normal subjects (n=145)	patients (n=5)
Deletion of *CYP21A*	4	–
Deletion of *CYP21B*	1	1
Large scale gene conversion	–	1
Duplication of *CYP21A*	17	–
Duplication of *CYP21B*	–	–
Triplication of *CYP21A*	1	–
Total	23	2

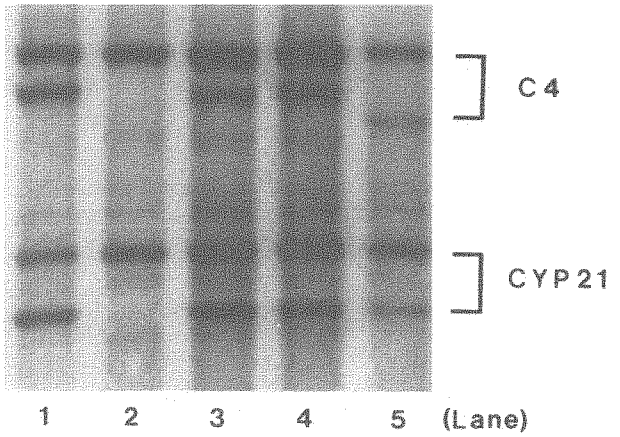

Fig. 2. Deletion patterns of the *C4* locus II and *CYP21* genes. Lane 2, no hybridization for these genes; 3,4 and 5, the intensities of *C4* locus II and *CYP21A* genes are half of the I those of the *C4* locus I and *CYP21B* genes.

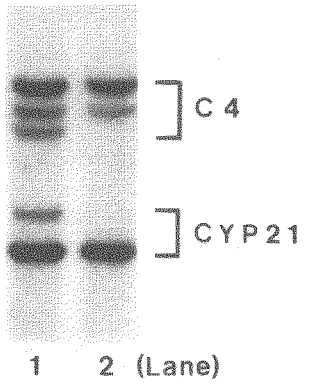

Fig. 3. Large- scale gene conversion (from *CYP21B* to *CYP21A*) in a CAH patient (lane 1), and another gene conversion from *CYP21B* to *CYP21A* and a hemizygous deletion of the *C4* locus II and *CYP21B* genes in the other patient.

In most of genetic disorders, point mutation or deletion is usually confined to patients or carriers. However, we detected a high frequency of duplication and/or deletion in a normal population. Therefore, duplication itself does not directly affect the 21-hydroxylase level. It is of interest that the frequency of *CYP21* duplication is far higher in our study than that in the Caucasian study, and patterns of deletion or duplication are different from those in previous reports (Collier *et al.*, 1989).

In conclusion, a high frequency of genetic alterations in the *CYP21* gene region may be involved in the pathogenesis of CAH and an evaluation of the copy number of the *CYP21* gene in CAH patients is mandatory.

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