Association study of four activity SNPs of CYP3A4 with the precocious puberty in Chinese girls

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Abstract

CYP3A4 plays an important role in the metabolism of a variety of endogenous and exogenous compounds. Earlier studies revealed that a high-activity variation of the CYP3A4 gene, CYP3A4*1B is significantly associated with the precocious puberty in girls. Other three variations, CYP3A4*4, CYP3A4*5 and CYP3A4*6, which were found in a study carried out in a Chinese population in Taiwan, were reported to down-regulate the enzymatic activity of CYP3A4. The four activity SNPs were typed in our study in two groups of Chinese girls: 176 girls with precocious puberty as the case group, and 192 normal girls as the control group. No variations of CYP3A4*1B and CYP3A4*4 were found in all the cases and controls. Heterozygous of CYP3A4*5 was found in five subjects of the 192 controls but none in the cases, heterozygous of CYP3A4*6 was found in two subjects of the controls but none in the cases. Fisher’s exact test showed that the variation of CYP3A4*5 was associated with the onset of puberty in Chinese girls (P-trend = 0.038), while the variation of CYP3A4*6 was not associated with the onset of puberty in Chinese girls (P-trend = 0.272). The result suggests that these mutations in the CYP3A4 gene have no contribution to the early onset of puberty in Chinese girls, but are related in some way with the puberty development in Chinese girls.

Keywords: Precocious puberty; CYP3A4; Genetic variation

Precocious puberty is a common development abnormal phenomenon characterized by early onset of puberty. Precocious puberty is defined as onset of puberty before 8 years of age in girls and 9–9.5 years in boys, although the normative data used to set those limits has been challenged recently [4]. Puberty is a complex trait affected by both genetic and environmental factors. The onset of puberty involves the activation of hypothalamic function and maturation of the pituitary–gonadal axis. But the exact mechanism of the onset of puberty remains a mystery of the human biology.

The abnormal pubertal development, in the form of either delayed or precocious puberty, has long-term implications on physical and psychological health of individuals. It has been well established that precocious puberty girls are of greater risk for breast cancer [14] and are often shorter in stature. Though the exact mechanism of pubertal development regulation is not completely revealed, advance in molecular genetics and technology has made it possible to understand it more deeply over the past decade, and there have been some studies carried out with precocious puberty samples in some populations [2]. Members of cytochrome P450 family play important roles in the oxidative and reductive metabolism of a variety of endogenous and exogenous compounds [13], for example the drugs, some endogenous steroids and harmful environmental contaminants. There are evidences proving that these genes take part in ovarian hormone metabolism and may affect pubertal development [10,19]. Kadlubar et al. [8] reported a mutation of the CYP3A4 gene, the
high-activity allele CYP3A4*1B has association with increased testosterone availability and early puberty in girls and Kadibhar et al. [9] also gave corresponding result about the relationship of allele CYP3A4*1B with the early onset of puberty in African American, Hispanic, and Caucasian girls. The high-activity variation of CYP3A4*1B up-regulates the gene expression and causes a disproportionate drop in testosterone levels, which may increase the estradiol/testosterone ratio and results in the hormonal cascade that accompanies early puberty. With the SSCP method, Hsheh et al. [5] reported that no CYP3A4*1B mutation was found in their study in a Taiwan population, but they have found some low activity variations in the exons of the gene, designated as CYP3A4*4, CYP3A4*5 and CYP3A4*6, which down-regulate the enzymatic activity of CYP3A4.

In this study, an economical and reliable PCR-RFLP method was used to type the four activity SNPs (CYP3A4*1B, CYP3A4*4, CYP3A4*5 and CYP3A4*6), which down-regulate the enzymatic activity of CYP3A4.

The cases consisted of 176 independent Chinese Han girls with precocious puberty at the age of 6–8.5. Eligible girls were identified in the pediatric clinic of the Children’s Hospital, Fudan University (Shanghai, China). Breast development was assessed using Tanner breast stages determined by special physicians based on standardized methods, all the girls had Tanner breast stage scores ≥2, most of the girls had vagina bleeding, and they also had an abnormal bone age ahead of their true age[12]. The results of standard intravenous gonadotropin releasing hormone (GnRH) stimulating tests showed that the peak level of luteinizing hormone (LH) in the patients was elevated dramatically. The average peak value of LH at 40 min after the injection was 32.3 [± or –] 18.6 mIU/ml, comparing with 2.9 [± or –] 2.4 mIU/ml in normal girls of the same age. The controls consisted of 192 independent healthy Chinese girls with the mean age of 18, and the age of menarche is above13.2 years. Informed consents were obtained from a parent or guardian of each girl in both case and control groups. Blood samples were collected from all the subjects and genome DNA was isolated from peripheral leukocytes using phenol-chloroform methods as described [7].

Gene sequence of CYP3A4 was obtained from the GenBank database (Accession No. AF209389).

Genotyping was carried out with an economical and reliable PCR-RFLP method developed in our lab. For the variations of CYP3A4*1B, CYP3A4*4 and CYP3A4*6, according to the sequence around the SNP site, we designed primers with mismatching bases at the 3’ end to introduce a restriction endonuclease recognizing site into the amplification product after two rounds of PCR. For the variation of CYP3A4*5, PCR-RFLP was carried out with the BamHI site in the sequence. Primers for amplification and the endonucleases used are summarized in Table 1, and the mismatching bases in the primers are marked with italic and bold letters.

In the first round of reaction, 1–5 ng of genomic DNA was added into a final volume of 5 μl containing 0.5 μl of 10 × Taq buffer, 0.5 μl of 10 × dNTP mixture, 0.4 μl of 25 mM MgCl2 solution, 1 μM of each of the forward and reverse primers and 0.5 U of Taq polymerase (TaKaRa). The reaction was performed as following: an initial denaturation of 5 min at 94 °C was followed by 14 cycles of 30 s at 94 °C, 30 s at 63 °C (decreased by 0.5 °C per cycle), and 50 s at 72 °C, then the reaction continued with 30 cycles of 30 s at 94 °C, 30 s at 56 °C and 50 s at 72 °C, and was followed by a final extension of 7 min at 72 °C. The products of the first round were diluted by 10 times as templates for the second round reaction.

In the second round, 1 μl of the diluted products of the first round was added into in a final volume of 30 μl containing 3 μl of 10 × Taq buffer, 3 μl of 10 × dNTP mixture, 1.8 μl of 25 mM MgCl2 solution, 3 μM of each of the forward and reverse primers and 2 U of Taq polymerase (TaKaRa). The reaction program was shown as following: an initial denaturation of 5 min at 94 °C was followed by 45 cycles of 30 s at 94 °C, 40 s at 56 °C, 10 s at 45 °C and 50 s at 72 °C, and then a final extension of 5 min at 72 °C.

Two microliters of the products were digested by corresponding endonucleases before electrophoresis on 3.0% agarose gel. PCR products of different genotypes would give bands of different lengths after digestion, as shown in Table 1. PCR products of all the heterozygous subjects found by PCR-RFLP were purified and sequenced using PE Biosoynms’ Capillary 3700DNA Analizers to confirm the genotyping results.

Statistical analysis: the descriptive statistics of the polymorphisms of the CYP3A4 gene on the onset of precocious puberty were calculated using Ab-STAT statistic software (Anderson-Bell, Boulder, CO). Allele frequencies of the four detected loci were calculated by gene counting method. Fisher’s exact tests were used to compare the genotype frequencies between the two subgroups, cases and controls. Difference was considered as significant if the P-trend value < 0.05 at 95% confidence interval.

None of the four variations were found in the 176 cases of precocious puberty Chinese girls, while in the 192 healthy Chinese girls, we found five heterozygous subjects of CYP3A4*5 and two heterozygous subjects of CYP3A4*6, as shown in Figs. 1 and 2, no mutant homozygous subject was found in all the subjects, and there was no variation of CYP3A4*1B and CYP3A4*4 detected in all the 368 Chinese girls including the cases and the controls. The frequencies of the variations of CYP3A4*5 and CYP3A4*6 in the controls were 0.013 and 0.005, respectively, which were similar to the results reported by Hsheh et al. [5] (see Table 2).

Fisher’s exact tests (one sided) were used to compare genotype frequencies between the two groups, and the P-trend values of CYP3A4*5 and CYP3A4*6 were 0.038 and 0.272, respectively, indicating that CYP3A4*5 is associated signifi-
Table 1

<table>
<thead>
<tr>
<th>Sequence around the SNP</th>
<th>1st round primer F</th>
<th>1st round primer R</th>
<th>2nd round primer F</th>
<th>2nd round primer R</th>
<th>Length of PCR product</th>
<th>Restriction endonuclease used</th>
<th>Length of digested product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4*1B</td>
<td>CATAGAGACGAGGGCA</td>
<td>5’ TGGCATAAAATCTATTAAATGCGCTCACAGC 3’</td>
<td>5’ GGGAAAGATCTTTAGAAGGACATCTTGTTTCATTTCAAGGC 3’</td>
<td>165 bp</td>
<td>HinIII</td>
<td>A 132 ± 31 bp G 160 bp</td>
<td>A 149 bp G 180 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4*4</td>
<td>ATGAGACGGCTGAGTCAG</td>
<td>5’ CGGAGACGCTGAGTGGAGAATCCCTCTG 3’</td>
<td>5’ CTCTACACGACTGACACTCTCAAATAAATA 3’</td>
<td>189 bp</td>
<td>NdeI</td>
<td>A 161 ± 26 bp G 180 bp</td>
<td>A 189 bp G 180 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4*5</td>
<td>TGGAAAGFTCTAAGAGAACGCACTGTCTGT</td>
<td>5’ GGGAAAGATCTTTAGAAGGACATCTTGTTTCATTTCAAGGC 3’</td>
<td>5’ CAGTTAATTATTCTAGAAGAATATTATTTAA 3’</td>
<td>291 bp</td>
<td>BamHI</td>
<td>A 219 ± 72 bp G 291 bp</td>
<td>A 291 bp G 291 bp</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4*6</td>
<td>TCAAGAGTGAAGATCTAC</td>
<td>5’ AGGACTCCGTGATTATTCTAGAAGAATATTATTTAA 3’</td>
<td>5’ GACCCACGCTGATTACTCGCTGATTAGG 3’</td>
<td>132 bp</td>
<td>EcoRI</td>
<td>A 106 ± 26 bp G 132 bp</td>
<td>A 132 bp G 132 bp</td>
</tr>
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<td></td>
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</tr>
</tbody>
</table>

cantly with the development of puberty, while CYP3A4*6 is not associated with the onset of puberty.

Recently, some genetic studies on the development of puberty have been done and a variety of genes and SNPs involved were identified. The candidate genes selected include genes involved in the metabolism of sex hormone [3,8,11], in the signal pathways [16] and in the metabolism of fat and protein [21]. However, no single gene has been identified as the puberty onset susceptibility gene for the majority of cases. The CYP3A4 gene is one of the genes which have been characterized as a candidate genetic marker associated with early onset puberty, especially the locus of CYP3A4*1B, an A/G point mutation in the 5' flanking region of the CYP3A4 gene, has a striking association with the onset of puberty as reported by Kadlubar et al. [9] in African American, Hispanic and Caucasian populations.

Though there have been three studies carried out in Chinese population about the variation of CYP3A4, they all reported that no variation of CYP3A4*1B was found [5,15,18]. However, since none of the three studies investigated the distribution in precocious puberty patients, the proportion of which is about 1% in Chinese children [1], it’s

Table 2

<table>
<thead>
<tr>
<th>Variation</th>
<th>Controls (n=192)</th>
<th>Cases (n=176)</th>
<th>Previously reported frequency</th>
<th>P-trend value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterozygous subjects</td>
<td>Allele frequency</td>
<td>Heterozygous subjects</td>
<td>Allele frequency</td>
</tr>
<tr>
<td>CYP3A4*1B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4*4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4*5</td>
<td>0</td>
<td>0.013</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4*6</td>
<td>2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data from the study of Hiseh et al. [5].
necessary to investigate if the variation exists in Chinese girls
with early onset of puberty.

In this study, for the first time we investigated whether the
four activity SNPs in the CYP3A4 gene were associated with
the regulating of onset timing of puberty in Chinese (Han)
girls with case–control analysis. The frequencies of the four
SNPs were reported to be very low in Chinese population,
but rare SNPs may also have contribution on the process of
puberty which is a complex trait regulated by many genes.
Though the scale of samples was relatively small, our result
may be able to indicate the direction of other studies with
more samples.

In the normal samples, we have found five heterozy-
gous subjects of CYP3A4*5, a variation in exon 7 caus-
ing a Pro218Arg amino acid change, and two heterozy-
gous subjects of CYP3A4*6, a variation in exon 9 causing
frame shift and forming an early stop codon. The two mu-
tations were both reported to decrease the activity of the
aromatase CYP3A4 [19], which catalyzes the 6β-, 2β- and
15β-hydroxylation of testosterone [6], leading to changes in
estradiol:testosterone ratio. Higher estrogen level in girls is
one index on the diagnosis of the onset of puberty [20], so
it could be explained why the low-regulating variations were
not found in the cases of early puberty. The result also ap-
proves and complements the conclusion of previous study of
Kadilbar et al., which revealed that girls with high-activity al-
lele of CYP3A4*1B were more likely to have early puberty.
With the results, it is reasonable to conclude that CYP3A4
does affect the process of puberty in some way in Chinese
girls, yet the mechanism remains to be investigated by further
study.

The fact that the variation of CYP3A4*1B which is sig-
nificantly related to precocious puberty has not been found
in Chinese population is due to the ethnic difference. As re-
ported, the allele frequencies also differ between some other
ethnic populations [8]. It well demonstrated that precocious
puberty is a complex trait regulated by many genes and af-
fected by some other factors including nutritious condition
and the environment, etc., and CYP3A4 is just one of them.
Variations or genes reported to be related with some trait in
some population maybe does not exist in other populations.
More studies need to be carried out in the future to finally
elucidate the mechanism of the early onset of puberty.

It was reported that the allele frequency of CYP3A4*4
was 0.0147 in 102 Chinese subjects of a Taiwan population
[5], yet no such variation was found in our study, that maybe
was because of the difference in genetic structure between
Chinese sub-populations [17].

Acknowledgment

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