Contributions of CAG repeat length in the androgen receptor gene and androgen profiles to premature pubarche in Korean girls

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Abstract. The CAG repeat length of the androgen receptor (AR) gene, which exhibits an inverse relationship to AR sensitivity, might influence the development of the pubarche along with hyperandrogenemia. There are ethnic differences in the AR CAG repeat length, however, no Asian studies on premature pubarche (PP) have been reported, including Korea. Our objectives were to examine the hormone levels and AR CAG repeat length, and to assess their contributions to PP in Korean girls. Subjects with PP (n=16) and normal pubarche (NP, n=16), and normal controls (NC, n=16) were enrolled. The levels of dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), 17-hydroxyprogesterone (17-OHP), and free testosterone (FT) were checked. The methylation-weighted (MW) average CAG repeat lengths were analyzed. The median ages at pubarche were 7.4 and 8.9 years in the PP and NP groups, respectively, and the levels of 17-OHP, DHEAS, and FT were similar in both groups. The PP group exhibited a higher DHEAS:DHEA ratio than the NP group (P=0.014). The medians of the MW average CAG repeat length of the AR gene were 22.4 for all subjects and did not differ among the PP (22.3), NP (22.4), and NC (22.2) groups. The AR CAG repeat lengths in the PP and NP groups did not correlate with DHEAS or FT levels. These results suggest that the AR CAG repeat length was not involved in the development of PP in Korean girls. However, excessive adrenal androgen levels, particularly those caused by increased sulfortransferase activity, might be important in the pathogenesis of PP.

Key words: Adrenarche, Pubarche, Androgen receptor, Trinucleotide repeats

PREMATURE PUBARCHE (PP) is characterized by the appearance of pubic and axillary hair prior to the age of 8 years in girls and 9 years in boys [1]. These physical manifestations are triggered by an increase in adrenal androgen levels, the onset of which is termed adrenarche. Adrenarche gradually begins between 6 and 8 years of age, thus approximately 2 years before the onset of gonadal maturation [1, 2]. Although concentrations and production rates of cortisol remain constant, the urinary excretion levels and circulating
concentrations of dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), and androstenedione (AD) increase progressively [2]; a DHEAS level greater than 40 μg/dL is usually considered to reflect adrenarche. Hormonal changes in adrenarche cause not only the development of axillary and pubic hair but also other signs including body odor, acne, oily hair, and a transient acceleration of linear growth and bone maturation [2].

Girls are more frequently affected by PP than are boys; the gender ratio is about 10:1 [3]. The overall prevalence of PP is not known precisely but ethnic differences exist. In the USA, 9.5% of Black girls and 1.4% of White girls have pubic hair of at least Tanner stage II at 6 years of age [4]. In Lithuania, 0.8% of girls have PP at 7 years of age [5]. In Turkey, 4.6% of girls younger than 8 years have PP [6]. No report on PP in Asia (including Korea) has yet been published, but the prevalence of PP is estimated to be lower in Korea than in Western countries.

DHEA, formed by the desulfation of DHEAS, is metabolized to AD and testosterone in specific tissues including androgen-dependent hair follicles and sebaceous glands [7]. As with all steroid hormones, the binding of androgens to the androgen receptor (AR) triggers a conformational change in the AR-androgen complex, which is then transported into the nucleus where it binds to DNA [7]. Numerous endogenous or exogenous signals that stimulate the secretion of adrenal androgen secretions have been proposed [1, 8, 9] but not all PP children exhibit increased androgen levels [10]. Therefore, the response of the pilosebaceous unit to androgens may also influence the development of the condition.

The AR gene is located on the X-chromosome at Xq11.2-12 and consists of an N-terminal transactivating domain, a DNA-binding domain, and a C-terminal ligand-binding domain [11]. The ligand-bound AR binds in turn to androgen-response elements in the promoter region of target genes, thus influencing transcription rates [7]. The response of target tissues to androgens is thus determined by functional aspects of the AR, including its sensitivity. The N-terminal domain of the AR gene contains a highly polymorphic region in which a variable number of CAG repeats in exon 1 encodes a polyglutamine tract [11]. An inverse relationship between CAG repeat length and AR sensitivity has been reported in several studies on both PP [11, 12] and other hyperandrogenic conditions [13]. The mean CAG repeat length varies ethnically, and has been reported to be relatively longer in Asians [14].

The aim of the present study was to examine the clinical and hormonal characteristics of Korean girls with PP. Based on the assumption that the AR sensitivity of PP girls might differ from that of girls with normal pubarche (NP), the CAG repeat length of the AR gene (adjusted for X-chromosome inactivation (XCI)) was investigated to identify its relationships with levels of androgens and to define factors that contribute to PP.

Methods

Subjects

Between October 2011 and November 2013, 48 subjects were enrolled in the present study. The inclusion criteria for the 16 girls with PP were as follows: presence of pubic and/or axillary hair; no sign of breast budding; and PP onset before 8 years of age. PP subjects were enrolled from six hospitals: five from Seoul National University Hospital (Seoul); four from Seoul National University Bundang Hospital (Seongnam); three from Bundang Jesaeng Hospital (Seongnam); two from Inje University Busan Paik Hospital (Pusan); one from Inje University Ilsan Paik Hospital (Goyang); and one from Eulji University General Hospital (Seoul). The inclusion criteria for the 16 girls with NP were as follows: presence of pubic and/or axillary hair; breast development or not; and onset after 8 years of age. The normal controls (NC) who had no signs of pubarche or gonadarche were eight girls and eight boys younger than 8 years and 9 years of age, respectively. The NP and NC subjects were enrolled from Seoul National University Hospital. Neither the PP nor NC subjects had any sign of gonadarche; the only difference between the PP and NC groups was the presence of pubic or axillary hair. Therefore, eight boys were included in the NC group because no hormonal differences between the genders are evident before the onset of gonadarche. Other signs of adrenarche, including oily hair, body odor, and acne, were also assessed. The pubic and axillary hairs were thoroughly inspected. Pubertal stage was evaluated via both inspection and palpation of breasts or testes, by two pediatric endocrinologists. A change in scalp or axillary odor by inspection and smell was considered to be an adult-type body odor. If
parents complained that their child’s hair was greasy, or needed daily washing, the hair was considered to be oily. No subject had taken any medication known to affect the adrenal axis for at least 3 months prior to the study. All subjects were ethnically Korean.

Anthropometric data were obtained for each subject. Height (cm) was measured twice to the first decimal place using a Harpenden stadiometer (Holtain Ltd., Crosswell, UK), and weight (kg) was measured to the first decimal place using a digital scale (150A, Cas Co. Ltd., Seoul, Korea). The body mass index (BMI: kg/m²) was calculated by dividing the weight by the height squared. The Z-scores of height, weight, and BMI were derived from Korean growth data published by the Korea Centers for Disease Control and Prevention. Waist circumference (WC) was measured horizontally around the level of the navel. Bone age (BA) was determined using the method of Greulich and Pyle.

Blood sampling and ACTH stimulation test

Blood was taken from all subjects early in the morning after at least 8 h of fasting. PP girls underwent a standard-dose adrenocorticotropic hormone (ACTH) stimulation test to measure 17-hydroxyprogesterone (17-OHP) levels to exclude nonclassic congenital adrenal hyperplasia. An indwelling catheter was inserted at least 10 min before sampling to allow adaptation. Then, 250 µg of tetracosactide acetate (Synacthen, Ciba Geigy, Basel, Switzerland) was administered intravenously to stimulate adrenal steroidogenesis and hormone release. Blood was collected immediately before and 1 h after injection of Synacthen. A peak concentration of 17-OHP of less than 15 ng/mL was considered normal [15].

Samples were centrifuged and separated, and the resulting sera samples were stored -20°C prior to analysis.

Hormone assays

The plasma concentrations of total testosterone (TT), AD, DHEAS, and 17-OHP were measured via solid-phase radioimmunoassay, using the Coat-A-Count assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation (CVs) of TT, AD, DHEAS, and 17-OHP were 5.0-10.0%, 3.2-9.4%, 3.8-5.3%, and 3.5-7.1%, respectively; and 5.9-12.0%, 4.1-15.6%, 6.3-11.0%, and 5.0-11.0%, respectively.

The plasma concentrations of sex hormone-binding globulin (SHBG) and DHEA were measured via enzyme-linked immunosorbent assay using commercial kits (Alpco Diagnostic, Salem, NH, USA). The intra- and inter-assay CVs of SHBG and DHEA were 3.0-8.6% and 3.8-6.9%, respectively; and 7.2-11.6% and 3.7-10.0%, respectively.

The plasma concentrations of free testosterone (FT) were calculated from the plasma concentrations of TT and SHBG using the following equation [16]: FT = \( \frac{TT - (N)(FT)}{(K_T)(SHBG) - (K_T)(TT) + (N)(K_T)(FT)} \), where FT is expressed in pmol/L; TT is nmol/L, SHBG is nmol/L; KT is the association constant of SHBG for testosterone (1.0 × 10⁹ L/mol); N is \((K_A)(C_A)+1\), where KA is the association constant of albumin for testosterone (3.6 × 10⁴ L/mol); and CA is albumin concentration (4.3 g/dL).

Plasma glucose levels were measured using an enzymatic reference method employing hexokinase, and plasma levels of insulin were measured using an electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, USA). The intra- and inter-assay CVs of insulin were 2.5-2.8% and 1.9-2.0%, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin levels using the following equation: HOMA-IR = glucose (mg/dL) × insulin (μIU/mL) / 405.

Assessment of steroidogenic enzyme activities

The activities of steroidogenic enzymes were indirectly estimated by calculating serum steroid concentration ratios because direct measurements were not available. The estimated enzyme activities were as follows:

- DHEAS to DHEA ratio: sulfotransferase activity
- AD to DHEA ratio: 3β-hydroxysteroid dehydrogenase (3β-HSD) activity
- DHEA to 17-OHP ratio: 17,20-lyase activity
- TT to AD ratio: 17β-hydroxysteroid dehydrogenase (17β-HSD) activity

CAG repeat length determination

Genomic DNA was isolated from peripheral blood leukocytes using Wizard DNA extraction kits (Qiagen, QIAGEN GmbH, Hilden, Germany). The total volume of each polymerase chain reaction (PCR) mixture was 50 µL and contained 0.05 µg genomic DNA, 0.03 µM each of upstream and downstream primers,
5 μL buffer (300 mM Tris-HCl [pH 9.0], 300 mM K⁺ and NH₄⁺, 20 mM Mg²⁺), 10 mM deoxynucleotide triphosphates, 1.5 U Taq DNA polymerase (iNtRON Biotechnology, Seongnam, Korea), and 32.7 μL distilled water. The oligonucleotide primers used for PCR were as follows: upstream primer, 6FAM-5’-TCC AGAATCTGTTCCAGAGGTGC-3’ and downstream primer, 5’-GCTGTGAAGGTTGCTGTTCAT-3’. Following initial denaturation (4 min at 94 °C), all samples were subjected to 35 cycles of PCR at 94 °C for 40 s, 64 °C for 40 s, and 72 °C for 1 min, with a final extension of 10 min at 72 °C.

The size of the PCR products was determined by estimating the peak height using Peak Scanner software ver. 1.0 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Samples from five homozygous girls and eight boys were cross-checked by direct sequencing.

X-chromosome inactivation analysis

An XCI assay based on the methylation pattern of the AR gene from blood leukocyte DNA has been described previously [11, 17]. The methylation-sensitive restriction enzymes HpaII and HhaI, sites which lie close to the AR gene CAG repeats, digest only unmethylated (active X-chromosome) DNA and thus render subsequent PCR amplification impossible. Therefore, post-digestion PCR products are indicative of the presence of methylated (inactive X-chromosome) DNA sequences only. The technique described previously was used [17, 18]. Briefly, a 300 ng DNA sample was digested at 37 °C for 1 h with 10 U HpaII (TaKaRa Bio Inc., Shiga, Japan) and 10 U HhaI (TaKaRa Bio Inc.) in a total volume of 20 μL. The reactions were stopped by denaturation prior to PCR, as described above. The sizes of digested PCR products were determined using the same method.

To compensate for unequal amplification of alleles, the values of digested samples were normalized to those of undigested samples as follows: inactivation percentage (%) = (peak height ratio of two alleles of digested DNA / peak height ratio of two alleles of undigested DNA) × 100. Thus, the methylation-weighted (MW) biallelic means of the CAG repeat length were estimated by multiplying each allele of a genotypic pair by the percentage of its total expression (100 minus the inactivation percentage) and summing these two adjusted values, as described previously [2, 11]. The skewing criteria for XCI varies among studies: 70% [18], 80% [19], and 90% have been employed [20]. In the current study, skewing of the X chromosome was defined if the percentage activity of a single allele was ≥ 80% in heterozygous samples [19].

Data analysis

All data are presented as medians with interquartile ranges. The Mann-Whitney U test or the Chi-squared test was used to explore differences between the two groups classified by onset and presence of pubarche. Wilcoxon’s signed-rank test was used to compare paired data from the ACTH stimulation test in PP girls. Spearman’s correlation coefficients were calculated to explore possible associations between two continuous parameters. All hormonal variables were highly skewed and logarithmic transformation was performed prior to the logistic regression analysis used to define risk factors associated with PP, after controlling for other variables. A comparison of CAG repeat lengths between the 16 PP girls and 205 healthy premenopausal females who had no features of polycystic ovarian syndrome in a study published previously [13] was also performed using Mann-Whitney U tests. All statistical analyses were performed using PASW Statistics 18.0 for Windows (Chicago, IL, USA). P-values < 0.05 were considered significant. For comparisons involving CAG repeat lengths, statistical powers (1 minus the β error probability) were calculated using G*Power 3.1.0 (University of Kiel, Kiel, Germany).

Ethics statement

This study protocol was approved by the Institutional Review Board of Seoul National University Hospital (approval number: H-1107-102-370) and the relevant boards of each participating hospital. Written informed consent was obtained from all patients and all of their parents at the beginning of the study.

Results

Clinical characteristics of the study subjects (Table 1)

The median age at examination did not differ between the PP and NC groups. The median age at diagnosis of pubarche (7.4 years in the PP group and 8.9 years in the NP group) differed significantly between the two groups (p<0.001). Neither birth weight nor gestational age differed among the three groups. Height, weight, and BMI Z-scores were
The AR CAG repeat length in PP girls

Although plasma ACTH levels were similar among the three groups, the plasma levels of 17-OHP, DHEAS, DHEA, AD, and FT were significantly higher in the PP group than the NC group (17-OHP, \( p = 0.001 \); DHEAS, \( p < 0.001 \); DHEA, \( p = 0.001 \); AD, \( p < 0.001 \); FT, \( p < 0.001 \)). The plasma levels of 17-OHP, DHEAS, DHEA, and FT were similar in the PP and NP groups. The plasma concentrations of DHEAS were not correlated with age at diagnosis in PP and NP subjects (\( r = 0.159, p = 0.384, n = 32 \)), even when the MW average CAG repeat length was corrected (\( r = 0.140, p = 0.444, n = 32 \)). Upon logistic regression analysis evaluating the plasma levels of DHEAS, DHEA, AD, and FT together, no single hormone was associated significantly with an increased risk of PP compared to NC. The plasma levels of SHBG, which were negatively correlated with BMI Z-scores (\( r = -0.502, p < 0.001, n = 48 \)) and WC (\( r = -0.502, p < 0.001, n = 48 \)), did not differ among the three groups. No biochemical values differed between genders in the NC group.

Stimulated (by ACTH) plasma levels of 17-OHP allowed nonclassic congenital adrenal hyperplasia to be excluded in all PP subjects. Plasma levels of DHEAS were not affected by 250 μg ACTH. However, the stimulated plasma levels of DHEA, AD, and FT were significantly higher than their basal levels (Table 3).

### Table 1: Clinical characteristics of subjects

|                      | PP (n = 16) | NP (n = 16) | NC (n = 16) |
|----------------------|------------|------------|------------|
| Age at examination (years) \(^{a, b}\) | 7.7 (7.1–8.3) | 9.1 (8.5–9.7) | 7.8 (7.2–8.4) |
| Birth weight (kg)     | 3.0 (2.8–3.3) | 2.9 (2.7–3.1) | 3.1 (2.8–3.4) |
| GA (weeks)            | 40.0 (38.5–40.0) | 40.0 (40.0–40.0) | 40.0 (39.2–40.0) |
| Height Z-score        | 0.49 (0.15–1.13) | 0.71 (-0.04–1.47) | 0.06 (-1.44–0.86) |
| Weight Z-score        | 0.66 (0.27–0.90) | 0.70 (-0.14–1.19) | -0.41 (-1.16–1.22) |
| BMI Z-score           | 0.27 (0.10–0.75) | 0.54 (-0.34–1.01) | 0.05 (-0.85–1.05) |
| WC (cm) \(^a\)       | 57.0 (55.0–60.0) | 64.3 (57.3–69.4) | 55.0 (52.0–65.5) |
| BA (years) \(^{a, b}\) | 9.0 (7.5–10.0) | 11.0 (10.0–11.5) | 7.5 (5.1–9.4) |
| BA-CA (years) \(^{a, b}\) | 0.7 (0.2–1.2) | 1.8 (1.2–2.4) | -0.1 (-2.0–1.5) |

Data are expressed as medians (with interquartile ranges). \(^a\) \( p < 0.05 \), Normal Pubarche group vs. Normal Control group. \(^b\) \( p < 0.05 \), Premature Pubarche group vs. Normal Pubarche group. Abbreviations: PP, premature pubarche; NP, normal pubarche; NC, normal control; GA, gestational age; BMI, body mass index; WC, waist circumference; BA, bone age; CA, chronologic age.

Biochemical characteristics of subjects (Tables 2 and 3)

Fasting glucose and insulin levels, and HOMA-IR did not differ among the three groups. Plasma insulin-like growth factor 1 (IGF-1) levels were lower in the NC group than the PP group but upon logistic regression analysis, the difference was not statistically significant after correction for other factors such as height Z-score and BA (\( p = 0.090 \)).

Although plasma ACTH levels were similar among the three groups, the plasma levels of 17-OHP, DHEAS, DHEA, AD, and FT were significantly higher in the PP group than the NC group (17-OHP, \( p = 0.001 \); DHEAS, \( p < 0.001 \); DHEA, \( p = 0.001 \); AD, \( p < 0.001 \); FT, \( p < 0.001 \)). The plasma levels of 17-OHP, DHEAS, DHEA, and FT were similar in the PP and NP groups. The plasma concentrations of DHEAS were not correlated with age at diagnosis in PP and NP subjects (\( r = 0.159, p = 0.384, n = 32 \)), even when the MW average CAG repeat length was corrected (\( r = 0.140, p = 0.444, n = 32 \)). Upon logistic regression analysis evaluating the plasma levels of DHEAS, DHEA, AD, and FT together, no single hormone was associated significantly with an increased risk of PP compared to NC. The plasma levels of SHBG, which were negatively correlated with BMI Z-scores (\( r = -0.502, p < 0.001, n = 48 \)) and WC (\( r = -0.502, p < 0.001, n = 48 \)), did not differ among the three groups. No biochemical values differed between genders in the NC group.

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Indirect indices of enzyme activities

Indirect indices of enzyme activities were compared between the PP and NP groups. The PP group had a higher DHEAS:DHEA ratio than did the NP group (PP vs. NP; DHEAS:DHEA ratio, 191.9 vs. 130.4, p=0.014). The AD:DHEA ratio was higher but the DHEA:17-OHP ratio was lower in the PP group than in the NP group (PP vs. NP; AD:DHEA ratio, 0.23 vs. 0.15, p=0.011; DHEA:17-OHP ratio, 4.13 vs. 6.86, p=0.006). The TT:AD ratio did not differ between the two groups.

Upon ACTH stimulation, the DHEAS:DHEA ratio, AD:DHEA ratio, and DHEA:17-OHP ratios decreased, but the TT:AD ratio did not change in the PP group.
The AR CAG repeat length in PP girls

In all subjects, the medians and interquartile ranges of the average and MW average CAG repeat length in the AR gene were 22.5 (21.0-23.9) and 22.4 (21.3-23.3), respectively. Calculations of skewness and kurtosis revealed that the distribution was less skewed and more leptokurtic after MW-base calculations of CAG repeat length (Fig. 1).

The MW average CAG repeat length in the PP group was no shorter than were those in the NP and NC groups (Table 4). The statistical power of each comparison of CAG repeat length was < 0.80, except for the comparison of the long-allele CAG repeat length between the NP and NC groups (power=0.87). No differences were found in the median numbers of CAG repeat lengths between the 16 PP girls and 205 healthy premenopausal females [13], whether analyzed using biallelic means (22.0 in PP vs. 23.0 in healthy premenopausal females; \( p = 0.139 \)) or separately for short alleles (21.0 in PP vs. 22.0 in healthy premenopausal females; \( p = 0.130 \)) and long alleles (24.0 in PP vs. 24.0 in healthy premenopausal females; \( p = 0.486 \)).

In PP and NP subjects, the MW average CAG repeat length was not correlated with age at diagnosis of pubarche \( (r = 0.211, p = 0.247, n = 32) \). BMI Z-score, BA advancement, and HOMA-IR did not exhibit any relationship with the MW average CAG repeat length in subjects with pubarche.

**Table 4** CAG repeat lengths of the androgen receptor gene in subjects

|                      | PP (n = 16)         | NP (n = 16)         | NC (n = 16)         |
|----------------------|---------------------|---------------------|---------------------|
| Long allele of CAG length \( a \) | 24.0 (21.3–26.8)    | 24.5 (23.0–27.0)    | 23.0 (20.3–24.0)    |
| Short allele of CAG length | 21.0 (17.5–22.0)    | 22.0 (21.0–23.0)    | 20.5 (17.0–22.8)    |
| Average CAG length \( a \)     | 22.0 (20.6–23.9)    | 23.0 (22.1–24.9)    | 21.5 (20.0–23.0)    |
| MW average CAG length    | 22.3 (20.7–23.2)    | 22.4 (22.1–24.8)    | 22.2 (20.3–23.2)    |

Data are expressed as medians (with interquartile ranges). \( a \) \( P < 0.05 \), Normal Pubarche group vs. Normal Control group. Abbreviations: PP, premature pubarche; NP, normal pubarche; NC, normal control; MW, methylation-weighted.

**Fig. 1** Distributions of average (A) and methylation-weighted average (B) CAG repeat lengths of the androgen receptor gene are shown. The skewness and kurtosis were -0.552 and 0.359 in A, respectively, and -0.350 and 1.390 in B, respectively.
**X chromosome inactivation**

Of the 40 girls, 35 (87.5%) were heterozygous for CAG repeats of different length. The XCI percentage of the nondominant allele tended to be higher in the PP group (71.9%) than in the NP (65.6%) and NC (60.4%) groups, but the difference was not statistically significant (PP vs. NP, \( p = 0.130 \); PP vs. NC, \( p = 0.490 \)). The number of subjects exhibiting skewed XCI was four, one, and two in the PP, NP, and NC groups, respectively. The methylation percentages of the short-allele CAG repeat (PP, 54%; NP, 38%; and NC, 59%) did not significantly differ between the three groups (PP vs. NP, \( p = 0.963 \); PP vs. NC, \( p = 0.148 \)). The distributions of the expression percentage of long and short alleles were not associated with their CAG repeat lengths.

**Correlation between CAG repeat length and androgen levels (Fig. 2)**

Subjects with pubarche (the PP and NP groups) were divided into the two groups based on the 90th percentiles of the plasma androgen levels from the NC group. The cut-off point for the plasma levels of DHEAS, DHEA, and FT were 730 ng/mL, 6.18 ng/mL, and 2.50 pmol/L, respectively. The MW average CAG repeat lengths did not differ between the two groups. Upon simple correlation analysis, the MW average CAG repeat length did not correlate with the plasma levels of DHEAS (\( r = 0.060, p = 0.745, n = 32 \)), DHEA (\( r = 0.108, p = 0.558, n = 32 \)), or FT (\( r = 0.223, p = 0.221, n = 32 \)), in such subjects.

![Fig. 2](image-url)

**Fig. 2** Comparisons of methylation-weighted (MW) average CAG repeat length between two groups with reference to the plasma concentrations of dehydroepiandrosterone sulfate (A), dehydroepiandrosterone (B), and free testosterone (C) in subjects with pubarche. When the 90th percentiles from the normal control group were used as the cut-off for plasma androgen concentrations, the MW average CAG repeat length did not differ between the two groups.
 Statistical significance was lacking for the XCI per higher in the PP than the NP or NC groups, although DHEAS:DHEA ratio and the XCI percentage were CAG repeat lengths than NP girls. However, the average CAG repeat length was apparent. Therefore, there was no evidence that PP girls might have shorter average CAG repeat length did not differ between PP girls and healthy premenopausal normal controls. No significant association between androgen hormone levels and the MW average CAG repeat length was apparent. Therefore, there was no evidence that PP girls might have shorter CAG repeat lengths than NP girls. However, the DHEAS:DHEA ratio and the XCI percentage were higher in the PP than the NP or NC groups, although statistical significance was lacking for the XCI percentage comparisons.

Biochemical changes during normal adrenarche are explained by increases in the activities of 17, 20-lyase and sulfotransferase, and decreasing 3β-HSD activity to make the adrenal androgens; DHEAS [21]. DHEA is a biologically active hormone that can be converted into AD [21]. However, the half-life of DHEA is short (30-60 min), and is affected by circadian rhythm. DHEAS has a longer half-life (9-11 h) than does DHEA, and is less affected by circadian fluctuations. From the increased sulfotransferase activity (presented as DHEAS:DHEA ratio in this study), produced DHEAS is delivered to the target tissue in a more stable manner. Thus, the level of DHEAS (rather than DHEA) is generally used as an index of biochemical adrenarche. Immunohistochemical experiments have revealed the gene expression pattern optimal for DHEAS production in the zona reticularis (ZR), and only the ZR expresses high levels of SULT2A1, which sulfates DHEA [21]. Therefore, increased level of plasma DHEAS could provide more adrenal androgens to target tissue which will transformed to DHEA by sulfatase in the tissue. Utriainen et al. also reported an increased plasma DHEAS:DHEA ratio in PP subjects compared with normal controls [22], which was consistent with the present study.

Many efforts have been made to identify factors that stimulate adrenarche; ACTH, IGF-1, insulin, cortisol, adrenal medulla, and obesity have been proposed to play roles [1, 8, 9, 23]. The higher IGF-1 levels and advanced BA in PP compared to NC girls observed in the current study, confirming previous findings [23, 24], might be interpreted as the underlying mechanisms of the early growth pattern exhibited by PP subjects. Fasting glucose and insulin levels, and the HOMA-IR were similar among all three groups in the present study, in contrast to a report that PP girls have a higher prevalence of metabolic syndrome and reduced insulin sensitivity compared to normal controls [24]. This might be because only four of the 48 subjects had BMI Z-scores over 1.65 (the 95th percentiles) and/or due to ethnic differences. ACTH is certainly required for adrenarche, as demonstrated by the fact that patients with hypopituitarism do not experience adrenarche [25]. However, the physiological dissociation of cortisol and androgens production renders any trophic effect of ACTH and cortisol on adrenarche difficult to establish. Also, ACTH levels in one-time morning samples may not adequately reflect hormonal status.

In one NP subject with pubarche aged 9.4 years, the plasma level of DHEAS was 226 ng/mL and the MW average CAG repeat length 21.0, thus 1.4 units shorter than the median value. No functional study was performed in vitro, but increased AR transcriptional activity in this subject is suspected. In the PP group, two of 16 girls had plasma DHEAS concentrations (256 and 385 ng/mL) below the adrenarcheal levels described in a previous report [21]. However, their MW average CAG repeat lengths were 23.0 and 22.2, respectively, suggesting that factors other than adrenarchal androgen concentration per se and the MW average CAG repeat length of the AR gene contribute to pubarche.

The normal distribution of the CAG repeat length has been reported to be 6.0-39.0 repeats, and approximately 10.0-30.0 in Caucasians [26]. Ethnic differences exist; the median lengths is 19.0-20.0 in African-Americans, 21.0-22.0 in White Caucasians, 22.0-23.0 in Asians, and 23.0 in Hispanics [27, 28]. The median and ranges of MW average CAG repeat length were 22.4 (16.0-27.8) in a total of 48 subjects. No data on the mean CAG repeat length in normal Korean children and adolescents are available, but this result was similar to those obtained in adult Korean males (23.0) [29] and females (23.0) [13], and slightly greater than those of normal children from different ethnic groups: 21.0 in the Netherlands [30] and 21.4 in Finland [11].

In contrast to the initial expectation, the MW average CAG repeat length did not differ among the PP, NP, and NC groups. Several explanations for this may
be considered. First, the median age at pubarche in the NP group was lower than that reported elsewhere. Although biochemical adrenarche begins around the age of 6.0 years [1], Marshall and Tanner [31] reported that the mean age at development of Tanner stage II pubic hair was 11.7 years among girls in 1969. Recent studies have suggested that the mean onset age of pubarche is around 10.0 years in both Denmark and Lithuania [5, 32] but 12.3 years, 1 year after breast budding, in Korea [33]. The median age at diagnosis of pubarche in NP girls was 8.9 years and only two girls were over 10 years of age (10.4 and 10.8 years) when they entered pubarche. Therefore, if PP girls were compared with NP girls for whom the pubarchea1 age was over 10 years, the results might have been different. Also, ethnic differences in pubertal timing might mean that selecting an onset age criterion of 8.0 years in girls might not be appropriate in Korea. An age younger than 9.0 years might be better as a PP inclusion criterion. Second, in studies evaluating significant differences in the average CAG repeat length in PP subjects compared to normal controls, the mean differences were 0.7 [11, 12] and 0.9 [34]. However, it appears that AR genes with 10.0-30.0 repeats encode functional receptors, as revealed by assays of AR protein levels [35]. Thus it is not clear whether mean differences below 1.0 unit lead to phenotypic differences in vivo. Third, AR sensitivity is not solely dependent on the CAG repeat length in exon 1. For example, exon 1 contains another polymorphic site featuring GGN repeats encoding a polyglycine tract. Direct sequencing of DNA from normal male controls yielded a mean GGN repeat length of 23.0, similar to other studies [28] (data not shown). The most common GGN length (23.0) was associated with the highest transactivation capacity [36]. Finally, the probability of false-negatives was rather high, which is attributable principally to small sample size. This study enrolled PP subjects attending leading medical centers over almost 2 years, but ultimately enrolled only 16 girls, which indicates that the prevalence of PP seems to be low in Korea. Nevertheless, the lack of a difference of the average CAG repeat length between PP girls and healthy premenopausal women might be a significant finding when considering the low prevalence of PP in Korea, although the history of PP in 205 healthy premenopausal normal controls from a previously published study [13] was not defined. In other words, the similar MW average CAG repeat length among PP, NP, and NC girls may be an expected result. However, a sufficient number of subjects to provide appropriate statistical power will be needed in an extended study.

Dose compensation of X-linked genes is achieved via transcriptional repression of one of the two female X chromosomes during early development [37]. Methylation is an important means of silencing gene expression during XCI which occurs randomly. Methylation-sensitive sites near the CAG repeats of exon 1 of the AR gene have been shown to correlate with XCI [17] and those are located in a highly polymorphic region [11] with a heterozygosity of 87.5% in the present study. A skewed XCI was found to play a role in the hypersensitivity of the AR to androgens in females with idiopathic hirsutism [38] and in children with PP [34]. In 35 heterozygous subjects, the XCI percentage and the number of skewed XCIs were higher in the PP than in the NP or NC groups, but it did not reach statistical significance. Additionally, the expression percentages of the long and short alleles were not associated with CAG repeat length. Any role for XCI in the pathogenesis of PP remains uncertain, as does whether skewed XCI, which is associated with less methylation of the shorter allele [38], plays any role. However, the absolute skewness of the average CAG repeat length fell to below 0.5 after methylation-induced inactivation. Therefore, methylation might function to hold the AR gene dose close to the median or mean value to ensure appropriate functioning.

This is the first study of PP in Asia (including Korea), and the findings are meaningful, despite the small number of subjects due to the low prevalence of PP. The CAG repeat length of the AR gene was not involved in the development of PP in Korean girls. However, excessive adrenal androgen levels, particularly caused by increased sulfotransferase activity, were important in the pathogenesis of PP. And finally, pathogenic mechanisms other than CAG repeat length or androgen levels might play a role, although further work will be required to confirm this.

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Competing Interest Declaration

The authors have declared that no competing interest exists.

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