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ARTICLE

Polymorphism of CAG and GGN repeats of androgen receptor gene in women with polycystic ovary syndrome

Chun Yuan [a], Chao Gao [a], Yi Qian [a], Ying Liu [a], Shi-Wen Jiang [b], Yugui Cui [a], Jiayin Liu [a,*]

[a] *State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing, China;* [b] *Department of Biomedical Science, Mercer University School of Medicine, USA*

* Corresponding author. *E-mail address:* jyliu_nj@126.com (J Liu).

Chun Yuan studied medicine at the Nanjing Medical University. She is a candidate PhD in the clinical center of Reproductive Medicine, First Affiliated Hospital of Nanjing Medical University. Her research field is reproductive epidemiology and neuroendocrinology.

Abstract One characteristic of polycystic ovary syndrome (PCOS) is hyperandrogenism, which may be related to the activity of androgen receptor (AR). This study was designed to investigate the polymorphism of CAG and GGN repeats in the AR gene in women with PCOS. The frequency distributions of CAG and GGN repeat alleles, as well as their X-inactivation patterns, were compared between 76 age-matched normal women (control group) and 80 women with PCOS (PCOS group). The expression of *AR* mRNA in the ovarian tissues of seven patients with PCOS and five normal women was also tested using real-time quantitative PCR. It was found that PCOS patients had significantly higher frequency of longer GGN biallelic mean (29.8%) and X-weighted biallelic mean (33.3%) than controls (6.1% and 3.2%, respectively, $P = 0.002$, $P = 0.003$). The odds ratio of the long GGN repeat length ($n > 16$) before and after X-chromosome inactivation (XCI) in the PCOS group was significantly higher than in controls ($P = 0.0001$, $P = 0.005$). AR-GGN repeat mRNA expression was higher in the ovarian tissue of controls compared with PCOS patients ($P = 0.022$). In conclusion, the data suggest that the GGN repeat polymorphism in the *AR* gene is associated with PCOS.

KEYWORDS: androgen receptor, endocrinology, hyperandrogenism, polycystic ovary syndrome, polymorphism

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Introduction

Polycystic ovary syndrome (PCOS), a common anovulatory condition, affects 6–8% of premenopausal women (Gluszak et al., 2012). The 2003 Rotterdam consensus workshop concluded that PCOS was a syndrome with two out of three criteria: (i) oligo- or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism; and (iii) polycystic ovaries (PCO) of ultrasound imaging (with the exclusion of other aetiologies) (Rotterdam, 2004). Hyperandrogenism often presents abnormal biochemical parameters (elevated serum concentrations of testosterone, free testosterone, androstenedione, dehydroepiandrosterone sulphate) (Gluszak et al., 2012), and/or clinical signs (acne, hirsutism, seborrhea, androgenic alopecia, virilization) (Das et al., 2013). The mechanism of hyperandrogenism is still unclear, although the ovarian origin of androgen is well studied (Schweighofer et al., 2012). Actually, there are significant differences in androgen concentration and androgen action among patients with PCOS. Clinical signs may not be apparent in some PCOS patients with biochemical hyperandrogenism, while clinical signs could be very apparent in other patients without biochemical hyperandrogenism. The so-called functional androgen excess in patients with PCOS was estimated to range from 4% to 14% (Sanchon et al., 2012). One of the mechanisms leading to this difference could be molecular mediation of androgen receptor (AR).

AR activity is physiologically modulated by its variably sized polyglutamine and polyglycine tracts in the N-terminal transactivation domain. The tracts are encoded by a highly polymorphic CAG and GGN repeat sequence in exon 1 of the *AR* gene located on the X chromosome (Bennett et al., 2010). The CAG repeat varies in length from 8 to 35 repeats, while GGN repeat, a complex repeat represented by $(GGT)_3GGG(GGT)_2(GGC)_n$, varies in length from 10 to 30 repeats (Faber et al., 1989). The in-vivo study showed that the shorter CAG repeat could be related to the increased transcription of androgen-responsive target genes (Sankar and Hampson, 2012). However, the effect of GGN polymorphism on AR activity remains unclear, as there were discrepancies in previous studies (Jaaskelainen, 2012). One in-vitro study indicated that in response to both testosterone and 5α-dihydrotestosterone, those *AR* with GGN10, GGN27 and GGN24 in the N-terminal showed significantly lower AR activities than the AR with GGN23 (Lundin et al., 2007). However, there were little epidemiological investigations on the association between GGN repeat length and female infertility (Panda et al., 2011). According to classic genetics, one X chromosome becomes inactive in every female cell. X-chromosome inactivation (XCI) causes transcriptional inactivation in two X chromosomes through a series of events, such as DNA methylation, which could induce some diseases due to inappropriate regulation (Lee and Bartolomei, 2013). Other than the microsatellite CAG and GGN repeat analysis, some studies investigated the XCI patterns in some cases (Rajender et al., 2013). However, CAG and GGN repeat polymorphisms in the Asian population with PCOS have not been studied in detail, and the XCI pattern of GGN repeat number has not been investigated.

The issues regarding the familial nature of PCOS and its potential genetic relevance, both autosomal and X-linked patterns, are yet to be defined in patients with PCOS. It is much more difficult to elucidate the aetiopathogenesis of PCOS because of its heterogeneity of clinical presentation and variable progression, such as the individual difference of androgen excess. It is true that the pathophysiological mechanism of PCOS involves the combined actions of genetic, environmental and epigenetic factors. The number variation of CAG/GGN repeat in the *AR* gene is correlated with the transcription of androgen-responsive genes, which is associated with susceptibility to many human diseases (Brokken et al., 2013; Peng et al., 2014). To the best of our knowledge, no studies have been published on the effect of *AR* gene polymorphism on the aetiopathogenesis of PCOS, especially androgen excess. This study was designed to investigate CAG and GGN repeat polymorphisms in the *AR* gene, the XCI pattern and AR expression in Chinese women with PCOS.

Materials and methods

Study population

This study recruited 156 Chinese Han women aged 21–34 years old comprising 80 PCOS cases (the PCOS group) and 76 age-matched healthy women (the control group), at the study centre from 2012 to 2015. All women provided their informed consent. The diagnosis of PCOS was based on the revised criteria of the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine in 2003 (two out of three criteria): (i) oligo- and/or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism; and (iii) polycystic ovaries; and exclusion of other aetiologies (e.g. congenital adrenal hyperplasia, androgen-secreting tumours and Cushing syndrome). Body mass index (BMI), androgen parameters, total testosterone (TT), sex hormone-binding globulin (SHBG), hirsutism (defined as a modified Ferriman–Gallwey score) and acne description were recorded. Blood (5 ml) was collected at 08:00 h.

Ovarian tissues from five women with normal menstrual cycles (women undergoing sex reassignment surgery) and seven PCOS cases (during surgical treatment) were collected after receiving signed consents. All volunteers had stopped hormonal treatment for at least 3 months.

This study was approved by the ethical review board of the First Affiliated Hospital of Nanjing Medical University on 16 April 2012 (reference number: 2012-SR-048).

Clinical and biochemical measurements

Whole blood was sampled on day 2–3 of the menstrual cycle or during the period of amenorrhoea in PCOS patients. Basal sex hormone concentrations were measured in all PCOS and control subjects; TT and SHBG were evaluated using radioimmunoassay kits (North Institute of Biological Technology, Beijing, China) using an automatic clinical chemistry analyser (Olympus AU5400). Free androgen index (FAI = TT/SHBG × 100%) was used to evaluate free testosterone concentrations.

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DNA extraction, amplification and GeneScan analysis

DNA was extracted from the case and control blood samples using a RelaxGene Blood DNA system (TianGen, China). CAG repeats were genotyped using fluorescent PCR-based assay. Genomic DNA (gDNA) was PCR-amplified using fluorescent-labelled primers that flanked the CAG and GGN repeats. The CAG repeat forward fluorochrome-labelled primer was 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′; the reverse primer was 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′. The GGN repeat forward hex fluorescein-labelled primer was 5′-CTGGAGAACCCGCTGGACTA-3′; and reverse primer was 5′-GCCCATTTCGCTTTTGACA-3′. PCR was performed using a GeneAmp 5333 Mastercycler (Eppendorf AG, Germany). Each reaction contained 100 ng DNA template, 0.2 μl DNA polymerase (Takara, Japan), 0.5 μl 5 pmol primers, 3 μl 2.5 mmol/l deoxynucleotides, 10 μl GC-rich buffer (Takara) and 3 μl sterile water in a final volume of 20 μl. The PCR conditions were one cycle at 95°C for 12 min, then 15 cycles at 94°C for 30 s, 63°C for 60 s with touchdown of 0.5°C per cycle, 72°C for 110 s, and finally, 24 cycles at 94°C for 30 s, 56°C for 60 s and 72°C for 110 s. PCR products confirmed on agarose gels were electrophoresed on an ABI PRISM 3730 Genetic Analyser (Applied Biosystems, Foster City, CA, USA), and the fragment size was analysed using GeneMarker V2.2.0 (SoftGenetics, State College, PA, USA).

X chromosome inactivation analysis

XCI analysis was performed according to the protocol described by Hickey et al. (2002) in the heterozygous subjects, where 400 ng gDNA was digested with 30 U *Hpa*II (Roche, Switzerland) and incubated in a 37°C water bath overnight, followed by incubation at 65°C for 15 min to denature the enzyme. About 2.5 μl digested and mock-digested products (the same incubation conditions but without adding *Hpa*II, here used as control) were subjected to PCR. The PCR primers, mixture and amplification conditions were the same as described above. PCR products were electrophoresed on an ABI Prism 3730 Genetic Analyser (Applied Biosystems). The peak area was used to calculate the volume of product using GeneMarker V2.2.0 (SoftGenetics). X-inactivation (relative methylation of each allele) and X-weighted biallelic mean, the ratio of the digested peak area to that of the undigested peak area, was proposed by Hickey et al. (2002). The X-inactivation ratio was evaluated by the degree of inactivation of the longer allele. Following X-inactivation analysis, the X-weighted biallelic mean was calculated, whereby each allele in a genotypic pair was multiplied by its percentage activation, and the two adjusted repeat values were added together.

Analysis of *AR* mRNA expression

The mRNA expression of the CAG/GGN repeats of *AR* in the ovarian tissue from seven PCOS patients and five controls was assessed. The mRNA expression analysis was carried out using quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was generated using random primers and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. SYBR Green I Master (Takara) was used for qRT-PCR and performed on a StepOnePlus thermocycler (Applied Biosystems). Calculations were performed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method. The averaged expression value was estimated following normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The AR and GAPDH primer sequences used were as follows: AR forward 5′-AGCCTATTGCGAGA GAGCTG-3′, AR reverse 5′-GCTTCACTGGGTGTGAAAT-3′; GAPDH forward 5′-GAAGGTCGGAGTCAA-CGGATTT-3′, GAPDH reverse 5′-CTGGAAGATGGTGATGGGATTTC-3′.

Immunohistochemistry assay

Paraffin-embedded ovarian tissues were sectioned (5 μm), and detection of AR protein was carried out using a two-step immunohistochemistry (IHC) procedure using 1:50 AR rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-816, Santa Cruz, CA, USA), and the 1:100 AP-labelled goat anti-rabbit IgG(H+L) as secondary antibody, then a DAB Horseradish Peroxidase Color Development Kit was used (Beyotime, A0239, China; Beyotime, P0203, China). The negative control was sections incubated with non-immune rabbit IgG (Beyotime, A0239, China) instead of the primary antibody.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA) was used to analyse the data; the median CAG and GGN repeat numbers were used as cut-off points for the PCOS case alleles. Differences of distribution and frequency of skewed inactivation (>80%) were analysed using Fisher's exact test. The chi-squared test was used to compare the percentage between PCOS and control group. The relationship of serum TT concentration and BMI to CAG and GGN repeat number was evaluated using simple linear regression and Spearman rank order correlation analysis. For all tests, data from transcription experiments were analysed using the Student's *t*-test; significance was set at 5%.

Results

Clinical features

Table 1 showed that PCOS patients suffered from significantly higher BMI and TT concentration ($P < 0.0001$). The percentage of overweight in PCOS patients was 38.75% (31/80), and there was no significant relationship between TT concentration and BMI ($r = -0.112$).

CAG/GGN allele distribution

The CAG and GGN repeats in the *AR* gene in the PCOS group and the control group were in the ranges 13–34 and 17–27 and

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Table 1 Comparison of clinical characteristics of polycystic ovary syndrome (PCOS) group and controls.

Parameter	*PCOS mean (SD), n = 80*	*Control mean (SD), n = 76*	*P-value*
Age	28.25 (0.26)	28,12 (0.29)	NS
BMI	24.06 (0.22)	22.5 (0.3)	<0.0001
TT(nmol/l)	2.388 (0.1352)	1.175 (0.083)	<0.0001

BMI = body mass index; NS = not statistically significant; TT = total testosterone.

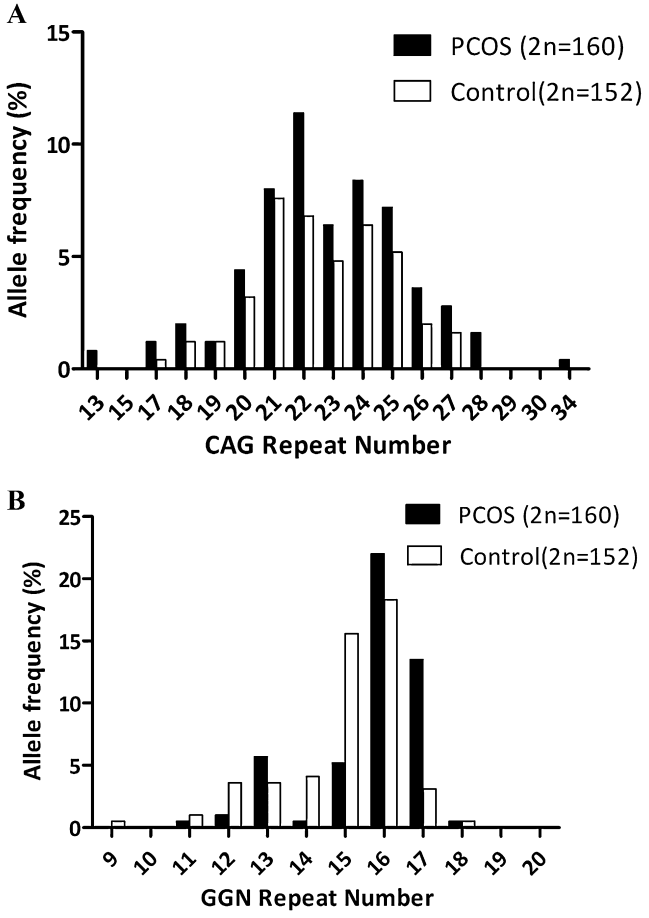

Figure 1 CAG and GGN allele distribution comparisons in patients with polycystic ovary syndrome (PCOS) (n = 80) and controls (n = 76). **A** indicates that CAG 22 and 23 were the most common alleles; **B** shows that alleles 15 and 16 were the most common in the patients with PCOS and controls, respectively. Based on the CAG or GGN allele, a cut-off point of no more than 22 or 16 divided both control distribution profiles into two approximately equal groups, respectively.

11–18 and 9–18, respectively. The frequency distributions of the total alleles (2N) of CAG/GGN repeats in the two groups are presented in Figure 1. The median CAG biallelic repeat values were 22 and 23 and the median GGN biallelic repeat values were 15 and 16 in the control and PCOS groups, respectively. Overall the median biallelic repeat values were 22 and 16 for CAG and GGN repeats, respectively. To evaluate

Table 2 Distribution of polycystic ovary syndrome (PCOS) patients and controls according to the length of CAG repeats calculated as the biallelic mean and X-weighted biallelic mean.

	CAG biallelic mean		*CAG X-weighted biallelic mean*	
	PCOS (%)	*Control (%)*	*PCOS (%)*	*Control (%)*
<22	25.0	35.6	26.0	35.6
≥22	75.0	64.4	74.0	64.4

χ^2 = 2.51, df = 1 (CAG biallelic mean); χ^2 = 0.07, df = 1 (CAG X-weighted biallelic mean): neither is statistically significant.

Table 3 Distribution of polycystic ovary syndrome (PCOS) patients and controls according to the length of GGN repeat calculated as the biallelic mean and X-weighted biallelic mean.

	GGN biallelic mean		*GGN X-weighted biallelic mean*	
	PCOS (%)	*Control (%)*	*PCOS (%)*	*Control (%)*
≤16	70.2	93.9	66.7	96.8
>16	29.8	6.1	33.3	3.2

χ^2 = 9.219, df = 1, P = 0.002 (GGN biallelic mean); χ^2 = 8.717, df = 1, P = 0.003 (GGN X-weighted biallelic mean).

the effects of CAG/GGN repeat lengths, the PCOS group and the control group were divided into subgroups: (i) two subgroups with shorter repeats (CAG ≤ 22, GGN ≤ 16); and (ii) two subgroups with longer repeats (CAG > 22, GGN > 16), according to the mean biallelic trinucleotide repeat lengths. As shown in Table 2, it was found that there were no significant differences of biallelic mean/X-weighted biallelic mean values of shorter CAG repeat subgroup (<22) and the longer CAG repeat subgroup (≥22) between PCOS and control groups (χ^2 = 2.51, df = 1; χ^2 = 0.07, df = 1, both not statistically significant). However, Table 3 showed that the PCOS group had significantly higher frequencies of the longer GGN biallelic mean (29.8%) and the X-weighted biallelic mean (33.3%) than the control group (6.1% and 3.2%, respectively) (χ^2 = 9.219, df = 1, P = 0.002; χ^2 = 8.717, df = 1, P = 0.003,). Figure 2 shows the distribution of CAG/GGN biallelic mean and X-weighted biallelic mean in the PCOS and control groups. In Figure 2B and D, the gel electrophoresis bands showed the difference of gDNA before and after enzyme digestion, which showed that the PCR amplification product of gDNA following *Hpa*II digestion (E+) band is dimmer than that following no *Hpa*II digestion (E−). In this study, all gDNA samples presented this phenomenon; however, only two PCOS samples are shown in the figure.

XCI analysis

XCI analysis showed that for the CAG repeats, 41.7% in the PCOS group and 22.8% in the control group followed the random XCI pattern (60%). Non-random XCI (60–80%) was 13.6% in the PCOS group and 12.1% in the control group, while skewed XCI (>80%) was 4.8% in the PCOS group and 2.9% in the control group (Figure 3A). Moreover, for the GGN repeats, 29.0% in

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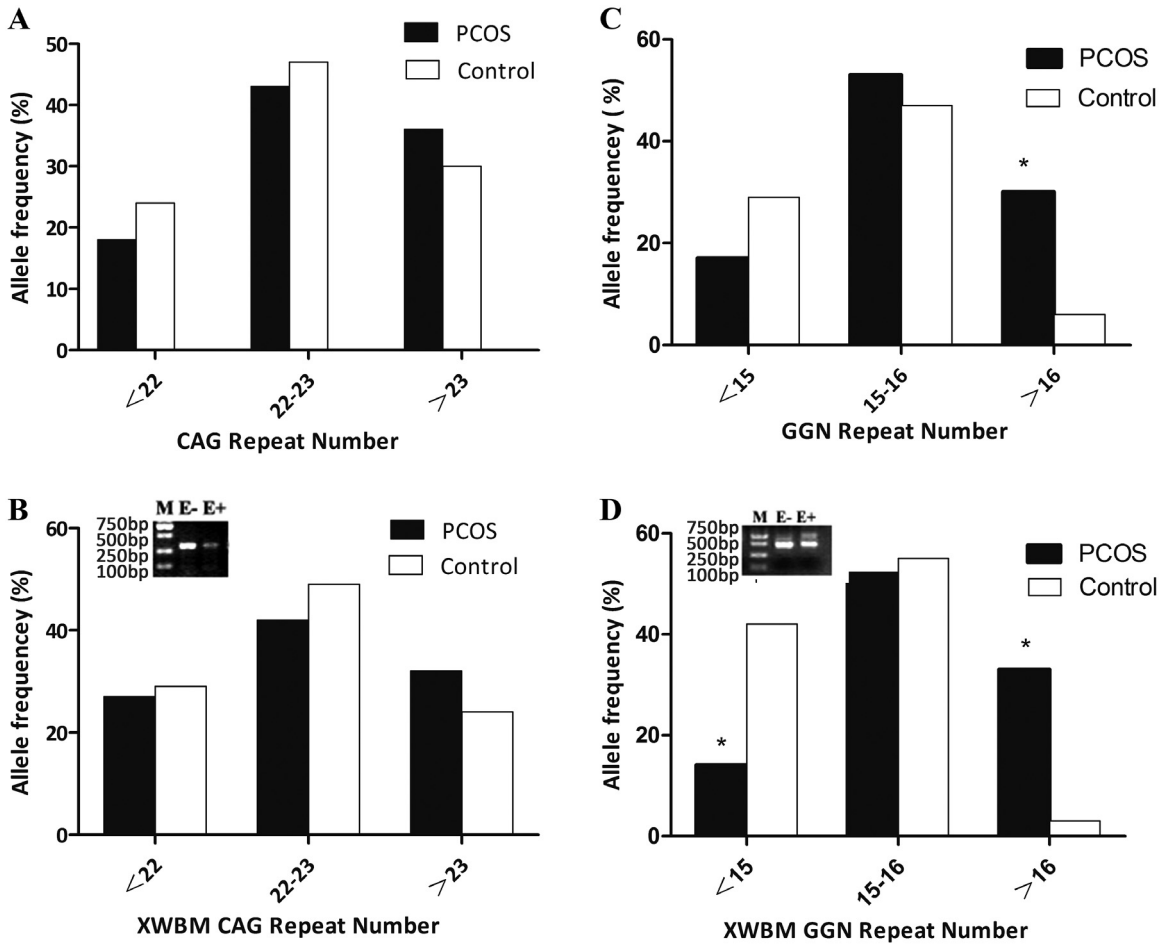

Figure 2 CAG/GGN allele patterns before and after X-inactivation analysis in controls ($n = 76$) and patients with polycystic ovary syndrome (PCOS) ($n = 80$). Agarose gel electrophoresis results showing that the PCR amplification product of gDNA following *Hpa*II digestion (E+) is a dimmer band than that following no *Hpa*II digestion (E−). (The two examples were from two PCOS cases, **B**, **D**). Both the CAG biallelic mean and X-weighted biallelic mean (XWBM) frequencies were not significantly different between the PCOS group and controls in all three subgroups ($\chi^2 = 0.39$, df = 1; $\chi^2 = 0.07$, df = 1) (**A**, **B**). Both the GGN biallelic mean and X-weighted biallelic mean values were significantly different between the PCOS group and controls for the longest (>16) repeat lengths ($\chi^2 = 9.219$, df = 1, $P = 0.002$; $\chi^2 = 8.717$, df = 1, $P = 0.003$, **C**, **D**), and the GGN biallelic mean and X-weighted biallelic mean values between the PCOS group and controls for the shortest repeat lengths (<15 group) were $\chi^2 = 1.812$, df = 1, ns; $\chi^2 = 4.493$, df = 1, $P = 0.032$. *$P < 0.05$.

the PCOS group and 38.0% in the control group were followed by the random XCI pattern. The non-random XCI were 7.0% in the PCOS group and 19.0% in the control group, and skewed XCI (>80%) was 2.0% in the PCOS group and 3.0% in the control group (Figure 3B). There was no significant difference in XCI of CAG and GGN repeat length between the PCOS group and the control group. However, the CAG and GGN repeat trends appeared to be exact opposites: for the CAG repeat non-random XCI and skewed XCI in the PCOS group were greater than those in the control group. Interestingly, for the GGN repeat the non-random XCI and the skewed XCI in the PCOS group were lower than those in the control group (Figure 3).

To estimate further the CAG and GGN repeat length in the PCOS group, the odds ratio (OR) of the shorter and longer repeats was caloculated. The OR value of the long GGN repeat length, before and after XCI analysis, in the PCOS group was significantly higher than that in control group ($P = 0.0001$, OR = 8.16, 95% confidence interval (CI): 2.20, 30.12; $P = 0.005$, OR = 15, 95% CI: 1.68, 133.92), suggesting that the longer GGN repeats ($n > 16$) could be a high risk factor of PCOS. However, there was no significant difference in the OR value of the CAG repeat length, before and after XCI analysis, between two groups (OR = 1.139, 95% CI: 0.55, 2.34; OR = 1.512, 95% CI: 0.676, 3.383).

Relationship between CAG/GGN repeats and androgen concentration

TT concentration and SHBG were used to calculate the FAI and to analyse the relationship between hyperandrogenism and the CAG/GGN repeat lengths. However, there was no significant association between FAI and the CAG/GGN repeat lengths. The Pearson correlation coefficients of the PCOS and control groups combined, before and after XCI respectively,

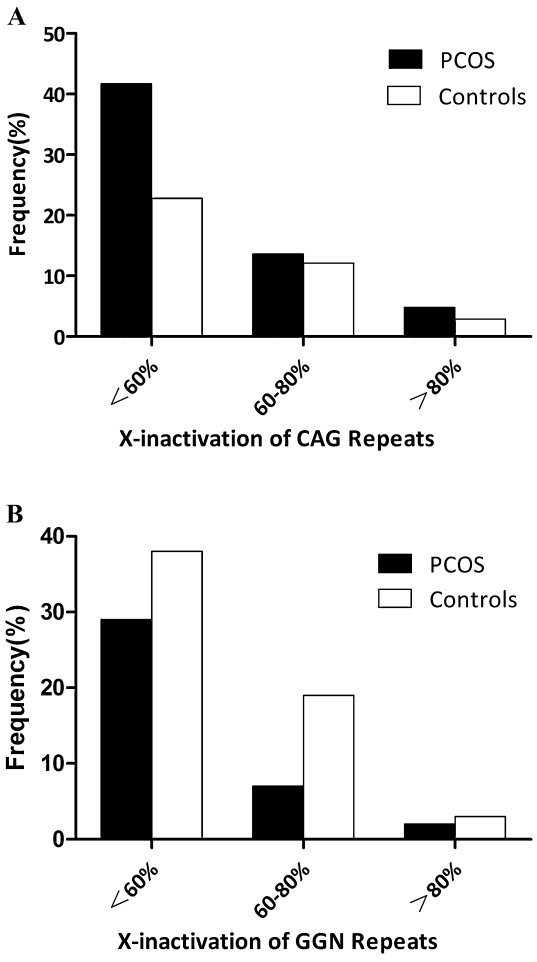

Figure 3 Comparison of X-inactivation patterns in controls ($2n = 152$) and patients with polycystic ovary syndrome (PCOS) ($2n = 160$). Inactivation was gauged by the relative methylation, normalized to 100%, of an allele in a genomic pair. Skewed inactivation was indicated when there was at least 80% inactivation of one allele. There were no significant differences between groups.

were 0.149 and 0.062 for CAG repeats and 0.220 and –0.170 for GGN repeats.

Expression of *AR* CAG/GGN repeats and *AR* mRNA

There was no significant difference in the mean expression level of CAG repeat mRNA in the ovarian tissues between PCOS cases and controls (Figure 4A). Interestingly, AR-GGN repeat mRNA expression was higher in the ovarian tissue of controls compared with PCOS patients ($P = 0.022$, Figure 4B). Moreover, there was a remarkable positive relationship between the expression level of CAG repeat mRNA and the CAG repeat length in the PCOS group, suggesting that higher expression level of CAG repeat could be associated with the development of PCOS ($r = 0.79$, $F = 19.2$, $P = 0.007$). However, this positive relationship was not found in the control group ($r = 0.14$, $F = 0.04$). When the PCOS cases and controls were put together, there was no significant correlation between the CAG repeat length and its expression level ($r = 0.115$, $F = 1.304$).

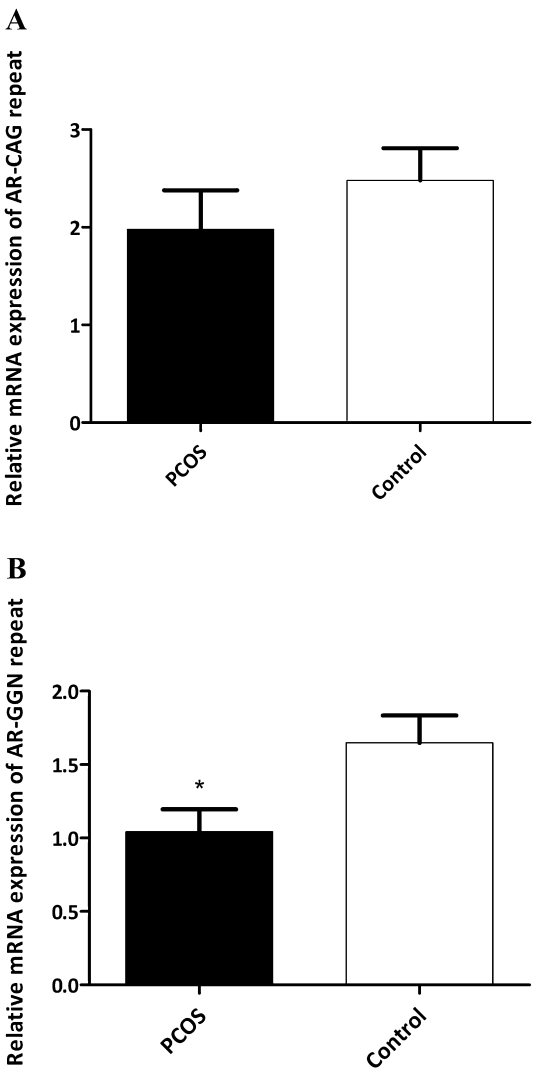

Figure 4 Relative mRNA expression of androgen receptor (AR) CAG/GGN repeats in ovarian tissue. AR CAG/GGN expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Panel **A** illustrates that the relative mRNA expression of the CAG repeat is not significantly different between patients with polycystic ovary syndrome (PCOS) ($n = 7$) and the controls ($n = 5$); in panel **B**, mRNA expression of the GGN repeat is significantly higher in the controls than in the patients with PCOS ($P = 0.022$), $^*P < 0.05$.

There was no significant difference in the expression level of *AR* mRNA between the PCOS group and the control group. The expression level of *AR* mRNA was positively associated with the CAG repeat length in the PCOS group ($r = 0.81$, $F = 20.97$, $P = 0.006$). However, this association was not found in the control group ($r = 0.31$, $F = 0.37$). Meanwhile, a correlation between the expression level of *AR* mRNA and the GGN repeat length was not found in the PCOS group or the control group.

Expression of AR protein in ovarian tissues

To support the above data, the expression and subcellular location of AR protein in the ovarian tissues were tested by

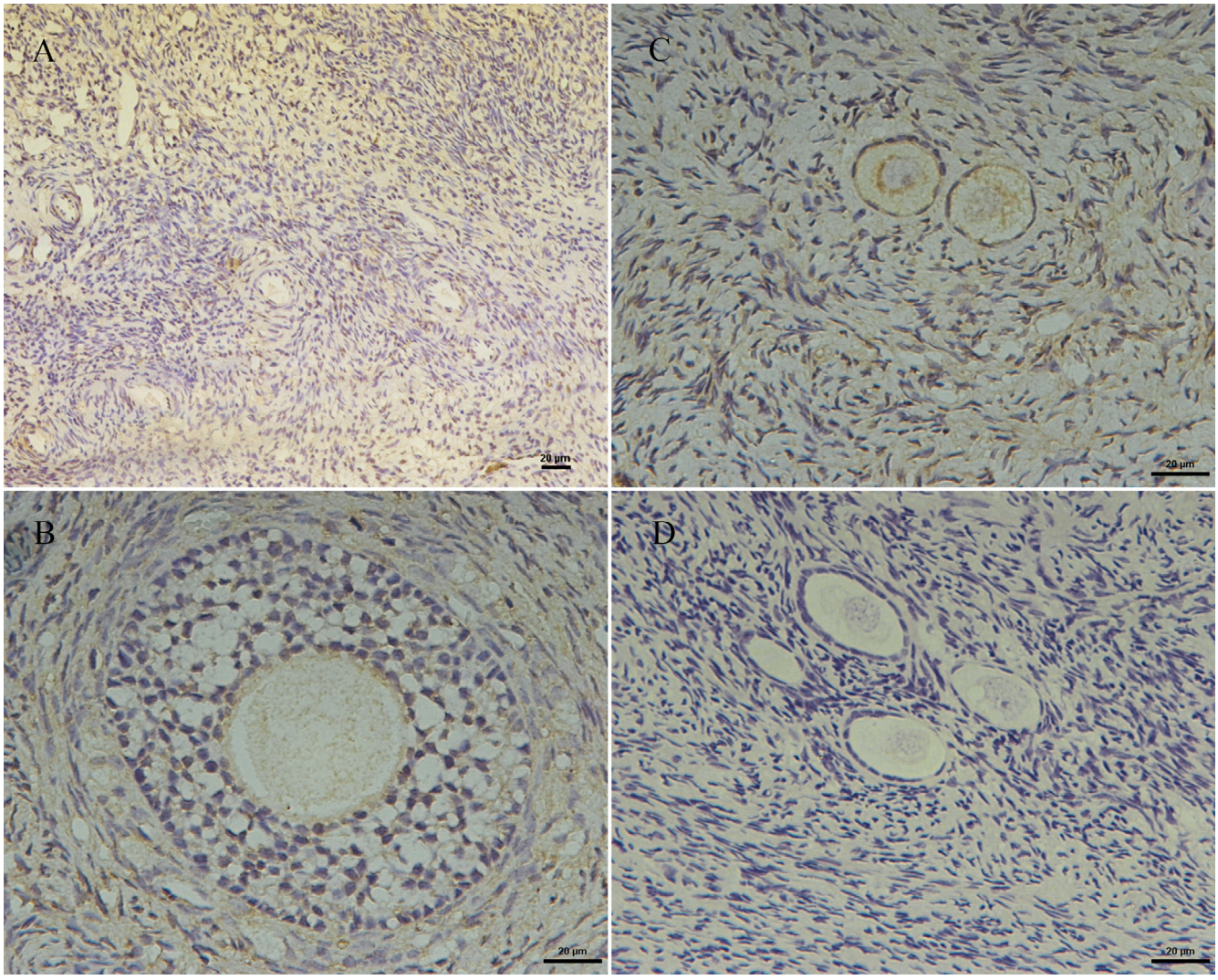

Figure 5 Immunohistochemical staining for androgen receptor (AR) protein in ovarian tissue from patients with polycystic ovary syndrome (PCOS). AR protein was expressed throughout the ovary sections (**A**), predominantly in the granulosa and theca layer of the small antral follicles (**B**, **C**, 3–5 mm diameter). (**D**) An adjacent section treated with non-immune rabbit IgG. Scale bar = 20 μm.

immunohistochemical staining. The expression of AR protein was evident in the ovarian tissues, especially in the granulosa and theca cells of the small antral follicles (**Figure 5**).

Discussion

In the present study, the hypothesis that some functional genetic variations in *AR* gene could be associated with the pathophysiological development of PCOS was proved. The CAG repeat in exon 1 of *AR* gene was not found to be associated with the development of PCOS, which confirms the results of previous studies (Peng et al., 2014; Zhang et al., 2013). Interestingly, it was found that there was a significant relationship between longer GGN repeats and PCOS development before and after XCI analysis. Therefore, polymorphism of some repeats in exon 1 of the *AR* gene, such as GGN repeat, may be one of the potential genetic factors of PCOS development.

XCI is the mechanism for dosage compensation of X-linked genes in females. The XCI analysis has long been of interest as a model for understanding gene regulation. The skewed XCI was found to be related to X-linked diseases and other disorders such as PCOS (Shah et al., 2008; Simmonds et al., 2014). This study found that there was no significant difference in XCI pattern between PCOS cases and controls, which means the frequency of non-random XCI was not increased in PCOS cases. The study result was consistent with that found by Shah *et al.* (Shah et al., 2008). Although the greater frequency of the active alleles with >22 CAG repeats in PCOS cases was reported by another study (Hickey et al., 2002), similar increased frequency in this study's PCOS groups was not found.

Two polymorphisms in exon 1 of the *AR* gene alter the primary protein structure: CAG repeat (polyglutamine) and GGN repeat (polyglycine). Some studies showed that CAG repeat expansions were responsible for a number of metabolic disorders, such as Kennedy syndrome (also known as spinal and bulbar muscular atrophy) (Chopra and Shakkottai,

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2014). Another in-vitro study also showed that those AR genes with more than 40 CAG repeats had lower transcription activity when compared with other *AR* genes with 25, 20 or no CAG repeats (Kazemi-Esfarjani et al., 1995). Therefore, the long CAG repeat should be associated with the decreased AR activity and hence disorders related to the reduced androgen actions. Similarly, Gao *et al*. estimated that the deletion of GGN repeat could result in 20–30% reduction of the transactivation potential of the *AR* gene (Gao et al., 1996). CAG repeat has been one of the most frequently studied polymorphisms of the *AR* gene in clinical studies on androgen metabolism disorders, while there have been few studies on the GGN repeat due to the technical difficulties in amplifying its GC-rich region. This study investigated the relationship between the variation of trinucleotide repeat length polymorphisms of CAG repeat and GGN repeat in the *AR* gene and the different phenotypes of PCOS.

The data suggested that there was no significant difference in CAG repeat length of *AR* gene between PCOS cases and control before or after XCI analysis. Xia *et al*. reported a significantly higher frequency of short $(CAG)_n$ alleles in the PCOS group, which suggested that the shorter alleles of the $(CAG)_n$ repeat in *AR* gene could enhance the susceptibility to PCOS by up-regulating *AR* activity or hyperandrogenism (Xia et al., 2012). Similarly, Schuring *et al*. asserted that the shorter CAG repeat length could be an independent risk factor for PCOS (Schuring et al., 2012). However, the meta-analysis of Wang *et al*. determined that the CAG repeat polymorphism was unlikely to be a major factor in the development of PCOS (Wang et al., 2012). It is necessary to investigate further the underlying association between the shorter CAG repeat length and PCOS development in the Chinese Han population.

To our knowledge, there were many studies on the relationship between CAG repeat length and some endocrine diseases (Song et al., 2012; Zhang et al., 2013), but few studies on the expression of *AR* mRNA related to CAG polymorphisms and/or on the combination of CAG and GGN repeats. In this study, it was found that there was no significant difference in CAG repeat length between PCOS cases and controls. However, there was a positive relationship between the transcriptional level of *AR* gene and the expression of CAG repeat length in the PCOS cases. Consistent with the results of this study, Nenonen *et al*. found that the expression of *AR* mRNA in the CAG 22 variant was higher than that in the CAG 16 variant or 28 variant, indicating that, within this CAG repeat range at least, the higher *AR* expression could compensate for functional difference (Nenonen et al., 2010). Some studies suggested that to a certain extent, there was an association between the increased activity of AR encoded by shorter CAG repeat polymorphisms and the development of PCOS, although there were also other conflicting results. For example, Ryan *et al*. showed that variation of the CAG repeat length in the *AR* gene was negatively correlated with the transcription level of androgen-responsive gene (Ryan and Crespi, 2013). Few studies focused on the association between AR activity and GGN repeat variants. Using an in-vivo model, Lundin *et al*. evaluated the transactivation activity of *AR* with the two most common GGN alleles in combination with the median CAG length (Lundin et al., 2007). It was shown that those AR with GGN10, GGN27 and GGN24 in N-terminal showed significantly lower AR activities than the AR with GGN23 (Lundin et al., 2007). In this study, it was found that the frequency of the longer GGN repeat in AR gene was significantly increased in PCOS cases, which suggested that the AR activity in those PCOS cases with the longer GGN repeat in *AR* gene could be increased and hence enhanced the effect of androgen.

The phenotype of hyperandrogenism in PCOS is actually more complex because of potential epigenetic effects of the XCI related to AR activity. Based on the dosage compensation theory, one of the two X chromosomes in female mammals is transcriptionally inactive (Wutz, 2011). This study firstly used the genomic mRNAs extracted from the ovarian tissues of PCOS cases and controls to evaluate the correlation between AR activity and CAG/GGN repeats. The results suggested that the longer GGN repeats ($n > 16$) could be a high risk factor of PCOS, before and after XCI analysis, whereas the CAG repeat length was not. However, there was no significant association between androgen concentration, and FAI, and the CAG/GGN repeat lengths, and this study could have some limitations, such as small sample size. Therefore, it is necessary to investigate further the potential correlation between clinical complex phenotypes and CAG/GGN repeats in Asian PCOS groups with hyperandrogenism.

In summary, it was found that the polymorphism of GGN repeat in the *AR* gene, other than CAG repeat, was associated with the development of PCOS in Chinese women of reproductive age. We thought that the AR activity in those PCOS cases with longer GGN repeats could be increased in the internal hyperandrogenic environment, which enhanced the effect of androgen. This study also provided potential evidence that the expression level of AR was epigenetically regulated by the polymorphism of trinucleotide repeat in the *AR* gene, which provided useful evidence of anti-androgen treatment for PCOS.

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