No Association of Androgen Receptor GGN Repeat Length Polymorphism With Infertility in Indian Men

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ABSTRACT: Androgens, acting through the androgen receptor (AR), play a role in secondary sexual differentiation from the prenatal stage to adulthood, including spermatogenesis. The *AR* gene has 2 polymorphic trinucleotide repeats (CAG and GGN) in exon 1. The CAG repeat length polymorphism has been well studied in a variety of medical conditions, including male infertility. Many of these studies have shown an association of the expanded CAG repeats with male infertility, although this is not true for all populations. The GGN repeat, in contrast, has been less thoroughly studied. Thus far, only 4 reports worldwide have analyzed the GGN repeat, alone or in combination with the CAG repeat, in male infertility cases. No such study has been undertaken on infertile Indian men. Therefore, we have analyzed *AR*-GGN repeats in a total of 595 Indian males, including 277 azoospemric, 97 oligozoospermic, and 21 oligoteratozoospermic cases, along with 200 normozoospermic controls. The analysis revealed no difference in the mean number or the range of the repeat between cases (mean = 21.51 repeats, range 15–26 repeats) and controls (mean 21.58 repeats, range 15–26 repeats). Furthermore, no difference was observed when azoospermic (mean = 21.53 repeats, range 15–26 repeats), oligozoospermic (mean = 21.46 repeats, range 15–26 repeats), and oligoteratozoospermic cases (mean = 21.48, range 19–26 repeats) were compared individually with the controls.

Key words: Androgen receptor, GGN repeat, male infertility, semen abnormality.

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Approximately 15%–18% of couples suffer from infertility, of which 50% is attributable to male factors. Although sperm count is affected by environmental factors, including lifestyle, food habits, and health status, it is largely dependent on genetic components. Hormonally, spermatogenesis is controlled by androgens (testosterone and dihydrotestosterone) and follicle-stimulating hormone (FSH) through their action on Sertoli cells (Tesarik et al, 1998). Androgens are dependent for their functions upon the androgen receptor (AR), a nuclear steroid hormone receptor. The receptor-testosterone complex signals the differentiation of Wolffian ducts during embryonic life and regulates the secretion of leutinizing hormone by the hypothalamic-pituitary axis, as well as spermatogenesis, after puberty. The receptor-dihydrotestosterone complex promotes development of the external genitalia and prostate during embryogenesis and is also responsible for the changes which occur at puberty in males (Haqq and Donahoe, 1998).

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The *AR* gene has been mapped to the long arm (Xq11-12) of the X-chromosome (Lubahn et al, 1988b; Yong et al, 1998). The gene consists of 8 exons and encodes a protein of 919 amino acids with 3 major functional domains: the N-terminal domain (NTD or transactivation domain), the DNA-binding domain (DBD), and the ligand-binding domain (LBD). The AR protein is a member of the nuclear receptor superfamily of ligand-activated transcription factors. Exon 1 of the gene consists of 2 polymorphic repeat (CAG and GGN) motifs, encoding variable lengths of polyglutamine and polyglycine stretches, respectively, in the N-terminal region (transactivation domain) of the AR protein (Lubahn et al, 1988a; Faber et al, 1989). CAG, a simple repeat, varies in length from 8 to 35 repeats, while GGN, a complex repeat represented by $(GGT)_3GGG(GGT)_2(GGC)_n$, varies in length from 10 to 30 repeats.

The CAG repeat has been well studied in a variety of medical conditions in addition to male infertility (Hickey et al, 2002; Sasaki et al, 2003; Yong et al, 2003). Expansion of the CAG repeat above the normal range has been found to be associated with adult onset of spinal and bulbar muscular atrophy (La Spada et al, 1991), characterized by undermasculinization and progressive neuromuscular degeneration. Expanded CAG repeat number within the normal range has also been

found to be associated with male infertility in some populations, but this is not true for all populations (Yong et al, 2003). Decreased CAG repeats, on the other hand, have been found to be associated with prostate cancer (Giovannucci et al, 1997; Mishra et al, 2005). The inverse correlation between CAG repeats length and AR transactivation potential has been demonstrated in vitro (Chamberlain, 1994, Kazemi-Esfarjani et al, 1995). We have earlier analyzed the CAG repeat polymorphism in 280 azoospermic and 201 fertile Indian men and found no correlation between the repeat length variation and infertility (Thangaraj et al, 2002).

Although deletion of the GGN repeat resulted in a 30% reduction in AR transcriptional activation in transfection assays (Gao et al, 1996), the GGN repeat has been less studied, mainly due to technical problems in the amplification resulting from the high GC content of this repeat. To our knowledge, only 4 studies on the GGN repeat in male infertility have been conducted to date (Tut et al, 1997; Lundin et al, 2003; Ferlin et al, 2004; Ruhayel et al, 2004). Hence the functional implications of variation in the GGN repeat are unclear. In particular, no study has been conducted on the role of the GGN repeat in male infertility among Indian men. Further, although normal variation of the CAG repeat has been well studied in many populations and worldwide polymorphism data exists, much less is known about the polymorphic variation of the GGN repeat, and nothing at all for Indian populations. Therefore, we have now analyzed the GGN repeat length in 395 infertile and 200 normozoospermic Indian men to understand the correlation between polymorphisms in this repeat and male infertility and to document the normal polymorphism of this repeat in Indian populations.

Materials and Methods

Subjects and Controls

A total of 395 infertile cases, consisting of 277 nonobstructive azoospermic, 97 oligozoospermic, and 21 oligoteratozoospermic men, aged between 25 and 45 years, were recruited for the study from the Institute of Reproductive Medicine (IRM), Kolkata. All the patients had a long period (3–8 years) of infertility, and known causes of male infertility such as obstruction of sperm release, cystic fibrosis, infections, and undervirilization were excluded by a team of expert andrologists. The obstruction of sperm release was ruled out by physical examination and the vasogram report for all the azoospermic cases. The infertile cases were subgrouped according to sperm concentration. Cases with no sperm were categorized as azoospermic, while cases with sperm counts less than 20 million/mL were categorized as oligozoospermic. Similarly, cases with both reduced sperm counts and deformities in the sperm shape were categorized as oligoteratozoospermia. The patients belonged to Indo-European, Austro-Asiatic, and Tibeto-Burman linguistic groups and originated from 6 states (West Bengal, Orissa, Bihar, Jharkhand, Uttar Pradesh, and Assam) of India. A total of 200 healthy normozoospermic men with the same ethnic backgrounds as the patients were recruited in the study as controls. All the control subjects had sperm counts greater than 20 million/mL and had fathered at least 1 child. Peripheral blood was obtained for karyotyping and molecular analysis, along with the family history and informed written consent of the subjects and the controls. Infertile men who had chromosomal abnormalities or Y chromosome microdeletions (Thangaraj et al, 2003) were not included in this study.

Genetic Analysis

DNA was extracted from peripheral blood by the method described in our earlier study (Thangaraj et al, 2002). The *AR*-GGN repeat was amplified with the primers: forward 5′ FAM-CCGCTTCCTCATCCTGGCACAC 3′ and reverse 5′ GCCGCCAGGGTACCACACATC 3′, flanking the repeat. PCR primers were designed using GeneTool software and synthesized using a 394 DNA/RNA oligosynthesizer (Applied Biosystems, Foster City, Calif). PCR reactions were set in a 10 μL volume with the composition 1.0 μL PCR buffer (10 ×), 1.0 μL $MgCl_2$ (25 mM), 1.0 μL dNTPs (10 mM), 5 pM of each primer, 0.5 units AmpliTaq Gold DNA polymerase, 0.4 μL DMSO (100%), 0.6 μL glycerol (100%), and 20 ng DNA. PCR reactions were performed under the following conditions: initial denaturation at 96°C for 12 minutes, followed by 40 cycles of 96°C for 1.5 minutes, 60°C for 1 minute, and 72°C for 3 minutes, with a final extension for 20 minutes at 72°C. The lengths of the PCR products were assessed by GeneScan analysis. For GeneScan, 3.0 μL of the PCR product was mixed with 0.2 μL of LIZ500 and 6.8 μL of formamide. After denaturation for 5 minutes at 95°C and cooling on ice for 5 minutes, the samples were run on a 3730 DNA analyzer (Applied Biosystems). The raw data were further analyzed using GeneMapper software to calculate the number of repeats. PCR and GeneScan were repeated for all the samples to confirm the number of repeats.

Statistical Analysis

The mean, median, and mode of the repeat number were calculated for all the sample categories using SPSS software (version 10; SPSS, Inc, Chicago, Ill). In addition to the analysis of all the patients as 1 group, and the controls as 1 group, we also analyzed the repeat length in various subgroups of the patients. The significance of the difference in the mean repeat length was compared for different subgroups of the samples against controls using an independent-samples t test. Two-sided P values of less than .05 were considered significant. In addition, the frequency distribution of the repeats was also calculated for all the 595 samples irrespective of their fertility status to illustrate the general distribution of the repeat in Indian populations.

GGN repeat length among different categories of patients and controls

Category	Total Samples	GGN Repeat			
		Range	Alleles	Mean	Mode
Controls	200	15–26	9	21.58 ± 0.07	22
All cases	395	15–26	9	21.51 ± 0.06	22
Azoospermic	277	15–26	9	21.53 + 0.07	22
Oligozoospermic	97	15–26	6	21.46 + 0.13	22
Oligoteratozoospermic	21	19–26	4	21.48 + 0.30	21, 22

Results

The GGN repeat number ranged from 15 to 26 with no significant difference in the mean repeat length or the range between the cases (mean = 21.51 ± 0.06 repeats, range 15–26 repeats) and the controls (mean = 21.58 ± 0.07 repeats, range 15–26 repeats) (Table). Similarly, the cases did not show any significant difference from the controls when individual patient subgroups (azoospermic, oligozoospermic, and oligoteratozoospermic) were examined (Table; Figure). Similarly, there was no significant difference in the mean repeat length between azoospermic and all oligozoospermic (oligozoospermic and oligoteratozoospermic) cases. The *AR* alleles with 21 and 22 GGN repeats were predominant, with a very low frequency of smaller or larger alleles in all categories of samples (Figure). *AR* alleles with 21 and 22 GGN repeats were found in almost the same percentage in the infertile cases (91%) and controls (93.38%). In the oligozoospermic cases, *AR* alleles with 16, 17, 20, 23, 24, and 25 repeats were not observed, while in oligoteratozoospermic cases the alleles with 15, 16, 17, 18, 20, 23, 24, and 25 GGN repeats were not observed (Figure). The distribution of *AR* alleles in all the 595 samples (irrespective of the abnormality status) showed a total of 9 alleles in the Indian populations, but more than 90% of individuals carried 21 or 22 GGN repeats.

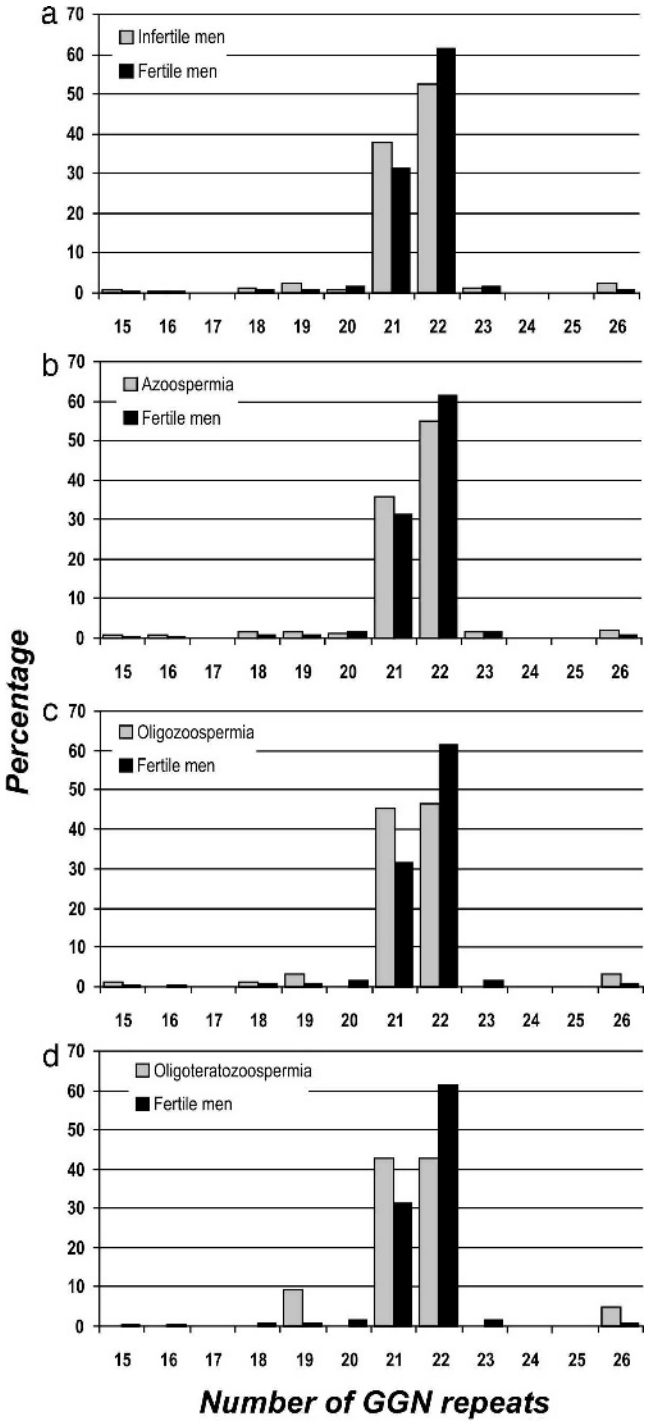
Discussion

In male infertility, much attention has focused on the analysis of spermatogenic genes in AZoospermia Factors (AZF) region. Many of the genes in this region exist in multiple copies, and the sperm count may be dependent on the number of functional copies of these genes. Although mutations in the known genes are a frequent cause of male infertility (Thangaraj et al, 2003), they still account for only ~20% of infertility cases (Thangaraj et al, unpublished data). Thus the majority of infertile cases remain unexplained. Mutations in the *AR* gene, including variation in the number of CAG repeats, have been found to be associated with male infertility in some populations but not others (Yong et al, 2003). AR-GGN repeat length polymorphism has been less studied in male infertility. Among the Indian populations, no earlier study has been conducted on GGN repeat length polymorphisms in male infertility.

In the present study, we have analyzed the *AR*-GGN repeat length polymorphism in a total of 595 men from India, and found it to be less polymorphic than the CAG repeat (Table). *AR* alleles with 21 and 22 GGN repeats predominate in Indian populations (Figure). An earlier study of an Italian population found *AR* alleles with 23 and 24 GGN repeats (17 and 18 GGC repeats) to be the commonest (Ferlin et al, 2004), which may reflect a geographical difference between the populations.

Furthermore, we found no association of the GGN repeat number with spermatogenic status. Indeed, unlike the varying correlation of the CAG repeat with male infertility, all studies on the GGN repeat thus far have shown no association with male infertility (Tut et al, 1997; Lundin et al, 2003; Ferlin et al, 2004; Ruhayel et al, 2004). However, studies on the GGN repeat in other diseases, especially prostate cancer, have given a more confusing picture: no association was detected in Scottish men (Tayeb et al, 2004), but a 12% increased risk was found in Chinese men with a repeat length less than 23 (Hsing et al, 2000) and an increased risk associated with shorter GGN repeats in other populations (Hakimi et al, 1997; Stanford et al, 1997), while, in contrast, an increased risk was associated with longer GGN repeats in yet another study (Edwards et al, 1999). The overall picture is thus still not clear, and further well-planned epidemiological studies are required to elucidate the role of the GGN repeat in AR function.

The *AR* gene has been shown to play role in final stages of sperm differentiation, and hence defects in the *AR* gene are more likely to cause reduced sperm counts along with sperm deformities (oligozoospermia or oligoteratozoospermia) rather than complete absence of the sperms (azoospermia) (Singh et al, 2006). Out of 395 infertile cases analyzed in the present study, 277 cases were azoospermic, and hence the results could be biased not to give a fair idea about the correlation of the



GGN repeat length distribution among all infertile men **(a)**, azoospermic cases **(b)**, oligozoospermic cases **(c)**, oligoteratozoospermic cases **(d)** versus normozoospermic individuals. The X-axis represents the number of repeats and Y-axis the frequency (%).

repeat length polymorphisms with oligozoospermia or oligoteratozoospermia. Therefore, we analyzed GGN repeat length for each subgroup of the cases versus controls. This revealed no association of repeat polymorphism with azoospermia, oligozoospermia, or oligoteratozoospermia (Figure). No difference was observed even between azoospermic and all oligozoospermic cases (oligozoospermic and oligoteratozoospermic). However, in the oligozoospermic cases *AR* alleles with 16, 17, 20, 23, 24, and 25 GGN repeats, and in oligoteratozoospermic cases the alleles with 15, 16, 17, 18, 20, 23, 24, and 25 GGN repeats, were not observed (Figure). These are the alleles with very low frequencies in the population; therefore the differences could be attributed to the lesser number of the oligozoospermic and even lesser number of oligoteratozoospermic cases in comparison to azoospermic or normozoospermic cases.

In all the cases included in this study, we have previously sequenced the whole *AR* gene and found no mutations (Singh et al, 2006). The present study, along with the previous one, thus demonstrates that the *AR* gene does not contribute significantly to male infertility in Indian populations. This conclusion contrasts with the observed decrease in the AR transcriptional activation upon deletion of GGN repeats in transfection assays (Gao et al, 1996), but it needs to be ascertained if the decrease in the number of GGN repeats parallels the decrease in the transactivation. However, it needs to be mentioned that the absence of correlation of the GGN repeat length with male infertility in the present study does not reflect absence of correlation between the repeat length and AR function. It has been found that longer CAG repeat resulted in decreased ability of AR to be coactivated by its coregulators (Heinlein et al, 2004), and it needs to be established if the GGN repeat affects the interaction of AR with its coregulators. Along with this, polymorphisms in other AR interacting genes, such as steroid receptor coregulator-1 (SRC-1) and transcriptional intermediary factor 2 (TIF-2), may also affect AR functions, spermatogenesis, and hence male fertility. In addition, the heavy use of agricultural and industrial chemicals, many of which have been proven to be endocrine disruptors, might contribute to the disruption of the androgenic functions, leading to decreased sperm counts or infertility. Thus the absence of a simple relationship between *AR*-GGN repeat length, likely transcriptional activation, and sperm count in India is readily explained.

In conclusion, *AR*-GGN repeat length polymorphism showed no correlation with male infertility in our study. Nevertheless, 80% of male infertility in India remains unexplained, and investigation of further genetic and nongenetic factors that may influence spermatogenic status is required. Although it seems from most of the studies that GGN repeat length doesn't affect the AR functions significantly, it would be premature to draw conclusions about the role of GGN repeat at this stage.

This is the first study addressing analysis of GGN repeat in male infertility in Indian populations and the fifth such study worldwide. More studies on GGN repeat, along with in vitro assays using *AR* alleles with different number of GGN repeats, will add to the wealth of knowledge about the role of GGN repeat in AR function and its correlation with male infertility.

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