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LENGTH VARIATION OF TRINUCLEOTIDE CAG REPEAT IN THE ANDROGEN RECEPTOR GENE IN HUMAN INFERTILE MALES

PRAFULLA S. AMBULKAR AND SUNIL S. PANDE*

Rajiv Gandhi Biotechnology Centre, LIT premises, RTMNU University, Nagpur-33 MS, India

ABSTRACT

Androgens are required for normal spermatogenesis, expansion of CAG repeats in the androgen receptor (AR) gene gives rise to human male infertility. We investigated length polymorphism of CAG repeats in the androgen receptor as a possible cause of impaired spermatogenesis in patients with idiopathic male infertility in our geographical central Indian population. The CAG repeat length in exon 1 of the androgen receptor gene was studied in 60 men with idiopathic azoospermia, 90 men with oligozoospermia and in 40 fertile men. The CAG repeat region was amplified by polymerase chain reactions (PCR). The number of CAG repeat was analysed by DNA sequencing. Hormone levels (FHS, LH and Testosterone) were also measured in patients with azoospermia and oligozoospermia. In the azoospermic males, two (3.3%) had short CAG repeats and five (8.3%) had long CAG repeats. In oligozoospermic men were also had two (2.2%) short CAG repeats and two (2.2%) had long CAG repeats. FHS and LH level were significantly higher in azoospermia group than in fertile control but unable to correlate with a length of CAG repeats. The prevalence of infertile men with long CAG repeats is moderately higher in azoospermic group but not in oligozoospermic group when compared with the fertile controls. Our results suggest that short and long CAG repeat within AR gene might be affected the spermatogenesis in males.

KEYWARDS: Azoospermia, AR gene, CAG repeats, Infertility.

SUNIL S. PANDE
Rajiv Gandhi Biotechnology Centre, LIT premises, RTMNU University, Nagpur-33 MS, India

***Corresponding author**

INTRODUCTION

Androgens are important determinant for normal spermatogenesis. Although, a man with normal serum androgens level have impaired spermatogenesis, malfunction of androgen receptor gene[1], environmental mutagens, addiction of tobacco smoking and alcohol intake also the cause of male infertility[2]. Androgens exhibit their physiological activities by binding to the androgen receptor. Therefore, abnormalities in the androgen receptor are the potential cause of impaired spermatogenesis in patients with idiopathic male infertility [1]. The androgen receptor gene has been mapped in the long arm of the X chromosome (Xq11-12). The androgen receptor gene contains 8 exons with a coding sequence of about 2760 base pairs (bp)[2]. The gene has two polymorphic sites in exon 1, characterized by different numbers of CAG and GGC repeats resulting in variable lengths of polyglutamine and polyglycine stretches[3]. Some research stated that longer CAG size seems to modulate the AR function. Expansion of the CAG repeats may loss the transactivation function of the androgen receptor gene, can lead to different pathologies associated with azoospermia, oligozoospermia, testicular atrophy and spinal bulbar muscular atrophy[4]. The expanded repeats are unstable and show a tendency to expand over generations. Clinical features of spinal and bulbar muscular atrophy include androgen insensitivity as evidenced by testicular atrophy, impotence, gynecomastia, and elevated serum gonadotrophin levels. However, short CAG repeat alleles are associated with prostate cancer and development of metastatic disease[5]. The molecular mechanism of these expansions or contractions of CAG repeat length and infertility are not yet know but are believed to involve either unequal crossover or single-strand slippage of the DNA polymerase during meiotic DNA replication[6]. Recent studies have provided evidence of CAG repeat expansion in the androgen receptor gene linking oligozoospermia and azoospermia with slowly progressing muscle weakness, low virilization, testicular atrophy, and reduced fertility[4]. The CAG repeats length variation may vary among infertile populations of different ethnic origin, allelic distribution of the CAG repeat in AR gene of various ethnic group was crucial to understanding the inter-individual variability in AR activity. The variability of CAG repeat length in the AR gene has been reported in men with defective sperm production in different worldwide population[7]. Thus, no consistent pattern of CAG repeat length has been found in patients with spermatogenic disorders. In the present study, we investigated CAG repeat length of the androgen receptor gene in Central Indian men with idiopathic azoospermia and oligozoospermia also discuss the correlation with impaired spermatogenesis with male oligozoospermic and azoospermic infertility.

MATERIALS AND METHODS

Patients and Control Subjects

A total of 150 men with impaired spermatogenesis ranging from idiopathic oligozoospermia (20 x 10^6/ml) to azoospermia as assessed by WHO (2010)[8] standard criteria, were recruited from the different hospitals, Fertility centres and Andrology clinics. Infertile men were investigated to exclude established causes of male infertility, such as sex/autosomes chromosomal abnormalities, testicular abnormalities, endocrinological disorders and infections, also who had microdeletions of the Y chromosome after an examination of 18 loci, including deleted in azoospermia (DAZ) and ribonucleic acid-binding motif (RBM), were also excluded from the study[9]. Controls were 40 men with proven fertility recruited from the hospitals and they had no previous infertility history or treatment. Blood was taken from the recruited subjects for DNA extraction and for the determination of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T).

DNA Isolation, Amplification, and Sequencing of CAG Repeats

DNA was extracted from peripheral blood lymphocytes from fertile and infertile men according to protocols. The CAG repeats in exon 1 of the AR gene were amplified by polymerase chain reactions (PCRs) using forward primer 5'-AGG GAA GTA GGT GGA AGA TTC AG-3' and reverse primer 5'- CTC

ATC CAG GAC CAG GTA GC-3'. The components for each PCR were 50–100 ng of genomic DNA, 5 µl 1X PCR assay buffer, 2 mM each primer, 200 mM dNTP, 1.5 mM MgCl2, and 1 U of *Taq* polymerase and volume adjust with triple distilled water (Merck Biosciences, Banglore) in a total reaction volume of 50 µl. The PCR was performed by Thirty cycles of amplification, initial denaturation at 94^0C for 1 minute, annealing at 58^0C for 1 minute, and extension at 72^0C for 1.3 minutes and final extension at 72^0C for 7 min. The PCR product was separated using agarose gel electrophoresis containing ethidium bromide at 60 V for 1.30 hrs. The exact number of the CAG repeats located in exon 1 was then determined after sequencing. Those samples with abnormal CAG repeat were further confirmed after two successive sequencing from the same sample.

Hormone analysis

In all patients and controls, serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) were measured by chemiluminesce-immunoassay (RIA) methods (Thyrocare, Mumbai). Total testosterone (T) was measured by commercially RIA. To determine whether any patient with recognized androgen insensitivity on the basis of their hormone profile, the androgen insensitivity index (ASI) was calculated by multiplying the serum concentrations of LH and T.

Statistical Analysis

All results were expressed as the mean $\pm$SD. Statistical analyses were performed by using openepi software (www.openepi.com). The Chi^2 test was used to evaluate the significance of the difference of the mean number of CAG repeats and the hormone levels between normal fertile control and infertile groups. The incidence of long or short CAG repeat length in infertile groups was compared with fertile control group by using student *t* test. Statistical significance was considered as a two-sided *p* value of <0.05.

RESULTS

The CAG repeat lengths in 60 non-obstructive azoospermic and 90 oligozoospermic patients were compared with 40 fertile control group (Table I). The mean CAG repeat length in azoospermic group was 32.8 $\pm$5.0 (mean $\pm$SD) with a range of 25–45 and the oligozoospermic group was 31.9 $\pm$4.9 (mean - $\pm$SD) with a range of 25-43. In the fertile control group, the mean CAG repeat length was 31.1 $\pm$4.7 (mean $\pm$SD) with a range of 24-41. There was no significant difference in the mean number of CAG repeat length among the three groups of men. We defined those men having <16 and >30 CAG repeats as short and long CAG repeat, respectively, because they are outside the range of CAG repeat found in our fertile controls. In the azoospermic group, five patients (P6, P21, P46, P79, P91) possessed long CAG repeat, and two patients (P39, P126) possessed short CAG repeat. In the oligozoospermic group, two patients (P27 and P71) possessed long CAG repeat and two patients (P16 and P62) possessed short CAG repeat (Table II). Statistical analysis of the incidence of azoospermic men with short or long CAG repeat is moderately increasing in the azoospermic group and in the oligozoospermic group when compared with the fertile controls. The distribution of the CAG repeat lengths is shown in Fig. 1. *Student t test* showed no difference in the distribution of CAG repeats length when compared between the fertile control group and the two infertile groups. Y chromosome microdeletion was done in both fertile and infertile men group. Among infertile males, seven azoospermic and four oligozoospermic patients with Y chromosome microdeletion were also included in the analysis. But, their CAG repeat length was within the range with fertile controls. In remaining patients, no Y chromosome microdeletions were detected in all azoospermic and oligozoospermic patients that showed abnormal CAG repeats suggesting that Y chromosome microdeletions and abnormal CAG repeats are independent contributors to male infertility.

Hormone analysis

Increase level of serum FSH is commonly found in non-obstructive azoospermia men, similar results are found in our study (Table 1). Most of infertile men showed an increase level of LH which leads to increase ASI in infertile patients. In Table 2 increasing levels of serum

FSH was observed in five patients (P06, P21, P46, P79, P91) with increased CAG repeat length. But same condition found in most of azoospermia men with normal CAG repeats. In azoospermia group high level of LH & ASI were observed in some patients with short & long CAG repeats in azoospermia group.

Table 1
Clinical phenotypes & molecular data of Azoospermia, Oligozoospermia & Normozoospermia

Patients groups	Number of patient	Age (yrs)	Hormone Concentration			ASI	CAG repeat
			FSH (IU/L)	LH (IU/L)	T (nmol/L)		
Azoo-spermia	63	25-45	22.62 ±16.38 (3.9-63.7)	8.2± 3.31 (2.9-42.1)	10.2 ±2.9 (0.34-32.1)	81 ± 21 (1-328.1)	24.26 ± 6.6 (14-34)
Oligozoo-spermia	90	25-43	12.7± 4.9 (1.9-31.8)	8.66± 3.8 (1.5-12.1)	13.91± (4.6-33.2)	49.2± 41.4 (6.9-283.7)	24.68± 5.01 (14-31)
Normozoo-spermia	40	24-41	5.85±3.1 (2.1-17.8)	4.61±2.66 (1.9-16.8)	4.74±2.21 (4.71-43.1)	63.41± 19.34 (12-253)	22.25± 2.04 (15-30)

Note: FSH: Follicle stimulating hormone; LH: Luteinizing hormone; T: Testosterone. The androgen insensitivity index (ASI) was calculated by multiplying the serum concentrations of LH and T. CAG repeat length is expressed as mean ± SD (range). Hormone and ASI values are expressed as median (range). * p < 0:05 significantly higher than normal fertile control group.

Table 2
Patients-wise clinical data and length of CAG repeats contraction or Expansion

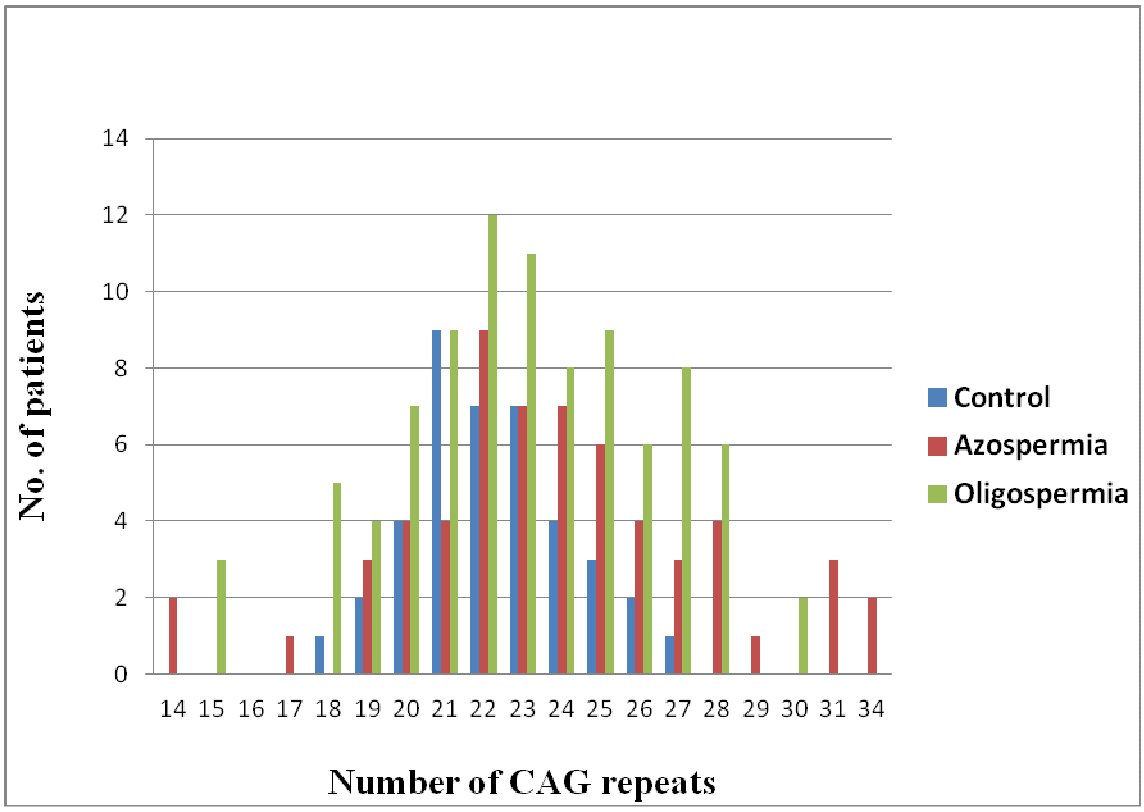
Patients No. Azoospermia	Age	FSH (IU/L)	LH (IU/L)	T (nmol/L	ASI(LH-T)	CAG repeat length
P06	31	29.4	11.4	13.2	150.5	31
P21	36	31.2	9.5	14.7	139.7	34
P39	29	19.8	13.2	9.4	124	14
P46	34	27.7	10.7	10.6	113.4	31
P79	30	29.7	10.8	5.1	55	34
P91	39	34.9	18.4	10.6	195	31
P126	42	18.5	8.3	9.6	79.6	14
Oligozoospermia						
P16	28	17.1	7.2	6.8	48.9	15
P27	34	23.7	9.4	11.4	107.2	30
P62	31	14.2	10.2	7.3	74.5	15
P71	31	16.4	10.7	10.2	109.1	30

Note: FSH-Follicle stimulating hormone; LH -Luteinizing hormone; T-Testosterone. Multiplying the serum concentrations of LH and T for calculation of androgen insensitivity index (ASI).

Table 3
Incidence of ethnic origin men with Abnormal CAG repeats

Ethnic Origin Azoospermia	CAG repeats	Incidence (%)	Reference
Hong-Kong	Long	11.4	6
Singapore	Long-Short	24	21
America	Long	27	7
Japanese	Long	9.8	17
Oligospermia			
Hong-Kong	Short	6.3	6
America	Long	--	7
Japanese	Short	15	17

Figure1
Distribution of CAG repeats length with in androgen receptor gene in normal fertile control group and in azoospermic and oligozoospermic patients



DISCUSSION

The expansion and contraction of CAG repeats in the AR gene and impaired spermatogenesis have been reported in population of azoospermic or oligozoospermic males. Studies on CAG repeats in infertile men have reported inconsistent results[10,11]. Some researchers were suggested decreased CAG repeats while other reported increased lengths are closely associated with impaired spermatogenesis in infertile men[12]. There are only few studies reporting the occurrence of long CAG repeats in a mixed population of Chinese– Indian and Australian–Chinese–Indian infertile patients. These conflicting results may be attributed to varying ethnic backgrounds (Table 3), as the stretches of CAG repeats appear to be longest in Asians, medium in whites, and shortest in Africans. Furthermore, this bias may also be related to limited sample sizes and inclusion criteria[7,13,14]. However, the relationship between length of CAG repeat and male infertility in Indian is still unclear as these studies did not define the range of CAG repeats in each ethnic group[7]. Moreover, our recent study demonstrated the relationship between the length of CAG repeats and impaired spermatogenesis in our Indian population. In this study, expansion and contraction of CAG repeats are found in the azoospermic males. The incidence of men with abnormal CAG repeat is higher in the azoospermic group but not in the oligozoospermic group when compared with CAG repeats in fertile controls. These results were inconsistent with other studies in which no association was found, but discordant with other studies that did find associations[13,15]. Due to small sample size some researchers could not correlated association in the oligozoospermia and CAG repeats length[10]. Azoospermic and oligozoospermic patients were also subjected Y chromosome microdeletions screening with 18 STS marker[9]. We did not find Y microdeletion in any of the infertile patients with abnormal CAG repeats. When we excluded the patients with Y chromosome deletions, the incidence of infertile men with abnormal CAG repeat becomes slightly higher, 11.7% (7/60) and 4.4% (4/90) in azoospermic and

oligozoospermic men, respectively. In a group of azoospermia, two males (3.3%) had short CAG repeat (<16) and five males (8.3%) had long CAG repeats (>30). In oligozoospermia, two males (2.2%) had short repeat and two (2.2%) had long repeat. These results showed that CAG repeat length and Y microdeletions are independent contributors to male infertility. In comparison with our studies, higher incidence of abnormal CAG repeats was reported in other populations. We suggest that the variability of the results by various investigators is mainly attributed to regional variations and differences in ethnic origin of the studied subjects. This is because lower incidence of abnormal CAG repeats was reported in non-obstructive azoospermic men from Japan and the absence of abnormal CAG repeats found in European infertile populations[16, 17]. Other factor might be due to patient's selection because patients with Y chromosome deletions were excluded in their studies. The expansion or contraction of CAG repeats may vary among populations of different ethnic origin. In oligozoospermic men, short CAG repeats are frequently found in Chinese and Japanese populations, whereas long CAG repeat is predominantly found in North American men[7,17]. In azoospermic men, both long and short CAG repeats are found in our population, whereas only long CAG repeats are observed in other populations[18]. The normal range of hormones concentrations of fertile normozoospermic men was slightly different from ASI presented in Table 1. High FSH level alone is not directly associated with abnormal CAG repeats because high FSH level was observed in majority of our non-obstructive azoospermic men with normal CAG repeats. This hormonal profile can be elucidated by two reasons. First, the reference ranges were taken from a heterogeneous normal healthy population with unknown fertility status. Second, these normal ranges are not derived from Indian men because of ethnic variation which is important in this condition[19]. The result of this study was shows no correlation between expansion/contraction length of CAG repeats and serum testosterone (T) level. On the other hand, we observed increased level of Luteinizing Hormones and higher ASI in azoospermic patients with short CAG repeats. High ASI is regarded as an indication of androgen insensitivity, which leads to elevation of LH and T as a result of impaired negative feedback control of the hypothalamic–pituitary–testicular axis[20]. Both LH levels and ASI were strikingly higher in azoospermic patients with short CAG repeats than in azoospermic patients with long CAG repeats. The serum LH levels in the upper normal range in the azoospermic patients with short CAG repeats may reflect resistance to androgen action. It has been suggested that short CAG repeats affected the androgen receptor function by suppressing the expression of the AR gene in patients with complete androgen insensitivity syndrome[20,21]. On the other hand, progressive expansion of the CAG repeat was shown to cause a linear decrease of transactivation function and this may subsequently lead to a failure of spermatogenesis[22]. Together, these data indicates that a suitable CAG repeat length is needed for the proper maintenance of the AR function.

CONCLUSION

In conclusion, our findings showed abnormal CAG repeat length in our azoospermic and oligozoospermic infertile population. Expansion and contraction of CAG repeat may be cause abnormal spermatogenesis in infertile males. Our strong consideration is that these changes occur in CAG repeat length in AR gene due to ethnic geographical variation in infertile males and this molecular alteration affect spermatogenesis in males.

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