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Association of Androgen Receptor Gene Polymorphisms at three SNPs and their Haplotypes with Severe Oligozoospermia Risk in Iraqi Patients

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Abstract: Infertility in otherwise healthy males be caused by a variety of disorders. These include acquired, as well as heritable conditions. Genetic causes of male infertility are of special interest to reproductive biology, because these disorders can be passed on to the offspring, single nucleotide polymorphisms are considered as one of reason of male infertility Androgens are critical steroid hormones that determine the expression of the male phenotype. Their actions are mediated by a single androgen receptor (AR) which, upon ligand binding, translocate to the nucleus to regulate the expression of androgen-responsive genes. Mutations in androgen receptor gene may lead to a disturbance in the function of the androgen receptor which, in turn, can lead to several forms of infertility. The aim of this study was to determine the frequency of rs962458(SNP1), rs6152(SNP2) and rs2361634(SNP3)and test their associations with severe oligozoospermia risk .This study was conducted using 50 severe oligozoospermia patients and 50 apparently healthy subjects (Control). The frequency of GA genotype of SNP2 was significantly ($p \leq 0.05$) increased in patients versus control (10 and 0%, respectively, OR=0.561, X2=4.329),while the frequency of GG genotype of SNP2 was significantly ($p \leq 0.01$) decreased in patients versus control(84 and 100%, respectively, OR=0.871, X2=6.524). The results of TAA and TAG haplotypes were not significant. The frequency of TAG/TAG haplotype combination was significantly ($p \leq 0.01$) decreased in patients versus control(78 and 100%, respectively, OR=1.072, X2=8.35),while TAG/TAA haplotype combination frequency was significantly ($p \leq 0.05$)increased in patients versus control(14 and 0%,respectively, OR=0.664,X2=4.69). In conclusion, both GA genotype of rs6152A>G and TAG/TAA haplotype combination may be correlated with severe oligozoospermia risk in Iraqi patients.

Key words: SNP, Androgen receptor,PCR, Infertility.

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Introduction:

Infertility is defined as the inability to conceive after at least 1 year of unprotected intercourse. In about one third of infertile couples, a male factor is the primary problem (1,2). Infertility is a common condition, affecting 15% of couples trying to conceive, the evaluation of infertility including an assessment of both the female and the male partner to discern the factors contributing to their difficulty in conceiving (3). Besides, infertility is a major health problem worldwide, affecting at least one in every eight couples, and affecting people both psychosocially and medically (4). It can be a devastating condition for couples who want to have children; it also

affects men and woman alike, as both genders report associated psychological distress, depression, and low self-esteem (5). It can either be primary or secondary; Iraqi study,that carried out during aperiod from January 2000 to May 2001 (6), studied causes of infertility for 250 couples, 193 (77.2%) of whom had primary and 57 (22.8%) secondary infertility. Male infertility was found in 36.8% of cases. These percentages give a strong indication of infertility among Iraqi couples. A male partner factor contributes to 50% of cases of infertility (7). About half of male infertility cases are due to defined reasons; including varicocele, infection, hormone imbalances, exposures such as drugs or medications, x-rays and tobacco use, blockage of the reproductive tract ducts, and previous surgery, genetic factors including chromosomal aberrations and single gene mutations (8, 9, 10, 11). A large proportion of infertile men fail to impregnate their female counterpart because of lack of sperm (azoospermia) or low sperm count (oligozoospermia); infertility may also be due to abnormal sperm morphology (teratospermia) and insufficient sperm motility (asthenozoospermia) (12). More than 90% of male factor infertility is characterized either by low number of sperm in semen or by production of poor-quality sperm (13). Oligozoospermia is a low sperm count which means that the men who have a sperm concentration of less than 15 million/ml are to be classified as oligozoospermic (1). It is a cause of human male infertility. A case where the number of sperm in the semen for the male1 to 5 million sperm/ ml is classified as "severe oligozoospermia" (14).

The development of the male phenotype and the initiation of spermatogenesis(events that lead to the production of male gametes) dependents on cellular phenomena that responds to androgens. The main androgens in mammals are testosterone and dihydrotestosterone, that are important for normal male sexual development before birth and during puberty. Androgen receptor (AR) are important for normal spermatogenesis, androgen receptors allowing the body to respond appropriatelyto these hormones (testosterone, dihydrotestosterone) (15). The androgen receptor helps direct the development of male sexual characteristics. Androgens and androgen receptors also have other important functions in both males and females, such as regulating hair growth and sex drive (16). The androgen receptor is encoded by a single-copy gene located in the long arm of the X chromosome (Xq11-12); it consists of eight exon, exon 1 of the AR gene containing a polymorphic sequence of CAG repeat, which usually varies in number from 10 to 35 (17). Mutations in the androgen receptor gene causes disturbances in the function of the androgen receptor that can lead to several diseases such as androgen insensitivity syndrome, which inturn can lead to infertility (18).

Materials and Methods

Subjects and sampling

Patients have been selected according to clinical and laboratory examination. Men with severe oligozoospermia were examined and diagnosed as related with infertility. Totally 50 blood samples were collected from severe oligozoospermia and 50 apparently healthy individuals (Control) during a period from 20

August 2015 to 20 October 2015. These were established from the Ministry of Higher Education and Scientific Research at Al- Nahrain University, High Institute of Infertility Diagnosis and Assisted Reproductive Technologies. They were clinically examined and evaluated by the consultant medical staff , and under the supervision of this staff,according to the results of semen analysis In the case of severe oligozoospermia,blood (2-3 ml) was also collected in EDTA tubes and stored in the freezer (-20°C) (19). Samples were collected according to WHO criteria (20). All the semen samples underwent semen analysis in accordance with the WHO laboratory manual for the examination of human seminal fluid, and the study was performed using these reference values (1).

Analysis of genes

Genomic DNA was extracted from whole blood of infertile and fertile males using wizard genomic DNA purification kit (Promega). Then, the extracted DNA was used for amplification of targeted fragments by using PCR. specific primers were used for each gene after inspecting them with Graphic program available on the web site of NCBI to check both the specify and the size of the product. All primers were supplied by Alpha DNA Company as a lyophilized product of different picomols concentrations. Primers were dissolved in a free nuclease distilled water according to the instruction of the manufacturing company to give a final concentration of (100 pmol/µl), which was used as a stock solution; 10 µl of this solution was added to 90 µl of free nuclease distilled water to obtain 10 pmol/µl of primer solution that is utilized in PCR technique. The sequences of these primers are listed in (Table 1).The PCR condition for amplification of SNPs fragment was as, 35 cycles of denaturation at 95 C, for 15 minute, annealing at 60 C for 30 sec and extension at 72 C for 30 sec . PCR-RFLP was applied for, the identification of these SNPs by using NspI, StuI and BstuI restriction enzymes, respectively, for 4 hours at 37 c.

Table (1): Sequences of primers used in this study .

Primers	Sequences 5´ → 3´	Band size/bp	Gene
Forword (F1)	CCGTAGCCTTCTGGAAAACATC	149*	Snp 1
Reverse (R1)	GTCACAATTTTACTTAAAAATGCGC		
Forword (F2)	CTCCGGACGAGGATGACTCA	256*	Snp 2
Reverse (R2)	TGGCGTTGTCAGAAATGGTCGAA		
Forword (F3)	ATGAGGTAAAGTTACAAACCTGG	398*	Snp 3
Reverse (R3)	AACATGGTCCCTGGCAGTCTC		

Sequencing

PCR products of androgen receptor gene were sent for sequencing to Macrogen Company (USA). The results of sequencing were analyzed by BLAST in NCBI.

Statistical analysis

The Statistical Analysis System- SAS (21) program was used to effect of difference factors in study parameters . Chi-square test was used to significant compare between percentage, and estimate of Odd ratio in this study.

Results and Discussion

As shown in (Table 4-1) , in both groups of control and patients, the age was within 20-40 years old. Within each age group there was no significant difference between control and patient groups and about 60% of samples which were within 26-35 years of age and the remainder 40% were within 20-25 and 36-40 years old in both groups. The periods of marriage within 5-8 years were significantly ($p<0.01$) higher in patient group in compared with control group (50 versus 24%, respectively), whereas, there were significantly ($p<0.01$) lower in patient group in compared with control group within 9-12 years of marriage (14 versus 40%, respectively). The ratio of smokers was in patient group significantly ($p<0.01$) higher than in control (62 versus 0%, respectively), and all control subjects were nonsmokers .

Samal *et al.* (22) indicated that infertility has been shown to be elevated by smoking. Greenlee *et al.* (23) reported that smoking and passive smoking are the risk factors for female infertility and stated that the metabolites of cigarette smoking are toxic to oocyte, sperm and embryos. Durphy *et al*. (24) studied the relationship between male cigarettes smoking and decreased chances of conception and found that the fertility outcome is unrelated to the number of cigarettes smoked by the male partner. Hull *et al.* (25) reported smoking having no effect on male infertility. These results indicate that 40% of severe oligozoospermic patients (who attending the high institute of infertility diagnosis and assisted reproductive technologies-Baghdad during the period from August to October 2015) were within 26-30 years old , 50% of patients were within 5-8 years after marriage (Table 2). In addition, the majority of patients were smokers (62%) and this may be one of the main causes of infertility. Sangita and Boramma (26) found that tobacco chewing and smoking affect semen volume, sperm motility and sperm count.

Table (2): Distribution of samples, in this study, according to the age groups, period of married and smoking.

Parameters	Control N (%)	Patients N (%)	Chi-square (X^2)
Age group (year)			
20-25	9 (18)	8 (16)	0.093 ns
26-30	23 (46)	20 (40)	1.277 ns
31-35	7 (14)	9 (18)	0.621 ns
36-40	11 (22)	13 (26)	0.621 ns
Period of married (year)			
1-4	18 (36)	18 (36)	0.000 ns
5-8	12 (24)	25 (50)	8.903 **
9-12	20 (40)	7 (14)	8.903 **
Smoking			
Yes	0 (0)	31 (62)	11.774 **
No	50 (100)	19 (38)	11.774 **

Genomic DNA extraction:

Genomic DNA was extracted from fresh blood by using Wizard genomic DNA Kit (Promega, USA) to obtain a pure DNA for PCR amplification. This method involves lysing of red blood cells and dissolving the undesirable contaminants such as protein and RNA, in addition to degrade the cell membrane of white blood cells. The results of DNA extraction showed that fresh blood samples yielded enough DNA concentrations for PCR amplification (Figure 1). The quantification of DNA by nanodrop revealed that the DNA concentration ranged between 80 – 120 ng /µl and purity range was between 1.8 – 1.9.

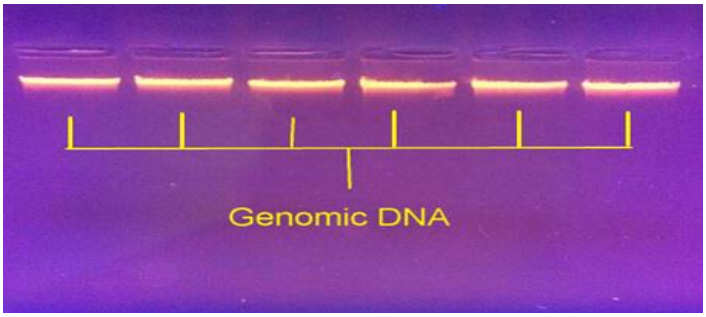

Figure (1): Gel electrophoresis of genomic DNA visualized under UV after staining with ethidium bromide on 0.5% agarose gel at 5 volt /cm for 30 minutes.

Androgen receptor gene analysis

SNP-1 (rs962458)

The androgen receptor gene polymorphism at rs962458 SNP, a 149 bp fragment in the upstream region of exon 1 (67525995 – 67526143 , *NC_000023.11*) was targeted for amplification by the forward and reverse primers that used by Saare *et al.* (27), using PCR (Figure 2). The rs962458 SNP is located within this fragment at 67526122 position (T). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of T allele to C allele at this

position create a site for *Bsh1236I* restriction enzyme (TGCG to CGCG). Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows: 149 bp for TT genotype; 23, 126 and 149 bp for TC genotype; 23 and 126 bp for CC genotype (Figure 3). The other name for this SNP is g.67526122 T>C.

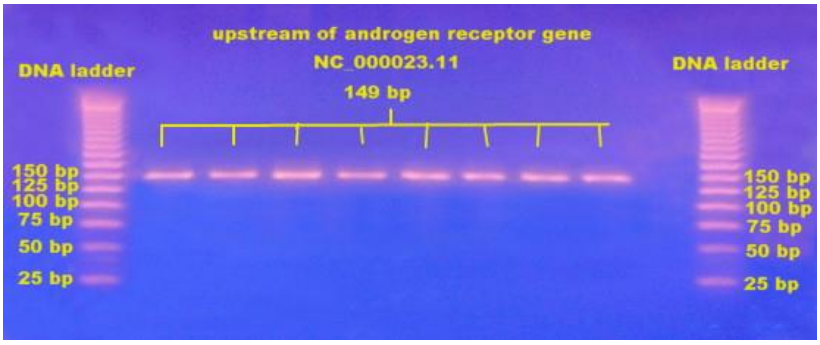

Figure (2): PCR product (149 bp) of targeted fragment (upstream of androgen receptor gene) flanking the rs962458T>C SNP (*NC_000023.11*) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours.

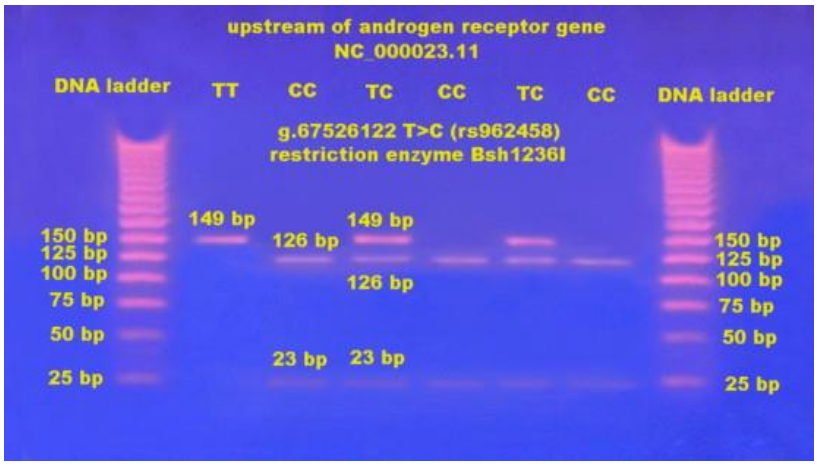

Figure (3): PCR product (149 bp fragment) of 5' promoter of androgen receptor gene digested with Bsh1236I restriction enzyme and electrophoresed on 2% agarose. The genotypes at 67526122 position (*NC_000023.11*) are: TT (149 bp), TC (23, 126 and 149 bp) and CC (23 and 126 bp).

(Table 2) shows the frequency of rs962458 SNP in severe oligozoospermic patients and control subjects. All homozygous (CC genotype) and heterozygous (TC genotype) mutations were noted in patient group only (10%) *versus* 90% of TT genotype, while only normal TT genotypes (100%) were noted in control group.

These results refer to a probable negative relation between the frequency of this SNP and the efficiency of spermatogenesis. Saare *et al.* (27) found no role for rs962458 SNP alone in the risk of infertility, but the effect of this SNP was within haplotype. As shown in (Table 2), C allele found in 5 out of 50 oligozoospermic patients, while this allele is not found in control subjects.

Table(2): Genotype and allele frequencies of g.67526122 (rs962458) SNP in the promoter region of androgen receptor gene (apparently healthy subjects[1] versus severe oligozoospermic patients[2]).

Genotype	Control[1] n(%)	Patients[2] n(%)	OR	Chi-square X^2
TT	50 (100%)	45 (90%)	0.752	4.327 *
TC	0 (0%)	2 (4%)	0.327	0.961 NS
CC	0 (0%)	3 (6%)	0.398	1.057 NS
Allele frequency (%)				
T	100	92		
C	0	8		

*** refer to a significant difference at 0.05 level. NS : No significant.**

SNP-2 (rs6152)

The androgen receptor gene polymorphism at rs6152 SNP, a 255 bp fragment in the exon 1 (6570 – 6824 , *NG_009014.2*) was targeted for amplification by the forward and reverse primers that used by Saare *et al.* (27), using PCR (Figure 4). The rs6152 SNP is located within this fragment at 6754 position (G). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of G allele to A allele at this position abolish a site for *StuI* restriction enzyme (AGGCCT to AAGCCT). Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows: 69 and 186 bp for GG genotype; 69, 186 and 255 bp for GA genotype; 255 bp for AA genotype (Figure 5). The other names for this SNP are G1733A and c.639G>A. This SNP is silent and did not change the amino acid (glutamic acid). (Figure 6) show the results of sequencing and blast for checking the PCR-RFLP results of rs6152G>A SNP.

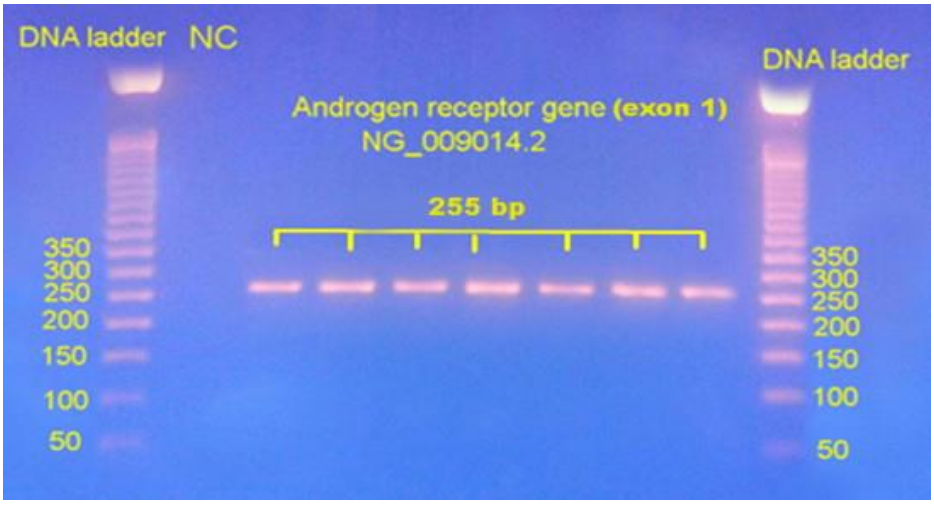

Figure (4): PCR product (255 bp) of targeted fragment (exon 1 of androgen receptor gene) flanking the rs6152G>A SNP (*NG_009014.2*) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours.

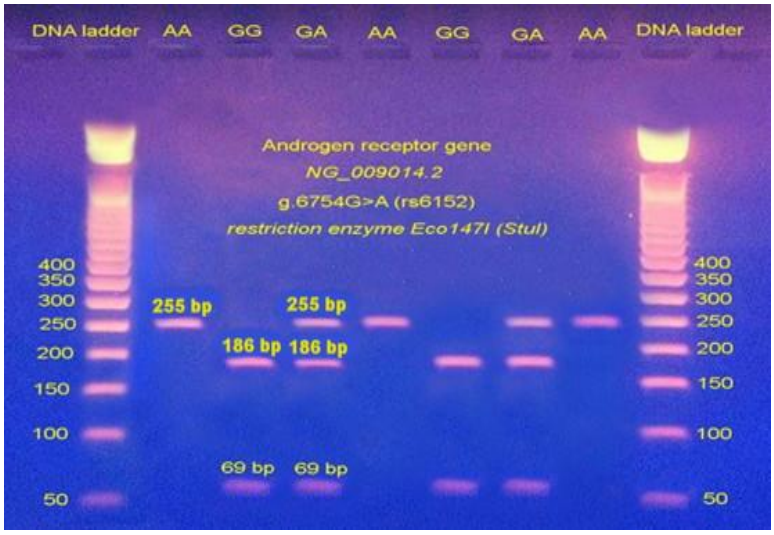

Figure (5): PCR product (255 bp fragment) in exon 1 of androgen receptor gene digested with StuI restriction enzyme and electrophoresed on 2% agarose. The genotypes at 6754 position (NG_009014.2) are: GG (69 and 186 bp), GA (69, 186 and 255 bp) and AA (255 bp).

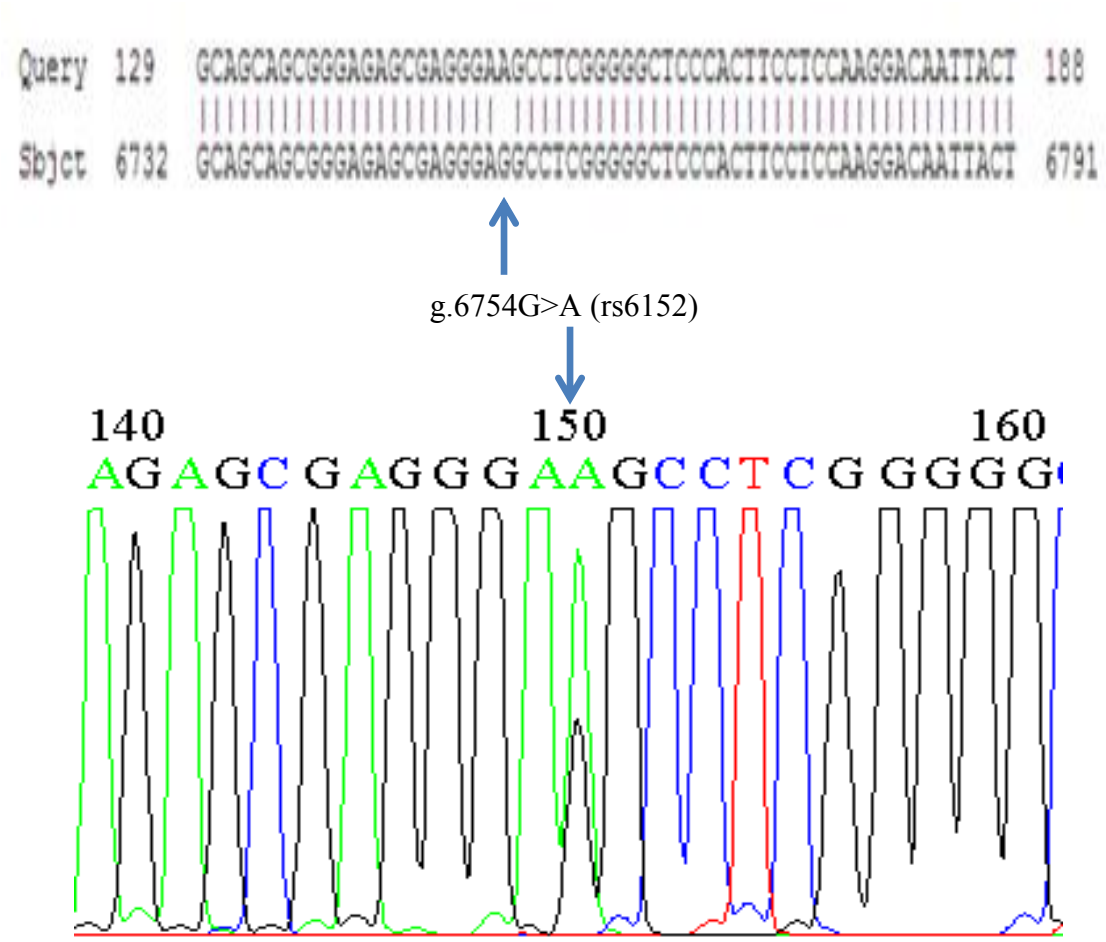

Figure (6): Electropherogram deicting the g.6754G>A (rs6152) SNP position and its flanks.

The distribution of genotype and allele frequency at 6754 site (rs6152) of androgen receptor gene presented in (Table 3). The GG genotype frequency was significantly ($p<0.01$) lower in oligozoospermic patients when compared with apparently healthy subjects (84 *versus* 100%, respectively). The rs6152 SNP was found in 16% of oligozoospermic patients (10% heterozygous and 6% homozygous), whereas, in control group was not found. The frequency of heterozygous mutations in oligozoospermic patients was significantly ($p<0.05$) higher than in control group (10% *versus* 0%, respectively). The frequency of A allele was 11% *versus* 0% in control group.

Table (3): Genotype and allele frequencies of g.6754G>A (rs6152) SNP in exon 1 of androgen receptor gene (apparently healthy subjects[1] versus severe oligozoospermic patients[2]).

Genotype	Control[1] n(%)	Patients[2] n(%)	OR	Chi-square X^2
GG	50 (100%)	42 (84%)	0.87	6.524 **
GA	0 (0%)	5 (10%)	0.56	4.329 *
AA	0 (0%)	3 (6%)	0.34	1.095 NS
Allele frequency (%)				
G	100	89		
A	0	11		

**** refer to a significant difference at 0.05 and 0.01 level, respectively.**

This SNP (rs6152) located between CAG repeat sequence encoding a poly-glutamine stretch and GGN repeat sequence a poly-glycine stretch in exon 1 of androgen receptor gene, which encodes the amino-terminal domain of androgen receptor involved in the transcriptional activation of downstream genes (28). Saare *et al.* (29) found that in Estonian men the rs6152 SNP with other SNPS in a haplotype appears to confer an increased risk for infertility. The results of the present study confirm the results of Saare *et al.* (29) that oligozoospermia was related with heterozygous rs6152 SNP. The functional effect of the SNP rs6152 has not yet been studied.

Kucerova *et al.* (30) confirmed that the androgen receptor gene polymorphism (SNP rs6152G>A) is associated with the development of androgenetic alopecia and the higher prostate-specific antigen in patients with benign prostatic hyperplasia. The common G allele of the rs6152 SNP has previously been associated with androgenetic alopecia in men (Hillmer *et al.*,(31); Ellis *et al.,* (32) ; Prodi *et al.,* (33)). In Chinese women, Peng *et al.* (34) indicated that polycystic ovary syndrome was found to be associated with androgen receptor gene exon1 synonymous SNP rs6152G>A. While, Henningsson *et al.* (35) observed that rs6152 SNP was not influenced by autism risk.

SNP-3 (rs2361634)

The androgen receptor gene polymorphism at rs2361634 SNP, a 398 bp fragment in the intron 1 (103852 – 104249 , *NG_009014.2*) was targeted for amplification by the forward and reverse primers used by Saare *et al.* (27), using PCR (Figure 7). The

rs2361634 SNP is locate within this fragment at 103970 position (A). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of A allele to G allele at this position create a site for *NspI* restriction enzyme (GCATAT to GCATGT). There is other site for this enzyme within this fragment. Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows: 54 and 344 bp for AA genotype; 54, 119, 225 and 344 bp for AG genotype; 54, 119 and 225 bp for GG genotype (Figure 8).

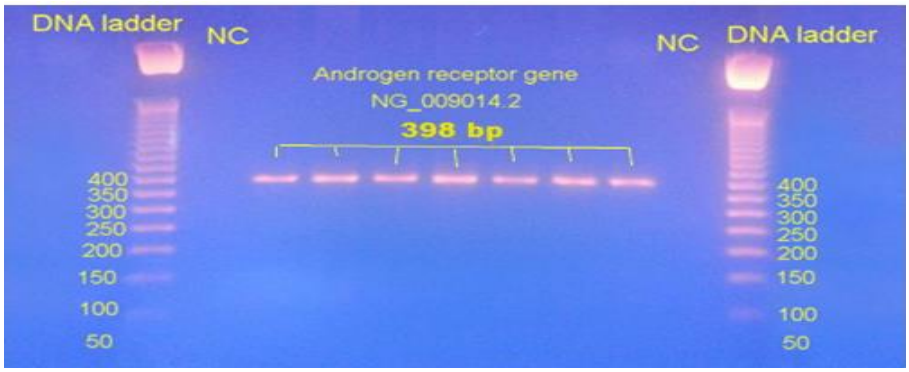

Figure (7): PCR product (398 bp) of targeted fragment (intron 1 of androgen receptor gene) flanking the rs2361634 (g.103970A>G, *NG_009014.2*) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours.

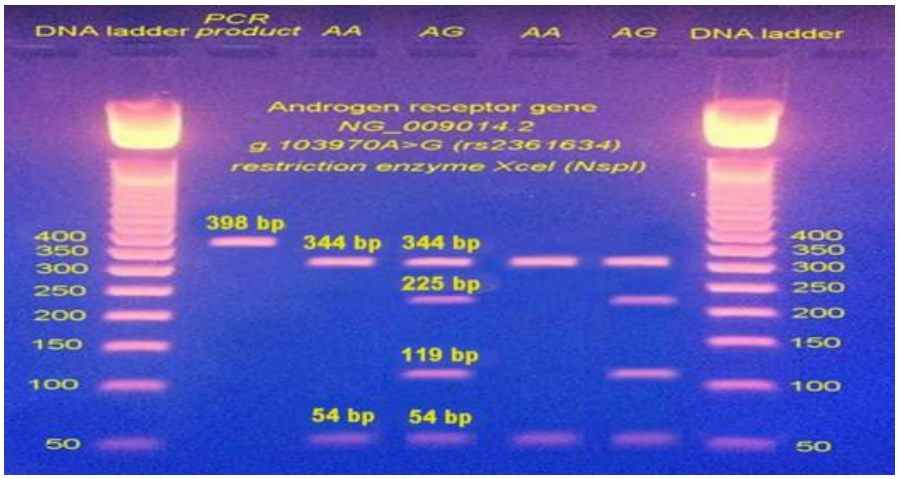

Figure (8): PCR product (398 bp fragment) in intron 1 of androgen receptor gene digested with *NspI* restriction enzyme and electrophoresed on 2% agarose. The genotypes at 103970 position (*NG_009014.2*) are: AA (54 and 344 bp), AG (54, 119, 225 and 344 bp) and GG (54, 119 and 225 bp).

The distribution of genotype, and allele frequency at 103970 site (rs2361634) of androgen receptor gene are presented in (Table 4). The frequency of this SNP was the same in both control and patient groups. Only one rs2361634 SNP was noted in patient group. These results may be attributed to the small sample size in this study, and this SNP may be observed in the large sample size.

In Chilean patients with primary spermatogenic failure, Bustamante *et al.*

(36) studied six SNPs in the androgen receptor gene.The rs2361634 SNP was one of these SNPs . They(Ibid) found no effect for this SNP as alone or within a haplotype on the incidence of primary spermatogenic failure. The results of this study as related with rs2361634 SNP are in agreement with the results of Bustamante *et al.* (36).

Table (4): Genotype and allele frequencies of g.103970 A>G (rs2361634) SNP in intron 1 of androgen receptor gene (apparently healthy subjects[1] versus severe oligozoospermic patients[2]).

Genotype	Control[1] n(%)	Patients[2] n(%)	OR	Chi-square X^2
AA	50 (100%)	49 (98%)	0.006	0.249 NS
AG	0 (0%)	1 (2%)	0.006	0.249 NS
GG	0 (0%)	0 (0%)	0.00	0.000 NS
Allele frequency (%)				
A	100	99		
G	0	1		

NS : No significant.

Haplotypes and haplotype combinations

The distribution of haplotypes (rs962458, rs6152 and rs2361634) in apparently healthy subjects *versus* oligozoospermic patients revealed that there were no significant differences between control and patient groups as related with TAA and CAG haplotypes, while, the frequency of TAG haplotype (defined by rs962458, rs6152 and rs2361634) was in oligozoospermic patients significantly ($p<0.05$) lower than in apparently healthy subjects (88 *versus* 100%, respectively).

Also, there were no significant differences between control and patient groups as related with TAA / TAA, TAG/CAG and CAG/TAG haplotype combinations. Besides, the percentage of TAG/TAG haplotype combination was significantly ($p<0.01$) decreased in oligozoospermic patients in compared with control (72 *versus* 100%, respectively).By contrast, the percentage of TAG/TAA haplotype combination was significantly ($p<0.05$) increased in oligozoospermic patients compared with control (14 *versus* 0%, respectively).

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