Molecular Association of Sexually Transmitted Infections and Methylation Pattern of *SNRPN* Gene Promoter among Males with Abnormal Semen

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Abstract

Infertility affects about 8-12% of couples worldwide, and male infertility accounts for a half of these cases, therefore, thisstudy was undertaken to focus on some factors affecting male's fertility such as DNA methylation and genital tract infections by sexually transmitted diseases. The study groups included infertile males as a patient group (n=63) in an age mean (32.28 ± 6.88 years) and fertile males as a control group (n=13)in an age mean (34.07 ±6.52years).

Seminal fluid was collected from all males attending a clinical laboratory for routine semen analysis, seminal fluid analysis was performed according to World Health Organization guidelines, all of the 63 infertile males was suffering from reduced sperm concentration (9.42 ± 8.70 million/ml), reduced progressive motility (2.89 ± 5.45%) and abnormal sperm morphology (27.06 ± 16.50%), while control group have a higher values thus, there was a highly significant difference in semen parameters between the two groups.

The DNA samples of the patients group (n=44) and control group (n=13) was subjected to bisulfite treatment to convert un-methylated C-residues to uracil, this step was performed to allow the qualitative detection of methylation at the promoter of Small nuclear ribonucleoprotein polypeptide N gene by Real time polymerase chain reaction using two sets of designed primers (methylated and un-methylated sets) allowing one set of the primers to anneal, and amplify the desired region.

The results of real time PCR for the detection of methylation in *SNRPN* gene promoter showed hyper methylation in the promoter of *SNRPN* in some samples (22.7%), a slight methylation in others (20.4%) and no methylation in other samples (56.8%) from infertile males, while all the 13 control samples showed no methylation.

Keywords: *Sexually Transmitted Infections , SNRPN genes , Infertility in man , Abnormal Semens*

Introduction

Paternity is one of the most desired aims in adulthood that could not be denied universally, many couples have life plans that include children, however, not all couples can achieve pregnancy spontaneously and many couples will require a medical help to resolve their infertility problems. Male Infertility defined as the inability of a male to achieve pregnancy in a fertile female after 12 months of unprotected intercourse; it is considered as a highly prevalent condition around the globe Approximately 15% of couples were suffering from this problem, male factor infertility responsible for 50% of cases [(2,3)]

Infertility considered as a multifactorial condition, due to the widespread of male infertility or subfertility which is known as the reduced fertility, and in major cases the main causes remain idiopathic, it is crucial to investigate factors affecting male fertility other than sexual disorders, endocrinopathies, obesity, drugs and ejaculatory dysfunction [(11)]. Many contributing factors

can lead to a reduced male fertility such as congenitaland acquired urogenital abnormalities, infectionsof the male accessory glands, increased scrotal temperature, endocrine disturbances, genetic abnormalities and immunological factors. In (40–60%) of cases the only abnormality is thesemen analysis and there is no significant history or abnormality on physical examination and endocrinelaboratory testing (idiopathic male infertility). The analysisof semen reveals a decreased number of spermatozoa (oligozoospermia), decreased motility (asthenozoospermia)and many abnormal forms on morphologicalexamination (teratozoospermia). Usually, these abnormalities come together and are described asthe oligo-astheno-teratozoospermia syndrome (OAT) [6].

Even though in (30-40%) of the cases the etiology of male infertility remains unknown and is called idiopathic male infertility. Nowadays many assisted reproductive technologies are used to obtain pregnancy, in these cases an adequate epigenetic diagnosis of male infertility is of major importance to evaluate if a genetic abnormality will be transmitted to the offspring. As well, there is a need for better diagnostic seminal biomarkers to assess the success rates of these assisted reproductive technologies. Male infertility is a complex, multi-factorial disorder and the underlying causes often remain unknown, therefore, it is crucial to investigate the probable causes and molecular mechanisms that lead to male idiopathic infertility such as gene mutations, proteome studies of spermatozoa from idiopathic infertile men, the role of epigenetics, post-translational modifications and sperm DNA fragmentation in infertile men [5 &13].

Small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene is an imprinted gene, which is normally methylated at the maternal allele and expressed in a mono allelic way through the paternal allele, abnormal imprinting of this gene due to the methylation may lead to abnormal function or silencing of the gene, hyper methylation of that gene at the promoter region have been observed to be linked with specific sperm abnormalities [4 &7].

On the other hand infections of the male reproductive tract have been documented for a long time as avital factor that interfere with sperm normal function, transport and productivity [1] . Different types of microorganisms have been associated with male infertility and the degree of association depends on the type of the infection caused.

A specific mechanism used by each microorganism to affect infertility, either directly by reducing sperms motility, or indirectly by causing an obstruction in the seminal tract *Chlamydia trachomatis,Neisseria gonorrhoeae, Mycoplasma genitalium and Trichomonas vaginalis*, have been detected in the semen of asymptomatic infertile males, these pathogens cause acute and chronic infections, the resultant inflammations in the male reproductive system, cause a damage in the function of spermatozoa that lead to a reduced sperm quality, decreased sperm count and motility [10].

Materials and Methods

The samples required in this study were seminal fluid from males suffering low fertility and difficulty to conceive, their age ranged from (20-52) years, attending clinical laboratories for routine seminal fluid analysis over a period of two months, November 2017 to January 2018.All the workwas done in Baghdad university/ Biotechnology department and Genome clinical laboratory (Baghdad/Al-kindi Street) for a period of 4 months.

The patient group consisting of males have no children and a problem in conceiving with a poor seminal fluid quality, the value of sperm count, motility and morphology were below the lower reference limits determined by WHO standard manual of 2010, their age ranged from (20-52) years old. By contrast, males having one child at least and a normal seminal fluid analysis were considered as a control group and their age ranged from (21-45) years old.

Genomic DNA isolation

DNA-sorb-AM nucleic acid extraction kit is used in this study to extract whole genomic DNA from seminal fluid. This kit is designed to extract and purify DNA manually from clinical materials such as seminal fluid, urine, discharges of urogenital tract, throat, etc. The lysis solution contains guanidine chloride that act as chaotropic agent to lyse the cells and denature cell proteins, the nucleic acid then sorbed on silica particles, washed and eluted using TE-buffer (Amplisens, Russia).

The concentration and purity of DNA are mostly evaluated semi quantitatively by gel electrophoresis technique. After genomic DNA extraction from seminal fluid samples, agarose gel Electrophoresis have been adopted in different concentrations according to the size of DNA, to check the presence and the integrity of the extracted DNA. For DNA integrity detection 1% agarose gel was used (1.0 gm of agarose dissolved in 100 ml of 1X TBE), for the checking of PCR products 2 % agarose gel was used 2 gm of agarose dissolved in 100 ml of 1X TBE) [8]

Human Beta-globin gene detection by PCR

Human beta-globin gene detection used in this study as positive internal control to assessand assure the presence and quality of human DNA. Primers for beta-globin gene were designed by [9] using in silico PCR amplification and were downloaded from the website, the primers provided in a lyophilized state by Alpha-DNA Company (Canada). The sequence of forward and reverse primers are F: 5`..AGTCAGGGCAGAGCCATCTA..3` , R: 5`..CCTCACCACCAACTTCATCC..3` .The amplification reaction were PCR mix 10 µl , primers forward and reverse 0.5µl , template DNA 4µl and nuclease free water 10µl to have total reaction volume 25µl. The PCR amplification were as Initial denaturation at 95 c° for 15 min , Denaturation at 95 c° for 15 sec for 40 cycle , annealing at 58c° for 25 sec , extension at 72 c° for 20 sec and final extension at 72 c° for 5 min [12]

Qualitative methylation-specific PCR (qMSP)

Qualitative methylation-specific PCR used to assess the presence of methylation in the promoter region of *SNRPN* gene where there is a high percentage of possibly methylated CpG islands. Methylation specific PCR (MSP) is based on the use of bisulphite treated DNA to assess the pattern of methylation.In conventional PCR only one set of primers is used.In MSP two sets of primers used, primer pairs are designed to be "methylated-specific" by including sequences complementing only unconverted 5-methylcytosines, or, on the converse, "un-methylated-specific", complementing thymine converted from un-methylated cytosine. The presence of methylation is determined by the ability of the specific primer to achieve amplification. In this study, the PCR system used was Real time thermal cycler (qPCR) by Biometera Company, Germany.

Methylated and Un-methylated primers design

The promoter sequence of *SNRPN* gene was downloaded using "Switchgear genomics" online free bioinformatics tool which identify functional elements in the human genome (http://switchgeargenomics.com) following the steps bellow:

i. After downloading the website, select search promoter bottom

ii. A box will appear to insert the gene name and click promoter then wait for processing.

iii. After a while the promoter sequence will appear with information's about it, http://switchdb.switchgeargenomics.com/productinfo/id _711827/

The sequence of *SNRPN* gene promoter is then copied to be used in another bioinformatics tool called "Methprimer" that issued for designing PCR primers for methylation mapping, at the website (http://www.urogene.org/methprimer/) to design methylated and un-methylated primers following the steps bellow:

i. Insert the promoter sequence of *SNRPN* gene in Bioinformatics Tool (MethPrimer – online)

ii. Click at the button "Pick MSP primers" and submit to determine theMethyl specific primers that suggested.

iii. Choose and select the best appropriate primers for the study.

In this study, another set of methylated and un-methylated primers is used to confirm the results of the designed primers. The primers used were designed by Using Meth primer to distinguish between methylated and un-methylated DNA after bisulphite treatment and designed primers are provided by alpha-DNA Company (Canada) and were prepared as) [10

M-MAM-d-*SNRPN* F: 5`..GACACAACTAACCTTACCCGCTCCATCGCG..3` ,
R: 5`..CGCGGTCGTAGAGGTAGGTTGGCGC..3`
U-MAM-d-*SNRPN* F: 5` CACCAACACAACTAACCTTACCCACTCCATCACA..3` ,
R: 5`..GTATGTTTGTGTGGTTGTAGAGGTACGTTGGTGT..3`.
M-d-*SNRPN* F: 5`..TTTGTATTGCGGTAAATAAGTACGT..3`
R: 5`..AAATACGTCAAACATCTCCGAC..3`

U-d-*SNRPN* F: 5`..TTTTTGTATTGTGGTAAATAAGTATGT..3` [4]
R: 5`..AACAAATACATCAAACATCTCCAAC..3`

Results and Discussion

Seminal fluid samples were collected from males suffering low fertility or problems in conceiving and attending the clinical laboratory for a routine seminal fluid analysis after their approval as mentioned in Appendix 1, each sample was collected after (3-4) days of sexual abstinence by masturbation, and examined according to WHO laboratory manual for the examination and processing of human semen. The samples with low sperm concentration (≤ 15 million/ml), sperm progressive motility (≤ 32%) and abnormal sperm morphology (≤ 30%) were considered as a patients group, and all the required information swere written in the questionnaire forma. Males with normal sperm concentration, motility and morphology, having one child at least were considered as a control group.

The number of patients with abnormal seminal fluid analysis results included in this study was (63) patient. Their sperm abnormalities were categorized into groups according to WHO reference guide (13).The number of control group was (13),all (63) patients were involved in the detection of STDs which include the detection of (*C.trachomatis/N.gonorrhoeae /M.genitalium and T.vaginalis*). By contrast, forreal time PCR for the detection of methylation in *SNRPN* gene promoter only (44) patient and (13) control were included, the age mean ± standard deviation of the patients group was (32.2 ±6.8) years and for control group was (34.07 ±6.5) years.

Genomic DNA extraction and evaluation

Genomic DNA extraction was achieved by using DNA-sorb-AM nucleic acid extraction kit to all seminal fluid samples (patients and control) by following the protocol mentioned previously, DNA concentration and purity was determined by using Nanodrop to measure both concentration and purity. All samples showed a concentration range of (50-250) ng/µl and a purity range from (1.5-2.00), since the components of semen are the spermatozoa and the seminal fluid, which is also called seminal plasma. This fluid is important for the survival of the spermatozoa, but may decrease the purity and quality of the DNA due to its fructose and protein content [5]. Sperm DNA may be inhibited if it is extracted through the standard techniques that are used for somatic cells due to the formation of disulfide bridges within and between the protamine, moreover, cell lysis step is difficult since the spermatozoa are protected by a disulfide bonds rich membrane [3].For all these reasons DNA extraction was a challenge in order to obtain a good quality DNA, many samples were excluded from the study for these reasons and only 63 patient and 13 control sample were extracted successfully. DNA concentration and purity was changeable depending on sample volume, handling techniques and sometimes the extraction protocol must be modified to acquire best results.

DNA integrity was detected by agarose gel electrophoresis subsequent the Nanodrop estimation. DNA with good integrity must be shown as a single clear band with no smears when visualized under UV light after ethidium bromide staining as showed in Figure (1).

Figure (1): Electrophoresis of the extracted genomic DNA from semen samples, on 1% gel of agarose for an hour at 90 voltages followed by ethidium bromide staining for 20 minutes and UV light visualization. Lane (1-9): extracted DNA, NC: Negative control of extraction.

Molecular detection of Beta globin gene by PCR technique

The desired β-globin gene region was detected in this study by using PCR technique for all samples as internal control for DNA extraction. It was an important step to check DNA integrity, and only samples positive for β-globin gene detection were involved in the following steps of this study, amplification was carried out using thermal cycler, the total reaction volume was 25 µl, gel electrophoresis was used to determine the successful amplification of the desired region, which appeared as a sharp bands with a specific molecular size(158bp) after staining with ethidium bromide and visualization under UV light, as shown in Figure (2).

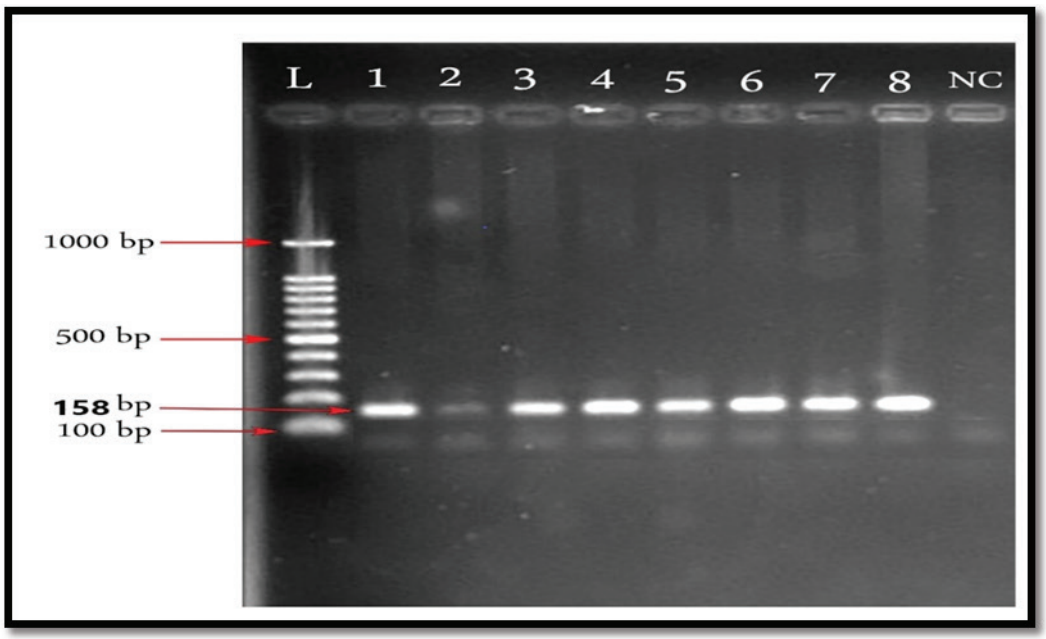

Figure (2): β-globin gene PCR product of 158 bp molecular size. Electrophoresis was applied as 2% agarose at 90 volt. For2 hr. followed by ethidium bromide staining for 20 min.then visualized by gel documentation system. L: DNA ladder (100-1000 bp), Lane (1-8): β-globin gene PCR product, NC: Negative control for amplification.

Methylation specific real time PCR for *SNRPN* gene detection

Methylation specific real time PCR(qMSP) is the technique used in the present study to detect the presence of methyl group at the promoter of *SNRPN* gene by using two sets of primers designed specifically to amplify a region with high CpG islands ratio in *SNRPN* promoter, a set of methylated primers and another set of un-methylated was used for each sample in a separated PCR reaction using real time thermal cycler (Biometra).

Optimization experiment was performed to check each primer set and to select the optimum annealing temperature by conventional PCR using standard human genomic DNA (Normal,Methylated and un-methylated), as shown in Figure (3).

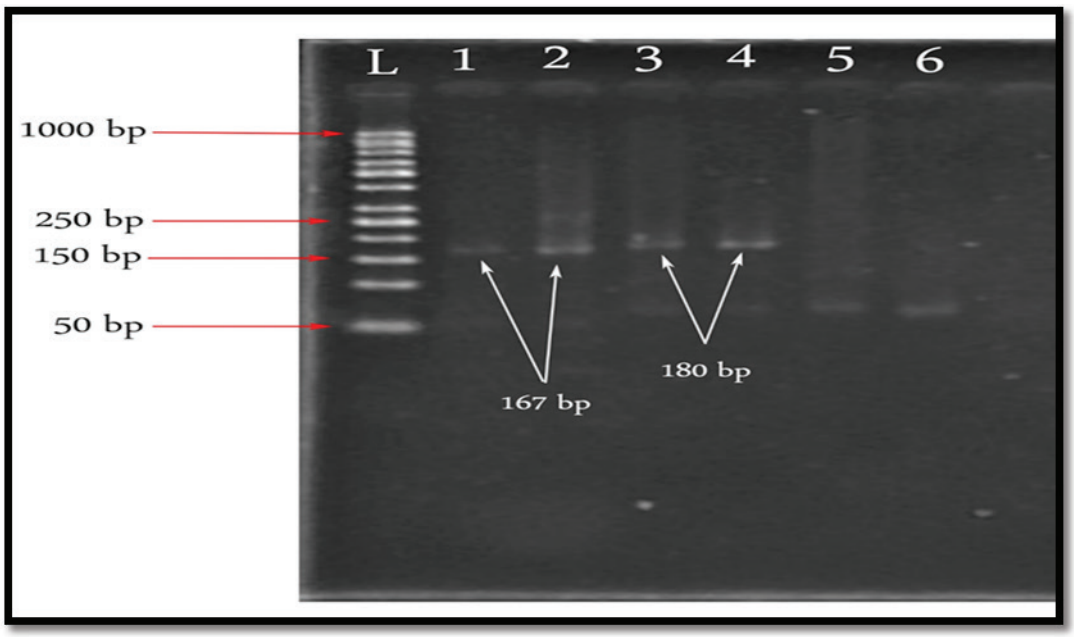

Figure (3): *SNRPN* gene PCR product for annealing temperature optimizationusing methylated, un-methylated and negative DNA control on 3% agarose at 70 vol. for 2 hours, visualized by gel documentation system after ethidium bromide staining for 20 minutes.

L: DNA ladder (50-1000bp).

Line 1: methylated control of *SNRPN* gene (167bp) at annealing temperature 56℃.

Line 2: methylated control of *SNRPN* gene (167bp) at annealing temperature 58℃.

Line 3: un-methylatedcontrol of *SNRPN* gene (180bp) at annealing temperature 56℃.

Line 4: un-methylated control of *SNRPN* gene (180bp) at annealing temperature 58℃.

Line 5, 6: Negative control.

Each DNA sample for the patients and controls was subjected to bisulfite treatment to convert un-methylated C-residues to uracil while keeping the methylated cytosine unconverted allowing one set of the primers to anneal, and amplify the desired region.

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Conflicts of Interest: Nil

Ethical Clearance: Nil

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