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Identification of a Methylation Pattern in the SNRPN Gene Promoter and its Association with Semen Abnormality Among Iraqi Males

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ABSTRACT

Infertility considered as a multifactorial condition; the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene is an imprinted gene. However, abnormal imprinting of this gene due to the methylation may result in abnormal function or silencing of the gene. Main aim of this study is to investigate the methylation present at the promoter of (SNRPN) gene and its role as a risk factor for male infertility. Sixty- three infertile males with age mean (32.28 ± 6.88 years) and 13 fertile males as a control age mean (34.07 ± 6.52 years) were investigated. Whole genomic DNA was extracted, DNA integrity was checked using β-globin gene as an internal control. The targeted region was amplified by polymerase chain reaction (PCR) technique. In addition, the SNRPN gene's promoter methylation was qualitatively detected using Real time polymerase chain reaction (qPCR) utilizing two sets of primers: methylated and un-methylated. Results reveled that all of the 63 infertile males were experiencing decrease in sperm concentration 9.42 ± 8.70 million/ml, reduced progressive motility 2.89 ± 5.45% as well as strange sperm morphology 27.06 ± 16.50%, while the values in the control group are normal. The results of the current investigation showed that the promoter of *SNRPN* was hypermethylated in some samples 22.7%, somewhat methylated in others 20.4%, and unmethylated in other samples 56.8% from infertile samples, while none of the 13 control samples had any methylation. These findings suggest that *SNRPN* gene may be associated with the negative changes in semen parameters, which could lead to male infertility.

KEYWORDS: Methylation; *SNRPN* gene; semen abnormality; male infertility.

الخلاصة

يعتبر العقم حالة متعددة العوامل؛ يعتبر الجين النووي الصغير متعدد الببتيد N (*SNRPN*) جين مطبوع. ومع ذلك، فإن البصمة غير الطبيعية لهذا الجين بسبب المثيلة قد تؤدي إلى وظيفة غير طبيعية أو إسكات الجين. ان الهدف الرئيسي من هذه الدراسة هو التحقق في المثيلة الموجودة في منطقة promoter الخاصة بالجين (*SNRPN*) ودورها كعامل خطر ومسبب للعقم في الذكور. تمت الدراسة في 63 من الذكور المصابين بالعقم بمتوسط العمر (32.28 ± 6.88 سنة) و13 من الذكور الاصحاء مع متوسط عمر (34.07 ± 6.52 سنة). تم استخراج الحمض النووي الجيني بالكامل، وتم فحص سلامة الحمض النووي باستخدام جين غلوبين β. تم تضخيم المنطقة المستهدفة بتقنية تفاعل البلمرة المتسلسل (PCR). بالإضافة إلى ذلك، تم اكتشاف وجود المثيلة في promoter الخاصة بالجين (*SNRPN*) باستخدام تفاعل البلمرة المتسلسل في الوقت الحقيقي (qPCR) باستخدام مجموعتين من primers: ميثيل وغير ميثيل. أظهرت النتائج أن جميع الذكور المصابين بالعقم البالغ عددهم 63 كانوا يعانون من انخفاض في تركيز الحيوانات المنوية 9.42 ± 8.70 مليون / مل ، وانخفاض الحركة 2.89 ± 5.45٪ وكذلك غير منتظمة الشكل 27.06 ± 16.50٪ ، بينما القيم في مجموعة الذكور الاصحاء كانت طبيعية. كما أظهرت النتائج ان *SNRPN* كان مفرط الميثيل في بعض العينات بنسبة 22.7٪ ، وميثيل إلى حد ما في 20.4٪ ، وغير ميثيل في عينات أخرى 56.8٪ من عينات غير قابلة للإخصاب ، بينما لم يكن لدى أي من عينات الذكور الاصحاء الـ 13 أي مثيلة. تشير هذه النتائج إلى أن جين *SNRPN* قد يكون مرتبطًا بالتغيرات السلبية في معايير السائل المنوي، مما قد يؤدي إلى العقم عند الذكور.

INTRODUCTION

Infertility is considered as a multifactorial condition because of the widespread male infertility also known as subfertility or decreased fertility. Other problems related could be brought on by a variety of things such as infections, hormonal imbalances, or structural flaws, which would then result in male infertility [1,2].

The genetic causes may account for about 15-30% of male infertility [3]. There are important genetic

factors that affect infertility such as Y chromosome microdeletions [4]. Understanding the genetic etiology of male infertility is crucial from a therapeutic standpoint in order to improve prognosis, therapy, and determine the likelihood of genetic abnormalities being passed on through natural or assisted reproductive methods [5].

Genomic imprinting is a form of epigenetic inheritance whereby the regulation of a gene or chromosomal region is dependent on the sex of the transmitting parent, DNA methylation, post-translational, histone modifications, and microRNA (miRNA) regulation that are all considered to be a part of the process of gene control known as epigenetics [6].

The small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene is an imprinted gene that normally expresses itself in a monoallelic fashion through the paternal allele and is methylated at the maternal allele. However, abnormal imprinting of this gene due to the methylation may result in abnormal function or silencing of the gene, and hypermethylation of that gene at the promoter region has been observed to be associated with certain sperm abnormalities [7,8].

Prader-Willi Syndrome (PWS) is a genetic disorder caused by the incorrect imprinting of the *SNPRN* gene, which is a maternally imprinted gene, PWS is caused by the lack of function of this gene on the paternal allele. In both oligozoospermic and individuals with aberrant protamine ratios, SNRPN was found to be excessively methylated [9].

Considering the recent increase of infertility globally and locally and since it is an idiopathic disorder, there is a growing need to improve diagnostic accuracy by using more advanced techniques, therefore, this study aims to Perform a qualitative detection of methylation status in SNRPN gene in promoter region by utilizing two primer sets: methylated and un-methylated using real time-PCR and its possible effect on quality of sperm and its role as a risk factor for male infertility.

MATERIALS AND METHODS

Sample collection and study groups

Patient samples were chosen based on the information provided by the questioner and the results of the analysis of the seminal fluid. The patient group ranged in age from 19-52 years consisting of childless males and have difficulty conceiving due to low-quality seminal fluid, the sperm count, motility, and morphology. Values were all below the WHO standard manual's 2010 lower reference limits. As opposed to males having at least only one child and a regular examination of seminal fluid was used as a control group with participants ranging in age from 29- 45 years old.

Seminal fluid analysis

All patients and control males had their seminal fluid collected by masturbating into sterilized specialized containers in the laboratory 3-4 days after the last sexual activity. The World Health Organization's standard manual was used to evaluate sperm quantity, shape, and motility [10]. Before analysis, the semen samples were liquefied at 37 °C for 30 minutes, an amount of 100-150µl transferred to in a 1.5ml Eppendorf tubes with fluid mixture 50µl mucolytic transport medium, semen kept in the freezer at –20 °C until DNA extraction.

Genomic DNA extraction and detection of human beta-globin gene by a conventional polymerase chain reaction (PCR)

The DNA-sorb-AM nucleic acid extraction kit (MoBiTec, Germany) is used to extract the full genomic DNA from seminal fluid, guanidine chloride, which acts as a chaotropic agent to lyse the cells and denature cell proteins, is present in the lysis solution. Determination of quantity and quality of DNA is done using nanodrop at 260/280 nm and gel electrophoresis.

Primer’s selection

The PCR amplification procedure utilized the following primers: beta-globin gene and methylated and Un-methylated primers sequence showed in Table 1.

Bisulfite conversion of DNA

The DNA methylation kit (Zymo Research, USA) was used to transformed unmodified cytosine (C) to uracil (U). While methylated cytosine left unaltered since it is protected by methylation. Then the converted DNA was used as a template to detect the methylation of the promoter of the *SNPRN* gene.

Table 1. Sequence and molecular size of PCR product

set	Name	Primers sequence (5' → 3')	Gene amplified	Reference
1	Hemoglobin subunit beta (*HBB*)	F:AGTCAGGGCAGAGCCATCTA R:CCTCACCACCAACTTCATCC	*158bp*	**[11]**
2	M-MAM-d-*SNRPN*	F: GACACAACTAACCTTACCCGCTCCATCGCG R: CGCGGTCGTAGAGGTAGGTTGGCGC	*SNRPN*	**[8]**
	U-MAM-d-*SNRPN*	F: CACCAACACAACTAACCTTACCCACTCCATCACA R: GTATGTTTGTGTGGTTGTAGAGGTACGTTGGTGT		
3	M-d-*SNRPN*	F:TTTGTATTGCGGTAAATAAGTACGT R: AAATACGTCAAACATCTCCGAC		**In this study.**
	U-d-*SNRPN*	F:TTTTTGTATTGTGGTAAATAAGTATGT R: AACAAATACATCAAACATCTCCAAC		

Qualitative methylation-specific PCR (qMSP)

Qualitative methylation-specific PCR **(**qMSP**)** was used to determine whether the promoter of *SNRPN* gene has been methylated and there is a high percentage of CpG islands. The (qMSP) is highly specific and more sensitive than conventional PCR since it enables the detection of a methylated allele in a 10.000-fold excess of unmethylated alleles, thus representing a suitable method for cfDNA methylation analysis [12]. Cycling conditions of qMSP are listed in Table 2, after the optimization of the annealing temperature of the primers using a gradient of temperatures.

Table 2. Cycling conditions of qMSP.

Steps	Temperature	Time	No. of cycles
Denaturation 1	95°C	15 min.	1
Denaturation 2	95°C	20 sec.	40
Annealing	58°C	20 sec.	
Extension	72°C	15 sec	
Final extension	72°C	5 min.	1

Statistical analysis

The impact of numerous factors on the research parameters was investigated using the statistical analysis system - SAS (2012) application. In this study, the least significant difference –LSD test (ANOVA) was used to make comparison of the means, and the chi-square test was utilized to compare percentages.

RESULTS AND DISCUSSION

Sample collection and seminal fluid analysis

Samples with sperm count ≤ 15 million/ml, progressive motility ≤ 32% and morphology ≤ 30% were regarded as a patient's group. Sixty-three patients with aberrant seminal fluid analysis results were included in this study [10]. All patients were not smoker, no medication was taken in addition to all patients went at least two weeks without taking any antibiotics. All subjects received a thorough explanation of the study's objectives, and each one signed a consent form. Control group were 13 fertile males, having one child as least, and their mean age was (34.07 ±6.5) years, the mean of sperm concentration was 38.46 ±9.43 million/ml, total motility 72.6 ±14.08%, progressive motility 52 ±13.63% and sperm morphology 56 ±17.75%. The difference between patients and control group were highly significant in the results of semen analysis as listed in Table 3.

Table 3. Clinical parameters of patients and control group

Variable	Mean ± SD		T-Test
	Patients (n=63)	Control (n=13)	
Age (years)	32.28 ± 6.88	34.07 ± 6.52	4.144 NS
Sperm count (million/ml)	9.42 ± 8.70	38.46 ± 9.43	5.356 **
Total motility (%)	22.62 ± 15.52	72.69 ±14.08	9.287 **
PR motility (%)	2.89 ± 5.45	52.3 ±13.63	4.506 **
Morphology (%)	27.06 ± 16.50	56.1 ±17.57	10.125 **

** (P<0.01), PR: Progressive motility, NR: Non-progressive motility, NS: not statistically significant.

Genomic DNA extraction and detection of beta globin gene by PCR technique

The genomic DNA were completely extracted using DNA-sorb-AM nucleic acid extraction kit and Nanodrop. The DNA concentration and purity of all samples were assessed. The concentration and purity ranges were 50-250 ng/l and 1.6-2.00 respectively. DNA integrity was detected by agarose gel electrophoresis as showed in Figure 1.

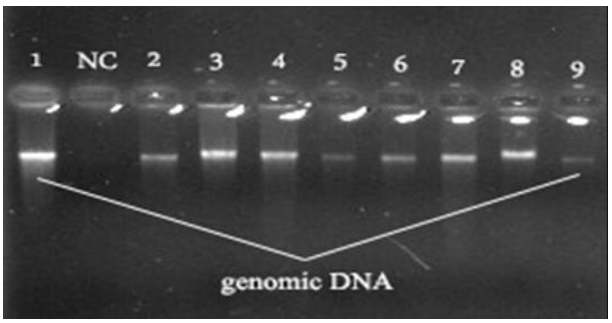

Figure 1. Genomic DNA from semen samples was extracted and electrophoresed on a 1 % agarose gel for an hour at 90 volts, followed by 20 minutes of ethidium bromide staining and UV light viewing.

In this investigation, the targeted -globin gene region was found using PCR technique as internal control to check DNA integrity. In the subsequent stages of this study, only samples that had been detected to contain the globin gene were used., Figure 2 showed sharp bands of β-globin gene with a particular molecular size (158bp) following ethidium bromide staining under UV light.

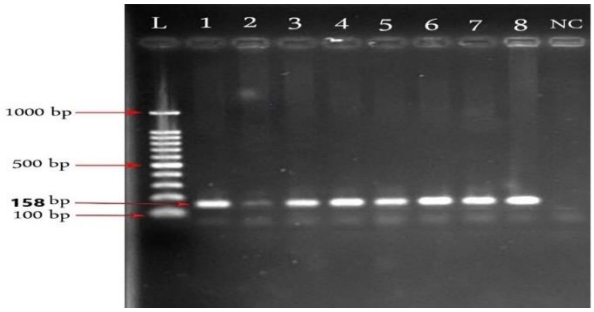

Figure 2. The PCR product of β-globin gene with 158 bp molecular size. Electrophoresis was applied as 2% agarose at 90 volts for 2 hr. L: DNA ladder (100-1000 bp), Lane (1-8): β-globin gene PCR product.

Methylation-specific Real-time PCR for the identification of the *SNRPN* gene

The *SNRPN* gene's promoter was checked for the presence of methyl groups by utilizing two primer sets created particularly to amplify a CpG-rich area islands ratio in *SNRPN* promoter region using real time thermal cycler (Biometra). The methylated samples in the reaction with methylated primers showed an amplification curve, which indicated the presence of methylation as shown in Figure 3.

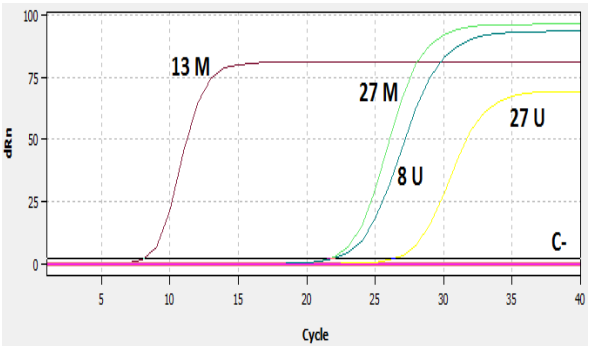

Figure 3. Methylation Specific Real Time PCR for both Methylated and un- methylated patterns.

13M: sample no.13 with methylated primers shows an amplification curve.

The reaction with un-methylated primers showed no amplification curve and vice versa, some samples showed an amplification curve in both reactions which means that there is a partial methylation in the selected region of the promoter.

Cycle threshold (Ct) values were used to record the amplification. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, the lower the Ct level the greater the amount of target nucleic acid in the sample.

As showed Figure 3, Real time PCR were used for identification of methyl group in *SNRPN* gene promoter regain included 44 from total 63 patients and 13 as a control. The results showed 19 of 44 (43.18%) patients were methylated, 25 of 44 (56.8%) were un-methylated, all the 13 (100%) control group were un-methylated.

The results of the current investigation showed that the promoter of *SNRPN* was hypermethylated in some samples (22.7%), somewhat methylated in others (20.4%), and unmethylated in other samples (56.8%) from infertile samples, while none of the 13 control samples had any methylation as showed in Table 5.

Table 5. Distribution of Sample study According to Methylation results

Group	Methylation	No. (%)
Patients (No. 44)	Positive	19 (43.18%)
	Negative	25 (56.82%)
Control (No. 13)	Positive	0 (00.00%)
	Negative	13 (100.00%)

The analysis of *SNRPN* promoter methylation showed a difference in each study group which is statistically significant (P <0.01), and these results are compatible with a study done by [13] which indicate the presence of hypermethylation in the promoter of *SNRPN* gene in the samples with specific sperm abnormalities while the promoter of SNRPN gene was hypomethylated in the control samples. Besides, the results of the present study are in agree with the meta-analytic study by [1], which confirmed the association between male infertility and increased *SNRPN* methylation.

A study by [14] suggested that imprinted genes like *SNRPN* and abnormal methylation have both been linked to the spermatozoa of infertile males. In addition, similar result found by [9] as *SNRPN* was hyper methylated in oligozoospermic patients as well as in patients with abnormal semen parameters and protamine ratios. It's interesting to note that human embryos born through ART and spermatozoa from asthenozoospermia patients both contain imprinting abnormalities at the SNRPN gene and IGF2-H19 [15].

The Failure in the maintenance of imprinted gene like *SNRPN* by methylation patterns in the germline might occur, and this has been associated with low pregnancy rates, poor post-fertilization

development, and poor sperm quality [16]. Male infertility can be related and explained by many genetic factors, and the studies have been extended to involve epigenetics and genomic imprinting. DNA methylation has been closely related with male infertility as one of the epigenetic factors responsible for genomic imprinting to regulate gene expression in a way depending on parental origin [8].

Genetics and environment can both have an impact on imprints. Variation in the sequence underlying the assessed epigenetic regions may have an impact on epigenetic states for genetic effects [17]. Imprinted genes have an unusual function in germ cells, in reprogramming events. They are also a significant target for examining aberrant methylation mistakes in sperm from males with abnormal semen parameters [18].

CONCLUSIONS

The data of the present study suggested that epigenetic changes especially DNA methylation may be associated with reduced male fertility. In addition to the detection of methylation pattern at the promoter of the maternally imprinted SNRPN gene may be associated with the negative changes in semen parameters, which could lead to male infertility

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Informed Consent: All patients gave their written informed consents before inclusion.

REFERENCES

[1] D. Santi. S. De Vincentis, E. Magnani,. and G. Spaggiari, “Impairment of Sperm DNA Methylation in Male Infertility: A Meta-Analytic Study’’. *Journal of Andrology* .5 (4): 695–703. 2017.

[2] N. Garrido, and I. Hervás “Personalized medicine in infertile men. Urol”. *Clin. North Am*., 47, 245–255. 2020.

[3] C. Krausz,; A.R. Escamilla,; and C. Chianese, “Genetics of male infertility: from research to clinic” *Reproduction*. 150 (5):R159- 174, 2015.

[4] H. Dong,; Y. Wang,; Z. Zou,; L. Chen,.; C. Shen,; S. Xu,; J. Zhang,; F. Zhao,.; S. Ge,; Q. Gao,; H. Hu,; M. Song, and W. Wang,” Abnormal Methylation of Imprinted Genes and Cigarette Smoking: Assessment of Their Association with the Risk of Male Infertility” .*Reproductive Sciences*, 2016.

[5] O. Okutman, M.B. Rhouma, M. Benkhalifa et al. “Genetic evaluation of patients with non-syndromic male infertility” *J. Assist. Reprod. Genet*., 35, 1939–1951, 2018. doi: doi: 10.1007/s10815-018-1301-7.

[6] J. Stomper, J. C. Rotondo, G. Greve, and M. Lübbert, “Hypomethylating agents (HMA) for the treatment of acute myeloid leukemia and myelodysplastic syndromes: mechanisms of resistance and novel HMA-based therapies” *Leukemia*, 2021. doi: 10.1038/s41375-021-01218-0

[7] S.S. Hammoud, J. Purwar, C. Pflueger, B.R. Cairns, and D.T. Carrell, “Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility” *Fertility and sterility* .94 (5): 1728-1733, 2010.

[8] A. Botezatu,; S. Razvan,; S. Demetra,; V. I. Iulia, and A. Gabriela, “Methylation Pattern of Methylene Tetrahydrofolate Reductase and Small Nuclear Ribonucleoprotein Polypeptide N Promoters in Oligoasthenospermia: A Case-Control Study: *Reproductive* BioMedicine Online. 28 (2): 225–31, 2014.

[9] C. Nevin, and M. Carroll, “Sperm DNA methylation, infertility and transgenerational epigenetics” *Journal of Human Genetics and Clinical Embryology*.1, (4): 9-10, 2015.

[10] World Health Organization “Examination and Processing of Human Semen” World Health Edition, (10): 286, 2010.

[11] M. I. Mezaal “Molecular Detection and genotyping of human Papilloma virus and cervical cancer prediction among women with abnormal Pap smears in Baghdad” PhD. Dissertation. University of Baghdad, 2012.

[12] CA. Eads, K. D. Danenberg, K. Kawakami, L. B. Saltz, C. Blake, D. Shibata, P.V, Danenberg, , P.W. Laird, “MethyLight: a high-throughput assay to measure DNA methylation” *Nucleic Acids Res* 28(8):E32, 2000.

[13] Peng, H., Zhao, P., Liu, J., Zhang, J., Zhang, J., Wang, Y., et al. (2018). Novel epigenomic biomarkers of male infertility identified by methylation patterns of CpG sites within imprinting control regions of H19 and SNRPN genes. OMICS J. Integr. Biol. 22, 354–364. doi: 10.1089/omi.2018.0019

[14] S. Gunes, M. A. Arslan, G. N. T. Hekim, and R. Asci, “The role of epigenetic in idiopathic male infertility” *Journal of Assisted Reproduction and Genetics* . 33 (5):553-569, 2016.

[15] H. Lou, F. Le, M. Hu, X. Yang, L. Li, L. Wang, et al. “Aberrant DNA methylation of IGF2-H19 locus in

human fetus and in spermatozoa from assisted reproductive technologies. Reprod. Sci. 26, 997–1004. doi: 10.1177/1933719118802052

[16] J. C. Rotondo, C. Lanzillotti, C. Mazziotta, M. Tognon, & F. Martini, "Epigenetics of male infertility: The role of DNA methylation" *Frontiers in Cell and Developmental Biology*, 1822., 2021.

[17] M. Lorgen-Ritchie, A.D. Murray, A. C. Ferguson-Smith, M. Richards, G.W. Horgan, L. H. Phillips, & P. Haggarty, "Imprinting methylation in *SNRPN* and *MEST1* in adult blood predicts cognitive ability" *Plos one*, 14(2), e0211799, 2019.

[18] A. Minor, C. Victor, and M. Sai, "Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and post vasectomy reversal" *Reproduction*. 141(6):749-757, 2011.

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