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Effect of human Mesoderm-Specific Transcript Gene Methylation on Oligospermia and Azoospermia Related with Men Infertility in a Sample of Iraqi Patients

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Abstract: Male Infertility is a significant problem for human reproduction in recent years. Several studies focused on the role of epigenetics, including DNA methylation, in spermatogenesis and male infertility. The mesoderm-specific transcription (MEST) gene is a paternally expressed imprinted gene repeatedly linked with male infertility. This study aimed to investigate the effect of MEST gene methylation on oligospermia and azoospermia related to male Infertility. Methods**:** Semen samples were collected from 80 infertile patients (40 patients suffered from oligospermia and 40 patients suffered from azoospermia) from Kamal Al-Samarrai Hospital, Baghdad, also (40) semen samples were collected from healthy fertile normospermia men as a control group, with ages ranging between 20 to 50 years. Microscopic examinations of seminal fluid were performed using routine methods, and methylated DNA was detected by quantitative real-time polymerase chain reaction using by high-resolution melting technique(HMR) and which measured the melting temperature (Tm) of PCR products after bisulfite. Results: The results identified an increased percentages of methylated gene in Azoospermia patients group (47.5) % and Oligospermia patients group (45.0%) compared to the controls (12.5 %), however, the control group showed the highest percentage (75.0 %) of non-methylated gene Conclusion: It was shown that the methylation status of the MEST gene is statistically significantly associated with the clinical presentation of the parameters whether normal or infertile, as well as MEST gene methylation may be considered an important predictor for addressing male factor infertility. Therefore, suggested that male infertility may be linked to methylation of the MEST imprinted gene.

Keywords: MEST, oligospermia, azoospermia, infertility, methylation.

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Introduction

Male infertility is defined by the World Health Organisation (WHO) as the inability of a male to make a fertile female pregnant for a minimum of at least 1 year of regular unprotected intercourse. The male is solely responsible for about 20% of cases and is a contributing factor in another 30% to 40% of all infertility cases (1,2). There are multiple causes for male infertility, which can be broadly classified due to their general underlying aetiology. These include endocrine disorders (usually due to hypogonadism) estimated at 2% to 5%

of cases, sperm transport disorders (such as vasectomy) at 5%, primary testicular defects (which include abnormal sperm parameters(oligospermia: low sperm count or azoospermia :absence of sperm) without any identifiable cause) at 65% to 80% and idiopathic (where an infertile male has normal sperm and semen parameters) at 10% to 20% (3,).These are broad estimates only, as accurate statistics are unavailable due to general underreporting, cultural factors, and regional variations(4). A number of studies focused on the role of genetic (5) and epigenetic, including DNA methylation, (6) Methylation is the process of adding a methyl group (CH_3) to a molecule, such as DNA, RNA, or proteins. In DNA, this typically occurs at specific regions known as CpG islands and plays a vital role in regulating gene expression, chromatin structure, and various cellular functions. That has been linked with male infertility (7).

Aberrant methylation of genes such as MEST has been associated with poor sperm quality in idiopathic infertile men. The mesoderm-specific transcription (MEST) gene is a paternally expressed imprinted gene that has been repeatedly linked with male infertility (8). MEST hypermethylation correlates with low sperm counts and poor motility, suggesting a significant epigenetic influence on fertility (9).

Aim of study

This study aimed to investigate the effect of MEST gene methylation on oligospermia and azoospermia related to male infertility

Materials and Methods

Current case-control study was carried out in the laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduaten samples were collected from 80 patients with male infertility (oligospermia, azoospermia) and 40 healthy fertile male controls. from Kamal Al-Samaria Hospital in Bagdad city

Sample collection

An amount 0.5 ml of seminal fluid was drawn from everyone (primarily and secondary male infertility) who participated in this study and put in an Eppendorf Ice centrifuge tube taken The pellet 500 µl of trace elution (TE) DNA preservation directly was added DNA extraction and maintained at -8 °c until employed for the investigation.

DNA extraction and sodium bisulfite treatment

Extraction of DNA from semen samples was done using the DNA Extraction Kit (Promega) based on the manufacturer's guidance. The DNA extraction was also confirmed by gel electrophoresis. Then, the extracted DNA was stored at 20 °c. The sodium bisulfite treatment was applied to the extracted DNA using the Bisulfite modification genomic DNA. For all samples, the same amount of genomic DNA (1000 ng) was used for bisulfite treatment. In that process, unmethylated cytosines changed to uracil, whereas methylated cytosines were unaltered (7). Sensitivity assay for bisulfite modification was carried out. For this assay, untreated DNA was used as a template for PCR on set of MSP primers.

DNA methylation analysis DNA methylation analysis

The methylation was measured quantitatively at the Differentially Methylated Regions (DMRs), **(**DMRs) of the MEST gene by Methyl Edge™ Bisulfite Conversion System yields bisulfite-converted kits and qRT-PCR real-time methylation-specific PCR (MSP), employing primers were supplied from Promega and to discern methylated from unmethylated DNA.

Amount 20–50ng (20 ng) of bisulfite-converted template DNA was used for each real-time PCR. Amplicons for real-time PCR was 75–200bp.Primer sequences of the MEST gene are shown in Table (1) The analysis of samples was independently applied by two MSP reactions **(9)**, Program a thermocycler reaction was done as follows: 8 minutes at 98°C 60 minutes at 54°C Hold at 4°C . Following incubation, the samples were stored or on ice, protected from light for up to 20 hours until proceed(9).

Statistical Analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of difference groups (patients and control) in study parameters. T-test and least significant difference (LSD) was Used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of correlation coefficient between variables in this study.

Table (1): Primers of MEST gene

Primer	Sequence	Primer sequence 5'- - 3'
MEST GENE METHYLATION	F	(TTTAGGAACGTGAGGTTTGAGTC)
	R	(CCTACTACTCCCTACCTACCAACG)
MEST GENE UN METHYLATION	F	(TTTTTAGGAATGTGAGGTTTGAGTT)
	R	(CCTACTACTCCCTACCTACCAACAC)

Results and Discussion

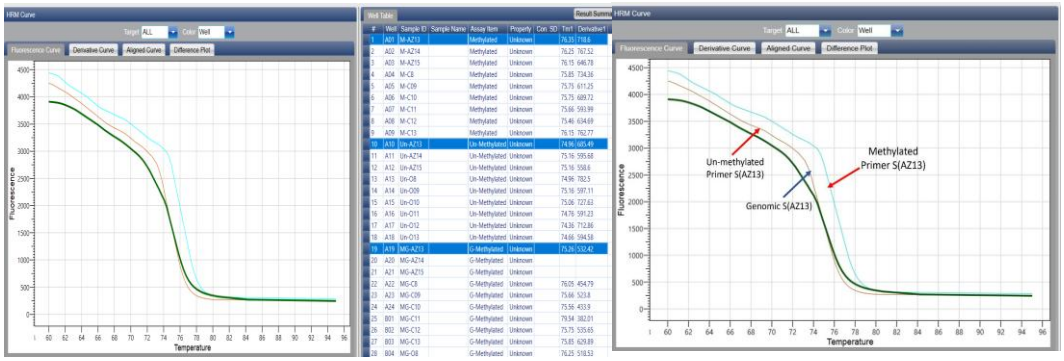

Figure (1): showed methylation , unmethylation by High Resolution Melting (HRM) analysis

The results indicated that there was very highly statistically significant association (p<0.001) between clinical presentation of the participants (Azoospermia Vs controls) with methylation status of the MEST gene, pointing to the effect of methylation of the occurrence of Azoospermia with the higher percentage of methylation (47.5 %) observed in the Azoospermia group while in the control group the higher percentage of non-methylated gene was noticed (Table2)

Table (2): Description of Azoospermia and control

Study groups	Frequency (%)			Total	P-value
	Methylated	Partially methylated	Non-methylated		
Azoospermia	19 (47.5)	15 (37.5)	6 (15.0)	40 (100)	**<0.001*** **
Control	5 (12.5)	5 (12.5)	30 (75.0)	40 (100)	
Total	49 (61.3)	20 (25.0)	11 13.8)	80 (100)	

-* Groups with are statistically different (p-value <= p<0.001)

- This groups by Chi-x.

The results revealed that there was very highly statistically significant association ($p<0.001$) between clinical presentation of the participants (Oligospermia Vs controls) with methylation status of the MEST gene, pointing to the effect of methylation of the occurrence of Oligospermia with the higher percentage of methylation (45 %) observed in the Azoospermia group while in the control group the higher percentage of non-methylated gene was noticed (75 %) (Table3)

Table (3): Description of Oligospermia and control

Study groups	Frequency (%)			Total	P-value
	Methylated	Partially methylated	Non-methylated		
Oligospermia	18 (45.0)	12 (30.0)	10 (25.0)	40 (100)	**<0.001*** **
Control	5 (12.5)	5 (12.5)	30 (75.0)	40 (100)	
Total	48 (60.0)	17 (21.3)	15 (18.8)	80 (100)	

The results showed there was no statistically significant association between the infertility indicators (Azoospermia and Oligospermia) from the one hand, and the methylation status of the MEST gene from the other side ($p=0.51$) stressing the critical role of the methylation mechanism in the infertility outcomes (Table 4)

Table (4) : Description of Azoospermia and Oligospermia

Study groups	Frequency (%)			Total	P-value
	Methylated	Partially methylated	Non-methylated		
Azoospermia	19 (47.5)	15 (37.5)	6 (15.0)	40 (100)	**0.51**
Oligospermia	18 (45.0)	12 (30.0)	10 (25.0)	40 (100)	
Total	37 (46.3)	27 (33.8)	16 (20.0)	80 (100)	

Collectively, it was shown that the methylation status of the MEST gene is statistically significantly associated with the clinical presentation of the parameters whether normal or infertile. The results clearly identified an increased percentages of methylated gene in Azoospermia (47.5) %and Oligospermia (45.0%) groups compared to the controls (12.5 %), however, the control group showed the highest percentage (75.0 %) of non-methylated gene (Table 5.).

Table (5) : Description of Azoospermia, Oligospermia and control.

Study groups	Frequency (%)			Total	P-value
	Methylated	Partially methylated	Non-methylated		
Azoospermia	**19 (47.5)**	**15 (37.5)**	**6 (15.0)**	**40 (100)**	**<0.001*** **
Oligospermia	**18 (45.0)**	**12 (30.0)**	**10 (25.0)**	**40 (100)**	
Control	**5 (12.5)**	**5 (12.5)**	**30 (75.0)**	**40 (100)**	
Total	**42 (35.0)**	**32 (26.7)**	**46 (38.3)**	**120 (100)**	

The study confirmed the presence of aberrant DNA methylation patterns in the MEST gene, which were closely associated with impaired sperm quality. These findings suggest that epigenetic modifications, particularly hypermethylation of the MEST gene, may play a crucial role in male infertility (**10**). However, the discussion did not sufficiently elaborate on the

biological role of MEST in spermatogenesis, nor did it explain how DNA methylation could affect the molecular mechanisms underlying sperm development. The MEST (Mesoderm-Specific Transcript) gene is a paternally expressed imprinted gene implicated in embryonic development and cell differentiation, including the regulation of testicular somatic cells and germ cell development. Aberrant methylation of MEST may disrupt the normal epigenetic reprogramming required for spermatogonial stem cell maintenance, meiosis, and sperm maturation, leading to reduced sperm production or function. The current study demonstrated a statistically significant difference in sperm parameters between normozoospermic (control) individuals and infertile patients (both azoospermic and oligospermic). Notably, methylation of the MEST gene was significantly higher in the azoospermia group, suggesting a strong association between MEST gene silencing and severe defects in spermatogenesis. In contrast, the control group showed a higher percentage of unmethylated MEST, consistent with normal gene expression and normal sperm production.

where results showed very highly significant between clinical presentation of the participants (azoospermia Vs control) with methylation of the MEST gene, pointing the effect of methylation of the occurrence of azoospermia with the higher percentage of methylation observed in the azoospermia group while in the control group the higher percentage of non-methylation gene was noticed.(11) These findings are in accordance with a retrospective study done by Pourmasumi et al. indicating a remarkable difference in sperm parameters between normozoospermia and infertile groups This study was in contrast with other studies using methylation as study of Amjadian *et al* (11) and Abdel Jail *et al* (12) when thy studied impact of DNA methylation of MEST gene on male infertility and found a negative correlation existed between MEST methylation with sperm parameter. The present study confirmed a clear difference in sperm parameters in the oligospermia and azoospermia groups compared to normal sperm in the control group, compared to the weak sperm in the oligospermia group, the oligospermia were characterized by reduced sperm counts, decreased motility, and abnormal morphology, confirming the clear impact on sperm quality in infertility cases. The results of the current study were consistent with the study of Rotondo *et al* (13), which investigated the role of DNA methylation in male infertility. As well as present results were agreed with research of Cannarella *et al* (14) who reported a systematic review and meta-analysis on sperm mesoderm specific transcript gene methylation status in infertile patients, however the current study confirmed a clear difference in sperm parameters in the oligospermia and the methylation ،which requires the need for further researches to understand male infertility to treat it at an early stage.

Conclusion

It was shown that the methylation status of the MEST gene is statistically significantly associated with the clinical presentation of the parameters whether normal or infertile, as well as MEST gene methylation may be considered an important predictor for addressing male factor infertility. Therefore, suggest that male infertility may be linked to methylation of the MEST imprinted gene.

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