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Association between promoter methylation of *MLH1* and *MSH2* and reactive oxygen species in oligozoospermic men—A pilot study

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Summary

MLH1 and *MSH2* are important genes for DNA mismatch repair and crossing over during meiosis and are implicated in male infertility. Therefore, the methylation patterns of the DNA mismatch repair genes *MLH1* and *MSH2* in oligozoospermic males were investigated. Ten oligozoospermic patients and 29 normozoospermic donors were analysed. Methylation profiles of the *MLH1* and *MSH2* promotors were analysed. In addition, sperm motility and seminal reactive oxygen species (ROS) were recorded. Receiver operating characteristic (ROC) analysis was conducted to determine the accuracy of the DNA methylation status of *MLH1* and *MSH2* to distinguish between oligozoospermic and normozoospermic men. In oligozoospermic men, *MLH1* was significantly (p = .0013) more methylated compared to normozoospermic men. Additionally, there was a significant positive association (r = .384; p = .0159) between seminal ROS levels and *MLH1* methylation. Contrary, no association between *MSH2* methylation and oligozoospermia was found. ROC curve analysis for methylation status of *MLH1* was significant (p = .0275) with an area under the curve of 61.1%, a sensitivity of 22.2% and a specificity of 100.0%. This pilot study indicates oligozoospermic patients have more methylation of *MLH1* than normozoospermic patients. Whether hypermethylation of the *MLH1* promoter plays a role in repairing relevant mismatches of sperm DNA strands in idiopathic oligozoospermia warrants further investigation.

1 | INTRODUCTION

Despite considerable efforts to determine the causes of male infertility, approximately 30% of these infertility cases are deemed idiopathic or unexplained (Groen et al., 2016). Recent studies have shown an association between idiopathic male infertility and epigenetic modifications, including promoter methylation in imprinted, reproduction-related and developmental genes in spermatozoa and might explain cases of idiopathic male infertility (Gunes, Arslan, Hekim, & Asci, 2016; Jenkins et al., 2016; Urdinguio et al., 2015).

The DNA mismatch repair (DMMR) mechanism is essential for maintaining cell genomic stability. DMMR proteins remove base substitution mismatches and insertion–deletion loops during replication and recombination. Mismatches occur during the DNA

replicationprocess by escaping the proofreading function of DNA polymerase III. These mismatches result in mispairing, including G-T or A-C. Mismatch repair (MMR) is also implicated in cell cycle arrest and apoptosis after DNA damage (Gunes, Al-Sadaan, & Agarwal, 2015). Therefore, defects in DMMR during spermatogenesis may disturb the integrity of the sperm DNA. Abnormally high amounts of reactive oxygen species (ROS) are detected in approximately 50% of the infertile patients (Homa, Vessey, Perez-Miranda, Riyait, & Agarwal, 2015). It is also known that high levels of ROS production lead to DNA damage and silencing of DMMR genes in spermatozoa (Homa et al., 2015; Vessey, Perez-Miranda, Macfarquhar, Agarwal, & Homa, 2014). Infertile men with high ROS levels in their ejaculate also present with a higher percentage of DNA-damaged spermatozoa than fertile males (Wang et al., 2003).

In an attempt to understand the relationship between the methylation status, seminal ROS and oligozoospermia, we hypothesised that transcriptional inactivation or silencing of DMMR genes during spermatogenesis may disturb this process, thus resulting in oligozoospermia. In addition, it has been hypothesised that high concentrations of ROS may cause hypermethylation of promoter DMMR genes and may result in downregulation of enzymes through transcriptional silencing (Min, Lim, & Jung, 2010). To investigate this hypothesis, we have compared promoter methylation patterns of MutL homolog 1 (*MLH1*) and MutS homolog 2 (*MSH2*) in spermatozoa from oligozoospermic men. Therefore, this study aimed at comparing the promoter methylation patterns of *MLH1* and *MSH2* genes in spermatozoa between oligozoospermic and normozoospermic men and to evaluate the role of seminal ROS levels on this possible association.

2 | MATERIALS AND METHODS

2.1 | Study group

The study protocol was approved by the Institutional Review Board of the Cleveland Clinic. Ten males with idiopathic oligozoospermia (sperm concentration $< 10 \times 10^6$/ml) attending the Andrology Clinic of Cleveland Clinic and 29 normozoospermic (sperm concentration $>15 \times 10^6$/ml) controls (donors) were enrolled in this study. Informed consent was obtained from all participants. Semen samples were collected in sterile containers after a period of ejaculatory abstinence of 2–4 days. The exclusion criteria included a history of maldescended testes, testicular trauma or surgery, absence of secondary sexual characteristics, presence of varicocele, genito-urinary infection, leukocytospermia, use of gonadotoxic medication, endocrine disorders and any other identifiable causes of male infertility.

After complete liquefaction of the ejaculate for 20–30 min at 37°C, all specimens were first assessed for volume, sperm concentration, total sperm count, sperm motility and round cell concentration according to World Health Organization (WHO) guidelines (World Health Organization, 2010). Sperm motility and concentration were assessed using a MicroCell counting chamber (Vitrolife, San Diego, CA). Samples were tested for leukocytospermia ($>1 \times 10^6$ WBC/ml) when the round cell concentration was $>1 \times 10^6$/ml (Groen et al., 2016).

Subsequently, specimens were assessed seminal ROS production and sperm methylation profiles of *MLH1* and *MSH2* promoters, as below.

2.2 | **ROS measurement**

Reactive oxygen species levels were measured within one hour of collection with a chemiluminescence assay using luminol [(5-amino-2, 3-dihydro-1,4-phthalazinedione (Sigma—St. Louis, MO, USA)], which reacts with various intra- and extracellular ROS. Liquefied semen aliquots of 400 μl were used for ROS measurement. Chemiluminescence measurement was performed by an Automat 953 Plus Luminometer (AutoLumat Plus LB 953, Oakridge, TN). The findings were reported as Relative Light Units/s/10^6 sperm (Agarwal, Ahmad, & Sharma, 2015).

2.3 | **Somatic cell lyses**

The aim of this procedure was to separate the germ cells from somatic cells such as leucocytes, lymphocytes and epithelial cells. This is essential as the DNA material from other cell types may interfere with the results of sperm DNA methylation. For somatic cell lysis, an aliquot of liquefied semen containing less than 10×10^6/ml sperm was pipetted into test tubes and centrifuged at 1500 × *g* for 8 min to remove the seminal plasma. After washing this pellet with 1 ml of 1X PBS, each sample was treated with 12 ml of somatic cell lysis buffer (0.1% sodium dodecyl sulphate [SDS], 0.5% Triton X-100 in 200 ml of distilled water) on ice for 30 min, Ostermeier, Dix, Miller, Khatri, & Krawetz, 2002). Afterwards, the supernatant was removed by centrifugation at 300 × *g* for 15 min. Samples were microscopically examined for ensuring complete somatic cell lysis. If necessary, the procedure was repeated.

2.4 | **DNA isolation**

After elimination of somatic cells, the DNA of sperm samples was isolated with Zymo Research Quick-gDNA™ MiniPrep (Irvine, CA, USA) according to the manufacturer's instructions (Yegin, Gunes, & Buyukalpelli, 2013). In brief, specimens were first incubated in 400 μl genomic lysis buffer for 5–10 min at room temperature, followed by centrifugation and two washing steps. Finally, sperm DNA was eluted with elution buffer and both the DNA concentration and its purity were measured using a NanoDrop spectrophotometer [Thermo Scientific 2000c (Waltham, MA, USA)]. DNA samples were stored at −80°C until bisulphite modification.

2.5 | **Bisulphite modification and methylation-specific polymerase chain reaction (MSP)**

Bisulphite modification of extracted DNA samples was carried out using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA). Approximately 500 ng DNA/20 μl were used for bisulphite modification (Yegin et al., 2013). MSP was run for the promoter regions of both *MLH1* and *MSH2* genes using methylated and un-methylated primer pairs. The primer sequences used in the analysis of *MLH1* and *MSH2* genes are listed in Table 1.

TABLE 1 Corresponding primers sequences used for amplification and amplicon length

Gene	Forward primer	Reverse primer	Amplicon length (bp)
MLH1	Methylation-specific polymerase (MSP)		
	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	124
	USP		
	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	115
MSH2	MSP		
	TCGTGGTCGGACGTCGTTC	CAACGTCTCCTTCGACTACACCG	143
	USP		
	GTTGTTGTGGTTGGATGTTGTTT	CAACTACAACATCTCCTTCAACTACACCA	121

Methylation-specific polymerase was performed with ZymoTaq™ DNA Polymerase (Zymo Research, Irvine, CA, USA). The reaction was carried out in a final volume of 50 µl containing 2 µl of bisulphite-treated DNA, 0.25 mm of each dNTP (Zymo Research), 0.5 µm of each of the primers and 2 units of *Taq* polymerase (Zymo Research).

After initial denaturation at 95°C for 10 min, the subsequent steps of denaturation at 95°C for 30 s, annealing at 60°C [*MLH1* and *MSH2,* both for methylated (M) and unmethylated (U)] for 45 s and extension at 72°C for 60 s were repeated for 40 cycles (Yegin et al., 2013). Final extension was performed at 72°C for 7 min. Half of the PCR products were electrophoresed on a 2.5% agarose gel. Methylated and unmethylated products of *MLH1* were identified by 115 bp and 124 bp, respectively, while *MSH2* methylated and unmethylated products were 121 bp and 143 bp respectively. Universal methylated and nonmethylated human DNA standards (IVD) (Zymo Research) were used as a positive control for methylation and unmethylation, water was used as a negative control for PCR. 100-bp DNA Ladder (New England BioLabs, Ipswich, Massachusetts, USA) was used as a marker. The gel images of methylation status of the *MLH1* and *MSH2* promoters in sperm samples are shown in Figure 1.

The gene methylation status was indicated as methylated when amplification products were detected in the reactions with the primers M or both M and U. Unmethylation status was indicated when amplification products were detected in reaction with the primers U only.

2.6 | Statistical analysis

For statistical analysis, MedCalc Statistical Software, Version 17.2 (MedCalc Software bvba, Ostend, Belgium), was used. After testing for normal data distribution using the Kolmogorov–Smirnov test, the nonparametric Spearman Rank correlation and Mann–Whitney *U* test were applied as needed. In addition, receiver operating characteristic (ROC) curve analysis was performed to demonstrate sensitivity (true positive rate) and specificity (false positive rate). A p -value <.05 was considered significant.

3 | RESULTS

Sperm concentration, sperm motility, ejaculate volume and seminal ROS levels for the 10 oligozoospermic men and 29 normozoospermic donors are depicted in Table 2.

Sperm concentration, motility and ROS levels were significantly different between the groups of oligozoospermic patients and normozoospermic donors (Table 2). The percentage of *MLH1* promoter methylation in patients with oligozoospermia was significantly higher than in normozoospermic men (p = .0013) (Table 3). Overall, 40% (4/10) of oligozoospermic infertile men had hypermethylation in their sperm DNA while none of the normozoospermic controls had methylation in *MLH1* promoter (Table 3) (Figure 1a). Additionally, there was a significant positive association between ROS levels and *MLH1* gene methylation (r = .384; p = .0159).

The percentage of *MSH2* promoter methylation was higher in oligozoospermic men (40%; 4/10) than normozoospermic controls (17.2%; 5/29), (Figure 1b) but the differences were not statistically significant (p = .1102). There was no correlation between ROS levels and *MSH2* promoter methylation (r = .279; p = .0859).

Receiver operating characteristic curve analyses were performed in order to investigate the predictive power of the sperm *MLH1* and *MSH2* promoter methylation to distinguish between oligozoospermic and normozoospermic men. While the calculation for *MLH1* was significant (p = .0275) with an area under the curve (AUC) of 61.1%, a sensitivity of 22.2%, specificity of 100.0%, a positive predictive value of 100.0% and a negative predictive value of 60.0% (Figure 1a-b), for *MSH2*, the test was not significant (p = .1372) with an AUC of 60.3%.

While significant negative associations between *MLH1* (r = −.417; p = .0083) and *MSH2* (r = −.400; p = .0116) promoter methylation could be found for sperm concentration, no correlations between *MLH1* (r = −.275; p = .0899) and *MSH2* (r = −.148; p = .3681) promotor methylation and sperm motility were observed.

4 | DISCUSSION

Spermatogenesis is a complex and sensitive process of proliferation and differentiation of male germ cells involving replication, mitosis, meiosis and spermatogenesis (Chocu, Calvel, Rolland, & Pineau, 2012; Nussbaum, McInnes, Willard, & Hamosh, 2007). ROS, exogenous agents and abnormal sperm chromatin packaging result in poor sperm development and sperm nuclear DNA damage. Although the integrity of the sperm genome and stability of the male germ cell are generally protected by DNA repair mechanisms, recent studies have shown an association between idiopathic male infertility and aberrant DNA methylation of the whole genome or some genes in human spermatozoa (Gunes et al., 2016).

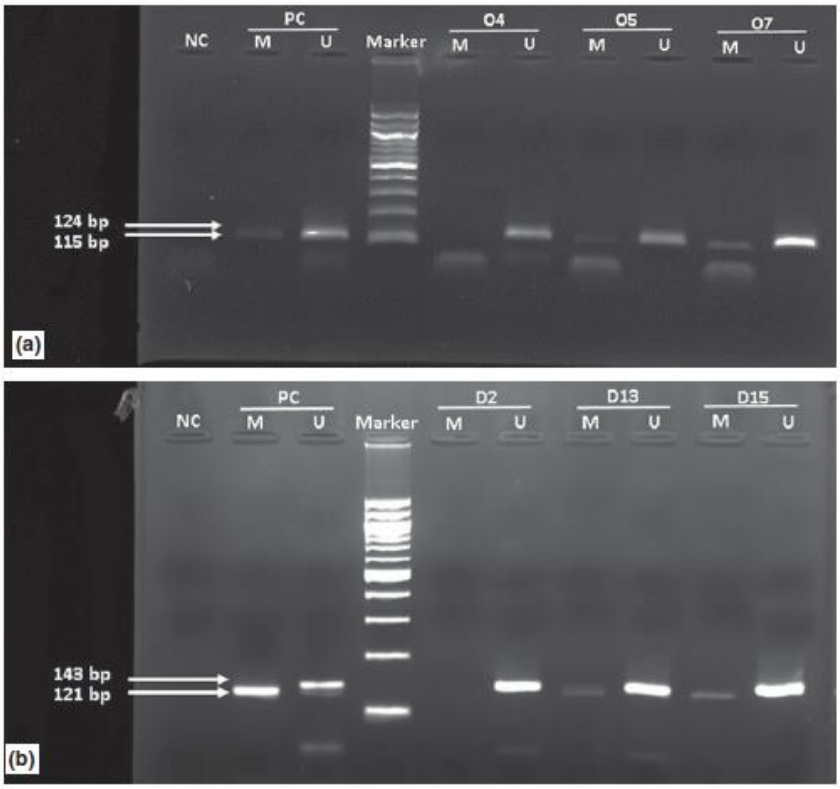

FIGURE 1a-b Methylation analysis of (a) *MLH1* and *MSH2* (b) promoters in semen samples of oligozoospermic infertile patients and control samples by methylation-specific polymerase. U indicates the presence of unmethylated genes; M indicates the presence of methylated genes. IVD was used as a positive control for both methylation and unmethylation, and ddH_2O was used as a negative control for PCR

TABLE 2 Semen parameters in oligozoospermic men and normozoospermic men

Parameters	Oligozoospermic (n = 10)	Normozoospermic (n = 29)	p
Volume (ml)			
Mean (±SD)	3.1 ± 0.4	2.8 ± 0.2	.6811
Range	1.4–7.0	1.2–5.8	
Concentration ($\times 10^6$/ml)			
Mean (±SD)	7.11 ± 1.2	53.19 ± 6.5	<.0001*
Range	0.84–13.0	20.0–151.2	
Motility (%)			
Mean (±SD)	37.18 ± 4.3	59.62 ± 2.2	.0002*
Range	8.0–59.0	33.0–59.0	
ROS (RLU/s/10^6 sperm)			
Mean (±SD)	1645.35 ± 694.33	104.19 ± 26.86	.0002*
Range	0.0–535.4	0.0–82,790.0	

$p < .05$ was considered significant by Mann–Whitney U test.
*Statistically significant.

TABLE 3 The methylation profile of *MLH1* and *MSH2* genes

	Oligozoospermic men (*n* = 10) (%)			Normozoospermic men (*n* = 29) (%)	*p* -value
	W	M	S		
MLH1	3 (30)	0	1 (10)	0	.0013*
MSH2	2 (20)	2 (20)	0	5 (17.2)	.1102

W, weak, M, moderate, S, severe.
$p < .05$ was considered significant by Mann–Whitney *U* test.
*Statistically significant.

In this study, we have analysed the methylation patterns of the *MLH1* and *MSH2* genes in sperm samples of oligozoospermic and normozoospermic men. Our results suggest a significant promoter methylation in the *MLH1* gene in oligozoospermic patients compared to normozoospermic controls. We also observed a significant positive association between elevated ROS levels and *MLH1* gene methylation, but not with *MSH2* promoter methylation. To the best of our knowledge, this is the first study indicating an association among promoter methylation of *MLH,* elevated levels ROS and oligozoospermia. *MLH1* is a DMMR protein involved in recombination between homologue chromosomes (Sun et al., 2007). Deficiency or absence of *MLH1* gene was reported to be associated with gametogenesis failure in humans due to meiotic arrest at pachytene level, thus resulting in reduced chiasma formation (Ferguson, Leung, Jiang, & Ma, 2009).

Infertile men with altered *MLH1* distribution were also shown to have unsynapsed autosomal meiotic chromosomes and increased sperm aneuploidy rates (Ferguson et al., 2009). Similarly, tagged polymorphisms in *MLH1* were shown to be associated with male infertility and sperm DNA damage (Ji et al., 2012). Recombination-induced double-strand breaks (DSB) and crossing over and then ligation of strands once again. Failure in ligation of these DNA strands may be deleterious during spermatogenesis and result in infertility (Cohen & Pollard, 2001; Marcon & Moens, 2005) .

Immunohistochemistry studies reveal genomic instability and defects in *MLH1* and *MSH2* in nonobstructive azoospermic men. The proteins are mislocalised or absent in both germ cells and somatic cells (Maduro et al., 2003). Infertile patients with high seminal ROS levels usually present with low sperm counts compared with fertile males (Agarwal, Mulgund, Sharma, & Sabanegh, 2014).

In addition to human studies, deletion of *MLH1* results in disrupted spermatogenesis, meiotic arrest and infertility in mice (Baker et al., 1996; Edelmann et al., 1999). In the mice, *MLH1* is located at meiotic crossing over sites and is involved in meiosis (Svetlanov & Cohen, 2004). Mukherjee and colleagues have reported that deletions of the *MLH1* gene result in microsatellite instability and infertility (Mukherjee, Ridgeway, & Lamb, 2010). *MLH1*-deficient and *MSH2*-knockout mice exhibit microsatellite instability and infertility

due to meiotic arrest at pachytene and disruption of normal chromosomal synapses (Baker et al., 1995; Mukherjee et al., 2010). *MSH2* is highly expressed in the mouse in spermatogonia and spermatocytes (Paul et al., 2007). In one study, an association between DMMR deficiency of *MSH2* gene in somatic cells and loss of some germ cells was reported (Ji et al., 2012). *MSH2*-knockout mouse models demonstrated elevated predisposition to ultraviolet (UV) radiation-induced skin cancer or tumorigenesis without abnormalities in spermatogenesis (Paul et al., 2007). Similarly, our results have demonstrated no association among oligozoospermia and *MSH2* promoter methylation in oligozoospermic men compared to normal controls.

Finally, it is important to note that the present study is limited by the small sample size as well as the nonquantitative nature of the detection of the methylation status. These results are preliminary and *part* of a *broader ongoing project* to assess the role of *MLH1* and *MSH2* genes in idiopathic male infertility and must be verified in larger cohort using other techniques.

Epigenetic alterations in spermatozoa can be associated with idiopathic oligozoospermia, abnormal sperm morphology and decreased progressive motility. The epigenetic profile of spermatozoa may also affect the health of offspring as epigenetic aberrations are heritable. Although the exact cause and effect relationship between epigenetics and male infertility have not been elucidated, further investigation of this area holds a significant potential and great promise for understanding the molecular mechanisms of infertility. An epigenetic approach during the male infertility investigation can be useful because unlike the genetic changes including mutations, DNA methylation and histone modifications are epigenetic changes, which are reversible (Enokida & Nakagawa, 2008). This reversibility makes epigenetic changes attractive candidates for infertility treatment.

In conclusion, our preliminary data indicate that the methylation pattern of *MLH1* gene may have a role in oligozoospermia. Large cohorts and well-defined phenotypes are required to reveal the possible role of the methylation pattern of *MSH2*. However, further studies are warranted to understand the molecular mechanisms involved in the development of oligozoospermia as male infertility factor.

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CONFLICT OF INTEREST
None of the authors declare competing financial interests.

AUTHORS' CONTRIBUTIONS
S. Gunes planned and conducted the experiment and wrote the article; A. Agarwal supervised, discussed, reviewed and edited the manuscript; R. Henkel analysed the results, reviewed and edited the manuscript; A. Metin Mahmutoglu participated in experiments; R. Sharma

involved in planning of the study and in its execution; S. C. Esteves reviewed and edited the manuscript; A. Aljowair participated in experiments; D. Emirzeoglu participated in experiments, A. Alkhani participated in experiments, L. Pelegrini participated in experiments; A. Joumah participated in experiments; E. Sabanegh reviewed the data and findings.

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