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rs1800734: A Risk Allele for Nonobstructive Azoospermia Related to Maturation Arrest

rs1800734: Matürasyon Arrestine Bağlı Nonobstrüktif Azoospermi İçin Bir Risk Aleli

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ABSTRACT

Background: Male infertility is increasingly being conceived as a biomarker of general male health status other than being a significant reproductive health issue. Nonobstructive azoospermia (NOA) patients have the worst health status impairment as well as highest risk of developing cancer. It's well known that cancer susceptibility gene, *MLH1* have roles in homologous recombination during meiosis. *Mlh1* mutant mice have azoospermia with meiotic arrest.

Aim: We aimed to identify risk alleles in *MLH1* gene in males with nonobstructive azoospermia related maturation arrest (MA) to investigate further whether this cancer susceptibility gene is also related to male infertility.

Patients and Methods: *MLH1* variants were genotyped in 42 patients with MA. The variants of interest then compared to GnomAD v.3.1.2 (non-cancer) male population database and to the repository exome data of a national genetic disease evaluation center (NGDEC).

Results: rs1800734 allele frequencies were significantly higher in the patient group compared to GnomAD (p<0.001) with odds ratio (OR):2.3 (1.5-3.5), and to the exome data of NGDEC (p=0.002) with OR:2 (1.3-3.1). Homozygous genotype for rs1800734 was significantly higher in the patient group compared to the GnomAD (p<0.001) with OR: 5 (2.24-11) and to the exome data (p=0.010) with OR: 3.3 (1.4-7.5).

Conclusion: In this study we found rs1800734 is a risk allele for NOA related to MA. It is known that rs1800734 is related to hypermethylation of *MLH1* promoter. As *MLH1* hypermethylation has been shown to be increased in oligozoospermic males, the same mechanism in carcinogenesis may also exist for azoospermia in the context of rs1800734.

Keywords: rs1800734, MLH1, nonobstructive azoospermia, maturation arrest, male infertility

ÖZET

Arka Plan: Erkek interfilitesi, önemli bir üreme sağlığı sorunu olmasının yanı sıra, genel erkek sağlık durumunun bir biyolojik belirteci olarak giderek daha fazla kabul görmektedir. Nonobstrüktif azoospermi (NOA) hastaları, en kötü sağlık durumunun ve kansere yakalanma riskinin en yüksek olduğu gruptur. Kanser duyarlılık geni olan *MLH1*'in, mayoz bölünme sırasında homolog rekombinasyonda rol oynadığı iyi bilinmektedir. *Mlh1* mutant farelerde, mayoz arresti ile birlikte azoospermi görülmüştür.

Amaç: Bu çalışmada *MLH1* geninin erkek infertilitesiyle de ilişkili olup olmadığını daha ayrıntılı olarak araştırmak amacıyla, nonobstrüktif azoospermiye bağlı matürasyon arresti (MA) olan erkeklerde *MLH1* genindeki risk alelerini saptamayı amaçladık.

Olgular ve Metodlar: MA olan 42 hastada *MLH1* varyantlarının genotipleri belirlendi. Önemi olan varyantlar daha sonra GnomAD v.3.1.2 (kanser dışı) erkek popülasyon veritabanı ve bir ulusal genetik hastalık değerlendirme merkezinin (UGHDM) ekzom veri deposu ile karşılaştırıldı.

Bulgular: rs1800734 alel frekansı, GnomAD'a (p<0,001) kıyasla hasta grubunda anlamlı olarak daha yüksek saptandı (odds oranı (OR): 2,3 (1,5-3,5)) ve UGHDM 'nin ekzom verilerine (p=0,002) kıyasla OR: 2 (1,3-3,1) idi. Rs1800734 için homozigot genotip, GnomAD (p<0,001) OR: 5 (2,24-11) ve ekzom verilerine (p=0,010) kıyasla OR: 3,3 (1,4-7,5) ile hasta grubunda anlamlı olarak daha yüksek saptandı.

Sonuç: Bu çalışmada, rs1800734'ün MA ile ilişkili NOA için bir risk aleli olduğu tespit edilmiştir. Rs1800734'ün *MLH1* promoter hipermetilasyonu ile ilişkili olduğu bilinmektedir. *MLH1* promoter hipermetilasyonunun oligozoospermili erkeklerde arttığı gösterilmiştir, bu nedenle rs1800734 açısından azoospermi için de karsinogenezde aynı mekanizma mevcut olabileceği düşünülmüştür.

Anahtar kelimeler: rs1800734, MLH1, Nonobstrüktif azoospermi, matürasyon arresti, erkek infertilitesi

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Introduction

Male infertility affects approximately 7% of men and nonobstructive azoospermia (NOA) is seen in 10-15% of infertile males [1]. Other than being a significant reproductive health issue, male infertility is increasingly being conceived as a biomarker of general male health status, since it has been associated with increased risk of cardiovascular and other chronic diseases, cancer development and increased mortality [2,3]. NOA patients have the worst health status impairment, highest risk of mortality as well as most substantial risk of developing cancer [3–5].

First suggestion of a possible link between *MLH1* as a cancer susceptibility gene and male infertility has been made by Nagirnaja et al. [6]. Pathogenic variants in *MLH1*, together with *MSH2*, constitutes 70–90% of a heritable cancer predisposition syndrome known as Lynch syndrome [7]. It is also known that *MLH1* is in the late recombination nodules of synaptonemal complex that is formed during recombination between homologues chromosome pairs synapse and recombine in meiosis [8]. Studies on mice revealed that *Mlh1* deficient spermatocytes arrest at first division of meiosis indicating the involvement of *Mlh1* in meiotic crossing over [9]. In another study by Edelmann et al. showed that *Mlh1* homozygote mutant mice had azoospermia and meiotic arrest in testicular histologic examination [10].

In humans, it has been reported that NOA patients have reduced MLH1 loci compared to patients with obstructive azoospermia (OA) [11]. Nudell et al. (2000) found increased microsatellite instability (MSI), which is a marker of *MLH1* and other mismatch repair proteins' deficiencies, in peripheral blood and testicular tissue samples of the patients with NOA in comparison to OA cases [12]. It is noteworthy that testicular tissue samples of these patients with NOA were compatible with meiotic arrest as in animal studies. Furthermore, Enguita-Marruedo et al. suggested that *MLH1* gene, among others, could be an interesting candidate to screen for variants in NOA patients with spermatogenic arrest [13].

To best of our knowledge, it has never been investigated in humans whether *MLH1* variants are associated with NOA specifically related to maturation arrest (MA). For these reasons, we aimed to identify risk alleles in ***MLH1*** gene in males with NOA who had MA in their testicular tissue samples.

Patients And Methods

Study population

239 patients with NOA who had been referred for testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) to a Reproductive Health Research Center between 2019-2020 included in the study. Detailed medical and family histories were obtained from each patient, including history of orchitis, testicular torsion, testicular trauma, testis tumors, cryptorchidism, mumps, and other urological diseases. All patients underwent thorough genital examination, and their testicular volume measurements were recorded by ultrasonography. Follicle stimulating hormone (FSH) (reference range 1.0–10.5 IU/mL) and total testosterone (reference range 2.0–12 ng/mL) levels were measured by chemiluminescent immunoassay (Beckman Coulter UniCel DxI 800 System, USA). Patients with history of orchitis, testicular torsion, testicular trauma, testis tumor, cryptorchidism, mumps, previous vasectomy, bilateral vas deferens agenesis, and other urological diseases, history of radiotherapy or chemotherapy, patients with abnormal karyotype results and Y microdeletions were excluded from the study. There were 42 NOA patients with maturation arrest (MA), 20 NOA patients with hypospermatogenesis (HS), 54 NOA patients with Sertoli Cell Only (SCO), 116 NOA patients with findings of atrophic testis (AT) and 7 NOA patients with normal spermatogenesis (NS) in their testicular histopathological examination.

The study proceeded with 42 NOA patients with MA to genotype *MLH1* variants. The variants of interest were then compared to GnomAD v.3.1.2 (non-cancer) male population database as well as to the repository exome data of a national genetic diseases evaluation center (NGDEC). GnomAD v.3.1.2 included 35,849 male individuals. The exome data consisted of 23,380 individuals of both sexes

on whom whole exome sequencing performed for various indications.

Acquisition of tissue samples

The TESE procedure was performed as previously described [14]. Briefly, a midline scrotal incision was made under sedation anesthesia supported by local anesthesia. The larger volume testicle was delivered first. The tunica albuqinea was opened with a transverse incision over an avascular area under microscopic magnification (x 8 power). Seminiferous tubules were observed with larger optic magnification (x 20 power) and more dilated opaque tubules were selected and excised. The samples were preserved in a sperm transport medium (G-IVF, Vitrolife, Gothenburg, Sweden). Randomized samples were taken from multiple areas with homogeneous tubule distribution. The collected samples were pre-examined by the embryologist in the operating room. In the meantime, the tubules were placed onto a petri dish and homogenized by shredding with the help of a scalpel and micro-scissors. The process continued until mature spermatozoa were found. If no spermatozoa were found, the same procedures were repeated in the contralateral testis. Afterwards, all the collected tissue suspensions were investigated in detail by centrifugation after enzymatic digestion with collagenase type IV (Invitrogen, cat # 17104-019) in the embryology laboratory. The procedure was considered positive if at least 1 motile spermatozoon was observed.

During the procedure, a testicular tissue sample was taken for biopsy and sent for histopathological examination in Bouin's solution. Biopsy results were interpreted in 4 groups according to histopathology findings: SCO (absence of germ cells in seminiferous tubules), MA (incomplete spermatogenesis), HS (all spermatogenesis steps containing spermatozoa, but marked decrease in germ cell number), NS (spermatogenesis intact) and AT (atrophic testis).

DNA isolation and sequencing of the *MLH1* gene

DNA isolation was carried out according to the manufacturer's protocol (PureLink™ Genomic DNA Mini Kit, K1820-01, Thermo Fischer). Polymerase chain reaction (PCR) was performed using DreamTaq Green PCR master mix (2X, Thermo Fischer) with the primers comprising the 5'UTR, 3'UTR and promoter regions as well as 19 exons and exon-intron junctions of the *MLH1* gene in Veriti™ thermal cycler. Next-generation sequencing was performed using Illumina MiSeq technology.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) for each variant was tested using the chi-squared (X^2) test and deviated variants were excluded from further analysis. *P* values, odds ratios (OR) and confidence intervals were calculated using SPSS 25.0 (IBM Corporation, Armonk, New York, United States) program. Variables were analyzed at 95% confidence level and ***p*** value less than 0.05 was considered significant.

Ethical approval and consent to participate

The study protocol was approved by an Ethics Committee (reference number: İ4-210-20) and performed according to the Declaration of Helsinki. All patients signed informed, written consent before participating in the study.

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Results

MLH1 variants in NOA patients with maturation arrest

There were twenty five variants detected of which only five of them were in Hardy-Weinberg equilibrium and only *MLH1*(NM_00249.4):c.-93G>A (rs1800734) reached significantly higher allele and genotype frequencies compared to both GnomAD and the repository exome data of NGDEC as shown in Table 1.

Minor allele frequencies of rs1800734 were significantly higher in NOA patients with MA (0,4167) compared to both GnomAD population database (0,2386) ($p<0.001$) and to the repository exome data of NGDEC (0,2623) ($p=0.002$). The odds ratios (OR) were 2.3 (1.5-3.5) and 2 (1.3-3.1), respectively.

We then examined whether homozygous or heterozygous genotypes for rs1800734 would pose an

Table 1. Distribution of *MLH1* allele frequencies

MLH1 Variant	Patients (I)	GnomAD (II)	NGDEC (III)	*p* Values	
				I vs II	I vs III
rs4234259	0.381	0.399	0.292	0.824	0.092
rs1799977	0.250	0.221	0.202	0.599	0.276
rs2286939	0.357	0.399	0.276	0.504	0.111
rs9876116	0.381	0.400	0.291	0.740	0.072
rs1800734	0.417	0.239	0.262	**<0.001**	**0.002**

NGDEC: national genetic diseases evaluation center

additional risk. Although heterozygous genotype frequencies were significantly higher in NOA patients with MA (GA/GG: 17/42, 40,5%) compared to the GnomAD (GA/GG: 12454/35849, 34.7%) and the NGDEC (GA/GG: 7733/23380, 33,1%) databases, the OR was not significant. Homozygous genotype frequencies, on the other hand, were significantly different between NOA patients with MA (AA/GG: 9/42, 21.4%) and both GnomAD (AA/GG: 2342/35849, 6.5%) and the repository exome data of NGDEC (AA/GG: 2265/2380, 9.7%), as shown in Table 2.

Discussion

In this study we found that rs1800734 risk allele results in an approximately 2-fold increased risk for NOA with MA, while homozygosity for rs1800734 constitutes 3-to-5-fold increased risk for NOA with MA. While Zhang et al. did not find any association between rs1800734 and male infertility, Zhao et al. found a statistically significant difference between severe oligozoospermia patients and controls but not with azoospermia [15,16]. These discrepancies may be the consequences of ethnic differences or may well be due to the lack of stratification of patients based on their testicular histopathological examination results.

Other than those studies, only human research that involved *MLH1* and male infertility is conducted by Gunes et al. [17]. Although their research had not involved investigation of any variants in *MLH1* gene, they found significantly higher *MLH1* promoter methylation in oligozoospermic patients compared to normozoospermic controls. Despite that there is no current data regarding the relationship between

Table 2. Comparison of rs1800734 genotype frequencies between NOA patient group and databases

	Patients (n=42)	**GnomAD (n=35849)**	**OR (95% CI)**	***p* Values**
rs1800734				
GA/GG	17 (40.5)	12454 (34.7)	1.802 (0.91-3.57)	**0.001**
AA/GG	9 (21.4)	2342 (6.5)	5.072 (2.24-11)	**<0.001**
	Patients (n=42)	**NGDEC (n=23380)**	**OR (95% CI)**	***p* Values**
rs1800734				
GA/GG	17 (40.5)	7733 (33.1)	1.839 (0.928-3.641)	**0.014**
AA/GG	9 (21.4)	2265 (9.7)	3.323 (1.467-7.530)	**0,010**

NGDEC: national genetic diseases evaluation center

rs1800734 and *MLH1* promoter methylation in infertility, there is abundance of evidence of this association in earlier cancer research in the literature.

Located in the core promoter region of *MLH1* gene, rs1800734, the transition of G allele to A -93 nucleotides 5' upstream of coding sequence has implications on methylation of CpG islands on the promoter of *MLH1* gene, as well as its expression and interactions with transcription factors as demonstrated in many studies in cancer research [18]. In the context of colorectal cancer, by Ma et al. indicated rs1800734 A allele changes methylation level of nine CpG sites with resulting promoter hypermethylation of *MLH1* [19]. They also showed that the A allele has significantly lower expression than those with G allele. They suggested that these results participated in the colorectal carcinogenesis among patients with microsatellite instability. The reduced expression of *MLH1* gene related to rs1800734 A allele has been supported by other studies as well [20,21]. In another study, the A/A genotype of rs1800734 was shown to be the most significant risk factor for increased methylation of the *MLH1* promoter region [22]. Rs1800734 related promoter hypermethylation of *MLH1* gene has been observed in other studies in the setting of cancer as well [23,24].

Promoter hypermethylation and resulting loss of expression might be related to lack or decreased binding of transcription factors to the critical regions and to the alleles residing in these critical regions of the gene. As an example, it has been shown that an important transcription factor TFAP4 has an allele-specific binding with higher affinity to G allele in the position of variant rs1800734, in which the binding is diminished when the A allele is present [25,26]. Furthermore, presence of A allele may create other binding motifs for different transcription factors. All these interactions or lack of them may change the promoter activity of *MLH1* gene [27].

These studies all confirm the association of rs1800734 with hypermethylation of *MLH1* promoter, reduced protein expression and increased microsatellite instability [28]. Considering the finding of *MLH1* hypermethylation in oligozoospermic males in the study by Gunes et al., the same suggested mechanism in carcinogenesis may also exist for azoospermia in the context of rs1800734 [17].

Although far from being a monoallelic cause of Lynch syndrome; as a risk allele, many studies revealed rs1800734 to confer increased risk for various cancers including colorectal cancer, endometrial cancer, glioblastoma, head and neck squamous cell carsinoma and gastric cancer [29–34]. The same might be true for the association of risk allele rs1800734 with NOA due to MA. This allele and *MLH1* variants in general, is far from being monoallelic cause of NOA. Rs1800734 is benign variant according to American College of Medical Genetics' variant classification; however, the increased risks rs1800374 confers for NOA with MA is substantial as a risk allele based on ClinGen risk allele definition [35,36]. In brief, rs1800734 is a low penetrance risk allele for NOA due to MA; however it is not a monogenic cause of NOA.

The main advantage of this modest research lies in the fact that it is the first study to suggest an association between a *MLH1* variant and maturation arrest in NOA patients. To best of our knowledge, there is no other study that has been conducted on NOA patients with detailed endophenotyping that includes testicular biopsy results and *MLH1* variants.

Due to selection of cases according to their testicular biopsy results, many patients were excluded from the study which resulted in a very small sample size that makes it a prominent disadvantage of this work. Additionally, we were unable to collect a select group of fertile controls; instead, allele and genotype frequencies detected in the patient group were compared to GnomAD population database and to the exome data of NGDEC. Although this may seem like a pitfall, it is expected these databases to include both fertile and infertile individuals; therefore, the significance of these differences in allele and genotype frequencies and resulting relative risks we found in this study might be even higher and more striking if there were a fertile control group. Nevertheless, due to the small sample size and the absence of data regarding population specific allele frequencies, direct conclu-

sions from this study cannot be made at this stage, emphasizing the need of further studies with a larger sample size and well-constructed control group.

This preliminary study has several implications for further studies. Confirmation of *MLH1* variants in larger sample sizes with matched controls with inclusion of testicular histopathology findings in cases would be valuable. Since this study did not include other testicular pathologies, comparison of *MLH1* variants between different testis biopsy results would be worthy to determine the exclusivity of *MLH1* variants to maturation arrest. Lastly, there is a large gap in male infertility literature whether rs1800734 would have the same effect on methylation status of *MLH1*, expression levels and an increase in MSI as in functional studies in cancer literature. Overall, this study adds a new risk allele for NOA related to meiotic arrest and points to future studies to fill the gaps in the literature in NOA and meiotic arrest.

Conclusion

This study suggests that rs1800734 is a risk allele for nonobstructive azoospermia related to maturation arrest in spermatogenesis. The finding of a risk allele for male infertility in a cancer susceptibility gene as *MLH1* may support the association of male infertility with suggested increased risk of malignancy in these patients.

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