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Prevalence of Lynch Syndrome in Middle Eastern Women with Mismatch Repair Deficient Ovarian Cancer

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Abstract

Objective: Microsatellite instability (MSI) is a hallmark of Lynch Syndrome (LS). This study aims to determine the prevalence of LS and the optimal diagnostic method in women from Middle Eastern ethnicity with newly diagnosed epithelial ovarian cancer (EOC).

Methods: Women newly diagnosed with primary EOC (n=424) were retrospectively assessed for deficient mismatch repair (dMMR) by immunohistochemistry (IHC). All cases suggestive of LS by IHC underwent germline mutation testing in MMR pathway and additional exome sequencing.

Results: 2.8% (12/424) of EOCs showed mismatch repair deficiency by IHC. MSH2/MSH6 protein loss was detected in five cases (41.7%), only MSH6 protein loss in four cases (33.3%) and MLH1/PMS2 protein loss in three cases (25.0%). None of the three cases of MLH1 loss showed MLH1 promoter hypermethylation. Two cases (16.7%) were confirmed to have LS. No double heterozygous somatic mutations were detected. Germline pathogenic variants in high penetrance OC susceptibility genes were seen in two cases: one with *BRCA2* and another with *ATM* germline mutation. In addition, we found five cases harboring variants of uncertain signifycance (VUS) in other hereditary genes.

Conclusions: This is the first comprehensive study in a Saudi Arabian cohort of EOC screened for LS, which revealed LS prevalence of 2.8%. Although LS was identified in only two of 12 dMMR EOCs, an additional seven cases showed either pathogenic mutations or VUS, accounting for 75% of dMMR cases. Hence, our findings expand the mutational spectrum of dMMR and LS-related genes, which is important to define the most effective strategy for identification, prevention and therapy for Middle Eastern EOC LS.

Keywords: Epithelial ovarian cancer, Mismatch repair, Lynch syndrome, Immunohistochemistry, Whole exome sequencing

Introduction

Ovarian Cancer (OC) is the most lethal gynecological malignancies [1, 2]. In Saudi Arabia, OC ranked as the seventh most common cancer affecting women [3]. More than 20% of OC have been related to hereditary causes [4]. The leading heritable cause which accounts for more than 60% of hereditary OC is due to germline mutation in *BRCA genes* [5, 6]. Another cause of hereditary OC risk has been attributed to Lynch Syndrome [7, 8]. Lynch Syndrome is an autosomal dominant hereditary condition caused by germline mutation in one of the DNA mismatch repair (*MMR) genes*

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(MLH1, MSH2, PMS2, and MSH6) [9, 10]. Lynch syndrome (LS) also can be caused by an EPCAM deletion which is also recommended to be analyzed for the diagnosis of LS [11]. DNA MMR genes are responsible for maintaining genomic stability [12]. Dysfunction of DNA MMR results in accumulation of genetic mutation during cell replication which could lead to dysregulations of many oncogenes or tumor suppressor genes [13, 14]. The molecular hallmark of deficient MMR (dMMR) is the alterations in the repetitive sequence number of microsatellites which is known as microsatellite instability (MSI) [15]. Identifying the MSI status by immunohistochemistry (IHC) or molecular testing is important screening tool for LS. Recently, determining the MSI status is gaining more clinical importance not only for LS screening but as predictive marker that can affect therapeutic decisions [16-18].

The cumulative lifetime risk of OC for women with LS ranges between 4 and 17% [19, 20]. Early identification of LS allows women to be enrolled in cancer surveillance programs and decide the best prevention strategy for the patients and for at risk relatives [21]. LS could differ in the degree of penetrance and age of cancer onset depending on the mutated genes [22]. For example, the risk of OC in LS caused by *MSH2* pathogenic variant showed the highest risk among the four MMR gene variant. While *PMS2* showed the lowest risk [20]. The incidence of lynch syndrome diagnosis associated with OC is not clear [23]. There are several studies that have looked for dMMR incidence in OC using IHC for MMR protein expression or DNA for MSI. However, results differ from one study to another. These differences could be contributed to the cohort size, tumor histological types and ethnicity [24-27]. However, few studies have evaluated germline LS in OC. A recent study by Hodan and colleagues [28] assessed 308 OC, Fallopian and Peritoneal Cancer for dMMR/MSI and found 5.2% with dMMR, with almost all tumor being endometrioid. They subjected all the 12 patients with dMMR suggestive of LS to germline testing as well as multigene panel testing and suggested that germline testing for LS in addition to *BRCA1/2* for all women with OC would approach 100% sensitively for identifying LS.

Although tumor screening for MSI by IHC is the preferred approach to detect LS patients in comparison to the gold standard molecular technique for identifying germline variant in LS, this approach is still not common in Middle Eastern countries. Additionally, germline variant testing remains largely unavailable in the public health system. Therefore, the prevalence of LS among OC patients in Saudi population is still unclear. To clarify the prevalence of LS in Saudi women with OC, we conducted a retrospective study of 424 epithelial ovarian cancer (EOC) patients who underwent surgical treatment in a single institute in Saudi Arabia. The MMR deficiency was assessed in tumor samples, and next generation sequencing (NGS) was performed on patients' tumor and normal tissue samples. Our analysis provides comprehensive information about LS in EOC from this ethnicity.

Materials and Methods

Sample selection

Archival samples from 424 EOC patients diagnosed between 1989 to 2017 at King Faisal Specialist Hospital and Research Center (Riyadh, Saudi Arabia) were included in the study. Detailed clinico-pathological data were noted from case records and have been summarized in Table 1. Recurrencefree survival was computed from date of surgery for patients who underwent primary surgery to date of disease progression or recurrence (local, regional or distant). The median follow-up time was 20 months (range, 2–321 months).

	n (%)
Age	
Median	50.3
Range	17.0 – 90.0
Histopathology	
High grade Serous	234 (55.2)
Low grade Serous	73 (17.2)
Mucinous	64 (15.1)
Endometrioid	35 (8.2)
Clear cell	10 (2.4)
Undifferentiated	8 (1.9)
Histological Grade	
Grade 1	86 (20.3)
Grade 2	140 (33.0)
Grade 3	176 (41.5)
Unknown	22 (5.2)
pT	
T1	88 (20.8)
T2	37 (8.7)
T3	293 (69.1)
Unknown	6 (1.4)
pN	
N0	383 (90.3)
N1	35 (8.3)
Unknown	6 (1.4)
pM	
M0	346 (81.6)
M1	72 (17.0)
Unknown	6 (1.4)
Stage	
I	88 (20.8)
II	24 (5.6)
III	234 (55.2)
IV	72 (17.0)
Unknown	6 (1.4)
dMMR (IHC)	
Yes	12 (2.8)
No	412 (97.2)

Table 1: Clinicopathological variables for the patient cohort (n=424).

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Tumors were classified according to WHO Classification of female genital tumors (2020). International Federation of Gynecology and Obstetrics (FIGO) system was used for staging and grading of tumors. All samples were obtained from patients with approval from Institutional Review Board of the hospital. For the study, since only retrospective patient data and archived paraffin tissue blocks were used, a waiver of consent was obtained from Research Advisory Council (RAC) under project RAC# 2190 015. Our study conforms to the provisions of the Declaration of Helsinki.

Tissue microarray and immunohistochemical evaluation

All samples were analyzed in a tissue microarray (TMA) format. TMA construction was performed as described earlier [29]. Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representtative tumor regions of each donor tissue block and brought into recipient paraffin block using a modified semiautomatic robotic precision instrument (Beecher Instruments, Woodland, WI). Two cores of EOC were arrayed from each case. Immunohistochemical staining of MMR proteins (MLH1, MSH2, MSH6 and PMS2) were performed manually. The primary antibodies used and their dilutions are shown in Table 2. Tumor was classified as dMMR if any of the four proteins showed loss of staining in cancer with concurrent positive staining in the nuclei of normal epithelial cells. Otherwise, they were classified as proficient MMR (pMMR). IHC scoring was done by two pathologists, blinded to the clinico-pathological characteristics. Discordant scores were reviewed together to achieve agreement.

DNA extraction

DNA samples were extracted from formalin-fixed and paraffin-embedded tumor and non-tumor tissues utilizing Gentra DNA Isolation Kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocols as elaborated in the previous study [30]. The non-tumor tissues were selected from normal tissues adjacent to the tumor tissue or from normal tissues from other organ sites operated for an unrelated disease. The tumor and non-tumor tissues were confirmed by histopathological examination.

Next-generation sequencing

Whole exome sequencing (WES) was performed on 12 ovarian cancer samples using Illumina Novaseq 6000. Pre and post-alignment quality metrics were performed using FastQC (http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). High quality reads in fastq format were aligned to the reference human genome (GRCh37/ hg19) using burrows-wheeler aligner (BWA) [31]. Aligned BAM files, PCR duplicates and local realignment was carried out using Picard-tools and genome analysis toolkit (GATK) [32].

Variant calling

The variant calling was carried out using GATK, and called variants were annotated using ANNOVAR [33]. The variants were annotated with dbSNP, 1000 Genomes, ESP6500, Exome Aggregation Consortium (ExAC), Clinvar and other genome databases. Pathogenicity of the variants was checked according to the recommended guidelines by the American College of Medical Genetics and Genomics and the Association of Molecular Pathology (ACMG/AMP) [34] by which variants were classified into pathogenic (PV), likely pathogenic (LPV) and variants of uncertain significance (VUS) groups.

Validation of variants utilizing PCR, sanger and capture sequencing

To validate the variants identified by WES sequencing technology, Primer 3 software was used to design the primers for each mutation (available upon request). PCR was performed in a total volume of 25 µl with 20 ng of genomic DNA, 2.5 µl 10 x Taq buffer, 2.3 mM dNTPs, 1 unit Taq polymerase and 0.2 µM each primer and de-ionized water. The efficiency and quality of the amplified PCR products was confirmed by loading them on a 2% agarose gel. For Sanger sequencing, the PCR products were subsequently subjected to direct sequencing with BigDye terminator V 3.1 cycle sequencing reagents and analyzed on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Reference sequences were downloaded from NCBI GenBank. Sequencing traces were analyzed with the Mutation Surveyor v4.04 (Soft Genetics, LLC, State College, PA). Amplicons for the variants that failed to amplify by PCR due to sample quality, validation was performed by Capture sequencing utilizing Illumina platform with the custom designed panel as described previously [35]. All the quality metrics were applied as described above.

MLPA analysis for EPCAM deletion

For the cases showing MSH2 protein loss by IHC,

Antibody	Clone	Source	Antigen retrieval	Visualization system	Dilution
MSH2	FE11	Oncogene/ CalBiochem	Dako retrieval solution (pH 9)	Dako EnVision+	1:100 overnight
MSH6	44	BD Transduction Laboratories	Dako retrieval solution (pH 9)	Dako EnVision+	1:100 overnight
MLH1	G168-15	BD Pharmingen	Dako retrieval solution (pH 9)	Dako EnVision+	1:50 overnight
PMS2	C-20	Santa Cruz Biotechnology	Dako retrieval solution (pH 9)	Dako EnVision+	1:100 overnight

Table 2: Antibodies used for the mismatch repair immunohistochemistry assay.

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Multiplex Ligation-dependent Probe Amplification (MLPA) assay (Holland, Amsterdam, The Netherlands) was used to investigate the *EPCAM* gene deletion, according to the manufacturer's recommendation. The MLPA probes set used was SALSA MLPA ME011. Briefly, the DNA was denatured and hybridization was carried out overnight with probes. The hybridized probes were then ligated and proceeded for a PCR reaction. Ligation step increases the specificity of the PCR reaction. The products were separated by capillary electrophoresis on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) and resulting traces were analyzed with Coffalyser (MRC-Holland, Amsterdam, The Netherlands).

Microsatellite instability analyses

Microsatellite instability (MSI) analyses was performed as described earlier [36]. A panel of 5 pairs of microsatellite primers, comprising mononucleotide or dinucleotide microsatellites (BAT24, BAT26, DS123, D5S346, and D17S240), was used to determine tumor MSI status. Multiplex PCR was performed in a total volume of 24 mL using 50 ng of genomic DNA, 2.5 mL of 10X Taq buffer, 1.5 mL of MgCl2 (24 mM), 10 pmol of fluorescent-labeled primers, 0.05 mL of dNTP (10 mM), and 0.2 mL of Taq polymerase (1UmL-1; all reagents were from Qiagen Inc). Fragment analysis was performed using an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The samples in which the novel alleles or loss of allele were found at one of these loci and 2 of those 5 loci were classified as MSI-L and MSI-H, respectively, whereas samples without novel alleles at any of those loci were classified as MSS.

Bisulfite modification of DNA and real-time PCR (MethyLight) for quantitative DNA methylation analysis

Bisulfite modification was performed as described previously [37]. Real-time PCR for the determination of MLH1 Methylation status was performed as described previously [37]. We used an ABI 7900HT Fast Real Time Analysis System (Applied Biosystems) for quantitative real-time PCR. A set of forward and reverse primers and probe were used to amplify promoter region of *MLH1* gene and *COL2A1* (the collagen 2A1 gene) to normalize for the amount of input bisulfite-converted DNA. Primers and probes were previously validated and published [38, 39]. The percentage of methylated reference (PMR) as described [40] was calculated by dividing the MLH1:COL2A1 ratio of a sample by the MLH1:COL2A1 ratio of CpG methyltransferase treated human genomic DNA (assuming it was fully methylated) and multiplying it by 100. A PMR cutoff of 4 was established to distinguish methylation positivity (PMR >4) from methylation negativity (PMR ≤4). The PMR cutoff of 4 as previously described [41], was used for *MLH1*.

Results

Patient characteristics

Median age of the study population was 50.3 years (range: 17 – 90 years). Majority of the cases were high grade serous carcinoma (55.2%), followed by low grade serous (17.2%) and mucinous (15.1%) carcinomas. The tumors were predominantly grade 2 or 3 (74.5%) and stage III (55.2%). 8.3% (35/424) had lymph node metastasis and 17.0% (72/424) had distant metastasis at the time of diagnosis (Table 1).

Incidence of deficient mismatch repair (dMMR) by immunohistochemistry and their clinico-pathological characteristics

The incidence of dMMR in our cohort was 2.8% (12/424). For the dMMR group, mean age at diagnosis was 42 years. 33.3% (4/12) of the women were < 35 years at diagnosis. Majority of the women (75.0%; 9/12) presented with lower grade tumors (grades 1 and 2), whereas 41.7% (5/12) presented with lower stages (stage I and II). The histopathological types of EOC were serous (41.7%; 5/12), mucinous (50.0%; 6/12) and endometrioid (8.3%; 1/12) (Table 3). MSH2/MSH6 protein loss was detected in five cases (41.7%) (95% CI: 19.3% - 68.0%). MSH6 protein loss was detected in four cases (33.3%) (95% CI: 13.8% - 60.9%). MLH1/ PMS2 protein loss was detected in three causes (25.0%) (95% CI: 8.9% - 53.2%).

Analysis of MMR gene variants, *MLH1* promotor methylation and *EPCAM* deletion

In our cohort of 12 dMMR cases, MMR gene germline pathologic variants were observed in two cases, one in *MSH2* (c.C1147T:p.R383X) and other one in *MSH6* (c.3266dupT:p.E1090fs), representing 16.7% of all dMMR cases. The *MSH2* mutant case had a grade 1 endometrioid tumor at stage I and was also observed to have protein loss in MSH2/MSH6 by IHC and MSI-H status. The other case with *MSH6* frameshift mutation had grade 3 serous tumor at stage I and IHC protein loss in MSH6 protein and was identified to have MSI-H status. Furthermore, MMR gene single or double somatic pathologic or likely pathologic variants were not detected in all dMMR cases. Interestingly, two germline pathogenic variants in the hereditary cancer related genes other than MMR were observed in two cases, accounting for 16.7% of all MMR deficient cases. One case with MLH1/PMS2 protein loss was detected to harbor a *BRCA2* germline pathologic variant c.4176insG:p. A1393fs. This case had a grade 3 serous tumor at stage III with MSI-L. The other case with MSH2 and MSH6 protein loss was identified to carry *ATM* germline pathologic variant c.3601delT:p.F1201fs. This case had grade I mucinous tumor at stage I. However, microsatellite instability status was not available for this case due to the failure of PCR reactions.

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S.no	ID	Age	Histo-pathologic subtype	Stage	Grade	MMR IHC protein loss	MMR mutation (germline)	Other hereditary germline mutation	MSI PCR
1	OVA-275	47	Serous	Stage III	Grade 2	MSH2, MSH6		*BMPR1A (VUS)* c.G1243A:p.E415K	MSS
2	OVA-048	48	Serous	Stage III	Grade 3	MLH1, PMS2		*BRCA2 (PV)* c.4176insG:p.A1393fs	MSI-L
3	OVA-018	51	Endometrioid	Stage I	Grade 1	MSH2, MSH6	*MSH2* c.C1147T:p.R383X		MSI-H
4	OVA-167	38	Serous	Stage III	Grade 2	MSH6		*FANCM (VUS)* c.G1518A:p.M506I	MSS
5	OVA323	21	Mucinous	Stage I	Grade 1	MSH2, MSH6		*ATM (PV)* c.3601delT:p.F1201fs	
6	OVA384	57	Mucinous	Stage III	Grade 3	MSH2, MSH6			MSS
7	OVA400	58	Mucinous	Stage III	Grade 1	MLH1, PMS2		*BUB1B (VUS)* c.G826C:p.D276H	MSI-H
8	OVA-530	27	Mucinous	Stage I	Grade 1	MSH2, MSH6			MSI-L
9	OVA-557	28	Serous	Stage III	Grade 2	MLH1, PMS2		*FANCA (VUS)* c.C2140G:p.R714G *FLCN (VUS)* c.G170A:p.R57Q	MSS
10	OVA-577	44	Serous	Stage I	Grade 3	MSH6	*MSH6* c.3266dupT:p.E1090fs		MSI-H
11	OVA-579	28	Mucinous	Stage I	Grade 1	MSH6			MSS
12	OVA-642	60	Mucinous	Stage IV	Grade 2	MSH6		*BRIP1 (VUS)* c.A1555G:p.I519V	MSS

dMMR – deficient mismatch repair; EOC – epithelial ovarian cancer; IHC – Immunohistochemistry; MSI – microsatellite instability; PCR – Polymerase chain reaction; PV – Pathogenic variant; VUS – Variant of uncertain significance

Table 3: Clinico-pathological and mutation data for dMMR EOC cases (n = 12).

Moreover, six variants of uncertain significance (VUS) in the hereditary cancer related genes other than MMR genes were observed in five cases, including *BMPR1A* c.1243G>GA; p.E415K, *BRIP1* c.1555A>AG; p.I519V, *BUB1B* c.826G>GC; p.D276H, *FANCA* c.2140C>CG; p.R714G, *FANCM* c.1518G>GA; p.M506I, *FLCN* c.170G>A; p.R57Q. The case with VUS in *BUB1B* gene had MSI-H tumor and loss of MLH1 and PMS2 protein expression while other four cases had MSS tumor. In addition, five cases that were positive for MSH2 protein loss by IHC were tested for germline *EPCAM* deletions by MLPA and no *EPCAM* deletions were observed in any of these dMMR cases. Furthermore, *MLH1* MethlyLight analysis was performed, and no *MLH1* promotor methylation was detected in all MLH1 protein loss cases.

Microsatellite analysis

In the cohort of 12 cases with MMR deficiency detected by IHC, there were three MSI-H cases (27.3%), 2 MSI-L cases (18.2%), and 6 MSS cases (54.5%). One was determined to be non-interpretable due to failure of PCR reaction. Of the three MSI-H cases, two cases were identified to carry MMR germline pathogenic variants. Among two cases with MSI-L, one case was detected to have *BRCA2* germline pathogenic variant. Only one germline pathogenic variant in *ATM* gene was observed in one MSS case.

Discussion

It is widely accepted that restricting LS testing according to clinical parameter is not entirely accurate and that tumor-based testing is a better way of triaging woman for germline analysis [42, 43]. Understanding genetic differences in each population is important to implement successful molecular tests in the ethnic population being served. Assessment using genetic analysis is very challenging in Middle Eastern countries and is limited in public health sectors. We have successfully performed universal screening of 424 unrelated EOC cases of which 12 tumors (2.8%) were dMMR based on IHC results. Since none of the MSI tumor were found to be sporadic due to *MLH1* promoter methylation, all the 12 cases were selected for germline variant analysis. Exome sequencing was successfully performed in all the 12 LS suspected EOC patients. Only two patients (16.7%) were found to have germline MMR PV/LPV confirming LS. One of the cases with PV/LPV in *MSH2* was endometrioid EOC, while the other confirmed LS carrying PV/LPV in MSH6 was high grade serous EOC.

The results of this study show lower prevalence of confirmed LS in OC than a recent study where more than 65% of patient with dMMR OC were confirmed to have LS [28]. Also the results of this study are different from another

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recent study where LS could not be identified in all MSI-H OC patients [27]. These conflicting results can be attributed to sample size, histological types tested and ethnicity. Whether our study could reflect the true prevalence of LS among OC from this ethnicity is not clear, since there are no previous reports that assessed MMR status and germline mutations among women diagnosed with OC in Saudi Arabia. Family history, Amsterdam criteria, and Bethesda guideline have been used clinically to screen for LS genetic testing despite their low sensitivity to detect LS [9, 44]. In our cohort, none of the dMMR patient and none of the two patients carrying PV/LPV in LS gene met these criteria or had a family history of cancer. This may have significant impact on practice guidelines for LS in Middle Eastern population. Perhaps the use of tumor screening approach to select OC patients for LS testing is a more effective method than clinical history.

Using next-generation sequencing (NGS) for all the 12 EOC in this cohort, two of the patients were found to have PV in well-known high penetrance susceptibility genes. One patient was identified to have *BRCA2* PV with absent staining in MLH1/PMS2 proteins and another patient was found to have *ATM* PV with absent staining in MSH2/MSH6. Interestingly, both patients did not have family history of cancer. The identified *BRCA2* and *ATM* mutations may explain the staining pattern in these OC patients and may contribute to the MSI status. Furthermore, we identified six VUS in five of 12 patients tested for LS (41.7%). The functional impact of these genes and their contribution to the MMR pathway is unclear and further studies are needed to understand this challenging finding. Previous studies have also shown that somatic inactivation of both MMR gene alleles (double somatic or biallelic somatic) can be observed in >50% of suspected LS tumors [45-47]. In our cohort, none of the 12 patients with dMMR had double somatic mutations (including loss of heterozygosity) which could lead to biallelic MMR gene activation.

This study has several limitations. First, it is a single center study. Second, we did not conduct MSI analysis for all cases which might limit the comparison between MMR IHC and MSI status by molecular testing. In conclusion, this is the first comprehensive study in Saudi Arabian cohort of EOC screened for LS, which revealed LS prevalence of 2.8%. Interestingly, none of the Lynch suspected tumors could be clearly diagnosed as sporadic EOC due to absence of *MLH1* methylation and double somatic mutations. Although LS was identified in only two of the 12 dMMR EOCs, an additional seven cases showed either pathogenic mutations or VUS, accounting for 75% of dMMR cases. Our findings expand the mutational spectrum of dMMR and LS-related genes and are important to define the most cost-effective strategy for LS identification, prevention and therapy in Middle Eastern EOC.

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Conflict of Interest Statement

Authors declare no Conflict of Interests for this article.

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