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Original Research Article

Microsatellite instability in non-endometrioid ovarian epithelial tumors: a study of 400 cases comparing immunohistochemistry, PCR, and NGS based testing with mutation status of MMR genes

Nikola Hájková [1], Michaela Kendall Bártů [1], David Cibula [2], Jana Drozenová [3], Pavel Fabian [4], Oluwole Fadare [5], Filip Frühauf [2], Jitka Hausnerová [6], Jan Hojný [1], Eva Krkavcová [1], Jan Laco [7], Sigurd F. Lax [8,9], Radoslav Matěj [1,3,10], Gábor Méhes [11], Romana Michálková [1], Kristýna Němejcová [1], Naveena Singh [12], Simona Stolnicu [13], Marián Švajdler [14], Tomáš Zima [15], Wilson Glenn McCluggage [16], Ivana Stružinská [1], Pavel Dundr [1,*]

[1] Department of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[2] Department of Obstetrics and Gynecology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[3] Department of Pathology, Charles University, 3rd Faculty of Medicine, University Hospital Kralovske Vinohrady, Prague, Czech Republic
[4] Department of Oncological Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[5] Department of Pathology, University of California San Diego, San Diego, California
[6] Department of Pathology, University Hospital Brno and Medical Faculty, Masaryk University, Brno, Czech Republic
[7] The Fingerland Department of Pathology, Charles University, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic
[8] Department of Pathology, General Hospital Graz II, Graz, Austria
[9] Johannes Kepler University Linz, Austria
[10] Department of Pathology and Molecular Medicine, Third Faculty of Medicine, Charles University, Thomayer University Hospital, Prague, Czech Republic
[11] Department of Pathology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary
[12] Department of Cellular Pathology, Barts Health NHS Trust, and Blizard Institute of Core Pathology, Queen Mary University of London, London, United Kingdom
[13] Department of Pathology, George E. Palade University of Medicine, Pharmacy, Sciences and Technology of Targu Mures, Romania
[14] Šikl's Department of Pathology, The Faculty of Medicine and Faculty Hospital in Pilsen, Charles University, Pilsen, Czech Republic
[15] Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[16] Department of Pathology, Belfast Health and Social Care Trust, Belfast, United Kingdom

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ABSTRACT

Testing of microsatellite instability is not only used as a triage for possible Lynch syndrome, but also to predict immunotherapy treatment response. The aim of this study was to assess the frequency of mismatch repair deficiency (MMR-D)/microsatellite instability (MSI) in 400 cases of non-endometrioid ovarian tumors (high-grade serous, low-grade serous, mucinous and clear cell), to compare different methodological approaches of testing, and to assess the optimal approach for next generation sequencing (NGS) MSI testing. For all tumors, we evaluated immunohistochemical (IHC) expression of MMR proteins and assessed microsatellite markers by PCR-based method. Except for high-grade serous carcinoma, we correlated the findings of IHC and PCR with NGS-based MSI testing. We compared the results with somatic and germline mutation in MMR genes. Among the whole cohort, seven MMR-D cases, all clear cell carcinomas (CCC), were found. On PCR analysis, 6 cases were MSI-high and one was MSS. In all cases, mutation of an MMR gene was found; in 2 cases, the mutation was germline (Lynch syndrome). An additional 5 cases with a mutation in MMR gene(s) with MSS status and without MMR-D were identified. We further utilized sequence capture NGS for MSI testing. Employing 53 microsatellite loci provided high

Abbreviations: CAP, College of American Pathologists; CCC, clear cell carcinoma; ESMO, European Society for Medical Oncology; FFPE, formalin-fixed and paraffin-embedded; HGSC, high-grade serous carcinoma; IHC, immunohistochemical; LGSC, low-grade serous carcinoma; LS, Lynch syndrome; MBT, mucinous borderline tumor; MC, mucinous carcinoma; MMR, mismatch repair; MMR-D, mismatch repair deficiency; MMR-P, proficient mismatch repair; mSBT, micropapillary subtype of serous borderline tumor; MSI, microsatellite instability; MSI-H, high microsatellite instability; mut/Mb, mutations per megabase; NGS, next generation sequencing; PCR, polymerase chain reaction; PCR-FA, polymerase chain reaction and fragment analysis; TCGA, The Cancer Genome Atlas; TMAs, tissue microarrays; TMB, tumor mutation burden

* Reprint request: Pavel Dundr, Department of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Studničkova 2, 12800 Prague 2, Czech Republic.
E-mail address: pavel.dundr@vfn.cz (P. Dundr).

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sensitivity and specificity. Our study shows that MSI occurs in 7% of CCC while it is rare or absent in other nonendometrioid ovarian neoplasms. Lynch syndrome was present in 2% of patients with CCC. However, some cases with MSH6 mutation can evade all testing methods, including IHC, PCR, and NGS-MSI.

At A Glance Commentary

Hájková N, et al.

Background

The significance of microsatellite instability testing in ovarian tumors is increasing. This testing is not only used as a triage for possible Lynch syndrome, but also to predict immunotherapy treatment response. However, the optimal screening strategy to identify patients with ovarian carcinomas associated with Lynch syndrome or to detect microsatellite instable ovarian tumors has not yet been determined.

Translational Significance

Precision assessment of the frequency of mismatch repair deficiency and/or microsatellite instability in non-endometrioid ovarian tumors. Comparison of different methodological approaches of testing and assessing of the optimal approach for next generation sequencing (NGS).

Introduction

Testing of colorectal and endometrial carcinomas for mismatch repair (MMR) protein expression by immunohistochemistry (IHC) or for microsatellite instability (MSI) by PCR is a well-established screening method for Lynch syndrome (LS).[1-5] Some ovarian carcinomas are also associated with LS but the optimal screening strategy to identify LS in ovarian cancer patients has not been determined, yet.[2,6] Currently, the significance of MSI testing is increasing in a broad spectrum of tumors. MMR deficiency (MMR-D) and/or presence of high MSI (MSI-H) are predictors of a favorable response to immune checkpoint inhibitor therapy in solid tumors.[7,8] In parallel with predictive testing in the broad spectrum of solid tumors, the knowledge about noncanonical neoplasms which can be associated with LS is increasing.[9]

Methods used for MSI testing have been mostly validated for screening purposes in colorectal and endometrial carcinomas. However, due to technical or biological reasons there are challenges in MSI testing even in colorectal and endometrial cancer. As such, immunohistochemical testing of the mismatch repair machinery may give different results for a given germline or somatic mismatch repair gene missense mutation.[10,11] However, in other tumors the value of different approaches is not clear. The 3 main methods for MSI testing are immunohistochemistry (IHC), PCR based approaches and NGS testing. According to the European Society for Medical Oncology (ESMO) recommendation on MSI testing for immunotherapy in cancer, the preferable method of testing in the first line is IHC with 4 antibodies (MLH1, PMS2, MSH2, and MSH6).[12] PCR based approaches are for second line testing, in cases in which IHC is not evaluable or the results are inconclusive. However, these recommendations are for tumors belonging to the spectrum of cancer well-known to be associated with LS, including colorectal, endometrial, small intestine, urothelial, central nervous system and sebaceous gland neoplasms. For other tumor types, there are insufficient data to inform a recommendation. The ESMO comment on NGS MSI testing is that it has the potential to become the method of choice for all tumor types.

In contrast, a recent College of American Pathologists (CAP) guideline for testing for possible immunotherapy with immune checkpoint inhibitors, considers IHC and PCR as equal and states that one of these methods should be used for colorectal, small bowel and gastroesophageal carcinoma.[8] This guideline states that for endometrial carcinoma, IHC is favored over PCR testing while for other tumor types, the optimal method of testing has not been established, so far. NGS may be used for colorectal cancer, but a validated MSI NGS assay is needed. For small bowel, gastroesophageal and endometrial carcinoma MMR-IHC, MSI-PCR and MMR-IHC, respectively, should be preferred over NGS. NGS is currently not the method of choice for primary isolated MSI testing. However, MSI could be evaluated by NGS panel testing together with the assessment of the mutation profile of the tumor and tumor mutation burden (TMB). However, the set of microsatellite loci analyzed by NGS have been established for colorectal and endometrial carcinomas and can differ in other tumors. Therefore, the optimal NGS approach covering all solid tumors has not been assessed, yet. Despite the recommendation for IHC testing using all 4 antibodies, first-line testing for only 2 MMR proteins (PMS2 and MSH6) can be used, if required completed by second-line testing of MLH1 and MSH2.[13]

A recently developed rapid digital PCR technique for microsatellite instability has been successfully used for colorectal, endometrial and gastric carcinomas and also a small number of ovarian carcinomas without histological characterization. This novel technique is easy to handle and fast and turned out comparable to other PCR based techniques and immunohistochemistry.[14-18]

According to the literature, MSI occurs in approximately 13% of ovarian endometrioid carcinomas (EC).[2,19-21] However, the published literature concerning MMR-D/MSI-H frequency in non-endometrioid ovarian tumors is equivocal.[4,22-25] The aim of our study was to assess the frequency of MMR-D/MSI-H in a broad spectrum of non-endometrioid ovarian tumors, to compare different methodological approaches for MSI testing, and to assess the optimal approach for NGS MSI testing using 3 different sets of microsatellite markers. Our sample set consisted of 400 primary ovarian epithelial tumors with serous, mucinous and clear cell morphology. For all tumors, we evaluated IHC expression of 4 MMR proteins and assessed the stability of microsatellite markers by the PCR-based method of 5 mononucleotides. Moreover, for all tumors except high-grade serous carcinoma (HGSC), we correlated the findings of IHC and PCR with NGS-based MSI testing. In all tumors in which NGS was performed, the results were also compared with mutation status in MMR genes. For tumors showing MMR-D/MSI-H status, non-neoplastic tissue was tested by NGS parallel to tumor tissue to exclude the possibility of LS.

Materials and methods

Samples

The archives of participating pathology departments were searched for cases diagnosed as low-grade serous carcinoma (LGSC), micropapillary subtype of serous borderline tumor (mSBT), clear cell carcinoma (CCC), mucinous carcinoma (MC) and mucinous borderline tumor (MBT). The sample set represents part of the sample set used in our previous studies.[26-28] About 296 selected samples were eligible for IHC, PCR, and NGS testing. These cases included 100 CCC, 75 LGSC, 29 mSBT, and 92 mucinous tumors (29 MC, 49 MBT, and 14 cases equivocal between MC and MBT as described in Dundr et al[26]). Moreover, 104 tubo-ovarian high grade serous carcinomas (HGSC) were selected from the archives of the Department of Pathology, 1st Medical Faculty and General University Hospital in Prague. These cases were analyzed only by IHC and PCR methods. All LGSC, mSBT, HGSC and CCC cases were reviewed by at least 2 experienced pathologists and fulfilled the strict morphological and immunohistochemical criteria. The classification of mucinous tumors was based on the results of our previous study, which focused on the interobserver agreement and molecular analysis of

Table I
Patients and tumors characteristics

	OCCC	LGSC	mSBT	*mucinous tumors	HGSC†
n. of samples	n = 100	n = 75	n = 29	n = 92	n = 104
Age mean (range)	60.2 (34–82)	52 (19–83)	48 (25–85)	52 (17–83)	59.9 (36–81)
FIGO					
I	67	11	11	84	9
II	7	3	1	1	7
III	16	42	11	5	64
IV	1	2	0	1	22
NA	9	17	6	1	2

Abbreviations: HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma; mSBT, micropapillary variant of serous borderline tumor; OCCC, ovarian clear cell carcinoma.
FIGO classification was done according to WHO classification of female genital tumors.
* mucinous tumors comprise of 29 mucinous carcinoma, 49 of mucinous borderline tumor and 14 of cases equivocal between mucinous carcinoma and mucinous borderline tumor.
† HGSC cohort was tested only by MMR IHC and PCR-based method.

ovarian mucinous tumors.[26] Clinicopathological characteristics of the analyzed tumors are in Table I.

The study has been approved by the Ethics Committee of General University Hospital in Prague in compliance with the Helsinki Declaration (No. 2140/19 S-IV). The Ethics Committee waived the requirement for informed consent, as according to the Czech Law (Act. no. 373/11, and its amendment Act no. 202/17) it is not necessary to obtain informed consent in fully anonymized studies.

Immunohistochemical expression of mismatch repair proteins

Immunohistochemistry was performed on tissue microarrays (TMAs) using 4 μm thick sections of formalin-fixed and paraffin-embedded (FFPE) tissue. For construction of the TMAs, eligible areas of each tumor were identified and 2 tissue cores (each 2.0 mm in diameter) were taken from the donor block using the tissue microarray instrument TMA Master (3DHISTECH Ltd., Budapest, Hungary).

Immunohistochemical analysis (IHC) was performed with antibodies against MSH2 (clone FE11, ready-to-use, Dako, USA), MSH6 (clone EP49, ready-to-use, Bio SB, USA), MLH1 (clone ES05, ready-to-use, Dako, USA), and PMS2 (clone EP51, ready-tu-use, Dako, USA) using Dako Omnis (Agilent Technologies, Calif) with the EnVision FLEX system.

Loss of MMR protein expression was defined as a loss of nuclear staining in all tumor cells for any of the 4 MMR proteins with preserved positive internal control (nuclear expression of endothelial cells, lymphocytes and/or stromal cells). Weak nuclear staining (weaker in comparison to the internal control) in less than 5% of tumor cells was regarded as lost. Weak nuclear staining (weaker than the internal control) in more than 5% of tumor cells or negativity of both tumor cells and internal control on the initial TMA sections were regarded as equivocal. All cases with an equivocal result on TMA, with loss of expression of any MMR protein or with an MSI-H status detected by PCR and/or NGS were immunohistochemically reanalyzed on whole tissue sections. All immunohistochemical stains were scored by at least 2 experienced pathologists (P.D., M.B., K.N.).

Microsatellite instability (MSI) testing by PCR and fragment analysis (PCR-FA)

A pentaplex PCR reaction was performed with fluorescent labeled primers for the set of 5 quasi monomorphic mononucleotide microsatellite markers BAT-26, BAT-25, NR-21, NR-22, NR-24, followed by fragmentation analysis on ABI 3500 (ThermoFisher). Size of PCR products was evaluated in GeneMapper Software (ThermoFisher). MSI-high (MSI-H) phenotype was defined as the presence of 2 or more unstable microsatellite markers. Cases with 1 unstable marker (MSI-low) were included in a group of MSS tumors.

Capture DNA NGS

For the purpose of this study, samples used as a part of a large project that focused on rare epithelial ovarian tumors were included. For all samples (100 CCC, 75 LGSC, 29 mSBT and 92 mucinous tumors) the NGS sequencing was part of our previous studies, the methodology is detailed there.[26] DNA was extracted also from the adjacent non-neoplastic tissue (Magcore Genomic DNA FFPE One step kit; RBC Bioscience) for sequencing analysis to rule out a potential germline origin of MMR gene mutation in cases with detected MMR mutation in the tumor. The NGS capture panel used in the current and previous studies included 727 genes or gene parts (2097 kbp; NimbleGen, Roche). In this study, we focused only on detected mutations in mismatch repair genes *MLH1, PMS2, MSH2, MSH6*, and on evaluation of microsatellite markers as described below.

MSI testing by NGS approach

The set of NGS 17 loci included standard recommended Bethesda markers BAT-26, BAT-25, NR-21, NR-22, NR-24, D5S346,[29] and microsatellite loci in cancer-related genes TGFBR2, BAX, IGF-II. These are frequently targeted by microsatellite instability and their inactivation may contribute to tumor progression.[30] In addition, MONO-27, Penta-C, ACVR2A, BTBD7, DIDO1, MRE11A, RYR3, SEC31A were included. These are used in commercial kits for testing microsatellite instability in different types of tumors, in particular, colorectal and endometrial cancer (Promega, Idylla).[31] The NGS raw data were processed (including trim reads, mapping reads to GRCh38 reference genome) by QIAGEN CLC Genomics Workbench software (Qiagen). Loci track of our 17 microsatellite markers were imported to the module "Detect MSI status." Fifteen samples with optimal DNA quality and with known MMR (IHC) and MSS (PCR-FA) status were used for creating a MSI-baseline. Default setting of this module was used. The "Detect MSI Status" module measures the statistical variation of the length distribution of each microsatellite locus and determines the stability of each locus by comparing the statistical variation of the tested sample with the normal baseline samples.

To increase the specificity of MSI detection in ovarian tumors, we identified in our targeted custom panel (2097 kbp) all microsatellite markers (1–5 bp in length and comprising 5 repeats or more) by comparison with a set of genome-wide microsatellite markers described by Hause et al.[32] The algorithm for selection of ovarian-specific microsatellite loci is shown in Fig 2. Out of 10,249 microsatellite loci identified (with sufficient read depth) 36 ovarian-tumors specific loci were

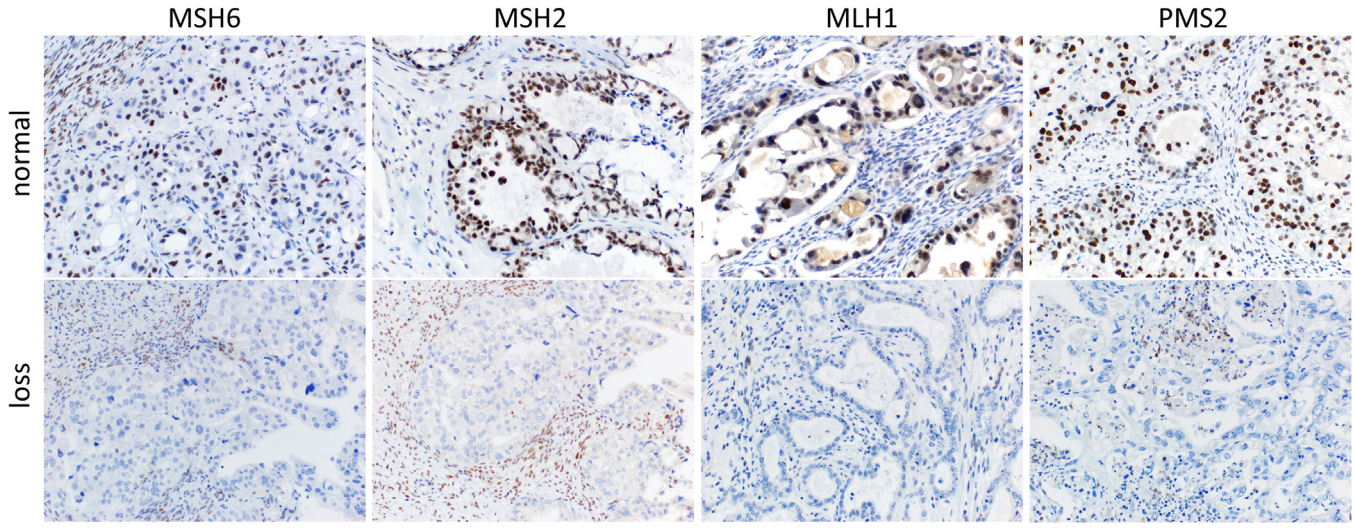

Fig 1. Examples of immunohistochemical staining for the MMR proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table II
Spectrum of the most unstable markers in MSI rare EOCs

Locus coordinates (GRCh37)	Nucleotide repetitive motif	gene (exon or adjacent intron region)
chr.15:41803530-41803544	(C)5	LTK (exonic)
chr.17:30293145-30293168	(T)14	SUZ12 (intronic)
chr.10:27333117-27333138	(A)12	ANKRD26 (intronic)
chr.11:108141951-108141975	(T)15	ATM (intronic)
chr.11:112832387-112832410	(T)14	NCAM1 (intronic)
chr.13:48954278-48954298	(T)11	RB1 (intronic)
chr.15:41991032-41991056	(T)15	MGA (intronic)
chr.2:48032736-48032758	(T)13	MSH6 (intronic)
chr.3:138664706-138664720	(G)5	FOXL2 (exonic)
chr.8:30954240-30954284	(T)13c(T)6cttttgtttg (T)5	WRN (intronic)
chr.9:8341276-8341304	(A)12gg(A)5	PTPRD (intronic)

There are shown selected unstable loci, which was detected in at least in 5 MSI-high OCCCs.

selected. They were unstable in at least 4 out of 6 MSI-H samples in our sample set (previously evaluated by PCR-FA), while stable in all MSS and proficient mismatch repair (MMR-P) samples. The most unstable microsatellite markers identified in >5 MSI-H tumors are summarized in Table II.

Statistical analyses

All statistical tests were carried out using the program R (version 4.0.2, https://www.r-project.org/) and /or Statistica (TIBCO). Association between age (continuous variable) and MSI/MSS status (dichotomous variable) was evaluated using the Mann-Whitney *U* test. To analyze the agreement among 3 methodical approaches (IHC, PCR-FA and NGS), the methods of inter-rater reliability was implemented using the package "irr" in the R program (available at http://CRAN.R-project.org/package=irr). The level of agreement among 3 methods was described using Fleiss Kappa coefficient (κ). Consistent with prior literature on the level of agreement (Landis and Koch 1977), kappa coefficients were interpreted as poor (0.01−0.20), fair (0.21−0.40), moderate (0.41−0.60), substantial (0.61−0.80) and almost perfect (0.81−1.00). The package "cutpointr" implemented in R software (https://github.com/thie1e/cutpointr) was used for the evaluation of an optimal cut-point value for sensitivity and specificity. All tests were 2-sided and a *P*-value of less than 0.05 was considered as significant.

Results

Among the whole cohort of 400 tumors, 7 MMR-D cases were found (Fig 1); 6 of these were MSI-H and 1 MSS on PCR analysis. All these cases were CCC. In all these tumors, mutation of an MMR gene was found. In 2 of these cases, the mutation was germline (*MLH1* and *MSH6*, respectively) and in 5 cases, the mutation was somatic (in 2 cases each *MSH2* and *MSH6*, in 1 case *MLH1*). In 5 of 7 cases, in which TMB was evaluable, high TMB (range 2−86) was found. The case with the highest TMB (TMB = 86) contained a concurrent *POLE* mutation. In addition, there were 5 cases identified with mutation in MMR genes but with a MSS status. Two of these cases showed a germline mutation of *MSH6*. Two MMR proficient cases with a somatic mutation of an MMR gene (1 case with *MLH1* and concurrent *PMS2* mutation, second case with *MSH2* mutation) showed high TMB, but both showed concurrent *POLE* mutation. The results are summarized in Table III.

Thirteen cases (8 CCC, 3 LGSC, 2 mucinous tumors) were MSI-low by PCR. These tumors were classified as MSS by using the NGS approach and immunohistochemistry showed proficient expression for all MMR proteins. The most frequently detected unstable microsatellite in these cases was NR-21 (in 8 of 13).

Pathogenic mutation or likely pathogenic mutation in at least one MMR gene was found in 12/296 (4 %) cases (10 OCC, 1 MBT, 1 LGSC; Table III). Out of those, 7 cases were MMR-D, 6 cases were MSI-H and 6 cases were MSS. Three MSS samples with mutation in the MMR gene had a concurrent mutation in the *POLE* gene and had also the highest TMB (range 51−86 mut/Mb; mean 67.7). TMB in 6 MSI-H tumors ranged between 2 and 26 mut/Mb (mean 17 mut/Mb).

The sequence capture NGS-based approach for MSI testing showed higher sensitivity and specificity using 53 selected microsatellite loci, based on the analysis of our MMR-D and MSS cases compared to using 17 microsatellite loci typical for colorectal and endometrial carcinoma and to analyzing all microsatellite loci present in our NGS DNA panel (Fig 2). Using the 17 microsatellite loci panel, the MMR-D cases showed between 18% and 65% of unstable microsatellites (mean 30%). The cut-off value for MSI-H status by this panel was 18% of unstable loci (sensitivity = 1, specificity = 0.97, AUC = 0.996). Using the 53 microsatellite loci panel the MMR-D cases showed 23%−79% of unstable microsatellites (mean 51%). The optimal cut-off value for MSI-H status by this panel was 23% of unstable loci (sensitivity = 1, specificity = 1, AUC = 1). Analysis of all microsatellite loci (10,249) showed a cut-off value of 5% for MSI-H (sensitivity = 1, specificity = 0.65, AUC = 0.807).

Table III
List of cases with detected pathogenic mutation in MMR genes, including results of MMR IHC, PCR based method and NGS approach for evaluation of MMR-D/MSI status

		IHC					PCR	NGS	Mutation analysis		
dg.	age at dg.	MLH1	PMS2	MSH2	MSH6	MMR-D/ MMR-P	MSI/ MSS	53 loci	MMR genes [G]_Germline [S]_Somatic	*POLE* gene	TMB (mut/Mb)
		% of positively stained nucleus									
CCC	44	98	95	0	0	MMR-D	MSI	55%	[S] *MSH2*:c.2375_2378del,p.(N792fs)	no	NA
CCC	44	1	3	95	40	MMR-D	MSI	45%	[G] *MLH1*:c.116+1G>A,p.?	no	2
CCC	39	100	70	0	45	MMR-D	MSI	66%	[S] *MSH2*:c.1386+1G>A,p.?	no	14
CCC	45	1	1	100	90	MMR-D	MSI	79%	[S] *MLH1*:c.1459C>T,p.(R487*)	no	26†
CCC	45	55	85	55	10	MMR-D	MSI	55%	[S] *MSH6*:c.1610_1613del,p.(K537fs)	no	25
CCC	59	100	100	90	5	MMR-D	MSI	55%	[G] *MSH6*:c.1238G>C,p.(W413S)	no	18†
CCC	66	100	85	90	5	MMR-D	MSS	23%	[S] *MSH6*:c.1630G>T,p.(E544*)	c.1376C>T,p.(S459F)	86
CCC	55	100	60	70	45	MMR-P	MSS	0%	[S] *MLH1*:c.1896+1G>A,p.? and [S] *PMS2*:c.1882C>T,p.(R628*)	c.1366G>C,p.(A456P)	66†
CCC	68	90	95	25	55	MMR-P	MSS	8%	[S] *MSH6*:c.1805C>G,p.(S602*)	no	7
CCC	48	100	100	100	95	MMR-P	MSS	6%	[S] *MSH2*:c.1447G>T,p.(E483*)	c.1231G>C,p.(V411L)	51
MBT	41	90	80	90	20	MMR-P	MSS	4%	[G] *MSH6*:c.2351_2352del,p.(N784fs)	no	1†
LGSC	46	80	20	90	35	MMR-P	MSS	8%	[G] *MSH6*:c.3226C>T,p.(R1076C)	no	1

Abbreviations: CCC, clear cell carcinoma; dg, histological diagnosis; IHC, immunohistochemistry; LGSC, low-grade serous carcinoma; MBT, mucinous borderline tumor; MMR, mismatch repair; MMR-P, proficent mismatch repair protein staining; MMR-D, deficient/loss of mismatch repair protein staining; MSI, microsatellite instable; MSS, microsatellite stable; mut/Mb, mutation per megabase; NGS, Next generation sequencing; PCR-FA, Polymerase chain reaction and fragment analysis; TMB, tumor mutation burden.
NA: data are not available.
reference sequences for *MSH2*: NM_000251.2; *MLH1*: NM_000249.3; *MSH6*: NM_000179.2; *PMS2*: NM_000535.5; NM_006231.2, *POLE*: NM_006231.2.
* termination codon.
† TMB in this samples could be higher because of the purity of tumors cell of the input DNA under 40%.

As the best result of both specificity and sensitivity was reached by the 53-loci panel, this panel was selected for further analysis of methods agreement.

The agreement between evaluation of MMR/MSI by different methods (IHC/PCR-FA/NGS−53 loci panel) was almost perfect (Kappa = 0.949, Z = 27.9, $P < 0.001$). Only 1 case showed discrepancy in the PCR approach (MSS) vs NGS and IHC (MSI in both methods).

Clinicopathological correlations

The average age of MMR-D CCC patient was significantly lower compared to MSS CCC patients (mean/median age 48.9/45 years versus 61.5/62 years, $P < 0.05$). All 7 MMR-D cases were FIGO stage I.

There was no significant difference for the onset of the disease between patients with germline (n = 4) and somatic (n = 8) MMR gene mutations (age 47.5 and 51.2, respectively, $P = 0.610$).

Discussion

It has been shown that 15%−25% of all ovarian carcinomas are related to hereditary factors, of which 10%−15% are associated with LS.[33,34] For patients with LS, the estimated lifetime risk of developing ovarian carcinoma is between 6% and 12% and up to 38% (10%−38%) for those with germline *MSH2* mutation.[2,35,36] Several studies have shown that the ovarian tumor types associated with LS are mostly endometrioid carcinoma followed by CCC, the latter accounting for about 12%−14% of ovarian carcinomas associated with LS.[2,33,37] In HGSC, germline pathogenic variants have been described in approximately 25% of cases, mainly in genes involved in the DNA damage response pathway such as *BRCA1, BRCA2, BRIP1, PALB2, RAD51, ATM*, and *CHEK2*.[34,38]

According to the literature, the frequency of MMR-D/MSI in ovarian endometrioid carcinomas is lower compared to endometrioid endometrial carcinomas and occurs in about 12% of cases (range 0% −22.9%).[2,4,20,21,39-42] The published literature concerning the frequency of MSI in nonendometrioid ovarian tumors is equivocal. In low-grade and high-grade serous carcinomas, the frequency of MSI is absent to very low with a range of 0%−4.2% (average 0.3%).[4,25,43-48] The only exception is a study by Segev et al[20] on 504 serous carcinomas in which 13.7% of cases were found to be MSI-H using a PCR method. However, the results could be potentially influenced by using a PCR based method with 2 mononucleotides and 3 dinucleotide microsatellites. We think that using dinucleotide microsatellites could overestimate MSI in ovarian tumors, as it has been shown that a panel of 5 mononucleotide repeats is more suitable.[49] Our results support the low frequency of MSI in ovarian serous tumors, low-grade and high-grade, as in our cohort of 208 tumors all were MSS. Only 2 other studies analyzed SBT (together 70 cases), all of them showing MSS, which is in concordance with the results of our study.[25,48] Concerning MC, MSI has been shown in the range of 0%−27.3% (average 6.7%).[4,19,22,25,43,45-47,50] In most studies, however, the frequency was 0%, with the highest frequency (27.3%) in the above-mentioned study by Segev et al.[20] One might speculate that in the Segev study also seromucinous tumors were included which are related to endometrioid tumors and were not studied by us. Our results support the finding that MSI is rare in ovarian MC, as all our mucinous tumors were MSS. For CCC, the published literature shows an MSI frequency of 0%−25% (average 5.4%), which concurs with our finding of 7%.[2,4,21,23,24,39,42,51-55]

Altogether, we detected 12 patients with class 4/5 mutation of any MMR genes, including 10 patients with CCC, 1 with LGSC and 1 with MBT. Mutations were somatic in 8 and germline in 4 patients, including 2 CCC, 1 LGSC and 1 MBT. In 3 patients with somatic MMR gene mutation (2 MSS, 1 MMR-D) a concurrent *POLE* mutation was found. All these mutations in MMR genes were classified as pathogenic or likely pathogenic (class 4/5) with the exception of 1 case with the germline *MSH6* variant NM_000179.2:c.1238G>C, p.(Trp413Ser). This variant is assessed as a variant of uncertain significance, according to the ClinVar database. However, our case with this variant was MMR-D/MSI-H, and along with the findings of 1 previous study, which detected this variant in 1 colorectal and 1 endometrial carcinoma associated with LS, this variant should be considered likely pathogenic (class 4).[56]

Our study showed a 100% specificity of MSI testing for the detection of MMR gene mutation. The sensitivity was rather low, as we found MMR-D/MSI-H status in only 7 of 12 (58%) cases. The 5

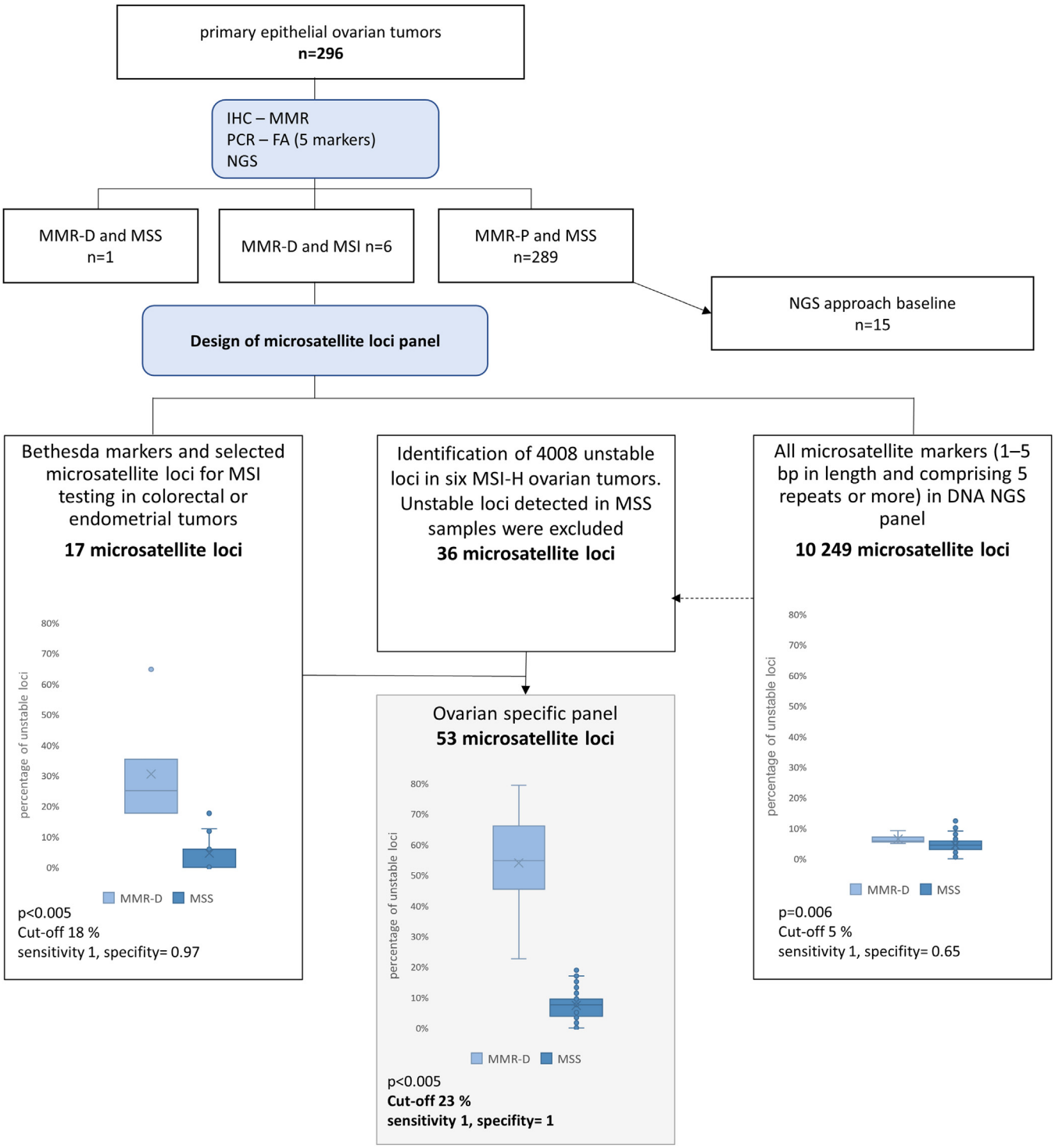

Fig 2. Design of NGS-based approach for the testing of microsatellite instability in ovarian tumors.

PCR-FA, polymerase chain reaction and fragment analysis (5 mononucleotide markers); IHC MMR, immunohistochemical analysis of 4 mismatch repair protein MLH1, MSH2, MSH6, and PMS2; NGS, next generation sequencing (capture DNA NGS, panel of 727 genes including *MLH1*, *MSH2*, *MSH6* and *PMS2*); MSI, microsatellite instability; MMR-D, deficient mismatch repair; MMR-P, proficient mismatch repair; MSS-baseline was calculated from randomly chosen set of MSS samples with sufficient read depth for automated analysis in CLC Genomic Workbench software (Qiagen). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

discordant cases included a somatic mutation of *MLH1* and concurrent *PMS2* in 1 case and of *MSH2* in the second case. Both occurred in *POLE* mutant tumors. All remaining discordant cases were *MSH6* mutated, 2 of them (LGSC and MBT) with germline mutation. Our results are similar to others showing that MSI testing can be false negative in up to half of *MSH6*-mutated cases and could lead to false-negative screening tests for some LS patients with a *MSH6* mutation.[1,9] The only discrepant case between IHC and PCR testing in our study was a case with a somatic *MSH6* mutation showing MMR-D, but a false negative result on PCR based testing.

It has been demonstrated that an NGS approach for MSI testing can be used as an alternative to IHC or PCR based methods, but one should be aware that the spectrum of microsatellite loci can differ among tumor types and different loci can be preferentially mutated in different tumors.[32,57,58] All previous studies focusing on unstable microsatellite loci in different tumor types used data from The Cancer Genome Atlas (TCGA). Some of these studies focused on the description of unstable loci occurring in stomach, colorectal and endometrial carcinomas, but ovarian carcinomas were not included.[32,57] One study analyzed 32 different tumor types. Although the results were promising, they failed to identify a set of unstable microsatellite loci with sufficient sensitivity and specificity, which could be used in ovarian and breast carcinoma.[58] These results further suggested that microsatellite loci are tumor specific and tissue-specific loci should be used for optimal NGS MSI testing, which was confirmed by our study. Comparing 3 sets of microsatellites, our study showed that 17 microsatellites typical for colorectal carcinoma and microsatellites selected based on the results of our MSI testing by IHC and PCR) have high sensitivity and specificity and could be used in routine practice. However, we are aware of the limitations of our study. The definition of an optimal microsatellite set for ovarian carcinomas would require validation on a confirmatory sample set, but that was not the goal of this study.

In conclusion, the results of our study show that MSI occurs in 7% of ovarian CCC. Absence of MSI in other tumor types suggests that occurrence of MSI in these tumors is unusual, which is concordant with the majority of the published literature. In our unselected population of CCC, 2% of patients had LS. Our results confirm that in addition to ovarian EC, also cases of ovarian CCC should be routinely tested for MSI as a part of LS screening and to identify candidate patients for immunotherapy. IHC testing of MMR protein expression seems to be superior to PCR based testing. NGS-MSI could be used in practice, but validation of microsatellite sets optimal for this testing in ovarian tumors is needed. Finally, our results suggest that germline mutation of MMR genes and possibly LS can rarely occur in ovarian tumors other than CCC and endometrioid carcinoma. However, some of these cases, particularly, with *MSH6* mutation would be missed by all testing methods, including IHC, PCR, and NGS-MSI.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest: The authors have no conflicts of interest that are relevant to the content of this article to declare. All authors have read the journal's authorship agreement.

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Author contributions are as follow: P. Dundr performed study concept and design. N. Hajkova collected and interpreted data and prepared manuscript, I. Struzinska prepared the manuscript. All authors participated on material preparation, data collection and/or data analyses. R. Michalkova provided statistical analysis. All authors read and approved the final paper.

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References

1. Hampel H, Frankel W, Panescu J, et al. Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. Cancer Res 2006;66:7810–7.
2. Kim SR, Tone A, Kim RH, et al. Performance characteristics of screening strategies to identify Lynch syndrome in women with ovarian cancer. Cancer 2020;126:4886–94.
3. Lynch HT, Lynch PM, Lanspa SJ, et al. Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. Clin Genet 2009;76:1–18.
4. Rambau PF, Duggan MA, Ghatage P, et al. Significant frequency of MSH2/MSH6 abnormality in ovarian endometrioid carcinoma supports histotype-specific Lynch syndrome screening in ovarian carcinomas. Histopathology 2016;69:288–97.
5. Ryan N, Wall J, Crosbie EJ, et al. Lynch syndrome screening in gynecological cancers: results of an international survey with recommendations for uniform reporting terminology for mismatch repair immunohistochemistry results. Histopathology 2019;75:813–24.
6. Concin N, Matias-Guiu X, Vergote I, et al. ESGO/ESTRO/ESP guidelines for the management of patients with endometrial carcinoma. Int J Gynecol Cancer 2021;31:12–39.
7. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015;372:2509–20.
8. Bartley AN, Mills AM, Konnick E, et al. Mismatch repair and microsatellite instability testing for immune checkpoint inhibitor therapy: guideline from the college of American pathologists in collaboration with the association for molecular pathology and fight colorectal cancer. Arch Pathol Lab Med 2022;146:1194–210.
9. Latham A, Srinivasan P, Kemel Y, et al. Microsatellite instability is associated with the presence of Lynch syndrome pan-cancer. J Clin Oncol 2019;37:286.
10. Hechtman JF, Rana S, Middha S, et al. Retained mismatch repair protein expression occurs in approximately 6% of microsatellite instability-high cancers and is associated with missense mutations in mismatch repair genes. Mod Pathol 2020;33:871–9.
11. Adeleke S, Haslam A, Choy A, et al. Microsatellite instability testing in colorectal patients with Lynch syndrome: lessons learned from a case report and how to avoid such pitfalls. Per Med 2022;19:277–86.
12. Luchini C, Bibeau F, Ligtenberg MJL, et al. ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumor mutational burden: a systematic review-based approach. Ann Oncol 2019;30:1232–43.
13. Aiyer KTS, Doeleman T, Ryan NA, et al. Validity of a two-antibody testing algorithm for mismatch repair deficiency testing in cancer; a systematic literature review and meta-analysis. Mod Pathol 2022;35:1775–83.
14. Gatius S, Velasco A, Varela M, et al. Comparison of the Idylla MSI assay with the Promega MSI Analysis System and immunohistochemistry on formalin-fixed paraffin-embedded tissue of endometrial carcinoma: results from an international, multicenter study. Virchows Arch 2022;480:1031–9.
15. Velasco A, Tokat F, Bonde J, et al. Multi-center real-world comparison of the fully automated Idylla microsatellite instability assay with routine molecular methods and immunohistochemistry on formalin-fixed paraffin-embedded tissue of colorectal cancer. Virchows Arch 2021;478:851–63.
16. Farmkiss L, Hopkins I, Jones M. Idylla microsatellite instability assay versus mismatch repair immunohistochemistry: a retrospective comparison in gastric adenocarcinoma. J Clin Pathol 2021;74:604–7.
17. Samaison L, Uguen A. Idylla MSI test combined with immunohistochemistry is a valuable and cost effective strategy to search for microsatellite instable tumors of noncolorectal origin. Pathol Int 2022;72:234–41.
18. Pecriaux A, Favre L, Calderaro J, et al. Detection of microsatellite instability in a panel of solid tumors with the Idylla MSI Test using extracted DNA. J Clin Pathol 2021;74:36–42.
19. Tajima Y, Eguchi H, Chika N, et al. Prevalence and molecular characteristics of defective mismatch repair epithelial ovarian cancer in a Japanese hospital-based population. Jpn J Clin Oncol 2018;48:728–35.
20. Segev Y, Pal T, Rosen B, et al. Risk factors for ovarian cancers with and without microsatellite instability. Int J Gynecol Cancer 2014;24:664–9.
21. Simila-Maarala J, Soovares P, Pasanen A, et al. TCGA molecular classification in endometriosis-associated ovarian carcinomas: novel data on clear cell carcinoma. Gynecol Oncol 2022;165:577–84.
22. Fraune C, Rosebrock J, Simon R, et al. High homogeneity of MMR deficiency in ovarian cancer. Gynecol Oncol 2020;156:669–75.
23. Bennett JA, Morales-Oyarvide V, Campbell S, Longacre TA, Oliva E. Mismatch repair protein expression in clear cell carcinoma of the ovary: incidence and morphologic associations in 109 cases. Am J Surg Pathol 2016;40:656–63.
24. Carter NJ, Marshall ML, Susswein LR, et al. Germline pathogenic variants identified in women with ovarian tumors. Gynecol Oncol 2018;151:481–8.
25. Lu FI, Gilks CB, Mulligan AM, et al. Prevalence of loss of expression of DNA mismatch repair proteins in primary epithelial ovarian tumors. Int J Gynecol Pathol 2012;31:524–31.
26. Dundr P, Bartu M, Bosse T, et al. Primary mucinous tumors of the ovary: an interobserver reproducibility and detailed molecular study reveals significant overlap between diagnostic categories. Mod Pathol 2023;36:100040.
27. Dundr P, Bazalova B, Bartu M, et al. The cytokeratin 17 expression in primary ovarian tumors has diagnostic but not prognostic significance. Virchows Arch 2022;481:201–12.
28. Nemejcova K, Bartu MK, Michalkova R, et al. A comprehensive immunohistochemical analysis of IMP2 and IMP3 in 542 cases of ovarian tumors. Diagn Pathol 2023;18:15.
29. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004;96:261–8.

30. Shima K, Morikawa T, Yamauchi M, et al. TGFBR2 and BAX mononucleotide tract mutations, microsatellite instability, and prognosis in 1072 colorectal cancers. Plos One 2011;6:e25062.
31. Zhao H, De Craene B, Sagaert X, et al. Association of a novel set of 7 homopolymer indels for detection of MSI with tumor mutation burden and total indel load in endometrial and colorectal cancers. J Clin Oncol 2018;36(15_suppl):e15654.
32. Hanse RJ, Pritchard CC, Shendure J, Salipante SJ. Classification and characterization of microsatellite instability across 18 cancer types. Nat Med 2016;22:1342–50.
33. Chui MH, Ryan P, Radigan J, et al. The histomorphology of Lynch syndrome-associated ovarian carcinomas: toward a subtype-specific screening strategy. Am J Surg Pathol 2014;38:1173–81.
34. Shah S, Cheung A, Kutka M, Sheriff M, Boussios S. Epithelial ovarian cancer: providing evidence of predisposition genes. Int J Environ Res Public Health 2022;19:8113.
35. Bonadona V, Bonaiti B, Olschwang S, et al. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. JAMA 2011;305:2304–10.
36. Lu KH, Daniels M. Endometrial and ovarian cancer in women with Lynch syndrome: update in screening and prevention. Fam Cancer 2013;12:273–7.
37. Helder-Woolderink JM, Blok EA, Vasen HF, et al. Ovarian cancer in Lynch syndrome; a systematic review. Eur J Cancer 2016;55:65–73.
38. Lhotova K, Stolarova L, Zemankova P, et al. Multigene Panel germline testing of 1333 Czech patients with ovarian cancer. Cancers (Basel) 2020;12:956.
39. Tanaka T, Takehara K, Yamashita N, et al. Frequency and clinical features of deficient mismatch repair in ovarian clear cell and endometrioid carcinoma. J Gynecol Oncol 2022;33:e67.
40. Vierkoetter KR, Ayabe AR, VanDrunen M, et al. Lynch Syndrome in patients with clear cell and endometrioid cancers of the ovary. Gynecol Oncol 2014;135:81–4.
41. Bennett JA, Pesci A, Morales-Oyarvide V, et al. Incidence of mismatch repair protein deficiency and associated clinicopathologic features in a cohort of 104 ovarian endometrioid carcinomas. Am J Surg Pathol 2019;43:235–43.
42. Leskela S, Romero I, Cristobal E, et al. Mismatch repair deficiency in ovarian carcinoma frequency, causes, and consequences. Am J Surg Pathol 2020;44:649–56.
43. Schmoeckel E, Hofmann S, Fromberger D, et al. Comprehensive analysis of PD-L1 expression, HER2 amplification, ALK/EML4 fusion, and mismatch repair deficiency as putative predictive and prognostic factors in ovarian carcinoma. Virchows Arch 2019;474:599–608.
44. Malander S, Rambech E, Kristoffersson U, et al. The contribution of the hereditary nonpolyposis colorectal cancer syndrome to the development of ovarian cancer. Gynecol Oncol 2006;101:238–43.
45. Xiao X, Dong DD, He WJ, et al. Mismatch repair deficiency is associated with MSI phenotype, increased tumor-infiltrating lymphocytes and PD-L1 expression in immune cells in ovarian cancer. Gynecol Oncol 2018;149:146–54.
46. Yamashita H, Nakayama K, Ishikawa M, et al. Relationship between microsatellite instability, immune cells infiltration, and expression of immune checkpoint molecules in ovarian carcinoma: immunotherapeutic strategies for the future. Int J Mol Sci 2019;20:5129.
47. Crosbie EJ, Ryan NAJ, McVey RJ, et al. Assessment of mismatch repair deficiency in ovarian cancer. J Med Genet 2021;58:687–91.
48. Jensen KC, Mariappan MR, Putcha GV, et al. Microsatellite instability and mismatch repair protein defects in ovarian epithelial neoplasms in patients 50 years of age and younger. Am J Surg Pathol 2008;32:1029–37.
49. Suraweera N, Duval A, Reperant M, et al. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. Gastroenterology 2002;123:1804–11.
50. Dellas A, Puhl A, Schraml P, et al. Molecular and clinicopathological analysis of ovarian carcinomas with and without microsatellite instability. Anticancer Res 2004;24:361–9.
51. Ge HJ, Xiao YX, Qin GQ, et al. Mismatch repair deficiency is associated with specific morphologic features and frequent loss of ARID1A expression in ovarian clear cell carcinoma. Diagno Pathol 2021;16:12.
52. Zhu J, Ke GH, Bi R, Wu XH. Clinicopathological and survival characteristic of mismatch repair status in ovarian clear cell carcinoma. J Surg Oncol 2020;122:538–46.
53. Lin SY, Hang JF, Lin YY, et al. Diffuse intratumoral stromal inflammation in ovarian clear cell carcinoma is associated with loss of mismatch repair protein and high PD-L1 expression. Int J Gynecol Pathol 2021;40:148–55.
54. Parra-Herran C, Bassiouny D, Lerner-Ellis J, et al. Mismatch repair protein, and POLE abnormalities in ovarian clear cell carcinoma: an outcome-based clinicopathologic analysis. Am J Surg Pathol 2019;43:1591–9.
55. Cai KQ, Albarracin C, Rosen D, et al. Microsatellite instability and alteration of the expression of hMLH1 and hMSH2 in ovarian clear cell carcinoma. Hum Pathol 2004;35:552–9.
56. Kiyozumi Y, Matsubayashi H, Higashigawa S, et al. Role of tumor mutation burden analysis in detecting lynch syndrome in precision medicine: analysis of 2,501 Japanese cancer patients. Cancer Epidemiol Biomarkers Prev 2021;30:166–74.
57. Cortes-Ciriano I, Lee S, Park WY, Kim TM, Park PJ. A molecular portrait of microsatellite instability across multiple cancers. Nat Commun 2017;8:15180.
58. Long DR, Waalkes A, Panicker VP, Hause RJ, Salipante SJ. Identifying optimal loci for the molecular diagnosis of microsatellite instability. Clin Chem 2020;66:1310–8.