



Retained mismatch repair protein expression occurs in approximately 6% of microsatellite instability-high cancers and is associated with missense mutations in mismatch repair genes

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Abstract

Immunohistochemistry for mismatch repair protein expression is widely used as a surrogate for microsatellite instability status—an important signature for immunotherapy and germline testing. There are no systematic analyses examining the sensitivity of immunohistochemistry for microsatellite instability-high status. Mismatch repair immunohistochemistry and microsatellite instability testing were performed routinely as clinically validated assays. We classified germline/somatic mutation types as truncating (nonsense, frameshift, and in/del) versus missense and predicted pathogenicity of the latter. Discordant cases were compared with concordant groups: microsatellite instability-high/mismatch repair-deficient for mutation comparison and microsatellite stable/mismatch repair-proficient for immunohistochemical comparison. 32 of 443 (7%) microsatellite instability-high cases had immunohistochemistry. Four additional microsatellite instability-high research cases had discordant immunohistochemistry. Of 36 microsatellite instability-high cases with discordant immunohistochemistry, 30 were mismatch repair-proficient, while six (five *MLH1* and one *MSH2*) retained expression of the defective mismatch repair protein and lost its partner. In microsatellite instability-high tumors with discordant immunohistochemistry, we observed an enrichment in deleterious missense mutations over truncating mutations, with 69% (25/36) of cases having pathogenic germline or somatic missense mutations, as opposed to only 19% (7/36) in a matched microsatellite instability-high group with concordant immunohistochemistry ($p = 0.0007$). In microsatellite instability-high cases with discordant immunohistochemistry and *MLH1* or *PMS2* abnormalities, less cells showed expression ($p = 0.015$ and $p = 0.00095$, respectively) compared with microsatellite stable/mismatch repair-proficient cases. Tumor mutation burden, MSI sensor score, and truncating mismatch repair gene mutations were similar between microsatellite instability-high cases with concordant versus discordant immunohistochemical expression. Approximately 6% of microsatellite instability-high cases have retained mismatch repair protein expression and would be missed by immunohistochemistry-based testing, hindering patient access to immunotherapy. Another 1% of microsatellite instability-high cases show isolated loss of the defective gene's dimerization partner, which may lead to germline testing of the wrong gene. These cases are enriched for pathogenic mismatch repair missense mutations.

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Introduction

Mismatch repair protein expression via immunohistochemistry and microsatellite instability status are integral parts of the management of many patients with solid cancers. Microsatellite instability testing is performed using DNA, either by polymerase chain reaction or next generation sequencing. Microsatellite instability polymerase chain reaction measures the degree of indel mutations involving microsatellite loci via a set of five mononucleotide microsatellites in the tumor vs. normal DNA [1]. Microsatellite instability assessment via next generation sequencing with

MSIsensor is performed via assessment of all available microsatellites covered by a given panel [2]. We have previously validated use of MSIsensor with our institutional next generation sequencing assay, MSK-IMPACT [3]. Immunohistochemistry is used to assess the presence or absence of mismatch repair protein expression, and complete absence of MLH1, PMS2, MSH2, and/or MSH6 usually correlates with the presence of microsatellite instability-high status in the DNA. The National Comprehensive Cancer Network universally recommends microsatellite instability testing/mismatch repair protein immunohistochemistry screening for all patients with colorectal carcinoma as well as various other types of solid cancer [4]. In addition, patients with stage II–III microsatellite instability-high/mismatch repair-deficient colorectal carcinoma are managed differently than mismatch repair-proficient/microsatellite stable colorectal carcinoma as the former do not benefit from 5-fluorouracil adjuvant therapy [5]. More broadly, mismatch repair protein loss or microsatellite instability-high status signifies eligibility for immune checkpoint inhibitor treatment [6], as well as the possibility of Lynch syndrome [7] in all types of solid malignancies. Patients with microsatellite instability-high status and advanced malignancies are often eligible for immune checkpoint inhibitor therapy based solely on microsatellite instability status, whereas microsatellite instability-high or mismatch repair-deficient status in itself is not diagnostic of Lynch syndrome but is generally a prerequisite before further work-up with clinical, personal, and family history, as well as germline sequencing to rule in or out the possibility of Lynch syndrome. It is important that mismatch repair-deficient/microsatellite instability-high status is detected when present so that patients do not miss an opportunity to be treated with immune checkpoint inhibitors such as pembrolizumab.

Mismatch repair protein expression status corresponds with DNA-based microsatellite instability (microsatellite instability) testing results over 90% of the time [7]. Due to the high concordance rate and certain advantages that immunohistochemistry has over microsatellite instability testing, including turnaround time, knowledge of which mismatch repair gene is abnormal, use of only four slides, and sensitivity in low tumor purity cases; many institutions perform mismatch repair protein immunohistochemistry rather than microsatellite instability testing.

However, in certain circumstances, mismatch repair protein expression status and microsatellite instability status are discordant. Low tumor purity affects microsatellite instability results, usually by resulting in an underestimation of the degree of microsatellite instability. Neoadjuvant therapy can induce loss of MSH6 expression in cases with microsatellite stable status [8]. Even rarer, authors have described isolated microsatellite instability-high cases with proficient (some

degree of nuclear expression) mismatch repair protein expression [9–11], as well as *MLH1* germline mutant Lynch syndrome cancers with retained MLH1 expression [12, 13]. Here, we systematically review 443 microsatellite instability-high cases also assessed by mismatch repair protein immunohistochemistry and report the incidence, molecular, and clinicopathologic features of mismatch repair-proficient yet microsatellite instability-high cases.

Methods

Selection criteria

Selection criteria for this study included formalin-fixed paraffin embedded tumor samples with MSK-IMPACT results from January 2014 to December 2018, microsatellite instability-high status by MSIsensor, and mismatch repair protein immunohistochemistry results available.

Microsatellite instability testing

The microsatellite instability status of all cancers sequenced with MSK-IMPACT from January 2014 to December 2018 was reviewed. MSK-IMPACT is a hybridization capture based assay that assesses somatic mutations, copy number, structural variants in 468 genes in its current iteration (410 in v5 and 341 in v3) against a patient's matched blood sample [14]. Microsatellite instability status is also routinely assessed using a modified and clinically validated version of MSIsensor v0.2 (github: <https://github.com/ding-lab/MSIsensor>) [3]. MSIsensor is a bioinformatic program that interrogates all available microsatellite loci with coverage of at least 20× against a matched normal. The median number of loci assessed by each version of MSK-IMPACT was 1152 in v3, 1241 in v5, and 1581 in v6. Each available locus is evaluated with a goodness of fit test to determine whether the locus is stable or unstable loci. The percentage of unstable loci are then expressed as a score [2].

Tumor mutation burden determination

We used all nonsilent exonic and splicing (within the ± 2 bp of intron/exon boundary) mutations (single nucleotide and insertions/deletions) that were reported to the patients and divided the total number of bases where we report mutations in each version of the panel: v3: 896,665; v5: 1,016,478 and v6: 1,139,322.

Mismatch repair protein immunohistochemistry

Immunohistochemical results for mismatch repair proteins (MLH1, PMS2, MSH2, and MSH6) were reviewed for

tumors that were microsatellite instability-high on MSK-IMPACT; and slides for any immunohistochemistry results signed out as “retained,” “equivocal,” “weak,” or “faint” were further reviewed. Mismatch repair-deficient status was defined as complete loss of nuclear expression of mismatch repair protein(s) within the tumor as per prior studies [11]. Mismatch repair-deficient cases with discordant patterns of germline or somatic mutations (ex: isolated PMS2 deficiency with *MLH1* hypermethylation and negative germline results) were also reviewed. When available, immunohistochemistry slides were reviewed for cases with discordant mismatch repair statuses and the percent of tumor cells expressing each mismatch repair protein was estimated. A 3:1 control set of immunohistochemistry slides from mismatch repair-P/microsatellite stable cases was also assessed, and the percent of tumor cells expressing each mismatch repair protein was visually estimated by JFH for all tumor cells available and recorded from microsatellite instability-high cases with discordant mismatch repair protein expression statuses as well as the control set of mismatch repair-proficient/microsatellite stable cases.

MLH1 promoter hypermethylation status analysis

MLH1 promoter hypermethylation testing was performed via bisulfite conversion followed by pyrosequencing as previously described [15] on samples that were microsatellite instability-high with no known germline or somatic mismatch repair gene mutations.

All immunohistochemistry and molecular assays were clinically validated and performed in CLIA accredited laboratories.

Analysis of mismatch repair gene missense mutations

All coding regions of *EPCAM*, *MLH1*, *PMS2*, *MSH2*, and *MSH6* are covered in all versions of MSK-IMPACT. Because truncating mutations (frameshift, stop gain, and splicing) should result in a premature stop codon, nonsense-mediated decay of the transcript, and subsequent lack of mismatch repair protein expression, we theorized that deleterious mismatch repair gene missense mutations are a potential cause for retained mismatch repair protein expression in microsatellite instability-high cases. We therefore assessed whether deleterious mismatch repair gene missense and in-frame indel mutations were enriched in this subset, as compared with truncating mutations (frameshift, stop gain, and splicing). The pathogenicity of somatic missense mutations was predicted using Rare Exome Variant Ensemble Learner [16]. The number of cases with a pathogenic mismatch repair gene missense mutation was compared against a 3:1 control set of microsatellite

instability-high cases with concordant immunohistochemistry to investigate whether microsatellite instability-high cases with discordant mismatch repair protein expression statuses are enriched in mismatch repair gene missense mutations which lead to expressed but potentially dysfunctional proteins.

Statistics

A Mann–Whitney Test was used for comparison of percentage of tumor cells expressing mismatch repair immunohistochemistry, and the frequencies of mismatch repair gene mutations were compared with two-sided Fisher’s exact test.

Results

Incidence

Examination of 29,530 clinical cases sequenced with MSK-IMPACT assay revealed 582 (2%) tumors were microsatellite instability-high. We reviewed 443 microsatellite instability-high cases with available mismatch repair protein immunohistochemistry, which identified 32 cases (7.2%) with discrepant immunohistochemistry results. These 32 tumors included 17 colorectal carcinomas, nine endometrial carcinomas, and one each of various other cancer types (Table 1). Discordant mismatch repair immunohistochemistry occurred in 6.4% of microsatellite instability-high colorectal carcinomas and 4.9% of microsatellite instability-high endometrial carcinomas.

Four additional microsatellite instability-high colon cancers from Lynch syndrome patients with discordant mismatch repair protein expression statuses were also identified via MSK-IMPACT testing performed in the research setting, for a total of 36 discordant cases identified for further analysis. These cases were compared against mutation data from a 1:1 matched cohort of 36 microsatellite instability-high, mismatch repair immunohistochemistry concordant cases, and mismatch repair immunohistochemistry data from a 1:3 matched cohort of 108 microsatellite stable, mismatch repair-proficient cases.

Mismatch repair protein immunohistochemistry patterns and expression

Nuclear dot-like *MLH1* staining has been reported in the literature in cases with abnormal mismatch repair expression and *MLH1* germline mutations [17, 18]. In the current study, two microsatellite instability-high/mismatch repair immunohistochemistry discordant case showed nuclear dot-like *MLH1* expression (Fig. 1a): one of these cases had

Table 1 Characteristic of microsatellite instability-high tumor with discordant immunohistochemical mismatch repair protein expression results.

CbioPortal ID	Tumor type	MMR IHC	Germline MMR mutation	Somatic MMR mutation	REVEL	VAF	MLH1 promoter hypermethylation	Dysfunctional MMR gene
P-0020886	Cervical squamous	Retained	None	<i>MLH1</i> p.E605Q (c.1813G>C) <i>MLH1</i> p.E620K (c.1858G>A) <i>MLH1</i> p.E669K (c.2005G>A)	0.74 0.65 0.78	0.54 0.52 0.55	Absent	<i>MLH1</i>
CMO42	CRC	Retained	<i>MLH1</i> p.E102K (c.304G>A)	<i>MSH6</i> p.F1088Sfs*2 (c.3261delC)		0.17	Not Performed	<i>MLH1</i>
CMO43	CRC	Retained	<i>MLH1</i> p.E102K (c.304G>A)	<i>MLH1</i> p.X196_splice (c.588+1G>A)		0.29	Not Performed	<i>MLH1</i>
P-0005455	CRC	<i>PMS2</i> loss only	None	<i>MLH1</i> p.M35del (c.105_107delGAT) <i>MLH1</i> p.E414Rfs*77 (c.1240delG) <i>MSH6</i> p.F1088Lfs*5 (c.3261dupC)		0.14 0.18 0.28	Absent	<i>MLH1</i>
P-0013462	CRC	Dot-like <i>MLH1</i>	Not performed	None	–	–	Present	<i>MLH1</i>
P-0012115	CRC	Dot-like <i>MLH1</i>	None	<i>MLH1</i> p.P300Hfs*40 (c.899_963del CCCAGAATGTGGATGTTAATGT GCACCCACAAAGCATGAAGT TCACTTCTGCACGAGGAGAGC)		0.25	Not Performed	<i>MLH1</i>
P-0014258	CRC	Retained	<i>MLH1</i> exons 1–19 deletion	<i>MLH1</i> p.D41N (c.121G>A) <i>MSH2</i> p.C697Y (c.2090G>A)	0.90 0.85	0.25 0.12	Not Performed	<i>MLH1</i>
P-0016938	CRC	Retained	<i>MLH1</i> intron 7 34 bp insertion	<i>MLH1</i> p.Y157Tfs*3 (c.469delT)		0.17	Not Performed	<i>MLH1</i>
P-0025715	CRC	Retained	<i>MLH1</i> p.N306K (c.918T>A)	None	–	–	Not Performed	<i>MLH1</i>
P-0018118	CRC	<i>PMS2</i> loss only	None	<i>MSH6</i> p.R1076H (c.3227G>A)	0.84	0.22	Present	<i>MLH1</i>
P-0036383	CRC	<i>PMS2</i> loss only	<i>MLH1</i> p.E102A (c.305G>C)	None	–	–	Not Performed	<i>MLH1</i>
P-0024289	UEC	<i>PMS2</i> loss only	None	<i>MSH6</i> p.N897Ifs*9 (c.2690delA)		0.22	Present	<i>MLH1</i>
P-0019360	UEC	Retained	None	<i>MLH1</i> p.R425Gfs*66 (c.1272delT) <i>MLH1</i> p.G98D (c.293G>A) <i>MLH1</i> p.Y750* (c.2250C>G) <i>MLH1</i> p.F261Sfs*6 (c.782_785delTCAT)	0.98	0.16 0.17 0.32 0.22	Absent	<i>MLH1</i>
P-0034967	Small bowel carcinoma	Retained	<i>MLH1</i> p.I19F (c.55A>T)	None	–	–	Not Performed	<i>MLH1</i>
P-0006482	Breast carcinoma	Retained	None	<i>MSH2</i> p.F474_L481del (c.1420_1443del ITTTGATCCTAATCTCAGTGAATTA)		0.39	Absent	<i>MSH2</i>
CMO40	CRC	Retained	<i>MSH2</i> p.A636P (c.1906G>C)	<i>MSH2</i> p.V34Pfs*2 (c.2690delA)		0.33	Not Performed	<i>MSH2</i>
CMO41	CRC	<i>MSH6</i> loss only	<i>MSH2</i> p.R524H (c.1571G>A)	<i>MSH6</i> p.F1088Sfs*2 (c.3261delC)		0.38	Not Performed	<i>MSH2</i>
P-0010504	CRC	Retained	<i>MSH2</i> p.A636P (c.1906G>C)	<i>MSH2</i> p.E572del (c.1715_1717delAAG) <i>MSH6</i> p.F1088Lfs*5 (c.3261dupC)		0.20 0.17	Not Performed	<i>MSH2</i>
P-0017697	CRC	Retained	None	<i>MSH2</i> p.D603N (c.1807G>A) <i>PMS2</i> p.M136I (c.408G>A)	0.92 0.20	0.14 0.14	Absent	<i>MSH2</i>
P-0027324	CRC	Retained	<i>MSH2</i> p.R389* (c.1165C>T)	<i>MSH2</i> p.E749K (c.2245G>A)	0.98	0.24	Not Performed	<i>MSH2</i>
P-0020757	UEC	Retained	<i>MSH2</i> c.942A +3>T	<i>MSH2</i> p.P385Qfs*27 (c.1152_1153delinsT)		0.05	Not Performed	<i>MSH2</i>
P-0027238	Anaplastic thyroid	Retained	Not performed	<i>MSH2</i> p.S494* (c.1481C>G) <i>PMS2</i> p.R151H (c.452G>A)		0.56 0.33	Not Performed	<i>MSH2</i>
P-0004379	UEC	Retained	None	<i>MSH2</i> p.R621Q (c.1862G>A) <i>MSH6</i> p.E547D (c.1641A>C) <i>MSH6</i> p.K70N (c.210G>T) <i>MSH6</i> p.E877* (c.2629G>T) <i>MSH6</i> p.R178H (c.533G>A)	1.00 0.88 0.04 0.80	0.28 0.32 0.34 0.32	Not Performed	<i>MSH2</i> or <i>MSH6</i> (POLE p.V411L, ultramutated)
P-0030372	UEC	Retained	None	<i>MSH2</i> p.E580* (c.1738G>T) <i>MSH2</i> p.A272V (c.815C>T) <i>MSH2</i> p.D386Y (c.1156G>T) <i>MSH2</i> p.R524C (c.1570C>T)	0.89 0.98 0.85	0.15 0.12 0.30	Absent	<i>MSH2</i> or <i>MSH6</i> (POLE p.P286R, ultramutated)

Table 1 (continued)

CbioPortal ID	Tumor type	MMR IHC	Germline MMR mutation	Somatic MMR mutation	REVEL	VAF	MLH1 promoter hypermethylation	Dysfunctional MMR gene
				<i>MSH2</i> p.E859* (c.2575G>T)		0.23		
				<i>MSH6</i> p.R121H (c.362G>A)	0.54	0.14		
				<i>MSH6</i> p.E699K (c.2095G>A)	0.78	0.18		
				<i>MSH6</i> p.R922Q (c.2765G>A)	0.64	0.39		
P-0013010	CRC	Retained	<i>MSH6</i> p.Y1249Lfs*26 (c.3743_3744insT)	None	–	–	Not Performed	<i>MSH6</i>
P-0030908	CRC	Retained	<i>MSH6</i> p.N911* (c.2731C>T)	<i>MSH6</i> p.I871Lfs*2 (c.2611delA)		0.31	Not Performed	<i>MSH6</i>
				<i>MSH6</i> p.F1088Sfs*2 (c.3261delC)		0.33		
				<i>MSH6</i> p.R1331Q (c.3992G>A)	0.44	0.31		
P-0036500	CRC	Retained	None	<i>MSH6</i> p.T1219I (c.3656C>T)	0.93	0.36	Not Performed	<i>MSH6</i>
				<i>MSH6</i> p.F1088Pfs*3 (c.3260_3261dupCC)		0.21		
P-0028580	CRC	Retained	None	<i>MSH6</i> p.T1219I (c.3656C>T)	0.93	0.33	Absent	<i>MSH6</i>
				<i>MSH6</i> p.F1088Lfs*5 (c.3261dupC)		0.28		
P-0021117	UEC	Retained	None	<i>MSH6</i> p.T1219I (c.3656C>T)	0.93	0.24	Absent	<i>MSH6</i>
				<i>MSH6</i> p.F1088Lfs*5 (c.3261dupC)		0.20		
P-0021505	UEC	Retained	None	<i>MSH6</i> p.T1219I (c.3656C>T)	0.93	0.20	Not Performed	<i>MSH6</i>
				<i>MSH6</i> p.G1105Wfs*3 (c.3312dupT)		0.20		
P-0019598	UEC	Retained	None	<i>MSH6</i> p.F1088Lfs*5 (c.3261dupC)		0.16	Not Performed	<i>MSH6</i>
P-0001821	Uterine leiomyosarcoma	Retained	None	<i>MSH2</i> p.R214I (c.641G>T)	0.80	0.49	Not Performed	<i>MSH6</i>
				<i>MSH6</i> p.A1162P (c.3484G>C)	0.93	0.81		
P-0030265	CRC	Retained	<i>PMS2</i> p.R563* (c.1687C>T)	<i>MSH2</i> p.X359_splice (c.1076+1G>A)		0.22	Not Performed	<i>PMS2</i>
				<i>MSH6</i> p.C869Y (c.2606G>A)	0.33	0.20		
				<i>PMS2</i> p.R151Pfs*22 (c.448_451dupCCCC)		0.10		
P-0031050	CRC	Retained	Not performed	<i>PMS2</i> p.R107W (c.319C>T)	0.76	0.33	Absent	<i>PMS2</i>
P-0000449	Prostate	Retained	<i>PMS2</i> p.S46I (c.137G>T)	None	–	–	Not Performed	<i>PMS2</i>

MMR mismatch repair, IHC immunohistochemistry, REVEL Rare Exome Variant Ensemble Learner, VAF variant allele frequency, CRC colorectal C carcinoma, UEC uterine endometrioid carcinoma

MLH1 promoter hypermethylation, while the other had a truncating somatic *MLH1* mutation (p. P300Hfs*40). Interestingly, 1 of 108 microsatellite stable/mismatch repair-proficient cases also showed nuclear dot-like MLH1 expression, demonstrating that while nuclear dot-like (also called nucleolar or granular) MLH1 expression is rare, it is not specific for mismatch repair-deficient/microsatellite instability-high status.

While the majority of cases displayed a degree of expression of all four mismatch repair proteins (Fig. 1b), five cases with *MLH1* abnormalities displayed isolated loss of *PMS2* (Fig. 1c, d), while one case with *MSH2* abnormalities (both somatic and germline) displayed isolated loss of *MSH6*. The DNA level abnormalities in these six cases are specified in Table 1. The remaining 30 cases displayed varying degrees of expression of all four mismatch repair proteins, with the abnormal mismatch repair gene often showing a decreased percent of tumor cells with mismatch repair protein expression in comparison to a mismatch repair-proficient/microsatellite stable cohort (Fig. 2): the decreased percentage of tumor cells expressing

mismatch repair proteins was statistically significant for cases with abnormal *MLH1* ($p = 0.015$) and *PMS2* ($p = 0.00095$), yet cases with abnormal *MSH2* and *MSH6* did not show statistical significant decreased expression despite trends.

Tumor mutation burden and MSI sensor score comparison

To assess whether microsatellite instability-high cases with discordant immunohistochemistry have a lesser degree of microsatellite instability or lower mutation burden, we compared them against microsatellite instability-high cases with concordant immunohistochemical results. The median tumor mutation burden and MSI sensor score for microsatellite instability-high cases with concordant mismatch repair immunohistochemistry was 44.6 mutations/megabase and 29.96, respectively. The median tumor mutation burden and MSI sensor score for microsatellite instability-high cases with discordant mismatch repair immunohistochemistry were similar: 54.1 mutations/megabase and 27.08, respectively.

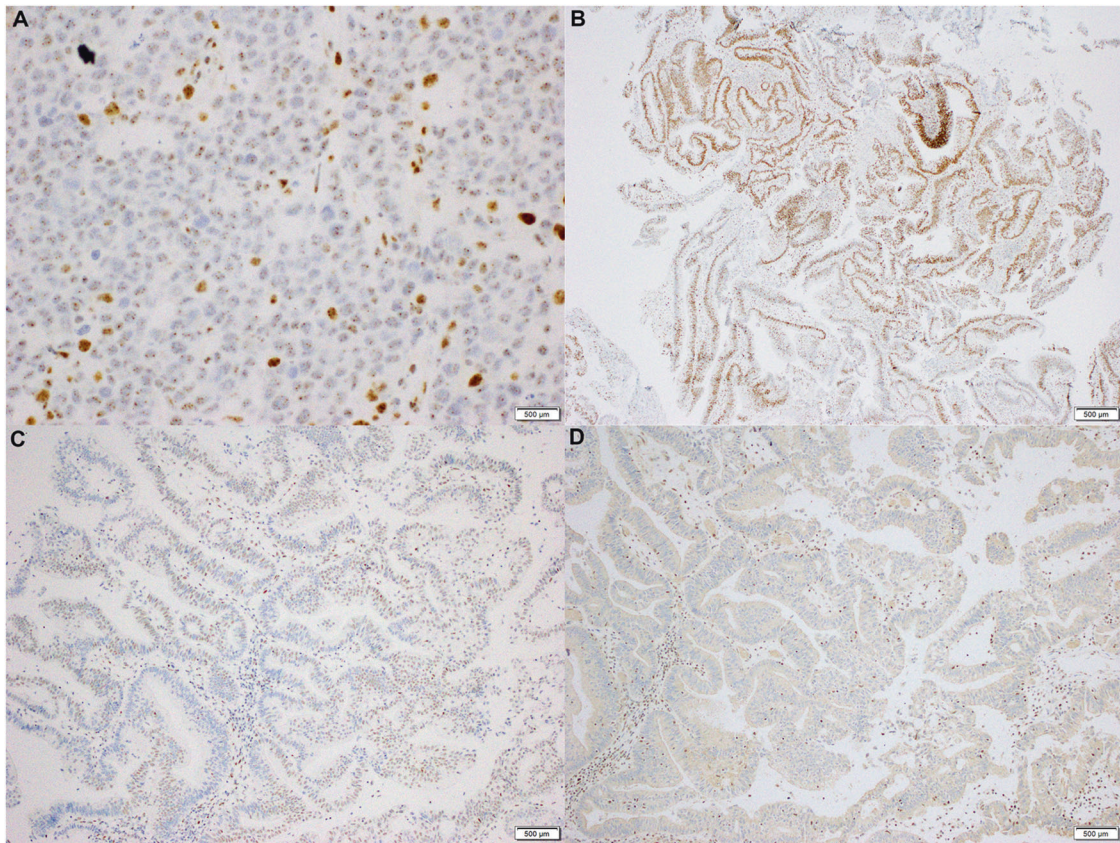


Fig. 1 Examples of abnormal/discordant mismatch repair protein immunohistochemistry. (a) A microsatellite instability-high colorectal carcinoma with a nuclear dot-like or “nucleolar” MLH1 expression pattern. No germline or somatic mismatch repair gene mutations were present, yet MLH1 promoter hypermethylation was present (MLH1 immunohistochemistry, Ventana, 400× original magnification). (b) A microsatellite instability-high colorectal carcinoma with retained MSH6 expression in ~75% of tumor cells. Germline mutation analysis was negative for mismatch repair gene mutations, yet two somatic mutations in MSH6 were present: p. F1088Pfs*3 (c.3260_3261dupCC) at 20.6% variant allele fraction (VAF), which is

truncating, and p.T1219I (c.3656C>T) at 36.4% variant allele frequency, which is a missense mutation that is predicted to be pathogenic (MSH6 immunohistochemistry, Cell Marque, 40× original magnification). (c) and (d) A microsatellite instability-high endometrial carcinoma with retained patchy MLH1 expression (c) and loss of PMS2 expression (d). Molecular analysis revealed no germline mismatch repair gene mutations, a single *MSH6* frameshift mutation at 22.4% variant allele frequency, and the presence of *MLH1* promoter hypermethylation (MLH1 immunohistochemistry: Ventana; PMS2 immunohistochemistry: BD Bioscience; each 400× original magnification).

Mismatch repair gene mutations

Deleterious somatic mismatch repair gene missense mutations were more common in microsatellite instability-high cases with discordant immunohistochemistry: 16 (44%) discordant cases harbored deleterious missense mutations in comparison to only 6 (17%) microsatellite instability-high cases in a matched cohort of concordant mismatch repair-deficient/ microsatellite instability-high cases ($p = 0.02$). Nine (25%) microsatellite instability-high cases with discordant mismatch repair protein expression harbored pathogenic germline alterations in a mismatch repair gene (Lynch syndrome), while 1 (3%) microsatellite instability-high/mismatch repair-deficient cases harbored a pathogenic germline missense mutation in a mismatch repair gene. Together, 25 (69%) microsatellite instability-high/mismatch repair immunohistochemistry discordant cases harbored either a

pathogenic germline or somatic mismatch repair gene missense mutation, while only 6 (16%) microsatellite instability-high/mismatch repair-deficient cases harbored a pathogenic germline or somatic mismatch repair gene missense mutation ($p = 0.0001$). The incidence of truncating mismatch repair gene mutations in microsatellite instability-high cases with discordant mismatch repair immunohistochemistry was not significantly different from that in microsatellite instability-high cases with concordant immunohistochemistry (Table 2).

Several mismatch repair gene missense mutations occurred repeatedly in multiple microsatellite instability-high cases with discordant immunohistochemistry. These recurrent missense mutations included two patients with germline *MLH1* p. E102K (c. 304G>A) mutations as well as a germline *MLH1* p. E102A (c. 305G>A), two cases of germline *MSH2* p. A636P (c. 1906G>C), and four cases with the somatic *MSH6* mutation p. T1219I (c. 3656C>T).

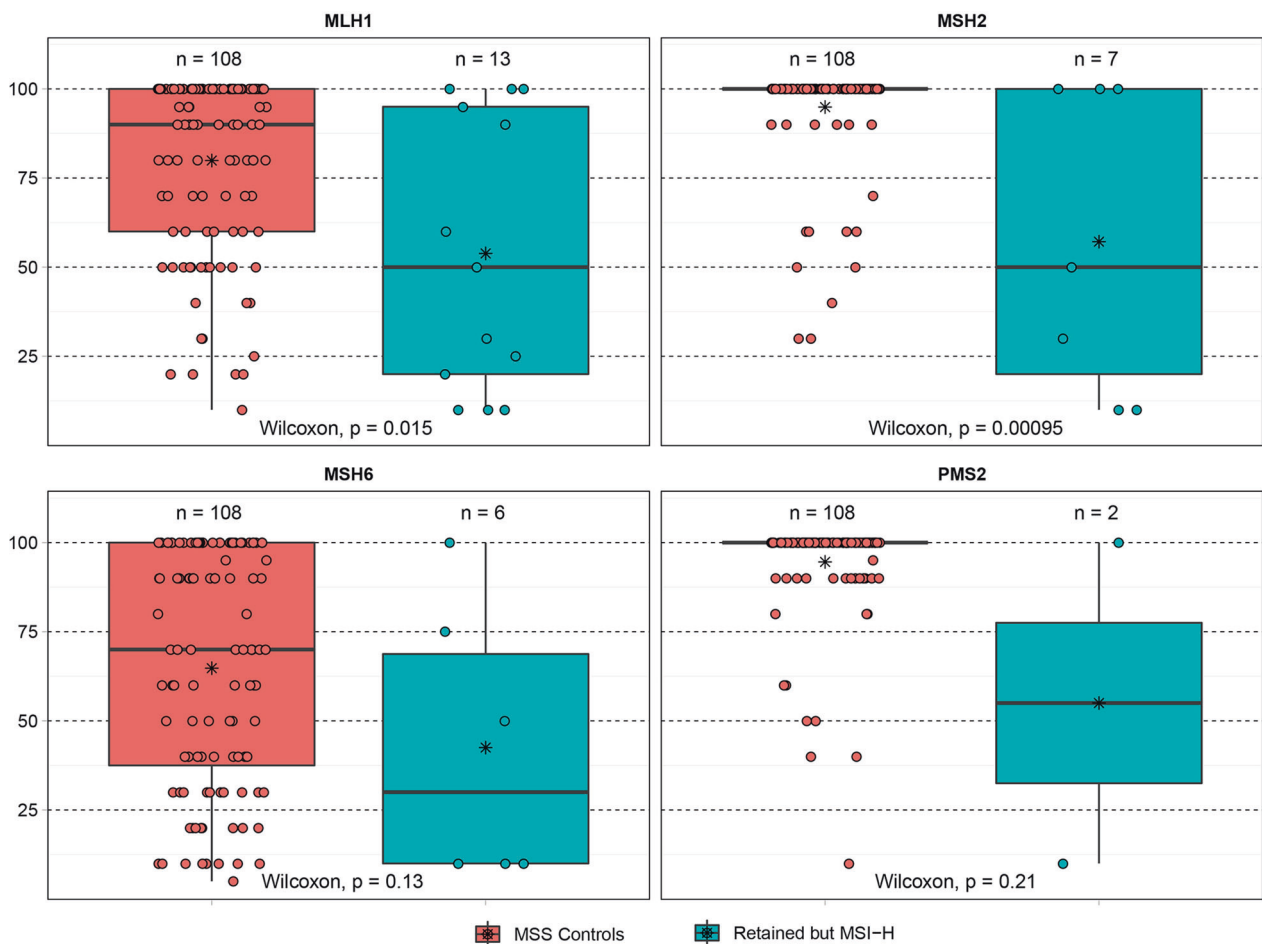


Fig. 2 Box plot of percentage of tumor cells expressing mismatch repair proteins by immunohistochemistry. The percentage of tumor cells expressing (a) MLH1, (b) MSH2, (c) MSH6, and (d) PMS2 was

in general lower for microsatellite instability-high cases with retained mismatch repair protein expression (right, green) in comparison to microsatellite stable cases (left, red).

Each case mentioned above had retained expression of the mutated mismatch repair gene.

Response to immune checkpoint blockade

Three patients with microsatellite instability-high/mismatch repair immunohistochemistry discordant cancers were treated with the anti-PD1 antibody pembrolizumab as monotherapy. All three patients benefited from treatment with best response, one each, of complete response, partial response, and stable disease. All three patients remained on pembrolizumab for over 12 months without progression of disease. Response data are summarized in Table 3.

Discussion

We found that ~7% of microsatellite instability-high cancers in our study population have discordant (either retained expression or different pattern of protein loss) mismatch

repair immunohistochemistry staining patterns, so the presence of microsatellite instability-high status would be missed with immunohistochemistry testing. These tumors are enriched in both germline and somatic mismatch repair gene missense mutations and often have a decrease in the percentage of tumor cells expressing the abnormal mismatch repair protein. Treatment with immune checkpoint inhibitors in three of the patients with discordant immunohistochemistry indicated prolonged clinical benefit, validating the identification of these cases as microsatellite instability-high by next generation sequencing, and highlighting the importance to properly classify these cases.

Mismatch repair protein expression is often interpreted as “retained” or “lost” with “lost” meaning complete loss of expression [11], and the literature to date has only scarcely dealt with mismatch repair gene missense mutations associated with patchy mismatch repair protein expression [11–13]. Our findings warrant caution in the interpretation of mismatch repair immunohistochemistry, especially when focal or when dot-like nuclear MLH1 expression is present.

Table 2 Comparison of mutation profiles in microsatellite instability-high cases with discordant versus concordant immunohistochemical mismatch repair protein expression results.

Number of cases with MMR gene mutations	Discordant MMR IHC	Concordant MMR IHC	<i>P</i> value
Pathogenic somatic missense mutations	16	6	0.02
Pathogenic germline missense mutations	9	1	0.01
Total cases with pathogenic missense mutations	25	7	0.0001
Truncating germline mutations	7	4	0.51
Truncating somatic mutations	20	18	0.64
Total cases with truncating mutations	27	22	0.2

MMR mismatch repair, IHC immunohistochemistry

Table 3 Pembrolizumab response in patients with microsatellite instability-high cancers and discordant immunohistochemical mismatch repair protein expression results.

CBioPortal ID	Time on pembrolizumab	Best response	Reason for discontinuation
P-0013462	35 months	Complete response	Stopped after 3 years/course completed
P-0012115	12 months	Partial response	Stopped after 1 year/course completed
P-0019598	24 months	Stable disease	Treatment is ongoing

Potential DNA-based reflex testing for microsatellite instability (next generation sequencing, polymerase chain reaction) may be warranted and may uncover microsatellite instability-high status that would otherwise have been missed, along with opportunities for immune checkpoint inhibition therapy. Particularly, colorectal carcinomas undergoing next generation sequencing testing benefit from having microsatellite instability testing integrated into the next generation sequencing test, even if mismatch repair immunohistochemistry shows proficient expression by clinical standards. In addition, when mismatch repair expression status and microsatellite instability polymerase chain reaction results are discordant, deciphering which test result is accurate is challenging. In these scenarios, certain findings including germline or somatic missense mutations in mismatch repair genes or patchy staining may help with clinical decision making (whether to manage the case as microsatellite instability-high/mismatch repair-deficient or mismatch repair-proficient/microsatellite stable). For example, rare next generation sequencing cases may show falsely elevated MSI sensor scores, resulting in a false microsatellite instability-high status. This may be due to low or borderline coverage. Other factors that elevate the MSI sensor score with the version we used include unmatched analysis (no matched blood sample, history of bone marrow transplant). Further scrutiny may reveal a problem with the next generation sequencing/microsatellite instability result in some cases. Yet in other cases, mismatch repair immunohistochemical expression is retained and microsatellite instability-high status is called confidently. In the latter scenario, presence of a mismatch repair gene missense mutation helps to verify the microsatellite instability-high result and explain the discordance.

Mismatch repair protein immunohistochemistry is often performed without microsatellite instability polymerase chain reaction/next generation sequencing in patients with early stage malignancies such as colorectal carcinoma as a screen for Lynch syndrome. The sensitivity of immunohistochemistry remains high in general, yet we have shown that for some patients with Lynch syndrome (including several with a pathogenic germline missense mutation), immunohistochemistry may still show retained mismatch repair protein expression. We recommend further work-up with a DNA-based microsatellite instability assessment method if tumor purity is sufficient when any of the five Revised Bethesda Guidelines criteria are met or if staining is focal and/or weak. If tumor purity and/or coverage is low, the degree of microsatellite instability might be underestimated, and germline sequencing should be offered. Patients with advanced (metastatic or unresectable) malignancies should be offered comprehensive molecular testing that covers targets for currently approved tumor agnostic therapies including *NTRK1-3* fusions and microsatellite instability status, as well as targets for their specific malignancy (for example, extended *RAS* mutation testing in colorectal carcinoma). If tumor purity or coverage is lower than a specific DNA-based assay's limit of detection for microsatellite instability-high status, mismatch repair immunohistochemistry should be considered based on the type of cancer (colorectal, endometrial, and esophagogastric all having relatively high rates of microsatellite instability-high) and the discretion of the oncologist and pathologist.

Rare studies have demonstrated the phenomenon of *MLH1* gene missense mutations (particularly germline mutations) resulting in retained MLH1 expression [12] and occasionally

isolated loss of PMS2 expression [13]. This study confirms that association on a larger scale, yet the mechanism behind the isolated loss of PMS2 expression in *MLH1* germline mutant Lynch patients has yet to be elucidated.

Abnormal dot-like patterns of *MLH1* expression may sometimes be associated with mismatch repair-deficient status, specifically deficient *MLH1* [17, 18]. However, we also identified this pattern of expression in the mismatch repair-proficient/microsatellite stable control group, indicating that dot-like “nucleolar” *MLH1* expression is not specific for abnormal *MLH1* function or microsatellite instability-high status. Since this phenomenon is relatively rare (occurring in only 1 of 108 mismatch repair-proficient/microsatellite stable cases in the current study), further testing for microsatellite instability is warranted.

The main limitation of this study is a relatively low number of discordant mismatch repair immunohistochemistry cases due to rarity, which decreases the power for statistical analyses and makes a retrospective approach necessary.

In conclusion, retained mismatch repair protein expression occurs in ~6% of microsatellite instability-high cases, while another 1% of microsatellite instability-high cases harbor isolated loss of the defective mismatch repair gene’s dimerization partner. Microsatellite instability-high cases with retained mismatch repair protein expression do not have a lower mutation burden or degree of microsatellite instability and benefit from immune checkpoint blockade. The majority of these cases harbor germline or somatic mismatch repair gene missense mutations and often express mismatch repair proteins in a lower percentage of tumor cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the institutional review board at Memorial Sloan Kettering Cancer Center.

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