

Evaluating Mismatch Repair Deficiency in Pancreatic Adenocarcinoma: Challenges and Recommendations



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Abstract

Purpose: Immune checkpoint inhibition has been shown to generate profound and durable responses in mismatch repair deficient (MMR-D) solid tumors and has elicited interest in detection tools and strategies to guide therapeutic decision-making. Herein we address questions on the appropriate screening, detection methods, patient selection, and initiation of therapy for MMR-D pancreatic ductal adenocarcinoma (PDAC) and assess the utility of next-generation sequencing (NGS) in providing additional prognostic and predictive information for MMR-D PDAC.

Experimental Design: Archival and prospectively acquired samples and matched normal DNA from $N = 833$ PDAC cases were analyzed using a hybridization capture–based, NGS assay designed to perform targeted deep sequencing of all exons and selected introns of 341 to 468 cancer-associated genes. A computational program using NGS data derived the MSI status from the tumor-normal paired genome sequencing data. Available

germline testing, IHC, and microsatellite instability (MSI) PCR results were reviewed to assess and confirm MMR-D and MSI status.

Results: MMR-D in PDAC is a rare event among PDAC patients (7/833), occurring at a frequency of 0.8%. Loss of MMR protein expression by IHC, high mutational load, and elevated MSIsensor scores were correlated with MMR-D PDAC. All 7 MMR-D PDAC patients in the study were found to have Lynch syndrome. Four (57%) of the MMR-D patients treated with immune checkpoint blockade had treatment benefit (1 complete response, 2 partial responses, 1 stable disease).

Conclusions: An integrated approach of germline testing and somatic analyses of tumor tissues in advanced PDAC using NGS may help guide future development of immune and molecularly directed therapies in PDAC patients. *Clin Cancer Res*; 24(6): 1326–36. ©2018 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a recalcitrant cancer with a challenging prognosis. It is currently projected to rise from the fourth to the second leading cause of cancer-related mortality in the United States by 2030 (1). Although surgery is the only curative option for PDAC, only 15% to 20% of cases are operable at diagnosis. Even following surgery, reported recurrence is high at approximately 80% to 85%, and the majority of patients after resection succumb to their disease (2, 3). As such, current

research is actively focused on identifying potential new treatment targets and subsets of PDAC patients that may benefit from a specific "targeted" approach. One recently identified subtype within the genomic landscape of PDAC is the mismatch repair-deficient (MMR-D) tumor (4–7).

Evaluation and therapy for MMR-D tumors is of particular contemporary relevance following the recent FDA approval of the programmed cell death-1 (PD-1) inhibitor, pembrolizumab, for the treatment of unresectable or metastatic, microsatellite instability-high (MSI-H) or MMR-D solid tumors that have progressed following prior treatment, and who have no satisfactory alternative treatment options, or with MSI-H or MMR-D colorectal cancer that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan (8). The catalyst for the FDA approval for pembrolizumab stems from the results of a pivotal phase II clinical trial conducted by Le and Diaz that demonstrated the efficacy of PD-1 inhibition in 12 different MMR-D cancer types, with objective radiographic responses in 53% of patients and complete responses in 21% of patients (9).

The advent of checkpoint inhibition as an effective therapy for MMR-D PDACs has been accompanied by numerous questions regarding the appropriate screening, detection tools, patient selection, timing and modality of testing, and initiation of therapy. Herein, we attempt to address these questions and report on the clinical course, prevalence, and treatment of MMR-D PDAC, in

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Translational Relevance

This report reviews the natural history, genomics, and pathology of mismatch repair deficient (MMR-D) pancreatic ductal adenocarcinoma (PDAC) and explores the prognostic and diagnostic utility of IHC, DNA sequencing, and computational microsatellite instability testing in guiding testing and therapy of MMR-D PDAC. In an analysis of $N = 833$ patients, we identified 7 (0.8%) MMR-D PDACs. The cases were associated with germline mutations in MMR genes, IHC loss of MMR protein expression, higher mutational loads, and elevated MSI sensor scores. MMR-D PDAC was noted to have an improved natural history relative to sporadic PDAC and favorable response to immune checkpoint inhibitors. Directed MMR-D testing with bioinformatics analyses from next-generation sequencing and parallel germline testing in the right clinical context of advanced PDAC represent a practical approach to biomarker identification and treatment selection.

the context of a single institution evaluation of the genomic landscape and pathology of MMR-D PDAC.

Materials and Methods

The study protocol was approved by the Institutional Review Board/Privacy Board and patients provided their written informed consent prior to study treatment and related procedures. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. We undertook a comprehensive single-institution review of $N = 833$ consecutive PDAC patients and all MMR-D PDAC tumors at MSKCC between 2006 and 2017. We queried the MSKCC institutional tumor registry and ICD billing database for PDAC with a pathologically or genetically confirmed diagnosis of MMR-D between January 1, 2006, and July 30, 2017. MMR-D status was evaluated through IHC, germline testing, or tumor and germline DNA sequencing using MSK-IMPACT testing. Clinical characteristics were extracted from the medical record.

Sequencing

The MSK-IMPACT assay was conducted as previously described (10, 11). DNA from formalin-fixed paraffin-embedded primary or metastatic tumors and patient-matched normal blood samples was extracted and sheared. Barcoded libraries from tumor and normal samples were captured, sequenced, and subjected to a custom pipeline to identify somatic mutations. Patients were also offered secondary germline analysis after consenting to tumor genetic analysis. Sequencing consisted of deep sequencing of all exons and selected introns of a custom 341 cancer-associated gene panel, with updated panels containing 410 and 468 genes. All exons tested had a minimum depth of coverage of $100\times$. To calculate mutational load, the number of somatic nonsilent protein-coding mutations with exclusion of copy number gene alterations and structural rearrangements were counted. For patients in whom the newer 410-gene or 468-gene panels were applied, the corresponding mutational load was calculated on the basis of the 341-gene panel via subtraction of mutations occurring in the 69 or 127 additional genes present on the 410- or 468-gene panels, respectively.

DNA MMR and microsatellite instability analyses

IHC staining of PDACs were performed using the standard streptavidin–biotin–peroxidase procedure. MMR IHC antibodies included MLH1 (Ventana, clone M1), MSH2 (Cell Marque, clone G219.1129), MSH6 (Ventana, clone 44), and PMS2 (BD Biosciences, clone A16.4). Detection systems were Bond Polymer Refine Detection (Newcastle Upon Tyne, UK) for MLH1 and PMS2, and iVIEW DAB Detection Kit (Roche Diagnostics) for MSH2 and MSH6. Tumors showing total absence of nuclear staining, with adjacent benign tissue showing nuclear staining, were scored as negative for expression of that protein. An experienced pathologist at MSKCC (JS) interpreted the histopathology and IHC results.

MSI analysis was performed using either the five microsatellite loci (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) by PCR reaction or MSI sensor analysis. With microsatellite instability PCR (MSI PCR) testing, fluorescently labeled products were detected and sized by capillary electrophoresis. Microsatellite instability at ≥ 2 loci was defined as MSI-H, instability at a single locus was defined as MSI-L, and no instability at any of the loci tested was defined as MSS. MSI sensor interrogated the length distribution of all genomic microsatellite loci included in the MSK-IMPACT capture region across tumor and matched normal (12). MSI-H was defined as $>10\%$ of the microsatellite loci showing microsatellite instability.

Statistical analysis

Descriptive statistics summarize the patient populations. A nonparametric Spearman correlation was used to calculate the correlation coefficient between the mutational load and the MSI sensor score.

Results

Patient characteristics

Out of 833 patients, we identified seven PDAC patients with MMR-D (0.8%) confirmed through IHC, germline testing, or germline next-generation sequencing (NGS) testing (Table 1). All seven patients had Lynch syndrome (LS). Five of the seven (71%) MMR-D PDAC cases had an extensive personal or family history of cancer. Patient 4 was identified to have a germline mutation in *PMS2* and notably had no prior personal or family history of cancer.

The 5 MMR-D PDAC patients with NGS data available all had a mutational load greater than 50, with a median mutational load of 53 (range, 48–81), an average mutational load of 59, and an average of 62.304 mutations per megabase (Mb). In comparison, the mutational load for the remaining PDAC patients were lower with a median mutation number of 4 (range, 0–14). MSI sensor testing of 785 available PDAC cases also revealed that the five MMR-D PDAC patients, with the exception of Patient 5, all had a MSI sensor score greater than 15.

We also identified two patients with LS who developed MMR-P PDAC. Patient 8 who had a germline *PMS2* mutation developed a tumor that retained intact MMR protein expression on IHC. MSK-IMPACT and MSI sensor score evaluations revealed a mutational load of 13 and 1.14, respectively. Patient 9 had a germline *PMS2* and *BRCA2* mutation and also developed a MMR-P PDAC. MSK-IMPACT and MSI sensor score showed a mutational load of 9 and 0.52, respectively.

Table 1. Case analyses of mismatch repair mutations in PDAC

Patient	Age at diagnosis (years)	Germine pathologic mutations	Stage	Mutational load (based on 341 panel)	MSI sensor score	Personal cancer history	Family history	Pathology	IHC	MSI-PCR	OS (m) as of 8/2017
1 ^a	45	<i>MLH1</i>	III	48	30.33	None	Meets revised Bethesda guidelines	Adenocarcinoma, moderately differentiated	Insufficient tissue	-	36 (alive)
2 ^a	75	<i>MSH2</i>	IIIB	53	24.06	Bladder ca, gastric ca, prostate ca, tubulovillous adenoma	Meets revised Bethesda guidelines	Adenocarcinoma, conventional ductal type with focal mucinous features, moderately differentiated, the tumor appears to arise in association with a pre-existing IPMN, tumor extends into perineural and vascular invasion is present, 14/24 nodes positive	MLH1: present MSH2: absent MSH6: absent PMS2: present equivocal NR-24: stable	BAT-26: stable NR-21: unstable BAT-25: stable MON-27:	134 (alive)
3 ^a	42	<i>MSH2</i>	IIIB	60	22.18	BCC, CRC, keratoacanthoma	Meets revised Bethesda guidelines	Adenocarcinoma, 12/17 nodes positive	-	-	320 (alive)
4	63	<i>PMS2</i>	IIIB	81	16.9	None	Does not meet revised Bethesda guidelines	Adenocarcinoma, moderately differentiated with mixed features of tubular and colloid carcinoma, the carcinoma arises in an IPMN intestinal type, tumor extends into peripancreatic soft tissues, no vascular invasion is identified, perineural invasion is present, 3/19 nodes positive	MLH1: present MSH2: present MSH6: present PMS2: absent	-	76 (alive)
5 ^a	88	<i>MSH6</i>	III	53	6.45	None	Meets revised Bethesda guidelines	Poorly differentiated ductal type adenocarcinoma exhibiting fused glands present in a necrotic background	-	BAT-26: stable NR-21: stable BAT-25: unstable MON-27: stable NR-24: stable -	2 (alive)
6	67	-	IIIB	-	-	CRC	Meets revised Bethesda guidelines	Intraductal papillary mucinous carcinoma with invasive tubular type adenocarcinoma, tumor extends into peripancreatic soft tissues, no perineural or vascular invasion seen, 1/13 nodes positive	MLH1: present MSH2: absent MSH6: absent PMS2: present	-	45
7	76	-	IIA	-	-	BCC, CRC, RCC, uterine ca	Does not meet revised Bethesda guidelines	Invasive adenocarcinoma, moderately differentiated, in situ carcinoma is identified, intraductal papillary carcinoma, tumor extends into peripancreatic soft tissues, no vascular invasion is identified, perineural invasion is present, 0/1 node positive	MLH1: present MSH2: absent MSH6: absent PMS2: present	-	63

(Continued on the following page)

Table 1. Case analyses of mismatch repair mutations in PDAC. (Cont'd)

Patient	Age at diagnosis (years)	Germline pathologic mutations	Stage	Mutational load (based on 341 panel)	MSIsensor score	Personal cancer history	Family history	Pathology	IHC	MSI-PCR	OS (m) as of 8/2017
8 ^a	68	PMS2	IV	13	1.14	Uterine ca, CRC	Meets Bethesda guidelines	Adenocarcinoma, moderately differentiated, associated with marked necrosis	MSH2: present MLH1: present PMS2: present MSH6: present	-	29
9	74	PMS2	IIA	9	0.52	None	Does not meet revised Bethesda guidelines	Ductal adenocarcinoma, conventional (tubular type), poorly differentiated, tumor extends into peripancreatic soft tissues, no vascular invasion is identified, perineural invasion is present, 0/11 nodes positive	-	-	38

Abbreviations: BCC, basal cell carcinoma; ca, cancer; OS indicates months of overall survival after diagnosis; RCC, renal cell carcinoma; m, months.

^aImmunotherapy treated.

The reported median mutational load using the 341-gene panel in MMR-P PDACs was lower compared with MMR-P colorectal cancers; four (range 0–14) vs. six (range 0–17), respectively. The median mutational load of 50 (range 20–90) in MMR-D colorectal cancer was similar to that in MMR-D PDAC [54 (range 48–81)]. Mutational load distribution of the 831 PDAC patients with NGS data is depicted in Fig. 1. Spearman's rank correlation analysis of mutational load and MSIsensor scores found a weak correlation ($\rho = 0.258$; Fig. 2).

Stadler and colleagues had previously reported three ultramutator phenotype colorectal cancer tumors with >150 mutations, each of which had a *POLE* P286R hotspot mutation (13). We found no ultramutator phenotype cases in our study. The PDAC cases with the highest mutation burdens were the five MMR-D PDAC cases. We also identified one patient with no prior germline testing that harbored a pathogenic *BRCA2* mutation with a high mutational load of 54 and MSIsensor score of 14.9, respectively. We present selected cases from Table 1.

Patient 1. A 45-year-old woman with known LS and an *MLH1* mutation was diagnosed with locally advanced PDAC in July 2014. The patient was treated with FOLFIRINOX and FOLFIRI in a neoadjuvant context with stable disease burden and declining tumor markers. She also received stereotactic body radiation (SBRT) 3,300 cGy in five fractions. In October 2015, she had re-emergence of back pain along with a progressive rise in CA 19-9 and enlargement of the mass in the pancreatic body and new small volume ascites. She was subsequently enrolled in a clinical trial with the combination of an anti-PD-L1 antibody and an IDO1 inhibitor in February 2016. Since initiation of immunotherapy, the patient's pain has resolved, CT scans have demonstrated a partial response and CA19-9 levels have durably normalized for over 22 months, to the present time.

Patient 2. A 75-year-old man with LS with a *MSH2* mutation underwent a distal pancreatectomy and splenectomy in May 2006 for AJCC stage IIB PDAC and completed 6 months of adjuvant gemcitabine and capecitabine. He was subsequently diagnosed with LS after germline testing. In March 2007, the patient enrolled in a clinical trial of vaccine therapy with irradiated allogeneic pancreatic tumor cells transfected with the granulocyte-macrophage colony stimulating factor gene (GVAX). In July 2010, a biopsy of enlarging retroperitoneal lymph nodes revealed recurrent/metastatic PDAC. He was treated with gemcitabine and capecitabine, retroperitoneal lymphadenectomy, and a course of 4,500 cGy in 25 fractions. During his treatment course for PDAC, the patient was also diagnosed with and treated for invasive bladder cancer and localized gastric cancer.

In June 2015, CT showed increased size of a left lower lobe nodule and increased retrocausal soft tissue adenopathy and rise in CA 19-9 consistent with metastatic PDAC. The patient enrolled in a clinical trial of anti-PD-1 therapy in patients with MSI-H tumors in September 2015. By RECIST, the patient has had a complete response to therapy and is completing 2 years of anti-PD-1 antibody therapy.

In February 2016, MRI findings showed a new mass around the prostate gland that was subsequently confirmed on biopsy to be locally advanced prostate cancer. The patient has been treated with androgen deprivation therapy (ADT) with good response. Of note, this latter malignancy was noted to have developed during anti-PD-1 therapy for his metastatic PDAC. NGS and IHC

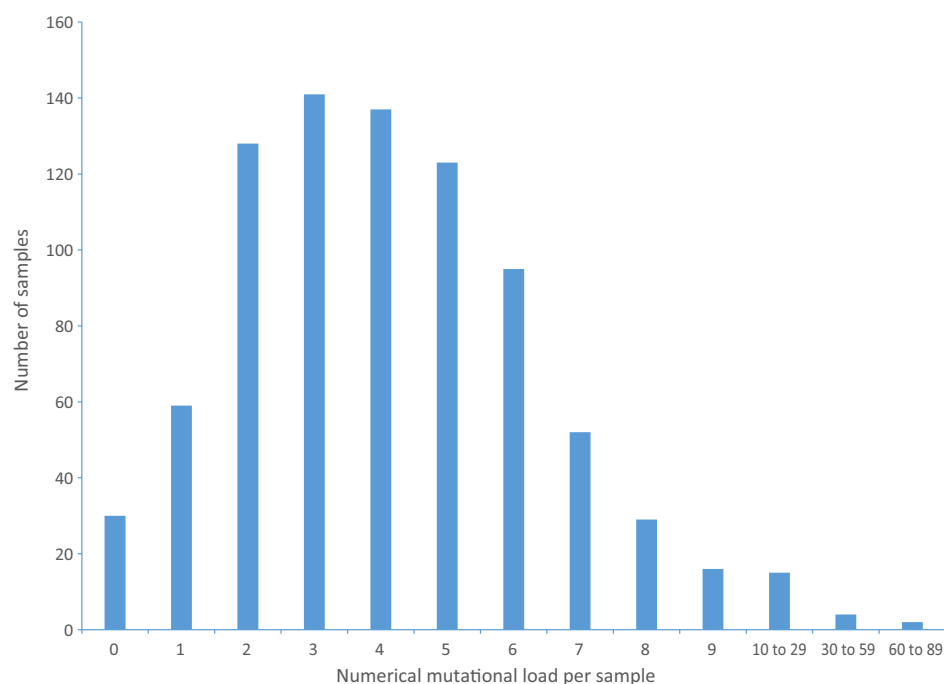


Figure 1. Distribution of mutational load among PDAC cases (N = 831).

confirmed that the prostate cancer had a high mutational burden of 73, high MSI sensor score of 23.91, and showed absence of MSH2 and MSH6 on IHC. Prostatic biopsy revealed a Gleason score of 9 (4+5) and 8 (4+4) in 5 of 13 and 4 of 13 specimens, respectively.

Patient 3. A 42-year-old woman with LS and an MSH2 mutation underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy (TAH/BSO) for a benign tumor of the right ovary and was incidentally found to have a mass in the pancreas in October 1990, which was confirmed by biopsy to be PDAC. She

subsequently underwent an exploratory laparotomy whereupon she was also found to have a separate right colorectal cancer. A distal pancreatectomy and hemicolectomy were done and the patient received adjuvant 5-fluorouracil and 4,000 cGy in 20 fractions. The patient was followed locally for 26 years with no evidence of recurrence of the colorectal cancer or PDAC.

In October 2016, she was found to have elevated liver function test values and further imaging revealed a biliary obstruction at the confluence of the right and left hepatic duct with a mass centered within the pancreatic head. The mass was confirmed to be locally unresectable PDAC with involvement of the portal vein

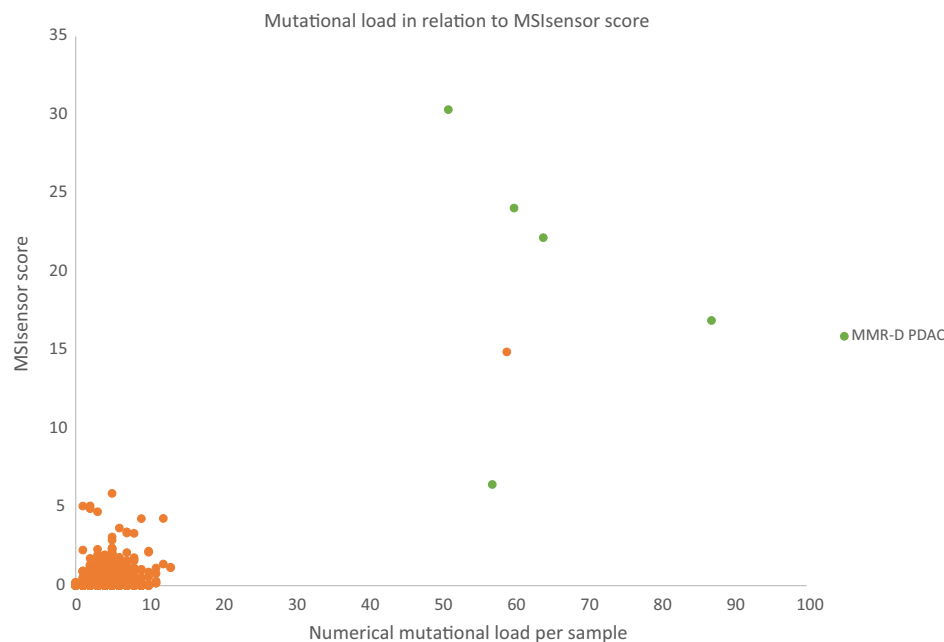


Figure 2. Mutational load in relation to MSI sensor score in PDAC cases (N = 785; ρ = 0.258).

and retroperitoneal and mesenteric lymph nodes. The patient was treated with neoadjuvant gemcitabine/nab-paclitaxel before commencing anti-PD-1 therapy. After initiation of immunotherapy, CA19-9 levels have normalized for the past 2 months.

Patient 4. A 63-year-old man with no known significant family or personal history of malignancy was diagnosed with an intraductal papillary mucinous neoplasm (IPMN) in 2006. In 2011, he was identified to have developed PDAC arising from the IPMN. He underwent a pancreaticoduodenectomy for AJCC stage IIB PDAC and received a combined modality therapy course of gemcitabine, capecitabine, and 4,500 cGy radiation in 25 fractions. In March 2012, imaging revealed new retroperitoneal lymphadenopathy biopsy confirmed as metastatic PDAC. The patient received FOLFOLX followed by maintenance chemotherapy with 5-fluorouracil and leucovorin until May 2013. The patient has been on observation since this time without disease progression. In 2016, MSK-IMPACT testing identified the patient as having a germline *PMS2* mutation with a mutational load of 81 and MSI sensor score of 16.9. IHC of his tumor also revealed loss of the *PMS2* protein.

Patient 5. An 88-year-old woman was diagnosed with locally advanced PDAC with large necrotic lymph nodes surrounding the celiac axis in June 2017. NGS of the PDAC revealed an *MSH6* mutation with a high mutational load of 53, but an intermediate MSI sensor score of 6.45. MSI PCR showed an MSI-L PDAC. Further germline testing confirmed a germline *MSH6* mutation. The patient subsequently reported a family history of one sister diagnosed with endometrial cancer and a nephew diagnosed with colorectal cancer in his forties. The family, however, was never previously tested for LS. After anti-PD-1 therapy was initiated, imaging has demonstrated significant regression of known disease and normalization of CA19-9 levels.

Patient 8. A 68-year-old woman with known LS with a *PMS2* mutation was found on surveillance MRI for branch duct IPMN to

have developed metastatic PDAC to the liver in June 2014. Her mutational load and MSI sensor score were 13 and 1.14, respectively. The patient was treated with courses of FOLFIRINOX, gemcitabine, and nab-paclitaxel. In March 2016, the patient enrolled in a clinical trial with the combination of a humanized monoclonal antibody targeting the CC chemokine receptor 4 (CCR4) and a PD-L1 inhibitor. In May 2016, CT imaging showed an increase in hepatic tumor burden and the patient was taken off trial. She continued to have progression of disease despite multiple lines of chemotherapy and died from her disease in December 2016.

Discussion

MMR-D in PDACs

Existing literature on MMR-D PDAC is limited and generally discordant with regard to its prevalence, implications for natural history, morphologic pathology, and pathogenesis. Because of different and evolving detection methods and sample sizes, reported rates of MMR-D in PDAC adjudicated through IHC and MSI testing have ranged widely from 0% to 75% (14–20) as outlined in Table 2. In the following discussion, we review the current literature on MMR-PDAC as well as place our own findings in context and provide recommendations for testing.

MMR-D PDAC patients have been reported to have a significantly prolonged survival time (17, 21). Nakata and colleagues reported that MSI-H PDAC compared favorably to MSI-L PDAC with survival times of 62 months vs. 10 months, respectively; $P = 0.011$ (17). In our study, we found MMR-D PDAC compared with MMR-P PDAC to have a tendency to be associated with IPMN and localized disease at presentation as well as a favorable natural history, with a mean survival time of 96.6 months (range 2 to 320 months). None of the MMR-D PDACs in our series presented *de novo* with metastatic disease. Limitations of small patient numbers notwithstanding, our data suggest that MMR-D PDAC represents a very different natural history compared with sporadic PDAC.

Table 2. Selected mismatch repair deficiency studies in PDAC

Author (year)	Cases (n)	Number of MSI/MMR-D PDAC (%)	Methodology	Selection	Lynch syndrome
Goggins (1998) (18)	82	3 (3.7%)	MSI PCR ^a	Unselected	—
Ghimenti (1999) (45)	21	0 (0%)	MSI PCR ^b , genetic analysis	Unselected	—
Wilentz (2000) (22)	18	4 (22.0%)	MSI PCR ^c , IHC	Selected (medullary histology)	—
Yamamoto (2001) (21)	103	16 (15.5%)	MSI PCR ^d , genetic analysis, IHC	Partially selected (3 LS pts added to series of $N = 100$ pts)	3
Nakata (2002) (17)	46	8 (17.4%)	MSI PCR ^e	Unselected	—
Tomaszewska (2003) (46)	30	0 (0%)	IHC	Unselected	—
Maple (2005) (23)	35	3 (8.6%)	MSI PCR ^f , IHC, genetic analysis	Selected (long-term survivors, ≥ 3 years)	2
Laghi (2012) (19)	338	1 (0.3%)	MSI PCR ^g , IHC	Unselected	—
Riazzy (2015) (47)	265	41 (15.5%)	IHC with TMA	Unselected	—
Connor (2017) (4)	255	4 (1.6%)	MSI PCR ^f , NGS, IHC,	Unselected	3
Eatrides (2016) (20)	109	24 (22.0%)	IHC with TMA	Unselected	—
Humphris (2017) (7)	385	4 (1.0%)	NGS, IHC, MSI sensor	Unselected	—
Hu (2018) (this study)	833	7 (0.8%)	NGS, IHC, MSI PCR ^g , MSI sensor	Unselected	7

Abbreviation: TMA, tissue microarray.

^aOne mononucleotide marker, four dinucleotide markers.

^bTen dinucleotide markers.

^cTwo mononucleotide markers, one dinucleotide marker.

^dTwo mononucleotide markers, three dinucleotide markers.

^eEight dinucleotide markers.

^fFour mononucleotide markers, five dinucleotide markers, one complex repeat marker.

^gFive mononucleotide markers.

MMR-D PDAC has also been reported to have a medullary histology associated with the wild-type *K-ras* gene (18, 19, 22). However, other reports have noted that MMR-D PDAC histology as being either well-differentiated or largely indistinguishable from MMR-P PDAC (17, 23). Pathologic analysis of the MMR-D PDACs in our study showed no specific uniform histologic features.

Reports have varied on whether MMR-D PDAC arise largely from germline or sporadic mutations. Humphris and colleagues interrogated 385 PDAC genomes and identified four (1%) MMR-D tumors (7). The authors identified private somatic events as the underlying cause of MMR-D in all four cases. In contrast, Connor and colleagues reported that of their four MMR-D cases, three had germline and one had a somatic mutation. Maple and colleagues also found that all 3 tumors with MMR-D in their study had some evidence of a germline mutation (23). *MLH1* promoter hypermethylation has also been reported as a cause of sporadic MSI-H in MMR-D PDAC. Yamamoto and colleagues detected *MLH1* promoter hypermethylation in six of their 13 sporadic MSI-H PDACs but none in the germline MMR-D and MMR-P PDAC (21). When the authors analyzed *MLH1* expression in 10 MSI-H PDACs, they found that the *MLH1* protein was barely detectable in any of the five tumors with *MLH1* promoter hypermethylation. They concluded that *MLH1* promoter hypermethylation was likely associated with sporadic MSI-H PDAC.

Our analysis of the genomic landscape of PDAC and MMR-D PDAC has identified MMR-D in seven (0.8%) of 833 patients. This is significantly less than that found in colorectal cancer where MMR-D was reported in 28 (13%) out of 224 patients (13). All of the MMR-D PDACs in our analysis were also found to have arisen in the context of LS, from germline mutations in MMR genes. In contrast, the majority of MMR-D colorectal cancers arise sporadically from somatic inactivation of the MMR pathway (24). Given the relative rarity of MMR-D PDACs, however, we cannot entirely exclude the possibility that MMR-D PDAC can also arise from somatic mutations as reported in previous studies (7, 21).

In addition, NGS analysis from the MSK data reveals that mutational load is not uniformly distributed in PDACs. Ninety seven percent of all sequenced PDACs had a mutational load of less than 9. All five MMR-D PDACs that underwent NGS testing had a mutational load above 48, ≥ 50 mutations/Mb, and with the exception of Patient 5, an MSI sensor score above 10. However, we did not find a strong correlation between the MSI sensor score and mutational load in the overall PDAC population ($\rho = 0.258$).

Immunotherapy in MMR-D PDACs

Among our study population, consistent with prior studies, MMR-D PDAC patients were found to have a favorable response to immunotherapy (4, 25). Patients 1, 2, and 8 had striking, durable and ongoing responses to immunotherapy and patient 3 has stable disease. The relative rarity of MMR-D PDACs and low mutational load of PDACs overall, however, has limited the use of immunotherapy in the clinical setting. One hypothesis for the better prognosis of MMR-D tumors to immunotherapy is that the increased mutational load in MMR-D tumors serves as a source for increased generation of mutation-associated neoantigens (MANA) that can be targeted by the immune system. This is consistent with reports of high numbers of MANA in immunogenic cancers, such as NSCLC and melanoma, that are responsive to checkpoint blockade (26, 27). Pharmacologic agents that target

DNA repair and replication to increase the overall tumor mutational load in MMR-P PDACs may potentially enhance tumor immunogenicity (28).

A number of other tumor-cell-intrinsic and tumor-cell-extrinsic factors contribute to determine response and resistance to checkpoint blockade therapy in MMR-D tumors. Of note, although Patient 2 responded to anti-PD1 therapy for his MMR-D PDAC, he also developed a MMR-D prostate cancer with a high mutational load while on immunotherapy.

Further use of NGS for both immunotherapy-resistant and responsive MMR-D tumors may play a key role in identifying potential therapeutic targets involved in cancer immunoeediting. For instance, NGS testing of Patient 1, who presented with a locally advanced PDAC, also identified a mutation in the $\beta 2$ -microglobulin (*B2M*) gene. *B2M* mutations in LS-associated MSI-H CRCs have been associated with immune evasion through MHC I loss (29). However, the significance of *B2M* and other mutations in MMR-D PDAC tumors in influencing clinical course and immunotherapy response is unknown and require further elucidation. As immunotherapy becomes increasingly used, NGS of may prove to be an invaluable tool in gaining temporal insight on the type and number of mutations involved in immune escape and response.

Detection of MMR-D tumors in PDAC

Unlike in colorectal cancer, where NCCN guidelines have recommended universal screening for LS in all colorectal cancers, PDACs are not routinely tested for MMR-D (30). As such, when PDACs are evaluated for MMR-D, IHC, and MSI PCR tests that were originally optimized for colonic cancer and not pancreatic cancer are typically used (31).

IHC evaluation assesses the absence of MMR protein expression on the tumor tissue (32). However, IHC interpretation can be limited by variations in tissue fixation and staining as well as adequacy of available tissue, often a particular challenge in PDAC. The extent of IHC measurement is also dependent on the specific panel of antibodies used for staining. The absence of IHC staining in a small biopsy sample may not be a reliable measure of protein loss in the entire tumor. IHC testing in extra-colonic tissues have been reported to have a weaker internal control compared to colonic tissues (32). Many extra-colonic tissues are less proliferative compared to colon tissue and as result, have a lower degree of MMR protein expression.

The MSI-H phenotype is a consequence of an increased incidence of insertions or deletions within microsatellite sequences caused by MMR-D. MSI PCR testing is used to detect MSI-H tumors by PCR amplification of a panel of five microsatellite markers, ("the Bethesda panel") consisting of two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123, and D17S250) repeats (33). An alternative panel is the MSI Analysis System, which replaces the dinucleotide markers with mononucleotide markers (NR-21, NR-24, and MONO-27). Tumors with instability in two or more of these markers are listed as MSI-H, whereas those with one unstable marker are described as MSI-L. Advantages of MSI testing include reproducibility and the potential to identify tumors with MMR-D in genes besides *MLH1*, *MSH2*, *MSH6*, or *PMS2*. Furthermore, MSI testing can detect tumors with defective MMR but intact staining secondary to a nontruncating missense mutation (34). However, in some LS tumors, MSI testing has been reported as negative despite evidence of MMR protein loss on IHC (35). One example is the

MSH6 mutant tumor, which will typically have a lower level of MSI compared to other mutants (36).

Newer tools for detecting MMR-D in PDAC include NGS and computational programs to quantify MSI (7, 10, 12). For example, the Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay performs targeted deep sequencing of all exons and selected introns in 341 to 468 key cancer genes. Causative germline variants in MMR-D tumors can also be detected through MSK-IMPACT, facilitating the distinction between sporadic and LS cancers (37). The frequency of mutations or mutational load generated in tumors can be quantified through MSK-IMPACT and has been shown to reliably detect MMR-D in colorectal cancer (13). Stadler and colleagues reported a sensitivity of 1.0 (95% CI, 0.93–1.0) and specificity of 1.0 (95% CI, 0.93–1.0) using a mutational load cutoff of ≥ 20 and < 150 for MMR-D detection in either a 341-gene panel or a 410-gene panel (13). NGS has also been used to identify distinct mutational signatures, including a MMR-D subtype, in PDACs (4, 38).

A number of computational programs such as MANTIS, mSINGS, and MSIsensor have been specifically developed to quantify MSI in tumor samples (12, 39, 40). For instance, MSIsensor computes and compares the length distributions of microsatellites per site in paired tumor and normal sequence data to quantify the percent of somatic sites with significantly different distributions. Compared to traditional MSI PCR testing, these programs allow for integration into existing NGS pipelines and assessment of more microsatellite loci. Middha and colleagues demonstrated that MSI status could be inferred from NGS data across multiple solid tumor types (41). The advantages and

Table 3. Advantages and disadvantages of mismatch repair deficiency testing methods in PDAC

Advantages	Disadvantages
Immunohistochemistry	
Widely available	Limited by variations in tissue fixation and staining
Inexpensive	Limited by antibodies available
Reproducible	Limited by biopsy size
Rapid turn-around time	
More sensitive than MSI PCR testing in detecting absence of <i>MSH6</i>	
MSI PCR testing	
Reproducible	Not able to detect specific gene that is mutated
Can detect MSI-H tumors that have intact MMR staining on IHC	In extracolonic tumors, may test negative despite positive IHC
Fast turn-around time	
Next-generation sequencing	
Can determine exact somatic and germline mutations	Expensive
Can also be used to assess level of mutational load and MSI	Not widely available
Identifies actionable mutations	Longer turnaround time

disadvantages of current testing methods for MMR-D in PDACs are summarized in Table 3.

Which diagnostic tests to use for evaluation of MMR-D/MSI-H PDAC?

Prior studies have largely identified MMR-D PDAC patients through MSI PCR using a range of microsatellite markers and selection criteria. However, in the context of immunotherapy

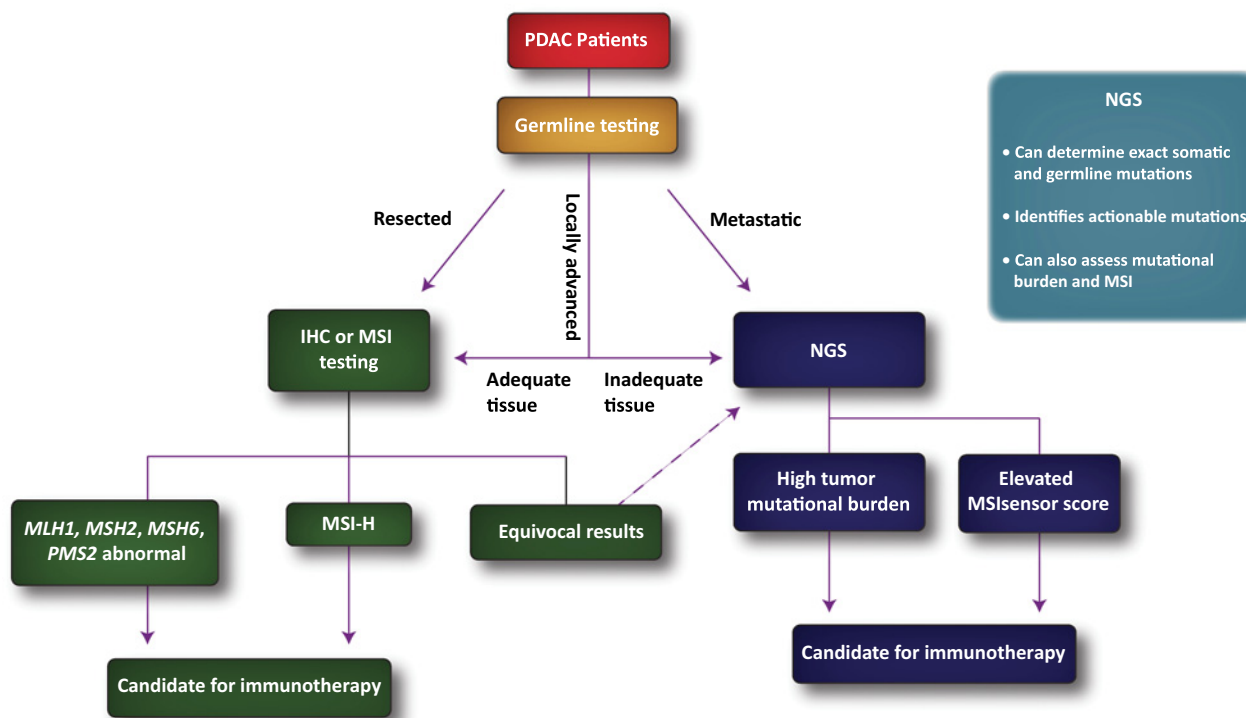


Figure 3. Suggested algorithm for evaluation of MMR-D in PDAC.

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response, IHC, PCR testing, and NGS are distinct methods that measure MMR-D, MSI, and mutational load, respectively. Currently, it is unclear which method and quantifier is optimally prognostic of clinical course and immunotherapy response. Herein, we propose a testing algorithm to evaluate MMR-D/MSI-H PDAC patients (Fig. 3). Of specific note, we recommend (i) all PDAC patients be considered for germline testing, and (ii) metastatic or locally advanced PDAC patients be considered for NGS testing, in the right clinical context of having an adequate performance status and being candidates for tumor-directed therapy.

We recommend that all PDAC patients be considered for germline testing for MMR-D and other germline mutations given the significant implications of the diagnosis of LS for the patient and the family as well as its effect on therapy choice. Recent studies have reported germline mutations occur in up to 21.5% of PDAC patients (42, 43). Germline testing also revealed MMR mutations in the case of Patient 4 despite the notable lack of any personal or family history of malignancy.

For patients with metastatic or locally advanced PDACs, we recommend that NGS be performed as a primary diagnostic test. Patient 5's case is illustrative of a scenario where NGS played a significant role in determining therapy. IHC to evaluate for the MMR-D status, PD-L1 status, and TILs was not feasible in this patient given insufficient tissue despite prospective tissue acquisition for both diagnostic and sequencing purposes. Both MSI PCR testing and MSIsensor identified an MSI-L tumor and low MSIsensor score. Patient 5 had an *MSH6* mutation, which has been reported to result in low or weak MSI in tumors. Using MSI PCR testing, this patient would likely not have been considered a candidate for immunotherapy. NGS testing, however, demonstrated that the patient had a high mutational load of 53, and the patient was started on anti-PD-1 therapy.

NGS allows for parallel assessment of mutational load, specific actionable mutations, and germline mutations within the tumor. As the overall costs for NGS decreases, we anticipate that NGS will become increasingly available in the future and arguably, ultimately the preferred choice for clinical testing given other useful information (44). If NGS shows that the tumor has a high mutational load and/or high MSIsensor score, the patient should be considered for immunotherapy with an unanswered question as to where immunotherapy should be sequenced related to cytotoxic therapy use.

For patients with resected PDACs, IHC or MSI PCR should be performed to determine MMR-D and MSI status after initial germline testing. If the results of either IHC or MSI PCR are equivocal, we recommend further confirmation with NGS. If either IHC or MSI PCR is abnormal and confirms the diagnosis of a MMR-D tumor, the PDAC patient should be considered a candidate for immunotherapy pending disease course.

For MMR-D PDAC patients who have had their tumors resected and have already completed standard therapy, we recommend surveillance. There is insufficient evidence at present to indicate that adjuvant immunotherapy would reduce the risk of recurrence; nonetheless immunotherapy would be a logical treatment at relapse.

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of MMR-D in PDAC is a rare event and from our comprehensive analysis is present at a frequency of <1% of all PDAC patients and is typically associated with a germline mutation in MMR genes, IHC loss of MMR expression, a higher mutational load and elevated MSIsensor score. These characteristics speak to an improved natural history compared with sporadic PDAC and MMR-D represents a robust biomarker in PDAC to predict for response to checkpoint inhibition. We also endorse strong consideration for routine germline testing in PDAC given the high frequency of germline findings beyond LS in PDAC and such testing represents arguably the most straightforward approach to a LS diagnosis in PDAC without extensive tissue sampling.

What form of testing (IHC vs. MSI-PCR vs. NGS) and which measure (MMR-D vs. MSI-H vs. mutational load) is used to determine initiation of immunotherapy is controversial and evolving. However, we would argue that bioinformatics analyses from NGS represents a pragmatic primary and complementary approach that facilitates simultaneous germline and mutational load evaluation. It offers the additional advantages of potential therapeutic target identification beyond MMR-D and is likely to emerge as a future standard.

Disclosure of Potential Conflicts of Interest

M.A. Lowery is a consultant/advisory board member for Agios. L.A. Diaz is an employee of and has ownership interests (including patents) at PGDx, and is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

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