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Hypermutation in pancreatic cancer

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Pancreatic cancer is molecularly diverse, with few effective therapies. Increased mutation burden and defective DNA repair are associated with response to immune checkpoint inhibitors in several other cancer types. We interrogated 385 pancreatic cancer genomes to define hypermutation and its causes. Mutational signatures inferring defects in DNA repair were enriched in those with the highest mutation burdens. Mismatch repair deficiency was identified in 1% of tumors harbouring different mechanisms of somatic inactivation of *MLH1* and *MSH2*. Defining mutation load in individual pancreatic cancers and the optimal assay for patient selection may inform clinical trial design for immunotherapy in pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival of less than 5% with therapies offering only incremental benefit¹, potentially due to the diversity of its genomic landscape²⁻⁴. Recent reports link high mutation burden with response to immune checkpoint inhibitors in several cancer types⁵. Defining tumors that are hypermutated with an increased mutation burden and understanding the underlying mechanisms in pancreatic cancer has the potential to advance therapeutic development, particularly for immunotherapeutic strategies.

Whole genome (WGS, n=180) and whole exome (n=205) sequencing of 385 unselected predominantly sporadic PDAC (Supplementary Table 1) defined an average mutation load of 1.8 and 1.1 mutation per megabase (Mb) respectively (Supplementary Table 2). Outlier analysis identified 20 samples with the highest mutation burden (5.2%, 15 WGS and 5 exome) (Table 1 and Supplementary Figure 1A), 5 of which were considered extreme outliers and classified as hypermutated as they contained ≥ 12 somatic mutations/Mb, the defined threshold for hypermutation in colorectal cancer⁶. Immunohistochemistry (IHC) for mismatch repair (MMR) proteins (MSH2, MSH6, MLH1 and PMS2) identified 4 MMR deficient tumors, all of which were hypermutated (n = 180, Figure 1).

KRAS mutation status and histopathological characteristics have been associated with MMR deficient pancreatic tumors⁷. Of the 4 MMR deficient tumors in our cohort, 2 were *KRAS* wild type; 3 had undifferentiated to moderately differentiated histology and one a signet-ring component. These features were not predictive of MMR deficiency in our cohort as 11 additional non-MMR deficient tumors had a signet-ring cell component or colloid histology and 131 of 347 assessable tumors had poorly- or un-differentiated histology.

Mutational signature analysis can detect MMR deficiency indirectly based on somatic mutations⁸. An MMR deficient signature dominated the MMR deficient tumors (with WGS), and was minimal in MMR intact tumors (Supplementary Figure 1). In addition, microsatellite instability (MSI), a hallmark of MMR deficiency in colorectal cancer, was detected in all three MMR deficient tumors with WGS using MSIsensor⁹ (Supplementary Table 2). MSI was not identified for the fourth MMR deficient sample potentially due to the reduced number of microsatellite loci in exome data.

The underlying causes of MMR deficiency in the 4 cases were private somatic events. For 2 cases *MSH2* was disrupted by different structural rearrangements, 1 case contained a missense *MSH2* mutation and the last, methylation of the *MLH1* promoter (Figure 1). The missense mutation caused an *MSH2* splice acceptor site mutation that alters the same nucleotide reported to result in a pathogenic skipping of exon 13 in germline studies¹⁰. Hypermethylation of the *MLH1* promoter is the predominant mechanism of MSI in sporadic colon cancer¹¹. The remaining hypermutated tumor contained an intact MMR pathway, and was a cell line (ATCC, CRL-2551) with an unidentified mutational signature; therefore the high mutation burden in this sample may be the result of long term cell culture.

The 15 samples (11 WGS and 4 exome) identified in the outlier analysis with high mutation burden, but not hypermutated (~4 - 12 mutations/Mb) contained no evidence of MMR deficiency. Mutational signature analysis of the WGS samples indicated homologous recombination (HR) repair deficiency as the most substantial (range 1.0 - 3.4 mutations/Mb) contributor to the mutation burden for 8 WGS mutation load outlier tumors. In support of a HR defect⁴; 7 of these tumors contained high levels of genomic instability

with >200 structural variants and mutations in genes involved in HR were present for 6/8 cases (Supplementary Table 2). In addition, one case that had undergone exome sequencing had a somatic *BRCA2* nonsense mutation that likely contributed to HR deficiency in this sample. A mutational signature associated with T>G mutations at TT sites previously described in other cancers, including esophageal cancer¹² was the major contributor (>3 mutations/Mb) in 2 samples. For these two and the remaining 4 cases, no potential causative event could be identified.

Although germline defects in MMR genes are well reported in pancreatic cancer¹³ in our cohort, they did not contribute to MMR deficiency even in those with familial pancreatic cancer or a personal or family history of Lynch-related tumors. A germline truncating variant was detected in *PMS2* for 1 case but did not have loss of the second allele, therefore had normal IHC staining and did not display a MMR mutational signature (Supplementary Table 2). MMR deficiency is important in the evolution in a small, but meaningful proportion of pancreatic cancers with a prevalence of 1% (4/385) in our cohort. This is consistent with recent studies using the Bethesda PCR panel¹⁴, and with previous estimates of MSI prevalence of 2-3%¹⁵. However, in tumors with low epithelial content that underwent exome sequencing, the sensitivity of somatic mutation detection is reduced, which will affect mutation burden and signature analysis. Whilst cognizant of small numbers, immunohistochemistry was the most accurate in defining MMR due to multiple genomic mechanisms of MMR gene inactivation. Multiple methods to define MMR deficiency may be required for clinical trials that aim to recruit MMR deficient participants for clinical trials examining checkpoint inhibitors or other therapies in pancreatic cancer. Homologous recombination deficient tumors, and those with a novel signature seen in esophageal cancer had an increased mutation burden, and need further evaluation as potential patient selection markers for clinical trials of checkpoint inhibitor and other therapies that target tumors with a high mutation burden.

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List of abbreviations:

APGI	Australian Pancreatic Genome Initiative
DNA	Deoxyribonucleic acid
HR	Homologous recombination
ICGC	International Cancer Genome Consortium
IHC	Immunohistochemistry
Mb	Megabase
MMR	Mismatch Repair
MSI	Microsatellite Instability
PDAC	Pancreatic Ductal Adenocarcinoma
SV	Structural Variant
WGS	Whole Genome Sequencing

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Figure Legends

Figure 1. Molecular mechanisms of MMR deficiency. IHC images (scale bars indicate 100µm) indicating loss of staining of MMR proteins in tumor cells (blue) and normal staining in adjacent cells (brown) with schematics of underlying genetic aetiology. A, *MHS2* absence due to a homozygous deletion (ICGC_0076). B, *MHS2* absence and a structural rearrangement disrupting the *MSH2* locus through insertion of a 27Kb DNA segment (ICGC_0548). C, *MHS2* absence and a missense acceptor splice site variant at exon 13 (ICGC_0090). D, *MLH1* absence through hypermethylation of the promoter region (ICGC_0297). The average beta value of 45 CpG sites in the promoter of *MLH1* for

pancreatic cancer (n=174) and adjacent non-malignant pancreas tissue samples (n=29) is shown.

Table 1. Clinical and histological features and proposed aetiology for highly mutated PDAC tumors (n=20).

Sample ID	Personal and family history of malignancy	Histology	Mutation Load (Mutations/Mb)	IHC result	MSIsensor score	KRAS mutation	Predominant mutation signature (mutations/Mb)	SV subtype (no of events)	Proposed aetiology
Hypermutation (extreme outliers)									
ICGC_0076†	None	Mixed signet ring, mucinous and papillary adenocarcinoma.	38.55	Absent MLH1 and PMS2	28.3	p.G12V	MMR (18.3)	Scattered (131)	MMR deficiency: >280kb somatic homozygous deletion over MSH2
ICGC_0297†	None	Undifferentiated adenocarcinoma	60.62	Absent MSH2 and MSH6	27.33	WT	MMR (33.4)	Scattered (75)	MMR deficiency: Somatic MLH1 promoter hypermethylation
ICGC_0548†	None	Ductal adenocarcinoma, moderately differentiated	30.13	Absent MSH2 and MSH6	17.47	WT	MMR (16.6)	Stable (49)	MMR deficiency: >27kb somatic inversion rearrangement disrupting MSH2
ICGC_0328†	None	Ductal adenocarcinoma	16.63	Normal.	3.2	p.G12D	Unknown (11.9)	Scattered (110)	Cell line with signature: etiology unknown.
ICGC_0090	1 FDR, father CRC	Ductal adenocarcinoma, moderately differentiated	12.9	Absent MSH2 and MSH6	0.21	p.G12C	N/A	N/A	MMR deficiency: Somatic MSH2 splice site c.2006G>A
Highly mutated tumors									
ICGC_0054†	None	Ductal adenocarcinoma, poorly differentiated	6.52	Normal	0.01	p.G12V	HR deficiency (1.3)	Unstable (310)	HR deficiency: No germline or somatic cause found.
ICGC_0290†	None	Ductal adenocarcinoma, poorly differentiated	6.54	Not Available	0.07	p.G12V	HR deficiency (3.1)	Unstable (558)	HR deficiency: Germline BRCA2 mutation c.7180A>T, p.A2394*. Somatic CN-LOH.
ICGC_0215†	2 FDR lung cancer, 2 FDR prostate cancer. Previous CRC and melanoma	Ductal adenocarcinoma, moderately differentiated	6.27	Normal	0.01	p.G12V	HR deficiency (1.9)	Scattered (111)	HR deficiency: Germline ATM mutation c.7539_7540delAT, p.Y2514*. Somatic CN-LOH.
ICGC_0324	None	Ductal adenocarcinoma, moderately differentiated	6.24	Normal	0	p.G12D	N/A	N/A	Undefined
ICGC_0034†	None	Ductal adenocarcinoma, poorly differentiated	6.09	Normal	4.02	p.G12D	HR deficiency (3.4)	Unstable (366)	HR deficiency: Germline BRCA2 mutation c.5237_5238insT, p.N1747*. Somatic CN-LOH.
ICGC_0131†	lung cancer after PC	Ductal adenocarcinoma, moderately differentiated	5.63	Normal	0	p.G12D	T>G at TT sites (3.0)	Focal (147)	T>G at TT sites signature: etiology potentially associated with DNA oxidation
ICGC_0006†	1 FDR, father lung cancer	Adenocarcinoma arising from IPMN, moderately differentiated	5.29	Normal	0.01	p.G12D	HR deficiency (1.2)	Unstable (211)	HR deficiency: Somatic BRCA2 c.5351dupA, p.N1784KfsTer3. Somatic CN-LOH.
ICGC_0321†	2 FDR, mother and cousin breast cancer	Ductal adenocarcinoma, poorly differentiated	4.79	Not Available	0	p.G12D	HR deficiency (2.1)	Unstable (286)	HR deficiency: Germline BRCA2 c.6699delT, p.F2234LfsTer7. Somatic CN loss- 1 copy.
ICGC_0309†	None	Adenocarcinoma arising from IPMN, moderately differentiated	4.74	Normal	0.03	p.G12V	T>G at TT sites (3.1)	Unstable (232)	T>G at TT sites signature: etiology potentially associated with DNA oxidation
ICGC_0005†	1 FDR, mother CRC	Ductal adenocarcinoma, poorly differentiated	4.72	Not Available	1	p.G12V	HR deficiency (1.1)	Focal (95)	HR deficiency: No germline or somatic cause found.
ICGC_0016†	None	Ductal adenocarcinoma, poorly differentiated	4.61	Normal	3.03	p.G12V	HR deficiency (1.7)	Unstable (447)	HR deficiency: potentially linked to Somatic RPA1 c.273G>T, p.R91S
ICGC_0046	1 FDR, brother PC	Ductal adenocarcinoma, poorly differentiated	4.3	Normal	0	p.Q61H	N/A	N/A	Undefined
GARV_0668†	None	Ductal adenocarcinoma, poorly differentiated	4.3	Not Available	2.19	p.G12V	HR deficiency (1.6)	Unstable (464)	HR deficiency: Germline BRCA2 c.7068_7069delTC, p.L2357VfsTer2. Somatic CN loss - 1 copy.
ICGC_0291	None	Ductal adenocarcinoma, well differentiated	3.84	Not Available	0.03	p.G12R	N/A	N/A	HR deficiency: Somatic BRCA2 c.7283T>A, p.L2428*
ICGC_0256	None	Ductal adenocarcinoma, poorly differentiated	3.72	Not Available	0.06	p.G12D	N/A	N/A	Undefined

† Sample sequenced by whole genome sequencing (WGS), other samples by exome sequencing
FDR, First degree relative; PC, Pancreatic cancer; CRC, Colorectal cancer; N/A, not applicable to exome data.