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Mismatch repair status may predict response to adjuvant chemotherapy in resectable pancreatic ductal adenocarcinoma

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Deficiencies in DNA mismatch repair have been associated with inferior response to 5-FU in colorectal cancer. Pancreatic ductal adenocarcinoma is similarly treated with pyrimidine analogs, yet the predictive value of mismatch repair status for response to these agents has not been examined in this malignancy. A tissue microarray with associated clinical outcome, comprising 254 resected pancreatic ductal adenocarcinoma patients was stained for four mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2). Mismatch repair deficiency and proficiency was determined by the absence or presence of uniform nuclear staining in tumor cells, respectively. Cases identified as mismatch repair deficient on the tissue microarray were confirmed by immunohistochemistry on whole slide sections. Of the 265 cases, 78 (29%) received adjuvant treatment with a pyrimidine analog and 41 (15%) showed a mismatch repair-deficient immunoprofile. Multivariable disease-specific survival in the mismatch repair-proficient cohort demonstrated that adjuvant chemotherapy, regional lymph-node status, gender, and the presence of tumor budding were significant independent prognostic variables ($P \leq 0.04$); however, none of the eight clinico-pathologic covariates examined in the mismatch repair-deficient cohort were of independent prognostic significance. Univariable assessment of disease-specific survival revealed an almost identical survival profile for both treated and untreated patients with a mismatch repair-deficient profile, while treatment in the mismatch repair-proficient cohort conferred a greater than 10-month median disease-specific survival advantage over their untreated counterparts ($P = 0.0018$). In this cohort, adjuvant chemotherapy with a pyrimidine analog conferred no survival advantage to mismatch repair-deficient pancreatic ductal adenocarcinoma patients. Mismatch repair immunoprofiling is a feasible predictive marker in pancreatic ductal adenocarcinoma patients, and further prospective evaluation of this finding is warranted.

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Pancreatic ductal adenocarcinoma is among the deadliest solid cancers, with little improvement in overall survival achieved in the past few decades.[1–3] Lack of effective screening modalities and late presentation with advanced stage disease result in fewer than 20% of patients being eligible for surgical resection.[4] Even in surgically-treated patients, the 5-year survival rate is lower than 20%,[5] highlighting the need for more effective and personalized systemic therapeutic approaches. Adjuvant chemotherapy is considered the standard of care for patients with resectable pancreatic ductal adenocarcinoma.[6,7] Pyrimidine analogs (gemcitabine or 5-fluorouracil (5-FU)) are the most commonly used agents, with gemcitabine demonstrating an improved survival compared with no treatment in CONKO-001,[7] and an improved toxicity profile compared with 5-FU in ESPAC-3.[8]

Molecular studies have shown that pancreatic ductal adenocarcinomas contain a high number of

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gene deletions, mutations, amplifications and rearrangements,[9,10] yet the contribution of these genetic abnormalities to tumor behavior and response to systemic therapy is not well understood. The DNA mismatch repair system is integral to maintaining genomic stability, and mismatch repair deficiency is observed in many malignancies.[11] Mismatch repair deficiency often has hereditary implications, but these tumors also have a genetic hypermutation signature,[12,13] and have been shown to follow a different natural history than their mismatch repair-proficient counterparts.[14,15] The mismatch repair-deficient phenotype may affect the natural history of malignancies through immunologic mechanisms, but this phenotype has also been associated with drug resistance. *In vitro* studies have shown that mismatch repair-deficient cells are resistant to certain alkylating, methylating and platinum-containing agents as well as select antimetabolites.[16] The mechanisms of drug resistance in mismatch repair-deficient malignancies include increased tolerance to DNA damage, which allows for accumulation of (and selection for) critical mutations, inability to induce cell-cycle arrest, and/or defective apoptotic signaling.[11,17] In fact, the clinical utility of mismatch repair status as a predictor of response to chemotherapy with 5-FU has already been shown in colorectal cancer (reviewed in Sinicrope and Sargeant[15]).

Pancreatic ductal adenocarcinomas are among the cancers that harbor mismatch repair-deficient phenotypes, with an estimated prevalence of 13–17% of tumors in some studies.[18–20] There is conflicting evidence regarding the prognostic value of mismatch repair status in pancreatic ductal adenocarcinoma.[18–22] One study, analyzing pancreatic ductal adenocarcinomas from 78 patients, found no prognostic significance associated with mismatch repair status.[19] In contrast, Dong *et al*[21] have shown that somatic variants in mismatch repair genes are positively correlated with increased overall survival, supporting earlier studies that suggested microsatellite instability (a surrogate marker of mismatch repair status) is prognostically important in pancreatic ductal adenocarcinoma.[18,20] Moreover, in contrast with colorectal cancer, the predictive value of mismatch repair status for treatment with pyrimidine analogs has not yet been studied in pancreatic ductal adenocarcinoma. We therefore tested the hypotheses that mismatch repair status influences the natural course of pancreatic ductal adenocarcinoma as well as its response to pyrimidine-based chemotherapy.

Materials and methods

Ethics Statement

Ethical approval for research on this retrospective cohort was obtained from the University of British Columbia Clinical Research Ethics Board (H12-03484).

Sample Collection and Immunohistochemical Assessment

A tissue microarray was constructed using duplicate 0.6 mm cores from the epithelial component of all available, resected, pathologically confirmed pancreatic ductal adenocarcinomas derived from the archives of the Vancouver Coastal Health Region between 1995 and 2014. All patients received primary surgery with a subset receiving adjuvant chemotherapy with a pyrimidine analog. Cores for the tissue microarray were obtained from areas of tumor as determined by routine microscopy on hematoxylin and eosin-stained sections. Cases were excluded if they lacked complete follow-up data or if clinico-pathologic variables were lacking.

Immunohistochemical Staining of Mismatch Repair Markers

Immunohistochemistry was performed on 4-μm-thick formalin-fixed paraffin-embedded sections of tissue microarrays or, in a subset, on whole tissue sections in the clinical laboratory of Vancouver General Hospital using the Ventana Discovery XT and the Ventana Benchmark XT automated system (Ventana Medical Systems, Tucson, AZ) as per the manufacturer's recommendations. Sections were mounted on charged glass slides and baked at 60 °C for 15 min. Heat-induced antigen retrieval was performed in Cell Conditioning solution CC1 (Ventana). Before antibody incubation, slides to be stained for PMS2 were additionally prepped with the Epitomics DAB prep kit. Slides were then incubated with MLH1 (mouse monoclonal antibody, 1:50 dilution, cat#: NCL-L-MLH1, clone ID:ES05; Leica Microsystems, Newcastle, UK), MSH2 (mouse monoclonal antibody, 1:1000 dilution, cat#: 286 M-16, clone ID:G219-1129; Cellmarque, Rocklin, CA), MSH6 (rabbit monoclonal antibody, 1:200 dilution, cat#: CLAC-0047, clone ID: EP49; Cedarlane Corporation, Burlington, ON, Canada), and PMS2 (rabbit monoclonal antibody, 1:20 dilution, cat#: CLAC-0049, clone ID:EP51; Cedarlane Corporation) for 32 min at room temperature. Antibodies were detected using the Ventana DABMap kit, counterstained with hematoxylin and treated with a proprietary bluing agent (Ventana); see Supplementary File—Supplementary Table S1. Positive and negative controls are routinely performed as part of clinical quality assurance; additionally, the laboratory at Vancouver General Hospital participates in an external quality control program (Canadian Immunohistochemistry Quality Control (cIQc), a provider of proficiency testing for Canadian clinical laboratories).

Interpretation of Immunohistochemical Stains

Mismatch repair protein expression was considered intact (normal) if any percentage of definite positive

nuclear staining of the tumor cells was detected on the TMA core. Cases that displayed the absence of staining for one or more mismatch repair proteins were selected for examination utilizing immunohistochemistry on whole sections. Each protein was considered lost (abnormal) if there was complete loss of nuclear staining in the tumor cells and if there was a positive internal control (intact nuclear staining of stromal elements such as inflammatory cells and/or endothelial cells). Cases showing a complete absence of nuclear staining pattern of both tumor cells and stromal elements were deemed uninterpretable and thus excluded from the study. For each immunohistochemical stain, both tissue microarray and confirmatory whole slide sections were scored independently by three pathologists (MR, BSS and DFS), who were blinded to clinical characteristics and patient outcomes. Divergent scores were reconciled by consensus conference. A case was considered to be mismatch repair deficient if any of the four mismatch repair proteins was completely absent. Cases were classified as mismatch repair proficient if all four proteins were present to some degree.

Clinico-pathologic Parameters and Outcome

Standard treatment, clinical and pathology parameters were collected from the British Columbia Cancer Agency which included: age at surgery, sex, adjuvant chemotherapy agents, lymphovascular invasion, perineural invasion, pathologic primary tumor stage, pathologic regional lymph-node status, and the presence of tumor budding, defined as single cells or non-glandular clusters composed of $<$ five cells present at the invasive tumor front. The primary outcome measure was defined to be disease-specific survival, where survival time was calculated as the difference between the date of last follow-up and the date of surgery, expressed in years. Patients were censored if they were alive at last follow-up or had died from a cause other than their pancreatic malignancy. Deaths attributable to treatment related toxicities or inter-current diseases were considered censored observations for this analysis.

Statistical Analysis

Heterogeneity for clinico-pathologic parameters across mismatch repair-deficient and -proficient cohorts was assessed with the following statistical approaches: continuous variables were examined using the Student's *t*-test after ensuring normality with the Shapiro-Wilk test and equal variances with O'Brien's method, two-level categorical comparisons were computed using Fisher's exact test, categorical comparisons with greater than two levels were quantified with the likelihood-ratio X^2 test. The temporal relationship between date of surgery and the use of adjuvant chemotherapy was modeled with nominal logistic regression. Univariable disease-specific survival comparisons were performed with the Kaplan–Meier method and differences assessed with the Log-Rank statistic. Multivariable survival analysis was performed with the Cox Proportional Hazards Model. A *P*-value of < 0.05 was considered as statistically significant for all analyses. All analyses were computed with JMP v12.0.1 (SAS Institute, Cary, NC).

Results

Of 277 initial patients, four patients were excluded due to missing data for one or more of the immunohistochemical markers in the mismatch repair panel, and an additional eight patients were excluded due to unattainable clinico-pathologic data, resulting in a final cohort of 265 patients (Supplementary File—Supplementary Figure S1). A mismatch repair-deficient profile was identified in 15% (41/265) of patients (Table 1). Histologically, areas with medullary-like features (such as syncytial growth pattern of poorly differentiated malignant cells with pushing borders) were identified in two of the mismatch repair-deficient patients, one with MSH2/MSH6 and the other a MLH1/PMS2-deficient immunophenotype.

Statistical assessments for heterogeneity of clinico-pathologic parameters across the mismatch repair-deficient and -proficient groups revealed no significant differences (Table 2).

Table 1 Resultant immunophenotypes for the 41 cases determined to be mismatch repair after full section confirmation

MLH1	*MSH2*	*MSH6*	*PMS2*	*Prevalence (%)*
Intact	Loss	Loss	Intact	64
Loss	Intact	Intact	Loss	25
Intact	Loss	Intact	Intact	5
Loss	Loss	Intact	Loss	2
Loss	Intact	Uninterpretable[a]	Loss	2
Loss	Uninterpretable[a]	Intact	Loss	2

[a]'Uninterpretable' is defined as lack of internal control staining (lymphocytes or stromal elements) in the tumor area with loss of MMR protein expression.

Table 2 Comparisons of demographics and clinico-pathologic covariates across the mismatch repair-proficient and -deficient cohorts

Clinical covariate	*MMR proficient*	*MMR deficient*	P-*value*
Mean age (95% CI)	66.4 (65.1–67.8)	64.5 (61.5–67.6)	0.26
Sex			
Male ($N=147$)	127 (57%)	20 (49%)	0.39
Female ($N=118$)	97 (43%)	21 (51%)	
Adjuvant chemotherapy			
Yes ($N=78$)	68 (30%)	10 (24%)	0.58
No ($N=187$)	156 (70%)	31 (76%)	
Lymphovascular invasion			
Yes ($N=149$)	122 (55%)	27 (66%)	0.23
No ($N=116$)	102 (45%)	14 (34%)	
Perineural invasion			
Yes ($N=242$)	204 (91%)	38 (93%)	1.00
No ($N=23$)	20 (9%)	3 (7%)	
Primary tumor stage			
pT1 ($N=2$)	2 (1%)	0 (0%)	0.22
pT2 ($N=12$)	12 (5%)	0 (0%)	
pT3 ($N=249$)	209 (93%)	40 (98%)	
pT4 ($N=2$)	1 (1%)	1 (2%)	
Regional lymph nodes			
pN0 ($N=66$)	56 (25%)	10 (24%)	0.71
pN1 ($N=197$)	166 (74%)	31 (76%)	
pNX ($N=2$)	2 (1%)	0 (0%)	
Tumor budding			
Absent ($N=29$)	27 (12%)	2 (5%)	0.27
Present ($N=236$)	197 (88%)	39 (95%)	

The median follow-up time in our study cohort was 1.3 years. Univariable assessment of disease-specific survival revealed an indistinguishable survival profile for both treated and untreated patients with an mismatch repair-deficient profile ($P=0.17$) (Figure 1a). Conversely, adjuvant treatment showed a highly significant effect in patients with mismatch repair-deficient pancreatic ductal adenocarcinoma, demonstrated by highly divergent survival profiles, with the treated cohort having a 14-month median disease-free survival advantage over their untreated counterparts ($P=0.0018$) (Figure 1b).

As shown in Table 3, none of the eight covariates included in the multivariable analysis demonstrated independent prognostic significance in the mismatch repair-deficient cohort, while in the mismatch repair-proficient cohort, half of the covariates demonstrated prognostic significance; adjuvant chemotherapy ($P=0.0010$), pathologic regional lymph-node status ($P=0.0002$), gender ($P=0.04$) and the presence of tumor budding ($P=0.04$).

A planned disease-specific survival analysis examining differences between the principal pyrimidine analog agents (5-FU ($N=19$) *vs* gemcitabine ($N=59$)) showed no significant agent-specific difference in either the mismatch repair-deficient or -proficient cohorts ($P=0.39$; $P=0.43$, respectively).

To illustrate the temporal treatment bias inherent to this retrospective cohort, nominal logistic regression was used to derive a more balanced cohort between treated and untreated patients in both cohorts (Supplementary File—Supplementary Figure S2). Based on this curve, it was determined that the first resected pancreatic ductal adenocarcinoma case which received adjuvant chemotherapy occurred on 3 November 2003. In an attempt to address temporal treatment bias, we excluded all cases before 3 November 2003 to establish a better balance in treatment allocation. Application of this exclusion criterion resulted in 72 untreated cases being excluded. The resultant univariable analysis indicated that adjuvant chemotherapy was still predictive of treatment response in the mismatch repair-proficient cohort ($P=0.0010$) and remained insignificant in the mismatch repair-deficient cohort ($P=0.08$).

Discussion

Our study sought to determine the prognostic and predictive significance of mismatch repair deficiency in pancreatic ductal adenocarcinoma, and found 15% of patients in our cohort to be mismatch repair deficient. In the literature, there are conflicting data regarding the incidence (and clinical significance) of mismatch repair-deficient pancreatic ductal adenocarcinomas. For example, screening with a panel of five mononucleotide repeats, Laghi *et al*[23] found only one microsatellite instable cancer among 338 consecutive pancreatic ductal adenocarcinoma cases (incidence 0.5%). Our cohort, however, showed a substantially higher incidence of mismatch repair deficiency, which is in agreement with other studies showing a prevalence of 13–17% mismatch repair-deficient profile as assessed by microsatellite instability[18,20] or immunohistochemistry on tissue microarray.[19] The reason for this wide variability in reported incidence of microsatellite instability/mismatch repair deficiency is not currently understood but small number of cases assessed in some studies, race-specific differences (Asian *vs* Caucasian), and diversity in methods to assess mismatch repair status may be contributing.

Additionally, we found that mismatch repair status was able to separate resected pancreatic ductal adenocarcinoma patients into two groups with a significantly different response to adjuvant chemotherapy. While the mismatch repair-proficient cohort showed a 10-month increase in disease-specific survival with gemcitabine or 5-FU treatment, no statistically significant survival advantage was observed in the treated mismatch repair-deficient cohort. This finding mirrors the existing data on predictive value of mismatch repair system in colorectal cancer and response to 5-FU-based adjuvant

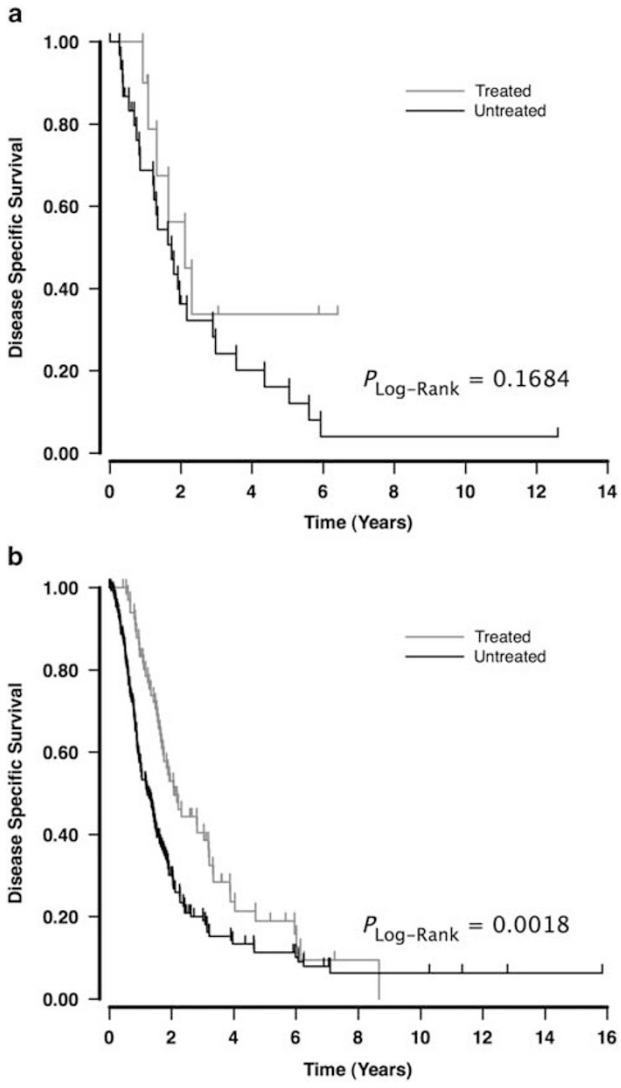

Figure 1 Univariable disease-specific survival using the Kaplan–Meier method. (**a**) Represents mismatch repair-deficient cases; (**b**) Represents mismatch repair-proficient cases.

chemotherapy (reviewed in Sinicrope and Sargeant[15]). Our analysis did not demonstrate a difference in disease-specific survival between 5-FU or gemcitabine treatment, in keeping with ESPAC-3 trial.[8]

Similar to what has been shown in colorectal cancer,[15] mismatch repair status may also have a prognostic value in pancreatic ductal adenocarcinoma. Since our study population included patients who did not receive adjuvant chemotherapy, we examined the influence of mismatch repair status on the natural course of untreated pancreatic ductal adenocarcinoma patients, following surgical resection. While the untreated mismatch repair-deficient group displayed a 6-month median survival advantage compared with the untreated mismatch repair-proficient group, this difference failed to achieve statistical significance ($P_{\text{log-rank}} = 0.51$). This result does little to resolve the ambiguity in the literature surrounding the prognosis of mismatch repair-deficient pancreatic ductal adenocarcinomas, with select reports showing no prognostic value,[19,22] and other studies suggesting mismatch repair status is a prognostic marker in pancreatic ductal adenocarcinoma.[18,20,21] The incongruent nature of the studies mentioned above may be explained by the relatively small sample size used and the heterogeneity in treatments applied across studies. Of note, this study and Ottenhoffe *et al*[19] used immunohistochemistry on tissue microarray to assess mismatch repair status, yet significant prognostic value has only previously been correlated to mismatch repair status as defined at the DNA level by microsatellite instability assessment[18,20] and other methods.[21]

The cohort used in this study is uniquely positioned to address treatment efficacy because of the large number of untreated cases, which serve as a control group. Unlike in mismatch repair-proficient pancreatic ductal adenocarcinomas, none of the clinico-pathologic covariates examined demonstrated independent prognostic significance in mismatch repair-deficient group. This may suggest that mismatch repair-deficient pancreatic ductal adenocarcinomas represent a distinct subset with a 'stable' natural course irrespective of the pathological stage at which they are diagnosed further highlighting the need for therapeutic agents that are effective against mismatch repair deficient-pancreatic ductal adenocarcinomas. Both FOLFIRINOX and gemcitabine in combination with nab-paclitaxel are being studied in the adjuvant setting in large phase III trials, and these combinations may ultimately demonstrate efficacy in this subgroup.

The present study has several limitations: Mismatch repair status was first assessed with immunohistochemistry in tissue microarray followed by confirmatory whole-slide immunohistochemistry in all cases with negative staining of any of the four mismatch repair proteins. It has been previously shown that immunohistochemistry of mismatch repair proteins is a feasible, very sensitive and highly specific marker of microsatellite instability especially when all four mismatch repair proteins are assessed (reviewed in Shia[24]). In our study, all immunohistochemistry was performed in a clinical laboratory, subject to ongoing internal and external quality control exercises. Mismatch repair status as determined by immunohistochemistry has been shown, by our group, to have a high degree of inter-observer consistency (unpublished data).

Another potential limitation is sample size: The combinatorial segmentation of cohorts based on multiple criteria results in increasingly small numbers for the resultant subgroups. This is a trend that is pervasive in personalized medicine where multiple biomarkers with minimal associations are used to define cohorts into discrete prognostic and predictive groups. This, combined with the fact that 'resectable' pancreatic ductal adenocarcinoma is not a common entity, makes it extremely difficult to generate definitive results. Assuming a prevalence of

Table 3 Multivariable disease-specific survival analysis for the mismatch repair-deficient and -proficient cohorts

Clinico-pathologic covariates	*Levels*	*Risk Ratio*	*95% CI*	P-*value*
MMR-d cohort				
Age at surgery	Entire range of regressor	1.79	0.18–25.24	0.6345
Sex	Male *vs* female	0.61	0.24–1.54	0.2853
Adjuvant chemotherapy	Treated *vs* untreated	0.80	0.27–2.09	0.6664
Lymphovascular invasion	Present *vs* absent	1.38	0.54–3.88	0.5052
Perineural invasion	Present *vs* absent	5.84	0.78–123.58	0.0904
pT stage	pT4 *vs* pT3	0.40	0.02–2.95	0.3990
Regional lymph nodes pN stage	pN1 *vs* pN0	2.05	0.70–6.48	0.1926
Tumor budding	Present *vs* absent	5.41	0.81–110.22	0.0870
MMR-p *cohort*				
Age at surgery	Entire range of regressor	1.29	0.58–2.84	0.5309
Sex	Male *vs* female	1.39	1.01–1.93	0.0416
Adjuvant chemotherapy	Treated *vs* untreated	0.56	0.39–0.80	0.0010
Lymphovascular invasion	Present *vs* absent	1.26	0.91–1.77	0.1681
Perineural invasion	Present *vs* absent	1.17	0.68–2.16	0.5893
pT stage	pT4 *vs* pT3	1.39	0.07–7.79	0.9852
	pT4 *vs* pT2	1.32	0.07–8.11	
	pT4 *vs* pT1	1.19	0.05–13.91	
	pT3 *vs* pT2	0.95	0.49–2.06	
	pT3 *vs* pT1	0.86	0.26–5.27	
	pT2 *vs* pT1	0.90	0.23–5.99	
Regional lymph nodes pN stage	pN1 *vs* pN0	2.00	1.38–2.94	0.0002
Tumor budding	Present *vs* absent	1.68	1.01–2.96	0.0451

Two cases with pNx status were excluded from the mismatch repair-proficient cohort for this analysis as the status of the regional lymph nodes was uncertain.

15% for mismatch repair-deficient phenotype, this study has an overall sample size adequate for the prognostic assessment of a single biomarker and the results presented here suggest that mismatch repair-deficient and -proficient groups differ in their treatment response as well as prognostic clinico-pathologic covariates.

Finally, the retrospective nature of our study poses some limitation as well. From 1988 to 2014, the number of patients receiving adjuvant chemotherapy increased, reflecting the results of major clinical trials, with the practice changing CONKO-001 trial first presented in 2007.[25] To account for the temporal treatment bias in our historical cohort, we excluded patients before 2003 in our analysis showing that mismatch repair status continued to predict the response to chemotherapy in this more contemporary subgroup.

In conclusion, our results show clear differences in the response to adjuvant chemotherapy between the mismatch repair-deficient and -proficient subgroups of pancreatic ductal adenocarcinoma. Since immunohistochemical analysis of mismatch repair system is an accessible and convenient method; this finding has the potential of rapidly changing the treatment algorithm for pancreatic ductal adenocarcinoma. While our results show a robust difference between mismatch repair-deficient and -proficient cohorts with respect to adjuvant chemotherapy, validation of these findings in specimens from previously conducted randomized trials with an untreated control arm is required.

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Disclosure/conflict of interest

DJR declares receiving honoraria from Sanofi and Celgene.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)