Published OnlineFirst October 20, 2020; DOI: 10.1158/1078-0432.CCR-20-1746

Improved Assessment of Response Status in Patients with Pancreatic Cancer Treated with Neoadjuvant Therapy using Somatic Mutations and Liquid Biopsy Analysis

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ABSTRACT

Purpose: To evaluate somatic mutations, circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in patients with Pancreatic ductal adenocarcinoma (PDAC) with pathologic complete response (pCR) to neoadjuvant therapy (NAT) and find their associations with outcome.

Experimental Design: Thirty-six patients with PDAC with pCR were identified from 2009 to 2017. Macrodissection was performed on resected specimens to isolate DNA from 332 regions of interest including fibrosis, normal duct, normal parenchyma, and undefined ductal cells (UDCs). Cell-free DNA and CTCs were also extracted. Next-generation sequencing was used to detect mutations of *KRAS, CDKN2A, SMAD4, TP53, GNAS*, and *BRAF*.

Results: *KRAS* mutation was detected in UDCs and fibrosis while *SMAD4, TP53*, and *GNAS* were only seen in UDCs. Patients with *TP53* mutation showed relatively worse overall survival (HR, 3.596, 95% CI, 0.855–15.130; $P = 0.081$). Five patients available for CTCs data were all positive for CTCs and seven of 16 patients with pCR were detected with ctDNA at surgery. We proposed a new concept of regression assessment combining genomic analysis of resected specimens and liquid biopsy data for PDAC, namely, molecular complete response (mCR). Three of six patients with mCR recurred as compared with six in 15 non-mCR patients. Seven of 15 non-mCR patients died during follow-up, while there was only one in six patients with mCR.

Conclusions: This study first reports that somatic mutations, CTCs, and ctDNA existed even in patients with PDAC with pCR to NAT, which could possibly predict early recurrence and reduced survival. The current regression evaluation system of PDAC needs to be reassessed at a molecular level.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease with an overall 5-year survival rate of only 9% (1). The fundamental biology of PDAC that underlies its high mortality rate is its propensity for early systemic spread, lack of clinically available tests for early detection, and relatively ineffective systemic therapy. Surgical resection of clinically localized primary cancer combined with systemic therapy remains the only treatment that can potentially result in cure. Unfortunately, more than half of patients present distant metastases at the time of diagnosis and are not surgical candidates (2). Moreover, among those selected patients who undergo a potentially curative oncologic resection, nearly 80% will develop a systemic relapse. With the advent of more effective multiagent therapies, such as FOLFIRINOX, neoadjuvant treatment has become the predominant treatment paradigm for localized disease. The benefits of this approach include selection of favorable "tumor biology," such that (i) only patients who will benefit the most from resection will undergo an operation, (ii) we can obtain early systemic control, and (iii) we can guarantee that all patients undergoing resection receive systemic therapy considering the fact that some patients would not be able to tolerate postoperative adjuvant chemotherapy.

Patients who present with localized disease but have tumors that involve major vessels are classified as stage III (American Joint Committee on Cancer 8th edition; ref. 3) and further stratified into either borderline resectable (BR) or locally advanced (LA) based on the extent of vessel involvement. In such patients, the chance of achieving a margin-negative (R0) resection with upfront surgery is not favorable. In addition to neoadjuvant systemic therapy, radiotherapy is also commonly integrated into the treatment plan, albeit with low-level evidence, to optimize the chance of an R0 resection. Several well-done retrospective studies have demonstrated that an R0 resection increases survival in a surgery-first approach (4).

The response rates of pancreatic cancer to different neoadjuvant chemoradiotherapy regimens is variable, but overall, efficacy remains relatively poor. In fact, the proportion of pCR of pancreatic cancer to neoadjuvant therapy is only 3%–11% (5). Although the response rate is rather low, the prognosis is significantly better for responding patients, mainly because patients with complete remission or minimal residual tumor have lower local recurrence, metastatic, and positive margin rates. A recent retrospective analysis of a cohort at Johns Hopkins

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Clin Cancer Res 2021;27:740–8

doi: 10.1158/1078-0432.CCR-20-1746

Published OnlineFirst October 20, 2020; DOI: 10.1158/1078-0432.CCR-20-1746

Translational Relevance

A pathologic complete response (pCR) in pancreatic ductal adenocarcinoma (PDAC) indicates significantly improved survival after resection. However, some of the patients with pCR still suffer from early recurrence and disease-specific mortality. These findings raise the possibility that pCR may not be equal to true complete response. This study found that somatic mutations, circulating tumor cells (CTCs), and ctDNA existed in patients with PDAC with pCR after neoadjuvant therapy. *TP53* mutation in resected specimens was a marginally significant risk factor for overall survival of pCR patients. These prognostic indicators may serve as a panel of indicators and biomarkers that predict recurrence and survival. This study indicates that the current regression evaluation system needs to be reassessed at a molecular level. Molecular parameters such as mutation of resected specimens, CTCs, and ctDNA might be needed for the regression evaluation system of PDAC in the future.

Hospital, which was the largest PDAC pCR cohort published to date, showed that patients with BR or LA PDAC who had a pCR after neoadjuvant chemoradiotherapy achieve significantly prolonged survival compared with those with a poorer response. However, some patients in the pCR cohort still suffer from some early recurrence and short survival, which indicates that pCR does not necessarily equate to complete eradication of disease. Using the current tumor regression grading system, patients with PDAC with pCR may still suffer from early recurrence and poor survival. As such, there is a need for an improved understanding of what a pCR represents on a biological level. A more detailed understanding of the biological status of a pCR may lead to an improved tumor regression grading system to better guide the completion of treatment and predict patient outcomes. Most importantly, this information may provide insight into the development of future treatments capable of achieving eradication of all disease.

Given this need, we sought to test the hypothesis that genomic analysis of resected specimens combined with liquid biopsy data can uncover and characterize the nature of subclinical disease in patients with pCR. To test this hypothesis, we evaluated the molecular features of resected PDAC pCR specimens with corresponding liquid biopsies.

Materials and Methods

Patients and biospecimens

A total of 479 patients with PDAC with neoadjuvant chemoradiotherapy were identified from a prospectively maintained database from 2010 to 2017, 36 of which were found with pCR (**Fig. 1A**). Twenty-six patients were with available formalin-fixed paraffin embedded (FFPE) blocks of resected specimens, 16 patients with longitudinal plasma samples for ctDNA detection, and five patients with longitudinal CTCs data (**Fig. 1B**).

This study was reviewed and approved by the Johns Hopkins Medicine Institutional Review Board. The informed consents were obtained from the patients by written. This study was conducted in accordance with the ethical guidelines of Declaration of Helsinki.

Patient and public involvement

Patients in this study or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this study.

DNA extraction

Slides of formalin-fixed resected specimens were macrodissected at regions of interest (ROI) of normal parenchyma, normal duct, undefined ductal cells (UDCs), and fibrosis, which were defined by two independent pathologists (**Fig. 2A–D**). Precursor lesions like PanIN1/2 were excluded before macrodissection. DNA was extracted from each ROI (at least three slides with 10-um thickness for each slide) using the Ion AmpliSeq Direct FFPE DNA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell-free DNA (cfDNA) was extracted from 2 mL of plasma using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific) with KingFisher Duo Prime Purification System (Thermo Fisher Scientific). Purified DNA was quantified by Qubit DNA Assay Kit (Thermo Fisher Scientific) in Qubit 2.0 Flurometer.

DNA sequencing

Next-generation sequencing was performed using the Ion GeneStudioS5 System (Thermo Fisher Scientific), according to the manufacturer's protocols. Six genes (*KRAS, CDKN2A, SMAD4, TP53, GNAS, BRAF*) were sequenced with an AmpliSeq Custom Panel covering the coding regions. A total of 4 ng of FFPE DNA (2 ng per primer pool) were used for library preparation with Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific). cfDNA was sequenced for six genes (*KRAS, CDKN2A, SMAD4, TP53, GNAS, BRAF*). A total of 10 ng of cfDNA (5 ng per primer pool) was used for library preparation with Ion AmpliSeq HD Library Kit (Thermo Fisher Scientific). cfDNA isolated from plasma of untreated patients with PDAC were used as a positive control. Libraries were eluted with low tris-EDTA and quantified by qPCR using QIAseq Library Quant Assay Kit (Qiagen). The postsequencing raw FASTQ files were launched in NextGENe (version 2.41; SoftGenetics) software for alignment to the hg19 human reference genome and single-nucleotide variant calling. Alignments were visually verified using Integrative Genomics Viewer (version 2.3; Broad Institute) and NextGENe Viewer.

CTC isolation and characterization

CTC isolation and characterization were performed after collecting 10 mL of peripheral blood of each time point. The blood was processed with the Isolation by Size of Epithelial Tumor Cells Assay (ISET, Rarecells) and characterized by immunofluorescent staining as described in our prior study (6). A combination of pan-cytokeratin (Bioss) and vimentin (Thermo Fisher Scientific) conjugated antibodies were utilized to assess epithelial and mesenchymal cell traits, respectively. CTCs were stratified as epithelial-type (pan-cytokeratin+, vimentin−, CD45−), mesenchymal type (vimentin+, pan-cytokeratin−, CD45−), and epithelial/mesenchymal-type (pan-cytokeratin+, vimentin+, CD45−), as described previously (7). Cells that express an epithelial phenotype were defined as eCTCs and cells that express a combined epithelial/mesenchymal phenotype were defined as mCTCs. A third population of cells with the purely mesenchymal phenotype (pan-cytokeratin−, vimentin+, CD45−) was not recognized in any of the patient samples.

IHC

IHC was performed with primary antibodies against Smad4 (Thermo Fisher Scientific) and p53 (Santa Cruz Biotechnology) using Ready-to-use IHC kit (Biotin free), One-Step HRP Polymer anti-Mouse, Rat and Rabbit IgG (H+L) with 3,3′-diaminobenzidine (BioVision). Briefly, slides were deparaffinized, pretreated with citric acid buffer for 30 to 60 minutes, and incubated with the primary antibody for 30 minutes at room temperature. Antibody binding was visualized

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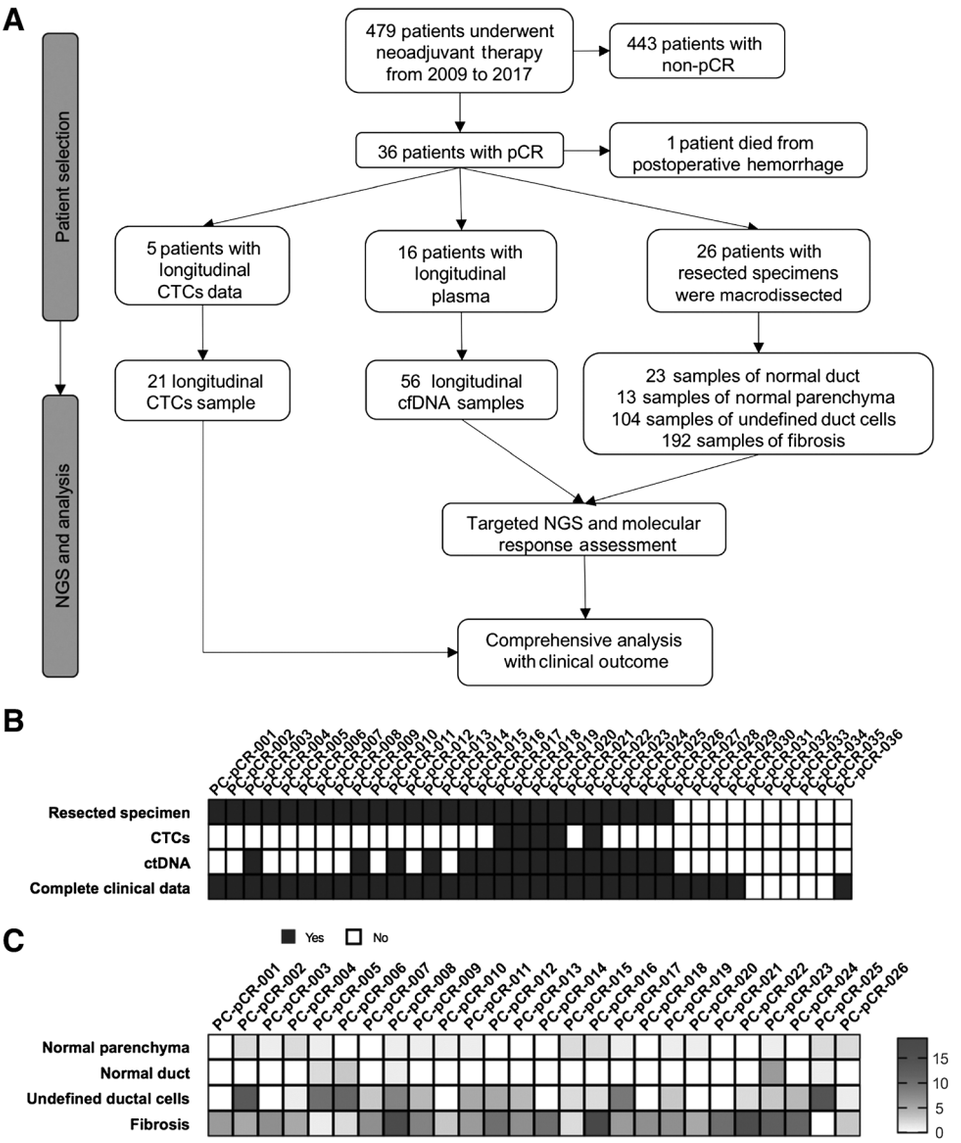

Figure 1.
Patients selection and data availability. **A,** Flowchart for study design. **B,** Data availability for all patients. **C,** ROIs availability for all patients.

with 3,3′-diaminobenzidine as dark brown precipitate. Counterstaining was performed with hematoxylin.

Definitions and statistical analysis

Continuous variables were summarized with means or medians and SDs or ranges, respectively. They were compared using an unpaired *t* test or Wilcoxon signed-rank tests as appropriate. Categorical variables were summarized using proportions or counts and were compared with χ^2 or Fisher exact test as appropriate. The radiological response was determined as any decrease in tumor size of metastatic lesion (if present) and primary tumor on CT or MRI scan reviewed by experienced radiologists in the Johns Hopkins Hospital. Disease-free survival (DFS) was defined as the time interval between the start date of neoadjuvant therapy and either date of recurrence or death, which came first or censored at last follow-up. Postdiagnosis overall diagnosis (OS) and postsurgery OS were defined as the time from the date of diagnosis or surgery to either death or censored at last follow-up. Molecular complete response (mCR) was determined as negative for tumor-related mutations of both resected specimens and plasma. Multivariable Cox regression was used to estimate the HR for survival. A P value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software version 21.0 (IBM).

Results

Characteristics of pCR cohort

Thirty-six patients were ultimately included in this study and the patient demographics and clinical characteristics are shown in Supplementary Table S1. The mean age was 61.4 years and 21 (58.3%) were male. Among this cohort, 23 patients were classified as LA pancreatic cancer (LAPC) and 11 as BR. Moreover, two patients were classified as metastatic prior to systemic therapy. Half of the patients (18, 50.0%) were treated with multiregimen neoadjuvant chemotherapy. Other single regimens included gemcitabine (four cases), FOLFIRINOX (12 cases), and Xeloda (two cases). Standard radiation including intensity-

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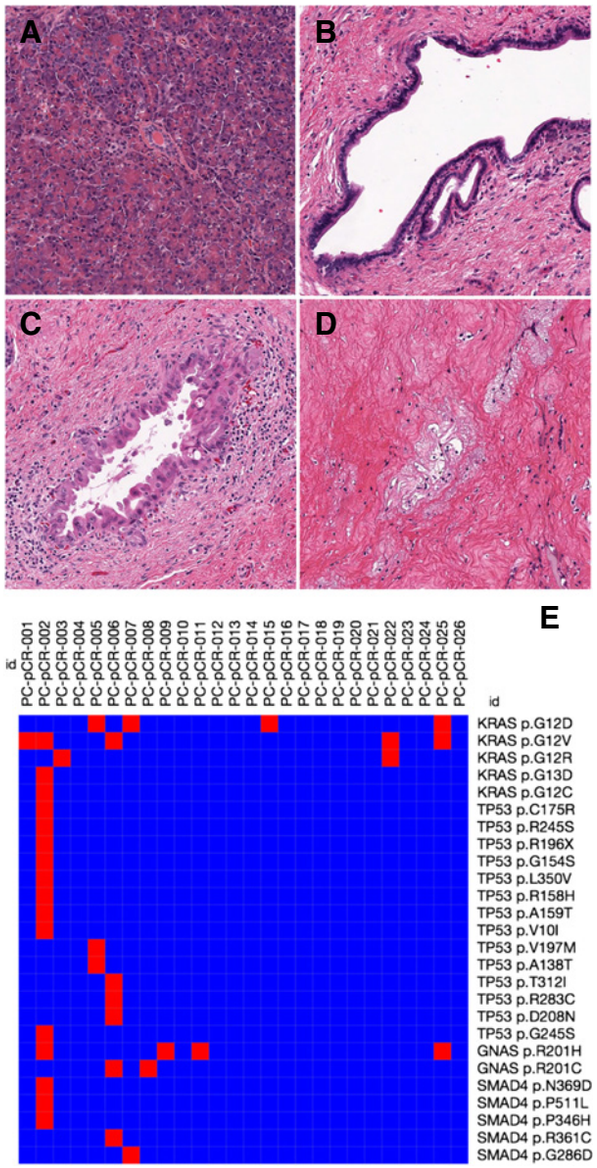

Figure 2.

Somatic mutations detected from the ROIs in the resected specimens. **A,** Normal parenchyma. **B,** Normal duct. **C,** UDCs. **D,** Fibrosis. **E,** Detailed mutations detected from all pCR patients.

modulated radiotherapy and stereotactic body radiotherapy were the most commonly used radiation modalities in 18 (50.0%) patients. The majority (29, 80.6%) of the patients underwent pancreaticoduodenectomy. One patient underwent a total pancreatectomy and six patients underwent distal pancreatectomy (Supplementary Table S1). Although all these patients are grade 0 based by the guideline of the College of American Pathologists (CAP), two of the patients were detected with cancerization (Supplementary Table S1).

Clinical predictors for survival

Six patients were excluded from the study because one patient died within 90 days after surgery due to postoperative complications and five more had incomplete follow-up (Supplementary Table S2). The remaining 30 patients had a median follow-up period of 44.4 months. Fifteen patients were found to have recurrence during follow-up. Four patients had local recurrences and four patients developed liver metastasis. Carcinomatosis was found in two patients. One patient was detected to have a lung metastasis and four patients had recurrence of multiple sites (Supplementary Table S3). Results of univariable analyses of predictors of OS and DFS are presented in Supplementary Table S2. Adjuvant therapy was the only significant factor associated with postdiagnosis DFS ($P = 0.009$) and postsurgery DFS ($P = 0.012$). The radiological response was not associated with better survival. There were too few patients to perform a meaningful multivariant analysis given the number of variables.

DNA sequencing for resected specimens

A total of 332 samples of ROIs were sequenced with a six-gene panel (**Fig. 1C**). These regions included 23 samples of normal parenchyma, 13 samples of normal duct, 104 samples of UDCs, and 192 samples of fibrotic tissue (**Fig. 2A** and **B**). Mutations were detected in UDCs and fibrosis but not in normal parenchyma or ducts. No *BRAF* or *CDKN2A* mutation were detected. *KRAS* mutation was detected in ROIs of both UDCs (25.0%, 26/104) and fibrosis (1.6%, 3/192). However, *TP53* (9.6%, 10/104), *SMAD4* (4.8%, 5/104), and *GNAS* (12.5%, 13/104) were only seen in UDCs (**Figs. 2E** and **3A–D**). Five patients demonstrated more than one pattern of mutation (patient No. 2, No. 5, No. 6, No. 22, No. 25; **Fig. 2E**). For these patients, mutually exclusive mutations were found for different ROIs of same patient. Three of these five patients recurred (No. 2, No. 5, No. 6) and died from disease progression during follow-up. The two patients with cancerization (patients No. 6 and No. 15) were both found to have mutations and recurred during follow-up (Supplementary Table S3).

Considering that genomic alterations have never been described in patients with pCR to NAT, the status of *TP53* was reevaluated using IHC (**Fig. 4B–E**). An analysis of the resected pCR specimens revealed that expression of p53 by IHC had a concordance of 83.3% with alterations in their respective genes. A total of 26 patients had both genomic data of resected specimens as well as clinical data. Univariate analysis was then performed to assess postneoadjuvant therapy and surgery survival (Supplementary Table S8). Younger age was shown to be associated with worse postdiagnosis OS (HR, 0.909, 95% CI, 0.833–0.993, $P = 0.034$). Adjuvant therapy was shown to be a risk factor for DFS postdiagnosis (HR, 5.667, 95% CI, 1.240–25.907, $P = 0.025$) and DFS postsurgery (HR, 5.214, 95% CI, 1.140–23.849, $P = 0.033$). When considering the mutations found in the resected specimens, patients with *TP53* showed a trend toward a worse OS and DFS postsurgery (**Fig. 3E** and **F**) although no significant *P* value was achieved. For subgroup analysis of patients with UDC samples sequenced, a trend for worse outcome of patients with mutated UDCs (**Fig. 3G** and **H**; Supplementary Table S7).

Longitudinal CTCs and ctDNA data

Five patients enrolled in the study were tracked longitudinally with CTCs. All five were positive for CTCs at surgery prior the abdomen incision (**Fig. 5A–C**; Supplementary Figs. S1 and S2; Supplementary Table S4). The CTCs were further classified on the basis of epithelial or mesenchymal phenotypes and four of five patients were detected with mCTCs at the time of surgery. The mCTCs remained positive during the follow-up for two patients (patient No. 18, No. 20) who recurred during follow-up (**Fig. 5B** and **C**). There was no apparent concordance

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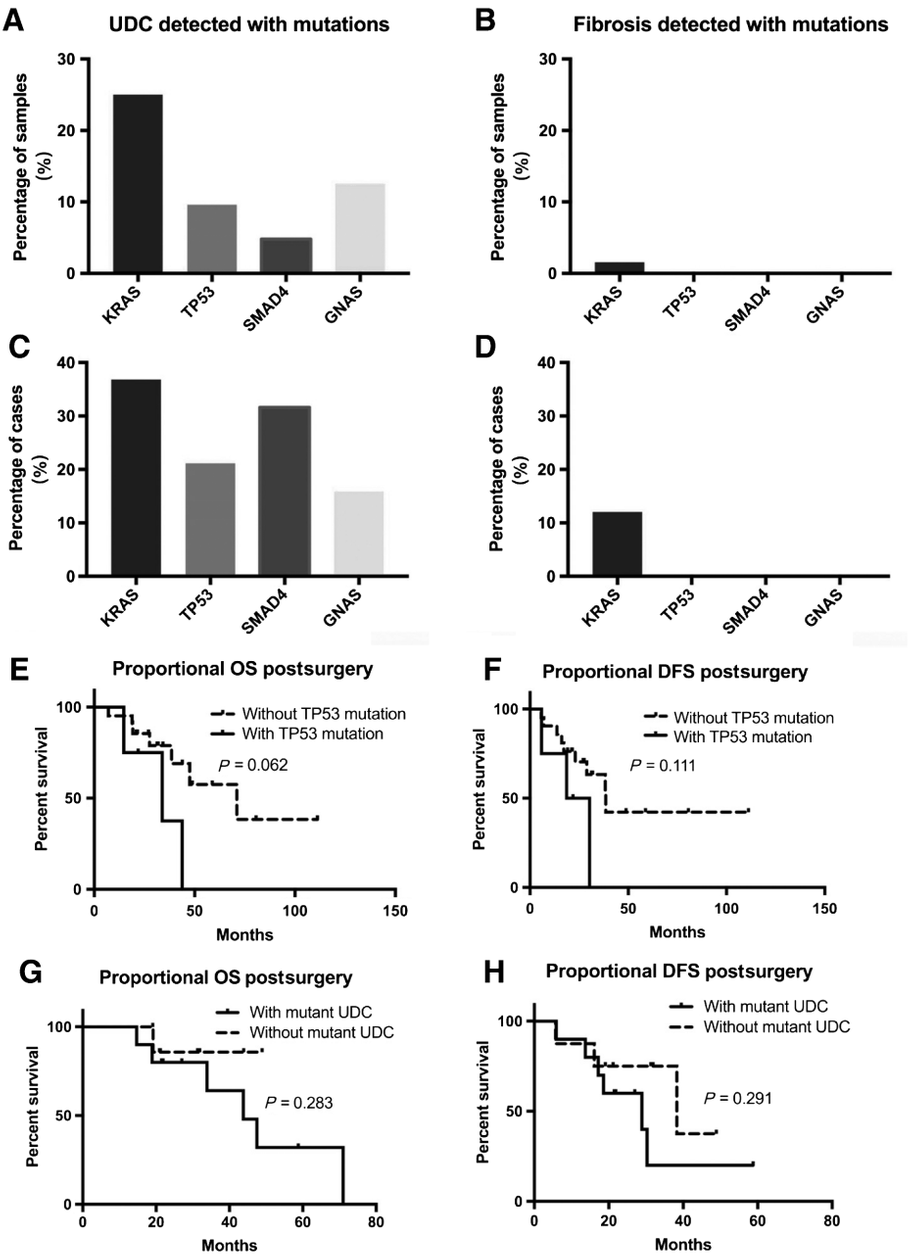

Figure 3.

Mutations detected in the resected specimens and their association with survival. **A** and **B,** Specific ROI DNA samples which were detected with somatic mutations. **C** and **D,** Specific cases (bottom) which were detected with somatic mutations. **E** and **F,** Comparison of OS and DFS postsurgery between pCR patients with and without TP53 mutation. **G** and **H,** Comparison of OS and DFS postsurgery between pCR patients with and without mutated UDC.

between CTCs and tumor-related mutations detected in resected specimens due to the fact that only one patient was positive for both CTCs and mutations in resected specimen (Supplementary Table S4). Sixteen patients were longitudinally tracked with ctDNA. Seven of these patients had ctDNA detected at the time of surgery, of which, six were found to have *KRAS* mutations. One of the seven patients were found with a *TP53* mutation (Supplementary Table S5). The concordance between ctDNA and mutation in resected specimen was not appreciable in three patients who were positive for both ctDNA and mutations in their respective resected specimen (patient No. 11, No. 15, No. 25; Supplementary Tables S4 and S5). Patient No. 15 and patient No. 25 were both positive for $KRAS^{G12D}$ mutation consistent with a mutation in the resected specimens while patient No. 11 had a different driver mutation in plasma compared with resected specimen ($KRAS^{G12D}$ *vs.* $GNAS^{R201H}$). Two of the three patients were found to develop recurrence during follow-up (patient No. 11 and No. 15). Patient No. 25 did not show recurrence in 19.1 months postsurgery.

CTCs and ctDNA dynamics

There were five pCR patients in the study with both longitudinal CTC and ctDNA data (No. 17, No. 18, No. 19, No. 20 and No. 22; **Fig. 5A–D**; Supplementary Fig. S2). Patient No. 17 (**Fig. 5A**) was negative for resected specimen mutations. In this patient, the peak quantity of ctDNA was 1 week after surgery. ctDNA then dropped remarkably after adjuvant chemotherapy. The total CTCs (tCTC) and ctDNA have remained positive 2 years after surgery. Patient No. 18 (**Fig. 5B**) was also negative for a mutation in the resected specimen and also showed no recurrence during the nearly 2 years

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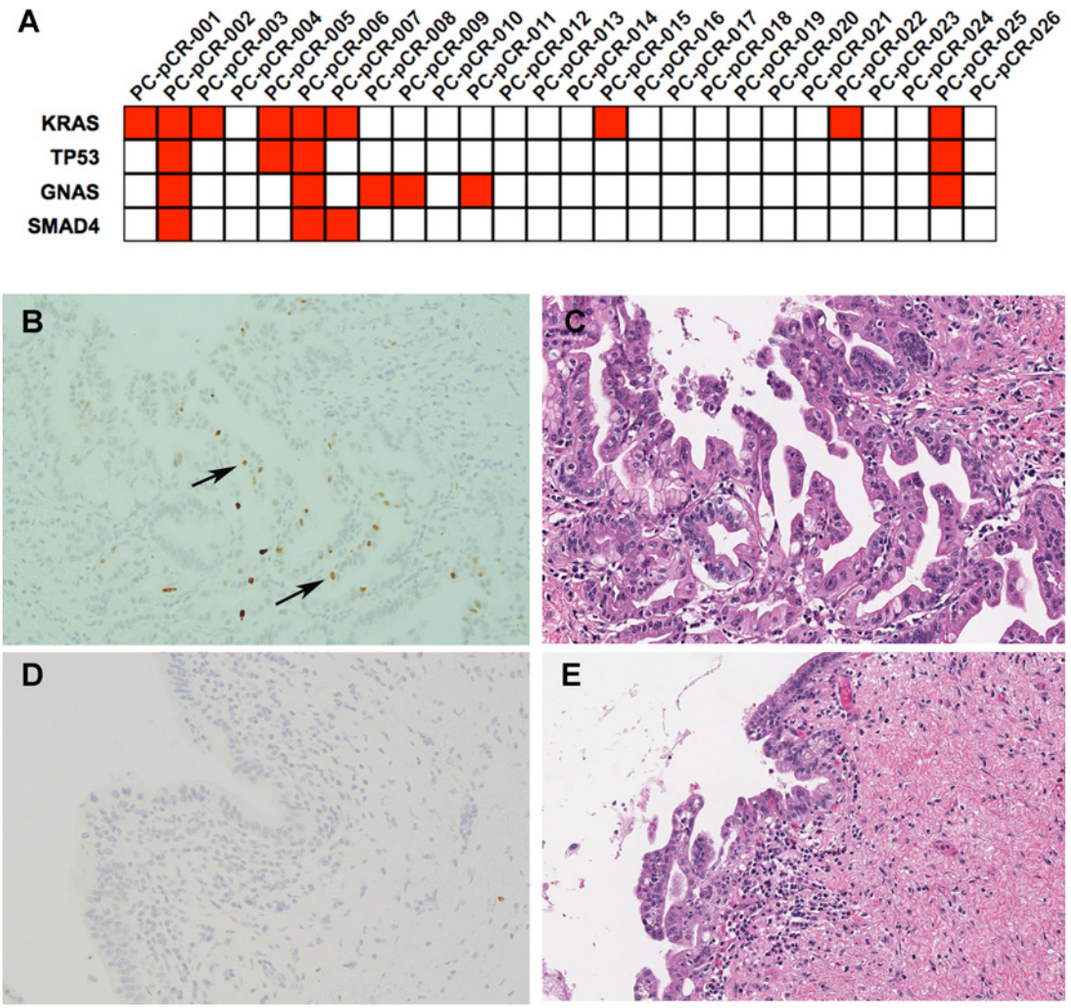

Figure 4.

Somatic mutations detected in the pCR patients and IHC validation. **A,** pCR patients with somatic mutations in the resected tumor specimens. **B–E,** Examples of concordance between IHC and next-generation sequencing with positive (**B** and **C**) and negative p53 (**D** and **E**; black arrows highlight TP53-mutated cells).

of follow-up. Here, tCTCs was positive for more than 1 year with no evident decrease after adjuvant chemotherapy. The ctDNA remained undetectable except in the last two follow-up dates when the patient was found to have a recurrence. Abnormal ctDNA was detected 75 days earlier than the radiological recurrence within the 3-month radiological follow-up interval.

Similarly, in patient No. 19 (**Fig. 5C**), no mutation was detected in the resected specimen. This has had no clinical recurrence 1 year out from surgery. In addition, both the CTC and ctDNA have dropped since surgery. The ctDNA stayed undetectable but tCTCs remains positive. Patient No. 20 (**Fig. 5D**) was also negative for a resected specimen mutation. This patient had a 1-year recurrence but only had two time points of data. The CTCs and ctDNA were both positive 1 week after surgery. Patient No. 22 (Supplementary Fig. S2) was detected with $KRAS^{G12V}$ and $KRAS^{G12R}$ mutations in ROIs with no adjuvant therapy and no recurrence. The CTC and ctDNA were negative 1 month after surgery; however, both of the CTCs and ctDNA showed up 1 year after surgery. The ctDNA of patients No. 24–26 (**Fig. 5E–G**) also showed the association with the CA19-9 to different degrees.

In this cohort, 15 of 36 cases suffered recurrence during follow-up. Among these recurrences, two patients were with CTCs data and were both found to have an increased level of CTCs during follow-up. Also, mCTCs were also present in the two patients. Six of the 15 patients had available for ctDNA data, of whom three patients were positive for ctDNA. Three of these six were had multiple time points of ctDNA data and two of them were found with increased level of ctDNA postoperatively before recurrence.

mCR

Six patients negative for both ctDNA and tumor-related mutations in resected specimens were identified as mCR (Supplementary Table S6). Fifteen patients positive for either of those two were classified as non-mCR. Significantly younger age was observed in mCR group compared with non-mCR group ($P = 0.035$). There is no statistical difference between two groups on stage ($P = 0.688$),

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neoadjuvant chemotherapy regimens ($P = 0.675$), modalities of neoadjuvant radiotherapy ($P = 0.285$), time of NAT administered ($P = 0.064$), radiological response ($P = 0.623$), type of operation ($P = 0.281$), adjuvant therapy ($P = 0.361$), and follow-up time ($P = 0.391$). Six of 15 patients of non-mCR group were found with recurrence during the follow-up while three of six patients of the mCR group showed disease progression. Seven of 15 non-mCR patients died during follow-up while only one of six patients with mCR died. However, no significant P value was achieved for analysis of OS or DFS postdiagnosis or surgery (Supplementary Fig. S3).

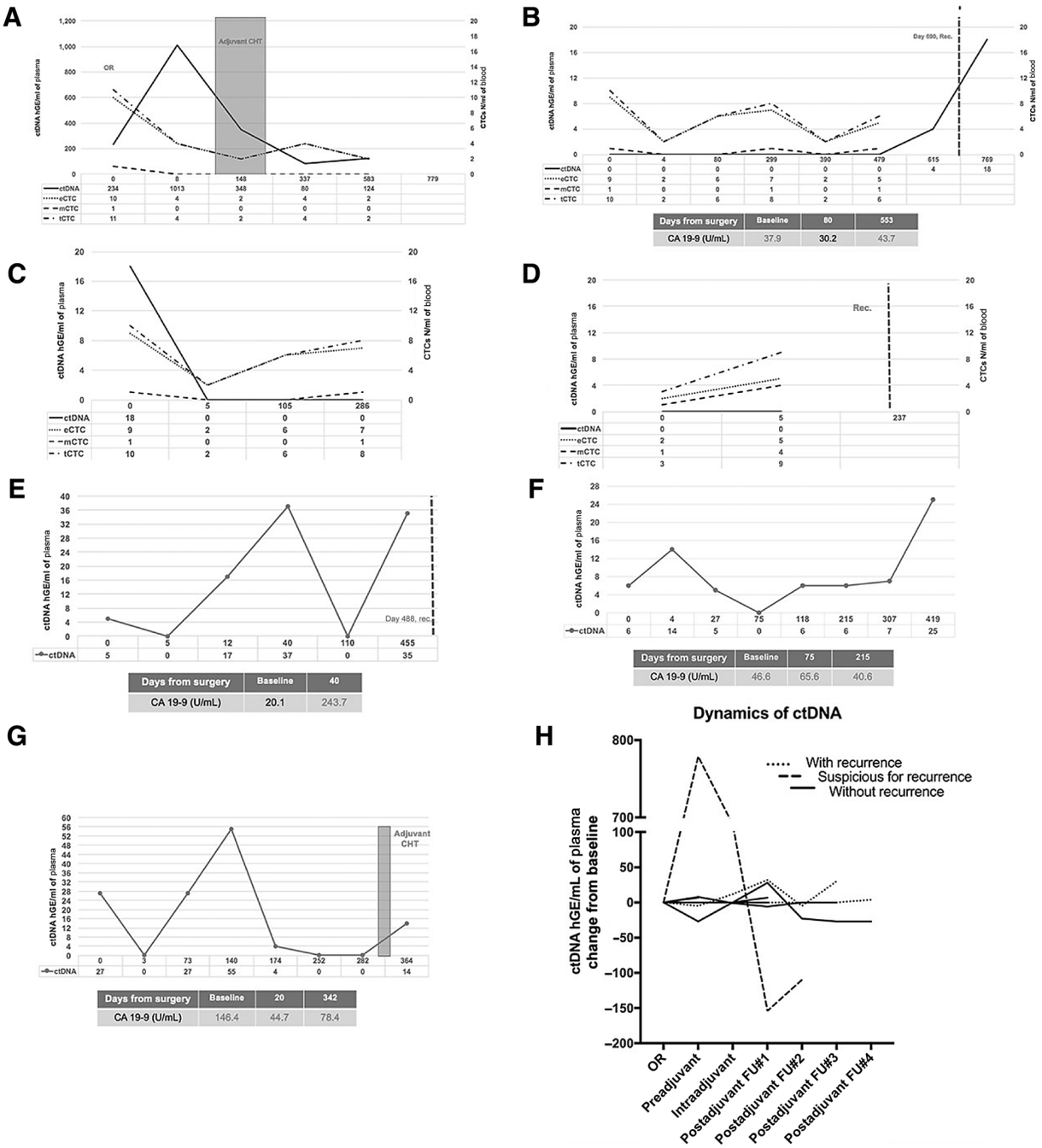

Figure 5.

CTCs and ctDNA dynamics. **A,** Patient No. 17. **B,** Patient No. 18: mCTC presented earlier than abnormal CA 19-9 before recurrence. **C,** Patient No. 19. **D,** Patient No. 20. **E,** Patient No. 24: ctDNA level dropped after surgery. ctDNA and CA 19-1 were both positive 40 days postsurgery. **F,** Patient No. 25: ctDNA and CA 19-1 were both positive during follow-up. **G,** Patient No. 26: ctDNA level dropped after surgery. ctDNA and CA 19-1 were both positive during follow-up. **H,** ctDNA were positive in all patients detected.

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Discussion

This study describes the somatic mutational landscape of resected PDAC with pCR to neoadjuvant therapy and the application of liquid biopsy in this specific subgroup for the first time. It was shown that residual somatic mutations remain in pCR "tumors" even though no viable cancer cells were found. Moreover, this is the first study that has reported on the presence of CTCs and ctDNA in patients with pCR after neoadjuvant therapy. This designates the value of liquid biopsy in pCR patients in terms of postoperative surveillance and prediction of outcomes.

Although neoadjuvant therapy is used widely in nonresectable patients with PDAC, the tumor regression grading system following neoadjuvant therapy is not standardized. The RECIST system, primarily based on radiological evidence, is the most commonly used method for evaluation of response at the clinical level. However, for PDAC, a radiographic response to neoadjuvant therapy is often insufficient to predict the response at the pathologic level because PDAC sometimes does not change in size. Radiologic restaging after neoadjuvant therapy has to be judged with caution when predicting tumor response and resectability, because inflammation induced by neoadjuvant therapy may mimic a solid tumor. In our pCR cohort of 36 patients, only 44.4% (16/36) had a reduction in tumor size. Furthermore, no additional survival benefit was found in pCR patients with a radiological response. Therefore, pathologic response evaluation is a more reliable measurement of response than radiology.

Currently, six pathologic assessment systems have been reported about the evaluation of pancreatic cancer following neoadjuvant therapy including Ishikawa and colleagues, 1989 (8), Evans and colleagues, 1992 (9), White and colleagues, 2005 (10), Le Scodan and colleagues, 2008 (11), Chatterjee and colleagues, 2012 (12), and CAP 2016 (13). Most systems are based on the evaluating the destruction of viable cancer cells and/or the extent of fibrosis induced by the treatment. The term "complete response" was defined in Le Scodan classification and CAP Protocol (2016), which is defined as "0% viable cancer cells in the resected primary specimens." However, a recent review on the pathology assessment of pancreatic cancer following neoadjuvant treatment showed that several inherent problems need to be addressed (14, 15) regarding the dispersed growth pattern of pancreatic cancer, identifying treatment-induced fibrosis from tumor-associated desmoplasia and sampling bias.

An accurate, standardized, and repeatable method for pathology examination of the residual cancer tissue following neoadjuvant therapy is needed to better compare publications on this topic and to establish pathways for the diagnostic and therapeutic management of these patients. In fact, although large cohorts showed that pCR is associated with improved survival (5), the survival of the pCR patients still vary remarkably from each other. This indicated that definition of pCR may not include all possible sources of residual tumor and the survival differences may not be strongly associated with the difference of the response in the primary tumor. Inherent problems of the pathological assessment system could be part of the reason. In addition, the absence of evaluating disease regression at a molecular level could also play a role. In this study, we performed genomic analysis on the resected pCR specimens and also the ctDNA, which provide the molecular parameters for the evaluation for pCR patients. It turned out that some patients with pCR did not achieve mCR, which could possibly explain cases of early recurrence and suboptimal outcomes with further validation.

In the era of personalized cancer therapy, patients with PDAC remain at considerably higher risk of relapse and death than patients with other cancer types, due to the aggressive nature of PDAC and the lack of novel targeted therapies. The patterns of recurrence have been discussed in our previous study, which reported no significant clinical prognostic factors for 29 pCR patients including the type of neoadjuvant therapy (16). The use of liquid biopsy to detect tumor-associated biomarkers in a variety of extractable body fluids has been described in numerous studies and is a promising biomarker of treatment response, disease progression, and survival. CTCs were also reported to be useful in monitoring clinical to chemotherapy (17). Bernard and colleagues demonstrated that an increase in exosome-associated DNA level after neoadjuvant therapy was significantly associated with disease progression (18). Unfortunately, the pathologic response data were missing in both studies. Because of the retrospective nature of most pCR studies, the absence of liquid biopsy data including CTCs and ctDNA makes it hardly possible to perform the matched analysis. Thus, the status of CTCs and ctDNA in pCR patients is still unknown. Our study for the first time reported the presence of CTCs and ctDNA in pCR patients. Although statistical conclusion could not be drawn due to the small sample size, the dynamics of CTCs and ctDNA were correlated with the recurrence and the survival of pCR patients.

Notwithstanding, our study has a number of limitations. Although it is the largest pCR cohort so far, the sample size is still too small to perform further statistical analysis. It is retrospective by design and not all patients received the same form of treatment. Also, there might be selection bias caused by sampling during the pathologic examination as discussed above. Because of the layer thickness of the slides, unknown pathologic status such as cancerization or even early cancer could be missed during slides selection. Cancerization, by definition, is that cancer cells that invade and grow along the lumina of nonneoplastic ducts, which is a transition between the normal duct epithelium and the cancer (19). Cancerization of the duct or residual tumor cells might be detected if the entire surgical resected specimens were examined slice by slice, even in pCR cases without cancerization of the duct. Of note, due to the nature of more prolonged survival of pCR patients, the follow-up of our cohort seems to be insufficient. However, the extensive use of neoadjuvant therapy started after 2010. The patients of our retrospective cohort were consecutively enrolled in the past 7 years. Thus, it is reasonable that some of the patients recruited more recently would have a relatively short outcome. The lack of a control cohort is also one of the limitations of this retrospective study. However, the patients included in this study were mostly at advanced stage. In this era of neoadjuvant therapy, it is uncommon to find a patient with borderline or LAPC treated with upfront surgery, especially at our institution, where a multidisciplinary tumor board discuss the treatment strategy for each patient. An additional study with larger sample size is needed for further validate the prognostic value of mCR.

In summary, pCR is a rare response status in patients with PDAC following neoadjuvant therapy but is associated with significantly improved survival. This is the first report to suggest that somatic mutations, CTCs and ctDNA exist even in patients with PDAC with pCR after neoadjuvant therapy. With further validation, these could hopefully be used to predict early recurrence and reduced survival. The current regression evaluation system of PDAC to neoadjuvant therapy needs to be reassessed at a molecular level. Accurate and standardized pathology examination of the residual cancer tissue after neoadjuvant therapy should be utilized primarily in prospective studies of neoadjuvant therapy with large sample sizes to offer more comparable data on pCR. Precision medicine with preoperative biopsy investigation, longitudinal liquid biopsy data, proper assessment of pathologic response, and genomic

analysis will give us a better understanding of the heterogeneity within patients' sensitivity to neoadjuvant therapy and further help us identify those patients who might benefit from some specific regimens. Thus, directing the remaining patients to novel targeted therapies, can be an effective strategy with near-term clinical impact for managing PDAC.

Authors' Disclosures

No disclosures were reported by the authors.

Authors' Contributions

L. Yin: Resources, data curation, formal analysis, writing-original draft, writing-review and editing. **N. Pu:** Resources, data curation, formal analysis, writing-original draft, writing-review and editing. **E. Thompson:** Resources, writing-original draft, writing-review and editing. **Y. Miao:** Resources, writing-original draft, writing-review and editing. **C. Wolfgang:** Resources, funding acquisition, writing-original draft, writing-review and editing. **J. Yu:** Conceptualization, resources, supervision, funding acquisition, methodology, writing-original draft, project administration, writing-review and editing.

Acknowledgments

This work was supported by the Nikki Mitchell Foundation and the Ben and Rose Cole Charitable Pria Foundation.

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Received June 9, 2020; revised August 10, 2020; accepted October 16, 2020; published first October 20, 2020.

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Published OnlineFirst October 20, 2020; DOI: 10.1158/1078-0432.CCR-20-1746

Clinical Cancer Research

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Clin Cancer Res 2021;27:740-748. Published OnlineFirst October 20, 2020.