FULL PAPER

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Clinical study of genomic drivers in pancreatic ductal adenocarcinoma

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Background: Pancreatic ductal adenocarcinoma (PDA) is a lethal cancer with complex genomes and dense fibrotic stroma. This study was designed to identify clinically relevant somatic aberrations in pancreatic cancer genomes of patients with primary and metastatic disease enrolled and treated in two clinical trials.

Methods: Tumour nuclei were flow sorted prior to whole genome copy number variant (CNV) analysis. Targeted or whole exome sequencing was performed on most samples. We profiled biopsies from 68 patients enrolled in two Stand Up to Cancer (SU2C)-sponsored clinical trials. These included 38 resected chemoradiation naïve tumours (SU2C 20206-003) and metastases from 30 patients who progressed on prior therapies (SU2C 20206-001). Patient outcomes including progression-free survival (PFS) and overall survival (OS) were observed.

Results: We defined: (a) *CDKN2A* homozygous deletions that included the adjacent *MTAP* gene, only its' 3′ region, or excluded *MTAP*; (b) *SMAD4* homozygous deletions that included *ME2*; (c) a pancreas-specific MYC super-enhancer region; (d) DNA repair-deficient genomes; and (e) copy number aberrations present in PDA patients with long-term ($\geqslant$ 40 months) and short-term ($\leqslant$ 12 months) survival after surgical resection.

Conclusions: We provide a clinically relevant framework for genomic drivers of PDA and for advancing novel treatments.

Pancreatic ductal adenocarcinoma (PDA) is projected to be the second leading cause of cancer deaths by 2020 and over the following decade (Rahib *et al*, 2014). Surgical resection remains the only modality capable of delivering curative outcomes for PDA patients. However, only ~20% of PDA patients are candidates for surgical resection, and of these ~80% are unable to achieve disease-free survival of >4 years. Most recurrences in surgically resected patients arise within 2 years and are almost universally fatal, typically within 1–2 years of recurrent disease (Neoptolemos *et al*, 2010; Oettle *et al*, 2013). Most patients are diagnosed with advanced metastatic disease adding to the dismal prognosis. Recent clinical trials have reported improved responses for subsets of patients highlighting the potential for discovery of somatic events in the variable landscape of PDA genomes that can be exploited for improved patient outcomes (Conroy *et al*, 2011; Von Hoff *et al*, 2013). However, the identification of drivers of clinical responses is challenged by the complexity and variability of PDA genomes and the heterogeneous cellularity and dense stroma present in tissue samples, as cancer cells represent on average only 25% of the cells within the tumour (Seymour *et al*, 1994; Evers *et al*, 2014). Furthermore, biopsies from patients who have received one or more prior therapies often contain high levels of necrosis and cellular debris.

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The diverse mutational landscape of PDA genomes has been catalogued (Waddell *et al*, 2015; Bailey *et al*, 2016). Integrated analyses of genomic data with associated expression patterns have identified subtypes that correlate with histopathological features and may drive clinical outcomes (Collisson *et al*, 2011; Biankin and Maitra, 2015). These genomic-based research studies of PDA typically rely on optimally collected resection specimens with relatively high (>40–50%) levels of tumour cells. Various approaches have been used to extend genomic studies to bio specimens with low tumour content and high levels of necrosis (Jones *et al*, 2008; Song *et al*, 2012; Moffitt *et al*, 2015). These include patient-derived cell lines and xenografts, *in silico* tumour purity determination based on sequencing reads for *KRAS* mutations, and algorithms designed to deconvolute single nucleotide polymorphism (SNPs)-based signatures prior to sequencing. However additional processing steps and reliance on specialised algorithms for biospecimen evaluation and data interpretation limits the application of these methods for PDA biopsies collected during clinical trials.

To address the challenges associated with routinely collected clinical biopsies, we have validated DNA content-based flow sorting of diploid, tetraploid and aneuploid tumour populations in PDA and other solid tumours (Ruiz *et al*, 2011; Holley *et al*, 2012). Our methods yield purified (>95%) tumour samples for whole genome analyses from a variety of clinical samples. These include fresh frozen and formalin fixed paraffin embedded tissues with low tumour content (<10–20%) and high amounts (>90%) of necrosis and debris. In this current study we applied our flow sorting-based methods to PDA biopsies collected from 68 patients enrolled in two Stand Up to Cancer (SU2C)-sponsored clinical trials. These included 38 surgically resected chemoradiation naïve tumours (SU2C 20206-003) and metastases, primarily from liver, from 30 patients with advanced disease who had progressed on prior therapies (SU2C 20206-001). Patients were followed prospectively and clinical outcomes including progression-free survival (PFS) and overall survival (OS) were observed. Here we show that by combining prospectively collected clinical data, clonal genomic analyses and employing novel information analytical tools for data integration, we can provide insights into the genomic landscapes and clinical phenotypes of primary and metastatic PDA. This integrated approach enables linkage of data to external sources and databases. Our initial results include the detection of homozygous deletions targeting known and novel tumour suppressor genes, the mapping of a PDA *MYC* amplicon cassette containing a cluster of pancreas-specific super enhancers, the identification of DNA repair defects including a BRCA-like copy number aberration signature, and the identification of genomic landscapes associated with improved and limited survival after resection.

METHODS

Clinical samples. Fresh frozen PDA samples were obtained under a WIRB protocol (20040832) for AACR/SU2C-sponsored clinical trials (20206-001 and 20206-003). All patients gave informed consent for collection and use of all the samples which were collected in liquid nitrogen and stored at −80 °C. All tumour samples were histopathologically evaluated prior to genomic analysis. All research conformed to the Helsinki Declaration (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/).

Metastatic patients 20206-001. The SU2C 20206-001 trial was a phase II study of therapy selected by molecular profiling in patients with previously treated metastatic disease. Eligible patients (⩾18 years of age) had a diagnosis of metastatic PDA and had received ⩾1 prior therapy for the treatment of metastatic disease. One or more metastatic tumours had to be measurable by CT scan and be accessible for a tumour biopsy. Other pertinent eligibility criteria were acceptable bone marrow, kidney and liver function, prior therapy must have been completed ⩾3 weeks before starting study and side effects of prior therapy must have resolved to ⩽grade 1. A Karnofsky performance status (KPS)⩾70 was required. All patients signed an institutional review board approved consent form before participating in any study-related activities. Patients who met eligibility criteria underwent a percutaneous CT or US guided biopsy of an accessible metastatic lesion using an 18 gauge needle with at least three passes. Treatment decision was determined by immunohistochemistry (IHC). Commercially available treatment regimens prescribed included FOLFIRI, FOLFOX, irinotecan and doxorubicin. The response (RECIST) was 9%. The median survival was 5.6 months (94% CI 3.8–7.2) and the 1-year survival was 20% (95% CI, 7–33%). A tissue from each metastatic core biopsy was pooled, then flash frozen before flow sorting and array-based comparative genomic hybridisation (aCGH).

Surgical resection patients 20206-003. The SU2C 20206-003 trial was a tissue acquisition protocol to support metabolomic and genetic profiling for predicting patient outcomes in early-stage resectable pancreatic cancer. Eligible patients (⩾18 years of age) had a diagnosis of primary pancreatic cancer and did not receive any prior therapies. Informed consent for research use was obtained from all patients at the enrolling institution (University of Pennsylvania) before tissue banking and study approval was obtained. PDA specimens and matched germline specimens (from peripheral blood) from patients were acquired for genomic analyses. Primary tumour samples for genomic analyses were selected from patients with resectable stage I, II, or III disease, and reviewed by histopathological assessment.

Flow cytometry and PDA biopsies. Biopsies were minced in the presence of NST buffer and DAPI according to published protocols (Rabinovitch *et al*, 2001; Holley *et al*, 2012). Nuclei were disaggregated then filtered through a 40 μm mesh before flow sorting with an Influx cytometer (Becton-Dickinson, San Jose, CA, USA) with ultraviolet excitation and DAPI emission collected at >450 nm. DNA content and cell cycle were analysed using the software program MultiCycle (Phoenix Flow Systems, San Diego, CA, USA).

Copy number analysis of flow sorted PDA biopsies. DNAs were extracted using Qiagen micro kits (Qiagen, Valencia, CA, USA). For each hybridisation 100 ng of genomic DNA from each sample and of pooled commercial 46XX reference (Promega, Madison, WI, USA) were amplified using the GenomiPhi amplification kit (GE Healthcare, Piscataway, NJ, USA). Subsequently 1 μg of amplified sample and 1μg of amplified reference template were digested with DNaseI then labelled with Cy-5 dUTP and Cy-3 dUTP respectively, using a BioPrime labelling kit (Invitrogen, Carlsbad, CA, USA). All labelling reactions were assessed using a Nanodrop assay (Nanodrop, Wilmington, DE, USA) prior to mixing and hybridisation to CGH arrays with 400 000 or 1 000 000 oligonucleotide features (Agilent Technologies, Santa Clara, CA, USA).

All microarray slides were scanned using an Agilent 2565C DNA scanner and the images were analysed with Agilent Feature Extraction (FE) version 11.0 using default settings according to our published data (Barrett *et al*, 2015). The aCGH data were assessed with a series of FE QC metrics then analysed using an aberration detection algorithm (ADM2; Lipson *et al*, 2006). The latter identifies all aberrant intervals in a given sample with consistently high or low log ratios based on the statistical score derived from the average normalised log ratios of all probes in the genomic interval multiplied by the square root of the number of these probes. This score represents the deviation of the average of the

normalised log ratios from its expected value of zero and is proportional to the height h (absolute average log ratio) of the genomic interval, and to the square root of the number of probes in the interval. The values for each sample were then imported into ISSAC's ILLUMINATIVE Analytics platform. For deletions a threshold of $\log_2\text{ratio} < -0.5$ was initially applied to identify regions of recurrent loss (Figure 1A). We then used a window of 10 kb and a threshold of $\log_2\text{ratio} < -2.0$ for ADM2 defined intervals with < -3.0 for individual probes to identify homozygous deletions. We used the root mean square (RMS) of the $\log_2$ratio genomic interval values with a sliding window of 0.5 Mb and a threshold of $\log_2\text{ratio} > 0.6$ to identify high level and recurring amplicons (Figure 1B). These data were integrated with *KRAS* (30 metastases) and cancer panel (5 primaries) targeted resequencing, and with whole exome sequencing (25 primaries).

Sequencing. From the needle biopsies in the 20206-001 trial, mutation analysis of selected regions for KRAS was performed using Sanger sequencing with M13-linked polymerase chain reaction primers. polymerase chain reaction products were bidirectionally sequenced using the BigDye Terminator v1.1 chemistry (Applied Biosystems, Grand Island, NY, USA), and analysed using the 3730 DNA Analyser (Applied Biosystems). Sequence traces were analyzed using Mutation Surveyor software v3.25 (Soft Genetics, San Francisco, CA, USA). Samples from the 20206-003 trial were processed for either whole exome ($n = 25$) or targeted resequencing with a 111 gene cancer panel ($n = 5$) according to our published methods (Sausen *et al*, 2015).

Super-enhancer analysis. Genomic intervals from the ADM2 derived CGH analyses were used to interrogate pancreas-specific features within the Super-Enhancer Archive (SEA) http://www.bio-bigdata.com/SEA/index.html (Wei *et al*, 2016). The response incorporated detailed (epi)genetic information, incorporating cell type specificity, nearby genes, transcriptional factor binding sites, CRISPR/Cas9 target sites, evolutionary conservation, SNPs, H3K27ac, DNA methylation, gene expression and transcription factor ChIP-sequence data.

Data integration and analysis. Genomic results, treatment reports and outcome data from trials 20206-001 and 20206-003 were fused and analysed using The Health Analytic (THA) powered by ILLUMINATIVE Analytics (ISSAC Corp). To perform intra- and inter-trial analysis, data were ingested, fused, analysed and reported-on, iteratively. Data were ingested via loose-heuristic parsing into a novel graph structure supported by SQL and fused and related across multiple dimensions via exact value matching (cardinal and categorical types), similarity assessment (real numbered values) and concept resolution (string types) (Talburt, 2011).

RESULTS

Tumour content of pancreas biopsies. There were 141 patients enrolled in SU2C 20206-003 trial. Biopsies from 65 resections were available for genomic analysis and processed for flow cytometry (Figure 1). Based on combined flow cytometry and copy number analysis we detected $\geqslant$1–5% tumour in the research biopsy in 38/65 (58.5%) of these optimally collected resected cases including two pancreatic neuroendocrine tumours (PNETs). Given the superior outcomes for patients with neuroendocrine tumours and the absence of *KRAS* mutations the PNET samples were analysed separately from the PDA cases (Jiao *et al*, 2011). Forty-nine patients were accrued for the advanced previously treated SU2C 20206-001 trial. One patient withdrew consent prior to a biopsy, and 48 were scheduled and underwent a biopsy without major complications. Thirteen patients did not start protocol therapy either due to insufficient tumour for analysis on biopsy ($n = 8$) or due to worsening cancer-related symptoms after biopsy which precluded further treatment ($n = 5$). A total of 35 metastatic PDA biopsies from treated patients were processed with flow cytometry and aCGH. In three of these cases there was no tumour detected whereas two additional cases failed quality control metrics in the aCGH analysis. A final total of 68 prospectively collected tumour biopsies (38 resected and 30 metastases) were included in this study. Tumour content in the biopsies varied extensively ($<5\%$ to $>50\%$). However, the use of flow sorted samples enabled objective thresholds for the identification of genomic lesions and for the discrimination of homozygous deletions and focal amplicons in each tumour.

Selected genomic lesions

Mutations. KRAS codon 12 mutations were detected by targeted resequencing in 27 of 28 (96%) metastatic PDA samples. Strikingly we detected a novel homozygous deletion affecting *RASA1*; a

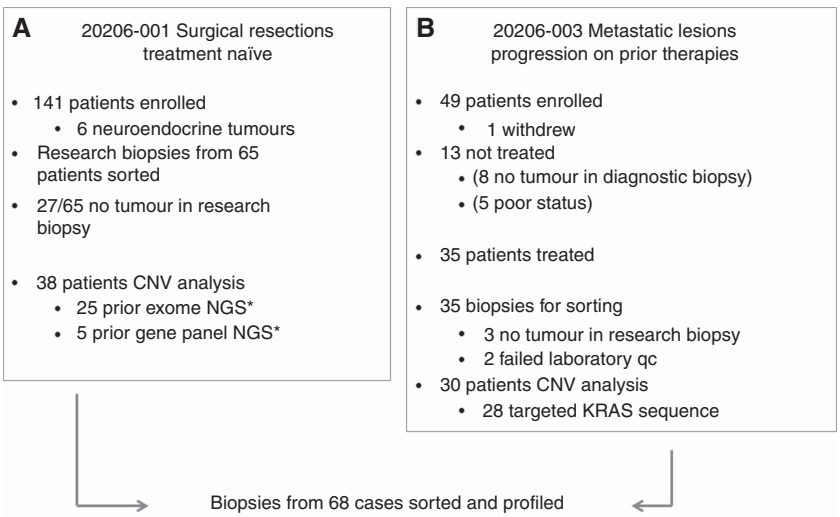


Figure 1. Summary of patients enrolled and samples profiled in SU2C clinical trials. (**A**) Copy number variant (CNV) analysis was performed on flow sorted primary pancreas cancer samples from 38 patients enrolled in SU2C 20206-003. Next generation sequencing (NGS) data were available from 25 of the 38 samples. (**B**) CNV analysis was obtained from 30 patients with metastatic pancreatic cancer. Targeted KRAS mutation analysis was done on 28 of these metastatic samples. *Sausen *et al*., Nat Commun **6**: 7686.

negative regulator of wild-type *KRAS*, in the one confirmed case that was *KRAS* wild type (Supplementary Figure 1). Thirty resected cases were sequenced including 25 by whole exome as previously described (Sausen *et al*, 2015). Twenty-seven (90%) of these had a detectable *KRAS* mutation. In addition, mutations in *TP53* (77% patients) and in multiple chromatin regulation and modification genes (*MLL1*, *MLL2*, *MLL3* and *ARID1A*) were detected in the resected patient samples (Sausen *et al*, 2015).

Copy number deletions. Two of the most frequent copy number aberrations were losses of 9p21 in 53/68 patients (78%) and 18q21 in 58/68 patients (85%) (Figure 2A). These included homozygous deletions targeting *CDKN2A* (9p21.3) in 37/68 cases (54%) and *SMAD4* (18q21.1) in 3/68 cases (4.0%), tumour suppressor genes with known roles in pancreatic cancer. The homozygous 9p21.3 deletions targeting *CDKN2A* varied in length from 76 kb to 7.6 mb and included methylthioadenosine phosphorylase (*MTAP*) which maps approximately 100 kb proximal to *CDKN2A* in 23/37 of these cases (62%) while 3/37 cases (8.0%) had homozygous deletions that extended only to the 3′ region of *MTAP* (Figure 3). The remaining 11/37 cases (30%) had at least one intact copy of *MTAP*. The three cases with *SMAD4* homozygous deletions also included the adjacent malic enzyme 2 (*ME2)* locus, a context that may be exploited through a synthetic lethal targeting of *ME3* (Dey *et al*, 2017). Homozygous deletions were also present in genes within common fragile sites including *FHIT*, *WWOX*, and in multiple tumour suppressor genes including unique deletions in *RB1*, *MAP2K4* and *MSH6* (Supplementary Figure 2).

The resected tumour with the *MSH6* homozygous deletion which was not associated with a germ line variant had over 400 somatic nucleotide variants (SNVs) in the exome. This level of SNVs is consistent with microsatellite instability (MSI). MSI represents a clinical phenotype that has been exploited therapeutically with immune checkpoint blockade in a variety of indications (Le *et al*, 2015). However, MSI PDAs are very rare and their clinical significance has not been validated (Laghi *et al*, 2012). Other novel homozygous deletions detected in our cohort included those within a series of chromatin and transcriptional regulators including *SMYD3*, *L3MBTL4*, *PRDM15*, *KLF13*, *ASXL2* and *MLL3*. The latter two genes also had mutations in one and four cases respectively highlighting their role and that of deregulated transcriptional programming in PDA. Additional homozygous deletions were detected in genes with a variety of known and putative biological roles including chromosome stability (*STAG1*, *FRY*), cell signalling

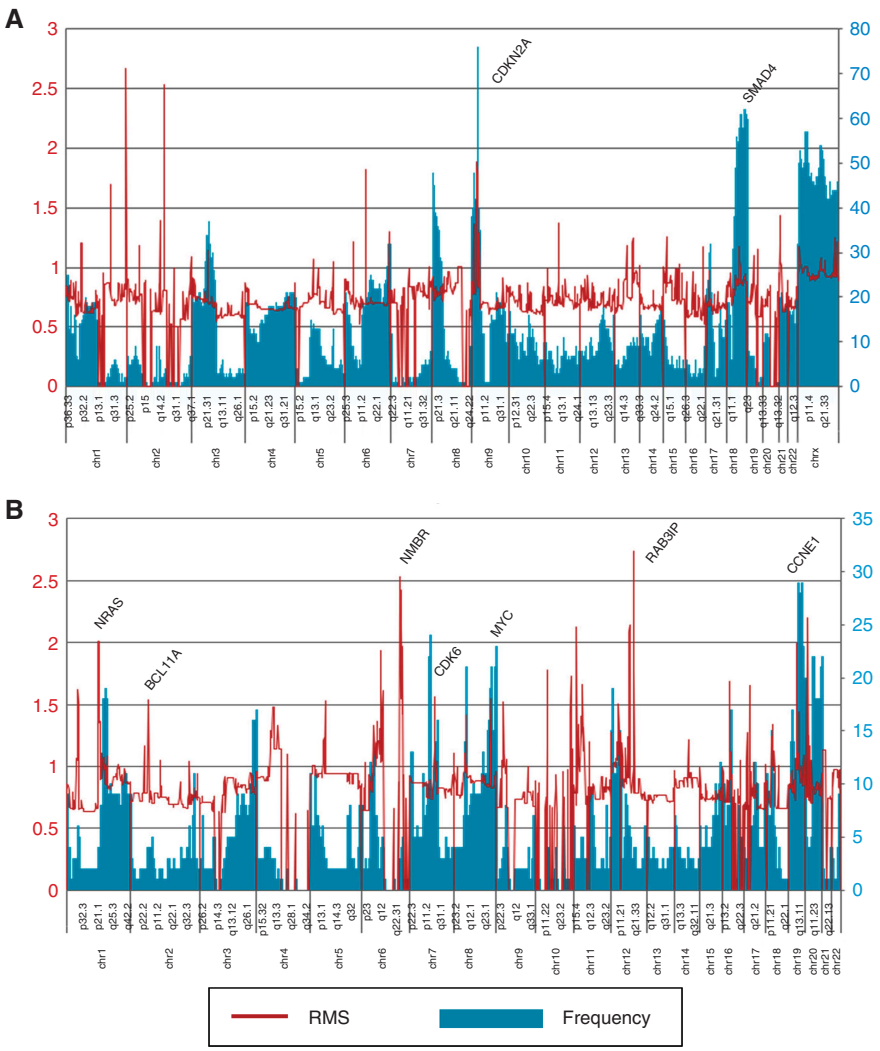
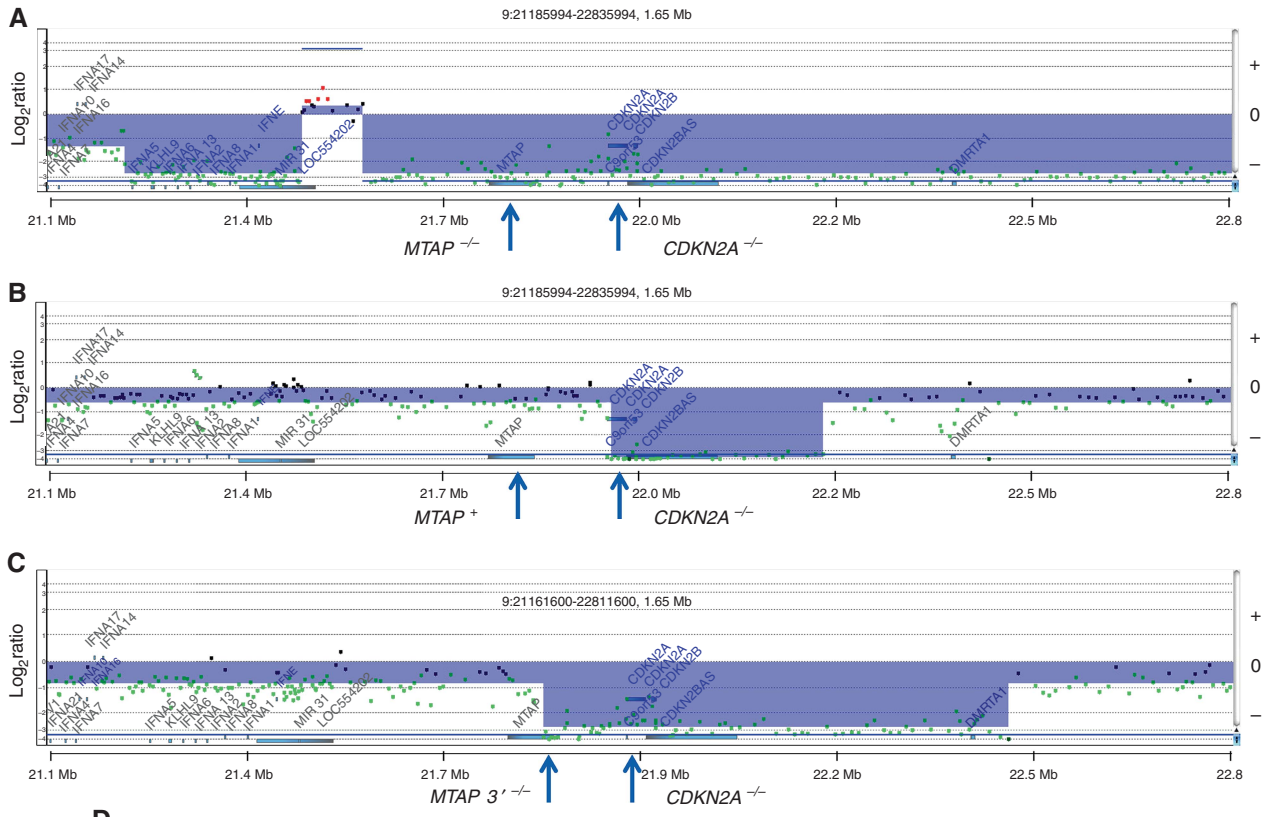


Figure 2. Genome-wide view of recurring copy number aberrations in PDA genomes. (**A**) Deletions: The root mean square (RMS) of the $\log_2$ratio genomic interval values with a sliding window of 0.5 Mb and a threshold of $\log_2$ratio < -0.5 was initially applied to identify regions of recurrent loss. A window of 10 kb and a threshold of $\log_2$ratio < -2.0 for ADM2 defined intervals with < -3.0 for individual probes was used to identify homozygous deletions. (**B**) Amplicons: The root mean square (RMS) of the $\log_2$ratio genomic interval values with a sliding window of 0.5 Mb and a threshold of $\log_2$ratio > 0.6 was used to identify high level and recurring amplicons.



D

9p21.3 deletions				
	CDKN2A $^{-/-}$	MTAP $^{-/-}$CDKN2A $^{-/-}$	MTAP $^{+}$CDKN2A $^{-/-}$	MTAP 3′ $^{-/-}$ CDKN2A $^{-/-}$
2026–001	17/30	8/17	6/17	3/17
2026–003	20/38	15/20	3/20	2/20
Total	37/68 (54%)	23/37 (62%)	9/37 (24%)	5/37 (14%)

Figure 3. Chromosome 9p24.1 homozygous deletions. Homozygous deletions were detected and mapped that include (**A**) *CDKN2A* and *MTAP* in patient 20206-003 041, (**B**) *CDKN2A* in patient 20206-003 039, and (**C**) *CDKN2A* and the 3′ region of *MTAP* in patient 20206-001 015. Blue shade areas denote ADM2 defined genomic intervals. (**D**) Summary of homozygous 9p21.3 deletions in the SU2C trials.

(*RGS7*), autophagy (*TRIM13*) and cell migration (*SLITRK6*, *SLITRK5*).

Copy number amplifications. The most frequent amplicons included chromosomes 8q24.3 in 21/68 (31%) patients and 19q12 in 29/68 (43%) patients (Figure 2B). These amplicons included focal events (RMS > 1.5) whose shortest region of overlap (SRO) targeted *c-MYC* and *CCNE1* respectively. We used the coordinates of the amplicon SROs to interrogate the SEA database and identified a pancreas-specific super-enhancer region that is co-amplified with the *c-MYC* driver oncogene (Figure 4). In contrast the SRO of the *CCNE1* amplicon did not contain any known pancreas-specific super-enhancers. Additional recurring high-level amplicons targeted chromosomes 12q15 in 4/68 (6.0%) of patients and 7q21 in 10/68 (15%) patients. The 7q21 amplicon included *CDK6* and was present in a genome with a co-occurring *CDKN2A* homozygous deletion suggesting these two selected oncogenic copy number events arose independently of each other and have non-overlapping cancer phenotypes (Supplementary Figure 3). Strikingly the two most focal 12q15 amplicons included *MDM2* but were present in cases with mutations in *TP53*, including the R175H gain of function, suggesting that a gene other than *MDM2* was the target of this selected event (Muller and Vousden, 2014). Notably the guanine exchange factor (GEF) *RAB3IP*, which can activate RAS superfamily members *RAB8A* and *RAB8B*, maps to the SRO of this amplicon.

Clinical outcomes for chemoradiation naïve surgical resection patients (20206-003). Fifty-three per cent of the evaluable patients were males with an age range of 47–85 (median 66 years). None of the individual recurring copy number aberrations was significantly associated with clinical outcomes in this trial. Furthermore there was no association with increased number of copy number aberrations and OS after surgical resection. However to investigate potential correlates of survivorship we examined those cases with the most positive and the most negative outcomes post surgery. There were six PDA patients who underwent surgical resection who survived at least 40 months after surgery followed by adjuvant chemotherapy (Table 1A). The genomic profiles for these six patients included the mismatch DNA repair phenotype associated with a *MSH6* homozygous deletion, two separate tumours with co-occurring high-level amplicons ($\log_2$ratio > 3.0) targeting *CCNE1* and *CTPS*, and two patients with a focal *GATA6* amplicon as the most significant copy gain in their tumour (Supplementary Figures 2, 4) (Figure 5A and B). These genomic profiles were unique to this subset of resected PDAs.

Seven PDA patients survived less than 12 months despite surgical resection and adjuvant treatment (Table 1B). The genomic

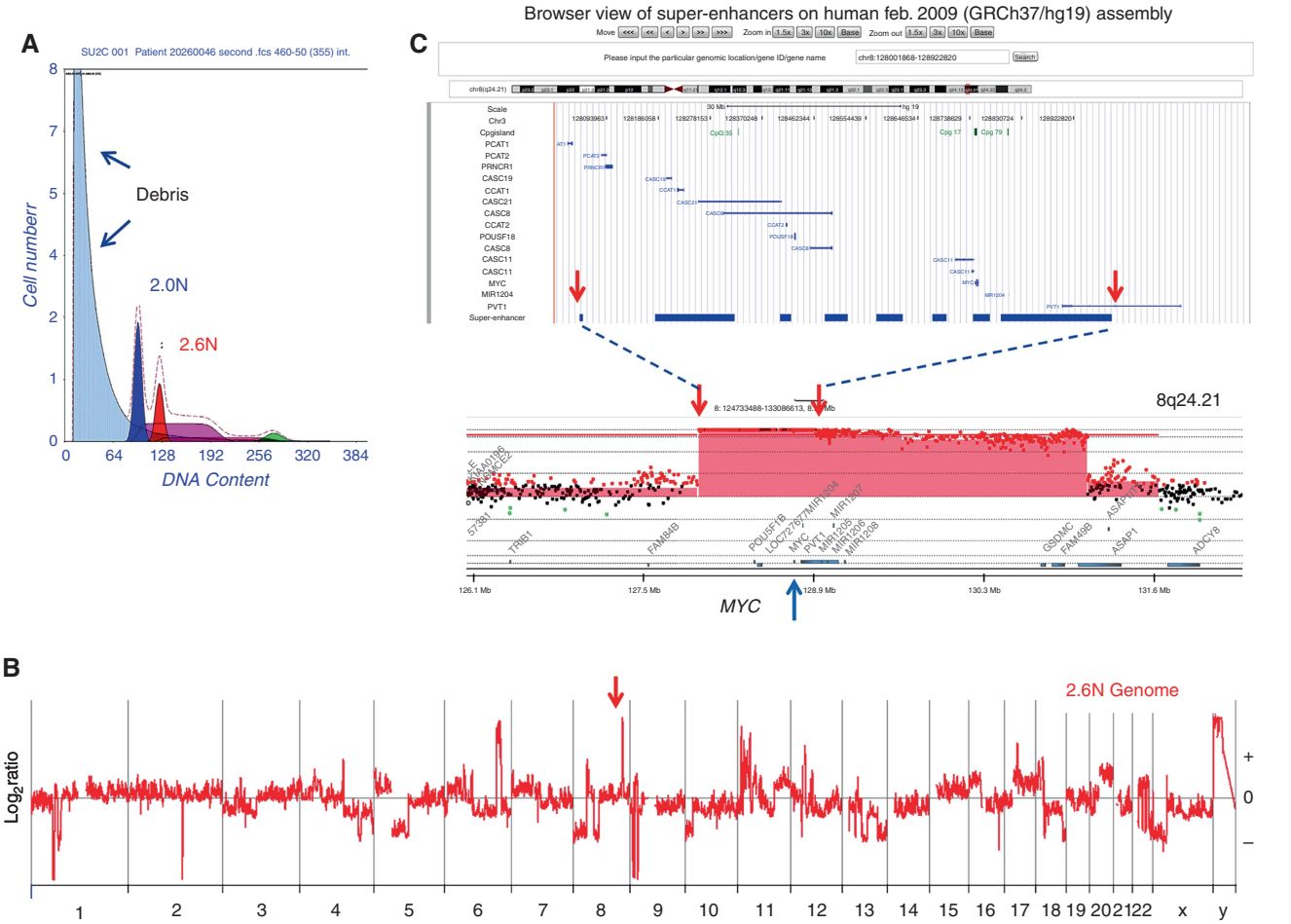


Figure 4. Shortest region of overlap (SRO) of 8q24.1 MYC amplicon and superhancer loci. (**A**) Flow sort histogram of biopsy from patient 2026-001 046. (**B**) Whole genome CNV plot of sorted 2.6N PDA population. (**C**) Pancreas-specific superhancer loci and SRO of MYC amplicon mapped using genome browser and genome build HG19. Red shaded areas denote ADM2 defined amplicon in 20206-001 046.

profiles of these seven short-term survivors shared heterozygous deletions of 18q spanning the *SMAD4* locus. These included two somatic mutations and a homozygous deletion consistent with previous studies associating inactivation of *SMAD4* with the development of a more aggressive cancer phenotype in the pancreas (Supplementary Figure 5) (Iacobuzio-Donahue *et al*, 2009). However each of the six long-term survivors also had heterozygous loss of 18q and 2/4 cases that were sequenced had somatic mutations in *SMAD4* suggesting that additional genomic and/or epigenetic events contribute to the aggressiveness of disease.

There were two cases (5%) of resected PDA that had *HER2* amplicons as their most significant copy number aberration. One of these cases subsequently developed multiple low-attenuation masses in the residual body and tail of pancreas with a large mass in the tail that spread around the spleen into the subdiaphragmatic region 11 months after surgery. The prevalence and clinical history is similar to previous reports of *HER2* positive PDA (Chou *et al*, 2013).

The outcomes for the six PNET patients were uniformly superior when compared to the PDAs with each surviving beyond 46 months after surgery. Five of the six patients with PNETs had no evidence of disease even without receiving any adjuvant therapy. The fifth patient relapsed at 13 months post surgery but achieved a complete response to chemotherapy and was reported alive after more than 60 months from time of surgery. The biopsies from two of these cases contained tumour cells that were sorted and profiled for copy number (Supplementary Figure 6). The two neuroendocrine genomes each had whole chromosome loss of chromosome 11 and gain of chromosome 5, two distinct features of this histologic subtype (Zhao *et al*, 2001). In addition we did not detect *KRAS* mutations in either of the two PNETs that were sequenced. Strikingly one of these sequenced tumours had a homozygous deletion of *SMYD3* (SET and MYND domain containing 3) histone methyltransferase.

Clinical outcomes for advanced previously treated patients (20206-001). The demographics of evaluable patients ($n = 35$) were males (55%), the majority had KPS of 70–80 and age range was 34–81 (median 63 years). Time from first diagnosis of metastatic PDA to biopsy ranged from 5.8 to 26.7 months (median 16.1 months). All patients had prior gemcitabine-based therapy with a median of two prior regimens (range 1–6). The time from patient signing consent to performing the biopsy was a median of 4 days (range 1–9 days) and from biopsy to availability of the IHC report was a median of 13 days (range 4–16 days). Time on study ranged from 0 to 8.6 months (median 1.3 months, 95% CI 1–1.6 months). All patients have died, with survival ranging from 0.4 months to 26.8 months. The median PFS was 2.4 months (95% CI 2.1–3.9 months) and median survival was 5.6 months (94% CI 3.8–7.2 months). The 1-year survival was 20% (95% CI, 7–33%) for all 35 patients. In the study population ($n = 35$) nine patients had stable disease and there were three partial responses (9%); these patients received FOLFOX (20206–001 023), FOLFIRI (20206-001 029) and nab-paclitaxel/capecitabine (20206-001 033) for a disease control rate of 35% (95% CI, 19–54%). The 30 metastatic tumour samples that were successfully sorted and profiled included 24 patients who received treatment. Tumour biopsies from two of the

Table 1. Survival SU2C 20206-003

Patient	Stage	Months	D/A[a]	Genomics	Age/Sex	Adjuvant
(A) Long term >40 months						
077	IIa T3N0	43	D	18q11 GATA6+++[b]	63 XX	Y
034	IIb T3N1	41	D	$MSH6^{-/-}$[c] MSI+	76 XX	Y
062	IIb T3N1	48	A	19q11 CCNE+++	70 XY	Y
038	IIa T3N0	41	D	1p34.2 CTPS+++	72 XX	Y
036	IIb T2N1	43	D	18q11 GATA6+++	77 XX	Y
035	IIa T3N0	51	A	$BRCA2^{969Q>X}$	72 XX	Y
(B) Short term ⩽12 months						
115	IIb	10	D	$18q^{-/+}$[d]	63 XX	?
039	IIa T3N0	12	D	$SMAD4^{-/-}$	65 XY	Y
090	IIb T3N1	8	D	$18q^{-/+}$	59 XY	Y
087	T3N1	12	D	$18q^{-/+}$	69 XY	Y
061	IIb T3N1	4	D	AKT2+++ SMAD4 insertion	56 XX	Y
021	IIa T3N0	9	D	$SMAD4^{361R>H}$	82 XX	N
042	IIb T3N1	8	D	$18q^{-/+}$	58 XY	Y

[a]D, dead; A, alive.
[b]+++ Amplified.
[c]−/− Homozygous deletion.
[d]−/+ Partial loss.

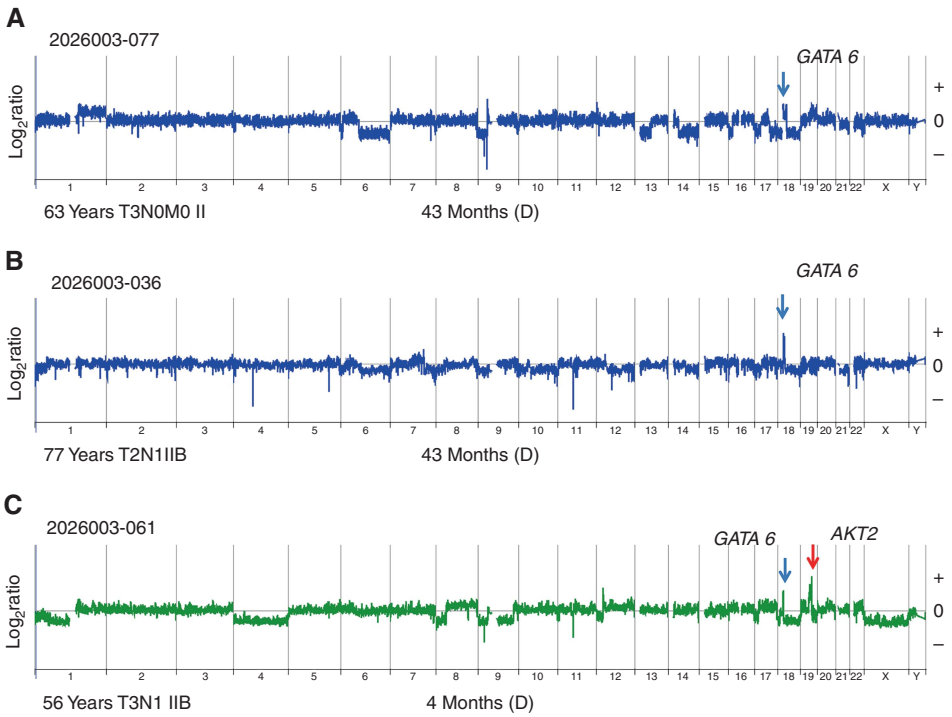


Figure 5. GATA6 amplification and long-term 20206-003 survivors. Patients (**A**) 077 and (**B**) 036 from 20206-003 trial had ⩾40-month survival post surgery. Each had a GATA6 amplicon as the most significant copy number aberration in the genomes of their PDA. (**C**) In contrast patient the PDA genome of patient 061, 4-month survival after surgery, had additional AKT2 amplicon that exceeded the height of the co-occurring GATA6 amplicon.

three patients who achieved partial responses were evaluated. In both cases the 1p34.2 amplicon (*CTPS*) and the 18q11 amplicon (*GATA6*) detected in primary tumours of long-term survivors were present as co-occurring aberrations in the metastatic PDA genomes (Supplementary Figure 7).

DISCUSSION

The genomic landscapes of primary and metastatic human pancreatic tumours have been surveyed in detail (Waddell *et al*, 2015; Bailey *et al*, 2016). However most of these studies do not include clinical trial biopsies and prospective outcomes. The two SU2C studies provide an opportunity to profile both newly diagnosed surgically resected patients (SU2C 20026-003) and those with advanced metastatic disease who had progressed on prior therapies (SU2C 20206-001). Our data provide a framework for developing new hypotheses for the drivers of clinical outcomes in PDA and the development of effective therapeutic strategies.

***CDKN2A* homozygous deletions.** Our detailed mapping of homozygous deletions targeting *CDKN2A* in flow sorted clinical samples (54% of cases) reveals that these events have been under

reported in conventional bulk tumour analyses (Waddell *et al*, 2015; Bailey *et al*, 2016). Recent reports suggest that co-deletion of *MTAP* with *CDKN2A* disrupts tumour methionine metabolism and creates a synthetic lethal condition that can be exploited clinically (Kryukov *et al*, 2016; Mavrakis *et al*, 2016). Given the high prevalence of *CDKN2A* homozygous deletions in both primary and metastatic lesions, PDA represents a favourable clinical model for future studies to test this therapeutic concept. Notably, homozygous deletions of *CDKN2A* may extend through *MTAP* (62%), include only the 3′ region (8.0%), or exclude *MTAP* altogether (30%) (Figure 3).This level of precision in genomic studies is needed to develop effective personalised therapies for patients with pancreatic cancer.

Genomic lesions targeting G_1/S regulators. The loss of G_1/S regulation is a hallmark of most solid tumours including PDA. This can arise as a consequence of multiple genomic and epigenomic lesions including deletions of CDK inhibitors, amplification of CDKs and disruption of cell cycle checkpoints. Accordingly, in addition to highly prevalent *CDKN2A* homozygous deletions we observed events such as homozygous deletions targeting *RB1* and amplification of *CDK6* in our cohort. The development of small molecule inhibitors of CDKs has shown promise in clinical trials (Asghar *et al*, 2015; Finn *et al*, 2015). Inhibitors with dual specificity for CDK4/6 are being advanced to target those tumours with deregulated G_1/S control. Elevated CDK6 activity is associated with different solid tumours (Mendrzyk *et al*, 2005; Ismail *et al*, 2011; Sherr *et al*, 2016). The role of CDK6 in regulating G_1/S is believed to be synonymous with CDK4. Therefore high level CDK6 amplification and homozygous deletion of *CDKN2A* should be mutually exclusive. Given the high prevalence of *CDKN2A* homozygous deletions PDAs represent a highly favourable clinical model for advancing CDK inhibitors. However the co-occurrence of these two highly selected lesions suggests that CDK6 amplification can drive additional tumour pathways beyond the cell cycle (Supplementary Figure 3). Notably, CDK6 has also been shown to have a transcriptional role in tumour angiogenesis (Kollmann *et al*, 2013).

The high definition detection and mapping of homozygous deletions identified other novel contexts that can be used to inform new therapeutic strategies. For example mouse studies have implicated *SMYD3* as an activator of MEK signalling suggesting that *SMYD3* represents a highly favourable clinical target for RAS-driven tumours including those arising in the pancreas (Mazur *et al*, 2014). Furthermore, the complete loss of SMYD3 function has no visible phenotype in mice. However the presence of a focal SMYD3 homozygous deletion in a PNET suggests a tumour suppressor role for this histone lysine methyltransferase in KRAS wild-type tumours that needs to be addressed in future clinical studies.

***MYC* amplification.** Mapping of recurring *MYC* amplicons identified an SRO for this frequently amplified driver oncogene that targets a pancreas-specific super-enhancer region. Super-enhancer specific amplicons have been identified from TCGA data sets for multiple cancers (Zhang *et al*, 2016). Notably an amplified super enhancer that drives MYC expression was mapped distal to the *PVT1* locus in lung adenocarcinoma and endometrial carcinoma. We did not detect this super-enhancer-specific amplicon in our flow sorted PDA data. Rather the PDA *MYC* amplicon consisted of a cassette-like structure that when highly amplified retained distinct core pancreas-specific super-enhancer regions spanning the *PVT1* locus and loci proximal to *MYC*. Copy number profiles of 30 established PDA cell lines also mapped the *MYC* PDA super-enhancer to this same amplified region in PSN1 cells which retained the most focal and the highest level MYC amplicon.

Recent preclinical studies have demonstrated that inhibitors of transcriptional activation-driven through super-enhancers may have clinical activity in a variety of tumours (Chipumuro *et al*, 2014; Christensen *et al*, 2014). Super-enhancers associated with *MYC* have been shown to be highly sensitive to small molecules that reduce levels of the transcriptional activator *BRD4* (Loven *et al*, 2013). The extensive occupancy and high density of BRD4 binding sites associated with the MYC super-enhancer in tumour cells creates an oncogenic dependency that may be exploited therapeutically. The presence of high level focal *MYC* amplicons in PDA that include pancreas-specific super-enhancers provides a biomarker and a target for emerging classes of compounds that aim to disrupt the ordered complexes that are required to drive *MYC* expression in tumour genomes. The extent of *MYC* amplification and expression in PDA may provide an increased therapeutic index for those patients with this amplicon. Of significant interest will be to follow the occupancy and access of the *MYC* super-enhancers with CHiP-seq and ATAC-seq assays using sorted tumour and non-tumour fractions from biopsies to be collected in our upcoming trials.

***GATA6* amplification.** Genomic aberrations, including both gains and losses, targeting 18q11 are frequent in PDA (Waddell *et al*, 2015; Bailey *et al*, 2016). Amplification of 18q11 that includes the GATA6 locus has been reported in gastrointestinal malignancies including those arising in the pancreas (Fu *et al*, 2008; Lin *et al*, 2012; Sulahian *et al*, 2014). Functional studies suggest that GATA6 can activate oncogenic signalling during development of pancreatic cancer (Zhong *et al*, 2011). However, GATA6 amplification has also been associated with a favourable prognosis after resection and adjuvant therapy (Martinelli *et al*, 2016a). GATA6 was also identified as a suppressor of $KRAS^{G12V}$-driven PDA and as a target in a sleeping beauty screen in KRAS-driven mouse models (Perez-Mancera *et al*, 2012; Martinelli *et al*, 2016b). In addition, recent studies suggest that GATA6 overexpression promotes epithelial cell differentiation while suppressing inflammatory pathways (Martinelli *et al*, 2016a, b). Thus the role of GATA6 in pancreatic cancer and its' clinical utility remain to be determined. Two of the long-term survivor cases in the 20206-003 trial had GATA6 amplification as the most significant (i.e. 'driver') copy number aberration in their tumour genomes (Table 1, Figure 5A and B). GATA6 driver amplicons were also present in two out of three patients with advanced metastatic disease who achieved partial responses after chemotherapy (Supplementary Figure 7). These data suggest that GATA6 driver amplicons may be exploited for improved clinical outcomes.

Homologous recombination deficient PDA. In addition to specific genomic lesions the overall burden and nature of copy number aberrations represents a potential clinical biomarker for PDA (Bailey *et al*, 2016). Specifically, the presence of elevated numbers of interstitial aberrations (IAs) throughout a genome is associated with a homologous recombination deficiency (HRD) in BRCA mutant tumours (Lord and Ashworth, 2016). A summary of the patients in the 20206-001 trial showed a range of <10 to >70 in the number of IAs in each sorted tumour population (Supplementary Figure 8). The IAs were defined by the ADM2 step gram algorithm as copy number aberrant intervals with intrachromosomal boundaries (Lipson *et al*, 2006). These included amplicons, deletions and homozygous losses in the tumour genome. Patient 006 had a pathogenic *BRCA2* germ line variant and a PDA genome with 54 IAs that included 20 of 22 autosomes. Notably the highest number of aberrations was observed in the sorted aneuploid population from patient 046. The latter had a stable disease response to an early PARP inhibitor prior to enrolment in the SU2C trial. Strikingly this patient was wild type for BRCA1 and BRCA2. The same HRD signature was observed in a subset of primary PDAs including a patient with a somatic

BRCA2^{Q969X} mutation (Supplementary Figure 2B). A fundamental hypothesis from these and other genomic data is that a subset of PDAs, both BRCA mutant and BRCA wild type, have a HRD phenotype and will be responsive to both targeted agents and broad-based chemotherapies that either disrupt DNA repair or increase the burden of DNA double-strand breaks. Our early phase clinical trial of newly diagnosed metastatic PDA combining cisplatin with gemcitabine and nab-paclitaxel has shown over 70% response rate including 30% complete responses (Jameson *et al*, 2017). These genomic signatures are being combined with strategies targeting other components of PDA tissues, including stroma, stem cells and immune cells, to improve the extent and duration of clinical responses.

CONCLUSION

We have developed a well-annotated clinical data set using purified flow sorted pancreatic cancer biopsy samples for genomic analyses. These include high resolution mapping of *CDKN2A* and *SMAD4* homozygous deletions and of *MYC* amplicons, DNA repair deficient tumours and targeted genomic lesions such as *GATA6* amplification in patients with positive outcomes in the adjuvant setting. These data provide hypotheses for current models of genomic drivers of this deadly disease as well as the framework for advancing novel treatments for patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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