## Supplemental Methods

## The Precision Medicine Workflow

Patients self-refer to the Patient Central call center at the Pancreatic Cancer Action Network. Those identified by a Patient Central Associate as being eligible and interested in enrolling in an IRB-approved registry trial are referred to Perthera. Eligibility requirements have become increasingly less restrictive since program inception, and are as follows as of May 2016.

*1. Diagnosis of a pancreatic malignancy*

*2. No signs of progression or impending need to change treatment, or understands that KYT cannot be used for immediate treatment planning (Note: It requires 25-45 days from receipt of tissue for the Perthera report to be completed and delivered to the patient and physician.)*

*3. Tissue is obtainable*

o *Newly obtained core biopsy, 4-6 cores with 18-20 gauge needle preferred*

*From accessible metastatic site*

*From primary if biopsy acquisition is deemed an acceptable risk*

o *Surgical specimen*

*If post-surgery, within 12 months of resection with no other source of tissue available*

*4. Patient is United States/Canada-based*

5. *Patient/close caregiver able to read/speak English or Spanish.*

*6. Patient is not imprisoned*

The Perthera precision medicine operation used in the KYT program utilizes an integrated workflow consisting of:

1. Consenting patient into an IRB approved registry study
2. Obtaining approval of the treating oncologist to coordinate biopsy acquisition and obtain previous treatment history
3. Coordinating tissue biopsy and distribution to CAP/CLIA accredited laboratories for multi-omic profiling
4. Data storage and standardization in HIPAA-compliant databases
5. Utilizing rules-based data analytics and integration of multi-omic data with previous treatment history. The rules for aggregating biomarker evidence and ranking therapies are derived from a knowledge database consisting of published cancer literature, clinical trial results, and treatment guidelines. The knowledge database is continuously updated by Perthera computational biologists and medical review panel (MRP) members.
6. Providing a cloud-based virtual tumor board to incorporate expert oncologist review of treatment rankings. The MRP for each case consists of 6 members: 2 medical oncologists with a focus on pancreatic cancer and 4 scientists, specifically cancer biologists or computational biologists. The MRP members provide a review of the data (both molecular and patient history) and of the report. They also enter new biomarker-treatment relationship information into the knowledge database.
7. Delivering final report to both treating oncologist and patient
8. Collecting treatment decision from treating oncologist, outcomes data collection and patient follow up.

***Biomarker Actionability***

To classify the therapeutic actionability of biomarkers, the following criteria were used:

* Highly actionable – published clinical trial evidence linking a biomarker to drug response in any cancer type
* Modified options – pre-clinical evidence or case studies linking a biomarker to drug response in any cancer type

The degree of “response” required to meet these criteria (particularly for the “highly actionable subgroup”) is not an absolute number, but reflects response/disease control rates that are well above what would otherwise be expected for “standard therapy” for that disease. For the highly actionable category, well known examples include:

* Response to PARP inhibitors in homologous recombination-deficient ovarian and prostate cancer1-3
* Response to immune checkpoint inhibitors (anti-PD-1) in MMR-deficient tumors4
* Response to ALK inhibitors in ALK rearranged non-small cell lung cancer5
* Response to NTRK inhibitors in NTRK fusion tumors6,7

***Legends for Supplemental Tables in Excel File***

**Table S1:** Genes and proteins on each panel used in this study.

**Table S2A:** Actionability status of each biomarker in this study.

**Table S2B:** Status of therapies (FDA approved vs. investigational) listed by highly actionable biomarker.

**Table S2C:** Details of the biomarkers in the “highly actionable subgroup” in which specific mutations for which there is clinical evidence demonstrating benefits of an appropriately targeted therapy. In some cases (e.g. AKT, we have made the assumption that molecular alterations in one gene, such as AKT1, are translatable to highly related genes, such as AKT2 and -3).

**Table S3:** Results of Fisher’s test (*P*-values and *Q*-values) for comparison to public datasets.

***PD-L1 staining methods***

We used Caris Life Sciences as the testing lab for PD-L1. The anti-PD-L1 antibody was the SP142 clone. Staining was read only from the cytoplasmic or membrane portion of tumor cells.

***Phosphoprotein profiling methods***

*Isolation of pure tumor cells via laser capture microdissection.* A total of 20 FFPE patients’ samples were submitted to Theranostics Health, Inc. for RPPA analysis. Sections (n=5) were cut at 5-µm thickness and mounted on uncharged glass slides. Adequate presence of a significant amount of tumor cells was verified by a certified pathologist on one Hematoxylin and Eosin stained slide. Before undergoing laser capture microdissection, slides were de-paraffinized in xylene, re-hydrated in 70% ethanol, and stained with Hematoxylin and Eosin. Tumor cells were isolated via direct visualization using an Arcturus XT automated system. A minimum of 10,000 microdissected tumor cells per sample were lysed in SDS-based buffer and boiled for 60 minutes before arrayed.

*Reverse phase protein microarray preparation and immunostaining.* Using a 2470 Aushon arrayer, samples were printed onto nitrocellulose covered slides (Sartorius Stedim Biotech, Göttingen, Germany) along with standard curves and internal controls. Samples, validation samples and process controls were printed in four technical replicates on each slide. To verify that each sample was in the linear dynamic range of the protein assay, a BSA serial dilution curve was added to the array to estimate the protein concentration of each sample.

Immunostaining was performed on an automated DAKO system using a commercially available Catalyzed Signal Amplification (CSA) kit (Dako, Carpinteria, CA). Samples were probed using primary antibodies targeting HER1 (Cell Signaling, Danvers, MA, Cat# 4267), HER1 (Y1068) (Cell Signaling Cat# 3777), HER2 (Dako Cat# A0485), HER2 (Y1248) (Novus Bio Cat# NB100-81960), HER3 (Cell Signaling Cat# 4754), HER3 (Y1289) (Cell Signaling Cat# 4791), SRC (Y416) (Cell Signaling Cat# 2101), MET (Y1234-1235) (Cell Signaling Cat# 3077), ALK (Y1604) (Cell Signaling Cat# 3341), VEGFR (Y951) (Cell Signaling Cat# 2471), FGFR (Y653-654) (Cell Signaling Cat# 3471), Ret (Y905) (Cell Signaling Cat# 3221), Ros (Y2274) (Cell Signaling Cat# 3078), PDGF Receptor Beta (Y751) (Cell Signaling Cat# 3161), AKT (S473) (Cell Signaling Cat# 4060), AKT (T308) (Cell Signaling Cat# 9275), mTOR (S2448) (Cell Signaling Cat# 9971), 4EBP1 (S65) (Cell Signaling Cat# 9451), 4EBP1 (T37/46) (Cell Signaling Cat# 9459), S6 Ribosomal Protein (S235-236) (Cell Signaling Cat# 4858), MEK1/2 (S217-221) (Cell Signaling Cat# 9121), ERK1/2 (T202 Y204) (Cell Signaling Cat# 9101), JAK2 (Y1007-1008) (Cell Signaling Cat# 3771), and STAT3 (Y705) (Cell Signaling Cat# 9145).

Signal detection was achieved using a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) coupled with a biotinyl-tyramide based amplification system. The Cy5 Streptavidin (KPL, Rockville, MD) fluorescent detection system was used. To quantify the amount of protein in each sample, selected slides were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) following manufacturer’s recommendation. A laser scanner was used to acquire images of antibody and Sypro Ruby stained slides (Genepix 4200 AL, Molecular Devices, Sunnyvale, CA). Images were analyzed using commercially available microarray software (Genepix Pro 6.1, Molecular Devices, Sunnyvale, CA).

*Normalization and scoring.* For each analyte tested, validation samples from commercial available cell lines were spotted to determine the linear range of quantification for the assay. The Normalized Fluorescence Intensity per unit of protein (NFU) is calculated by taking the measured fluorescence intensity of samples that are within the linear of quantification and normalizing them to the total protein fluorescence intensity within the same samples. Sample NFU values were compared to a representative population and reported as a “Score”. Scoring corresponds to the following values: “0” patient NFU value being below the population mean; “1” patient NFU value between the mean and 1 Standard Deviation (STD) above the mean; “2” patient NFU value between 1 and 2 STD above the mean; and “3” patient NFU value greater than 2 STD above the mean.

*Statistical analysis.*Testing for differences in mutation frequencies between clinical subgroups was performed using Fisher’s exact test. Correction for multiple testing was performed using the Benjamini-Hochberg method for controlling false discovery rate8. Survival analysis within and between groups was performed using Kaplan-Meier estimator in the *survival* package of the R statistical programming environment. Differences between groups were evaluated for statistical significance using the log-rank test and Cox proportional hazards models.

## Comparison of Mutation Frequencies to Published Datasets

Mutation frequencies of KYT patients were compared to four publicly available pancreatic adenocarcinoma datasets. These datasets consisted of primary tumor samples, mostly from surgical resections9-11. Data was downloaded from cbioportal.org12. on June 26, 2017. Only mutation data was used; CNV data was not. Thus, amplifications and deletions were removed from the KYT NGS data to ensure a valid comparison.



**Supplemental Figure S3.** KYT data from NGS profiling of 570 pancreatic ductal adenocarcinoma patients was compared to four publicly available datasets. Gene names are listed if Fisher’s test *P*-values were significant after multiple testing correction using the Benjamini-Hochberg procedure.

## Differences in Genomic and Proteomic Marker Frequencies Between Subgroups

**Supplemental Table S4: Marker prevalence in clinical subgroups.** For each clinical variable, patients were divided into two groups (listed in parentheses, group 1 vs. group 2) and the frequency of pathogenic mutations (NGS) or positive expression (IHC) was compared using Fisher’s exact test (*P*-values) followed by the Benjamini-Hochberg procedure8 for multiple testing correction (Q-value). All biomarkers with false discovery rate (FDR) less than 0.05 are displayed. No significant differences in biomarker frequencies were observed between males and females.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Clinical Variable** | **Marker** | **# Mutated or Positive in Group 1** | **# Mutated or Positive in Group2** | **P-value** | **Q-value** |
| **Age**  **(<62 vs. ≥62)** | KRAS (NGS) | 218/272 | 317/344 | 2 x 10-5 | 0.002 |
| **Histology**  **(Adenocarcinoma vs. Not Adenocarcinoma)** | KRAS (NGS) | 523/569 | 12/47 | 1 x 10-24 | 2 x 10-22 |
| MEN1 (NGS) | 0/569 | 6/47 | 1 x 10-7 | 8 x 10-6 |
| TP53 (NGS) | 425/569 | 17/47 | 2 x 10-7 | 8 x 10-6 |
| PGP (IHC) | 191/290 | 3/22 | 2 x 10-6 | 3 x 10-5 |
| PTEN (NGS) | 6/569 | 6/47 | 9 x 10-5 | 0.003 |
| RB1 (NGS) | 6/569 | 6/47 | 9 x 10-5 | 0.002 |
| DAXX (NGS) | 1/569 | 4/47 | 1 x 10-4 | 0.003 |
| MET (IHC) | 197/323 | 5/24 | 2 x 10-4 | 0.001 |
| CTNNB1 (NGS) | 4/569 | 5/47 | 2 x 10-4 | 0.004 |
| CDKN2A (NGS) | 237/569 | 9/47 | 0.003 | 0.042 |
| **Stage at**  **Biopsy**  **(Loc Adv vs. Met)** | TOP1 (IHC) | 30/119 | 226/332 | 6 x 10-16 | 1 x 10-14 |
| ERCC1 (IHC) | 23/129 | 154/344 | 4 x 10-8 | 3 x 10-7 |
| MET (IHC) | 36/91 | 166/256 | 4 x 10-5 | 2 x 10-4 |
| SMAD4 (NGS) | 20/178 | 107/438 | 2 x 10-4 | 0.02 |
| TP53 (NGS) | 110/178 | 332/438 | 0.001 | 0.044 |
| TLE3 (IHC) | 58/125 | 198/321 | 0.004 | 0.015 |
| **Tumor Location (Panc vs. Not Panc)** | TOP1 (IHC) | 47/139 | 209/312 | 6 x 10-11 | 1 x 10-9 |
| MET (IHC) | 42/102 | 160/245 | 4 x 10-5 | 4 x 10-4 |
| ERCC1 (IHC) | 36/148 | 141/325 | 6 x 10-5 | 4 x 10-4 |
| **Tumor Location (Liver vs. Not Liver)** | TOP1 (IHC) | 142/196 | 114/255 | 4 x 10-9 | 7 x 10-8 |
| TP53 (NGS) | 201/247 | 241/369 | 1 x 10-5 | 0.001 |
| ERCC1 (IHC) | 95/199 | 82/274 | 1 x 10-4 | 0.001 |
| CDKN2B (NGS) | 57/247 | 41/369 | 1 x 10-4 | 0.007 |
| MET (IHC) | 109/158 | 93/189 | 2 x 10-4 | 0.001 |
| TLE3 (IHC) | 127/191 | 129/255 | 0.001 | 0.005 |
| GNAS (NGS) | 3/247 | 24/369 | 0.001 | 0.043 |

## Phosphoprotein Expression by RPPA

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**Supplemental Figure S5: Phosphoprotein expression by RPPA in a subset of biopsies.** Levels of phosphorylated proteins were measured for a panel of 21 proteins across a subset of 20 patients. Raw signals were compared to the distribution from a reference population and binned according to the number of standard deviations (STD) from the mean, with “0” indicating less than the population mean, “1” indicating between the mean and 1 STD above the mean, “2” indicating between 1 and 2 STD above the mean, and “3” indicating greater than 2 STD above the mean. Each column corresponds to a patient, with patients ordered by ERK phosphorylation.

## Concordance Across Platforms

In the subset of 20 patients with phosphoprotein analysis, we examined the concordance between assay platforms for identical markers and for markers within the same pathway (Figure S2). RPPA revealed elevated HER2 expression in 2/20 tumors, with one of these having elevated HER2 phosphorylation as well (Figure S2A). These two patients were not HER2-positive by IHC. One patient in this subgroup had amplification of the *ERBB2* gene, but this finding was not confirmed by ISH; furthermore, this patient did not have overexpression of HER2 by IHC and RPPA and did not have HER2 phosphorylation by RPPA. These platforms may not be useful in selecting PDA patients for HER2 inhibitors such as trastuzumab and lapatinib.

RPPA measurements may help to prioritize therapeutic recommendations based on NGS and IHC assays by revealing which pathways are truly activated. In the subset of patients with RPPA measurements, 3/20 had amplifications of FGF-related genes (Figure S2B), but only one of these three had elevated FGFR1 phosphorylation. This may reflect differences in ligand specificity for FGFR1: the patient with elevated FGFR1 phosphorylation had amplification of *FGF3*, *FGF4*,and *FGF19*, whereas the patients lacking FGFR1 phosphorylation had amplification of *FGFR1* in one case and amplification of *FGF6* and *FGF23* in the other. Six of 20 patients had MET phosphorylation (Figure S2C), with 5 of these accompanied by a positive IHC test for MET expression. However, 8 of 13 patients that were MET-positive by IHC lacked MET phosphorylation.

In the 17/20 patients with *KRAS*-activating mutations, only 8/17 had elevated downstream phosphorylation of ERK (Figure S2D). Furthermore, one patient with wild-type *KRAS* had elevated phosphorylation of ERK, suggesting that factors other than *KRAS* mutation status may control the response to MEK or ERK inhibitors.

In the 12/20 patients with elevated phosphorylation of one or more of the proteins in the AKT/mTOR pathway (AKT, mTOR, 4E-BP1, and S6), only one had NGS alterations that could lead to activation of the pathway (Figure S2E). Additionally, expression of the tumor suppressor PTEN as measured by IHC was a poor predictor of pathway activation: 5/12 tumors with elevated AKT/mTOR pathway phosphorylation were PTEN-negative, whereas the other 7 were PTEN-positive (PTEN-positive tumors would be expected to have little phosphorylation of AKT and downstream proteins). Taken together, these results suggest that traditional NGS- and IHC-based assays may have limitations in determining which patients would respond to inhibitors of mTOR and AKT.



**Supplemental Figure S6: Concordance across molecular analysis platforms.** Multiple measurements of identical biomarkers or biomarkers within the same pathway often revealed differences for HER2 (A), FGF (B), RAS/MAPK signaling (C), and AKT/mTOR signaling (D). In the boxes corresponding to NGS markers, white space indicates wild-type whereas gray indicates that while no genetic alteration was observed, wild-type status could not be confirmed due to poor sample quality. For HER2 ISH data, gray indicates that amplified/not amplified status could not be confirmed due to poor sample quality.

**Supplemental Table S7: Cox proportional hazards models of PFS and OS in the actionability/matched therapy subgroups.** Inverse propensity weighted Cox regression was used to make two PFS and OS comparisons: 1) patients with highly actionable biomarkers versus patients with no highly actionable biomarkers; and 2) patients with highly actionable biomarkers receiving molecularly matched therapy versus patients with highly actionable biomarkers receiving unmatched therapy. Cox regression was also used to determine the significance of OS differences between these three groups (see Supplemental Figure S6).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **n** | **HR** | **95% CI** | ***P*-value** |
| **PFS in 2nd and 3rd line** |  |  |  |  |
| *Actionability* |  |  |  |  |
| No highly actionable mutations | 72 | 1.0 |  |  |
| Highly actionable | 35 | 0.65 | 0.45 – 0.93 | **0.02** |
| *Matched therapy* |  |  |  |  |
| Highly actionable, unmatched therapy | 17 | 1.0 |  |  |
| Highly actionable, matched therapy | 18 | 0.47 | 0.24 – 0.94 | **0.03** |
| **OS (biopsy to censoring/death)** |  |  |  |  |
| *Actionability* |  |  |  |  |
| No highly actionable mutations | 65 | 1.0 |  |  |
| Highly actionable | 29 | 0.95 | 0.62 – 1.5 | 0.83 |
| *Matched therapy* |  |  |  |  |
| Highly actionable, unmatched therapy | 13 | 1.0 |  |  |
| Highly actionable, matched therapy | 16 | 0.48 | 0.21 – 1.1 | 0.08 |



**Supplemental Figure S8: Progression-free survival of patients with highly actionable biomarkers in the 2nd and 3rd lines of therapy.** Progression-free survival was calculated from the time of treatment initiation.Patients receiving report-listed therapies in the 2nd **(A)** or 3rd **(B)** line of therapy were divided into three groups: those with highly actionable biomarkers who received molecularly matched therapy, those with highly actionable biomarkers who did not receive molecularly matched therapies, and those with no highly actionable biomarkers. Group sizes (n) and median progression-free survival (mPFS) are listed next to each survival curve. See Supplemental Table S7 for statistical comparison.



**Supplemental Figure S9: Overall survival of patients in the actionability/matched therapy subgroups.** Overall survival was calculated from the time of biopsy until censoring/death.Group sizes (n) and median overall survival (mOS) are listed next to each survival curve. See Supplemental Table S7 for statistical comparison.

**Supplemental Table S10: Patients excluded from outcomes analysis due to toxicity.** Eight patients were excluded from the outcomes analysis in Figures 5, S8, and S9. Treatment toxicity information is listed below for each of these 8 patients.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Drug** | **Group** | **Line of therapy** | **Time on therapy** | **Reason for stopping** |
| Gemcitabine + nab-paclitaxel | Unmatched (BRAF) | 2nd | 1 day | Tolerated one cycle poorly |
| nal-Irinotecan | Not actionable | 3rd | 1 day | Patient referred to hospice after one cycle |
| Irinotecan | Not actionable | 3rd | 7 days | Suspected Gilbert syndrome |
| Gemcitabine + nab-paclitaxel | Not actionable | 2nd | 3 months | Interstitial pneumonitis |
| Gemcitabine + docetaxel + capecitabine | Not actionable | 3rd | 4 months | Intolerance (poor appetite, neuropathy, nausea) |
| Gemcitabine + nab-paclitaxel | Unmatched (STK11) | 3rd | 1 day | Prior neuropathy exacerbated by one cycle |
| Everolimus + capecitabine | Matched (STK11) | 3rd | 6 weeks | Stomatitis, hand-foot syndrome |
| 5-Fluorouracil + nal-irinotecan | Not actionable | 3rd | 1 day | Patient referred to hospice after one cycle |

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