Supplemental Materials, Detection of Chemotherapy-Resistant Pancreatic Cancer Using a Glycan Biomarker

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The RNAseq data for 27 samples and the expression counts matrices have been submitted to NCBI GEO, [**Series GSE146722**](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146722)**.**

**The release date is April 2021, or upon publication.**

Supplemental Methods

Construction and sequencing of directional total RNAseq libraries

Libraries were prepared by the Van Andel Genomics Core from 500 ng of total RNA using the KAPA RNA HyperPrep Kit with RiboseErase (v1.16) (Kapa Biosystems, Wilmington, MA USA). RNA was sheared to 300-400 bp. Prior to PCR amplification, cDNA fragments were ligated to Bioo Scientific NEXTflex Adapters (Bioo Scientific, Austin, TX, USA). The quality and quantity of the finished libraries were assessed using a combination of Agilent DNA High Sensitivity chip (Agilent Technologies, Inc.), QuantiFluor dsDNA System (Promega Corp., Madison, WI, USA), and Kapa Illumina Library Quantification qPCR assays (Kapa Biosystems). Individually indexed libraries were pooled and 75-bp, paired-end sequencing was performed on an Illumina NextSeq 500 sequencer using a 150-bp HO sequencing kit (v2) (Illumina Inc., San Diego, CA, USA), with all libraries sequenced to a minimum of 40 million reads. Base calling was done by Illumina NextSeq Control Software (NCS) v2.0 and output of NCS was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.9.0.

RNAseq data processing

RNAseq libraries were generated using the KAPA RNA HyperPrep kit with RiboErase. Libraries were sequenced paired-end for 75 cycles on two Illumina NextSeq flowcells. Following demultiplexing, adapters and low-quality bases were trimmed using Trimgalore v0.4.2 (<http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/>). Trimmed data was quality controlled with FastQC v0.11.7 and then mapped with STAR v2.5.2b to the hg38 genome using the default settings. Raw gene counts (mean of 27M/sample) generated by STAR were imported into R v3.6.0. Genes with greater than 10 counts in 2 or more samples were retained and the rest removed in order to minimize multiple testing adjustments and remove genes that are unlikely to give meaningful information. A quasi-likelihood negative binomial generalized log-linear model was then fit to the filtered count data using the weighted trimmed mean of M-values to normalize for library size and composition biases.

For all differential expression contrasts, the gene set was further filtered so that, for the subset of samples being used in the comparison, a minimum of two samples have more than zero counts. A quasi-likelihood, negative binomial, generalized log-linear model was then fit to the filtered count data using the weighted trimmed mean of M-values to normalize for library size and composition biases. GSEA v3.0 was used to test for enrichment of various gene sets in glycan phenotype groups. Significance of enrichment was tested using 1000 phenotype permutations. All gene sets were run simultaneously to adjust for multiple testing. Heatmaps were made using the R package pheatmap v1.0.12. Samples and genes were clustered using the default method of the pheatmap function. Normalized counts were used, median-centered across genes.

Targeted genome sequencing

Libraries were prepared by the Van Andel Genomics Core from 50 ng of high-molecular-weight genomic DNA using the KAPA Hyper Prep Kit (v5.16) (Kapa Biosystems, Wilmington, MA USA). In brief, DNA is sheared to an average size of 200 bp, then end-repaired and A-tailed DNA fragments were ligated to to Bioo Scientific NEXTflex Adapters (Bioo Scientific, Austin, TX, USA). Libraries were amplified and pooled for targeted capture in batches of 16 libraries, using 31 ng of each indexed library. Capture baits were designed using IDT xGen Lockdown Probes and Reagents (Integrated DNA Technologies, Coralville, IA, USA) with minimal modification. Quality and quantity of the captured library pools were assessed using a combination of Agilent DNA High Sensitivity chip (Agilent Technologies, Inc.), QuantiFluor dsDNA System (Promega Corp., Madison, WI, USA), and Kapa Illumina Library Quantification qPCR assays (Kapa Biosystems). A 150-bp, paired-end sequencing was performed on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) at the Michigan State University RTSF Genomics Core. Base calling was done by Illumina RTA3 and output of NCS was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.9.0.

For all data, adapters and low-quality sequences (median Q<20) were removed with Trimgalore. Data was then mapped to hg38 using bwa mem. Following mapping, the median non-duplicate read depth for captured regions was 156x. From this high-depth data, variants were called using the best-practice workflow of the GATK4 tool Mutect2. Variants were then annotated using the command-line variant effect predictor tool from Ensembl using release 92. The annotated variant files were converted to mutation annotation format (MAF) using vcf2maf (<https://github.com/mskcc/vcf2maf>), and oncoplots and lollipop plots were generated using the R package maftools v1.6.15. To confirm specific mutations, eight samples were also re-sequenced paired-end for 300 cycles on an Illumina MiSeq.

Infinium QC array

DNA was quantified by Qubit fluorometry (Life Technologies) and 200 ng of DNA from each sample was processed by the VARI Genomics Core using the Illumina Infinium QC array v1.0 (Illumina), which contains some 16,000 SNPs focused on sex determination, ethnic ancestry, ADME, and genetic linkage markers. DNA was amplified, hybridized to the QC Array bead chip, and an extension reaction was performed using flurophore labeled nucleotides per the manufacturer’s protocol. Array beadchips were scanned on the Illumina iScan platform and probe specific calls were made using Illumina Genome Studio software.

Gene-expression classification and survival analysis

The gene classifier for sTRA included the top 14 up-regulated and top 14 down-regulated genes (p < 0.02 after multiple-testing correction) in sTRA-expressing cells versus sTRA-negative cells in the RNAseq analysis of 27 cell lines. We obtained the expression data (Z scores) for the gene list from The Cancer Genome Atlas (TCGA dataset provisions, <https://www.cbioportal.org/>)) and the International Cancer Genome Consortium (ICGC data portal <https://dcc.icgc.org/projects/PACA-CA>). For each case of PDAC, we calculated a score by subtracting the average of the down-regulated genes from the average of the up-regulated genes. The patients above the median score were classified as sTRA-signature positive. To classify patients as classical or basal in the ICGC data, we used the same algorithm but with a previously-identified gene list that included 23 classical-associated gene and 23 basal-associated genes (2). The Kaplan-Meier survival curves were generated with GraphPad Prism 6. The subjects who were alive at the end of the study or who died of other causes were censored.

References

1. Staal B, Liu Y, Barnett D, Hsueh P, He Z, Gao CF, et al. The sTRA Plasma Biomarker: Blinded Validation of Improved Accuracy over CA19-9 in Pancreatic Cancer Diagnosis. *Clin Cancer Res* **2019**;29:2745-54.

2. Moffitt RA, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, et al. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* **2015**;47:1168-78.

**Supplemental Tables**

**Supplemental Table 4. Antibody information.**

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| --- | --- | --- | --- | --- | --- | --- |
| **Antibody** | **Host** | **Clone** | **Target** | **Source** | **Cat. no.** | **Class** |
| goat anti-mouse IgG-HRP | Goat | Polyclonal | Mouse IgG | Santa Cruz Biotechnology | SC2005 | IgG |
| goat anti-Rabbit IgG-HRP | Goat | Polyclonal | Rabbit IgG | Santa Cruz Biotechnology | SC2004 | IgG |
| CA19-9 | Mouse | 9L426 | Sialyl Lewis A | US Biologicals | C0075-03A | IgG |
| TRA-1-60 | Mouse | TRA-1-60 | Terminal *N*-acetyl-lactosamine, type 1 | Novus Biologicals | NB100-730 | IgM |
| MUC5AC | Mouse | 45M1 | Lewis B blood group antigen | Abcam | ab212636 | IgG |
| MUC16 | Mouse | X325 | Lewis blood group antigen | Abcam | ab10033 | IgG |

Supplemental Figure Legends

**Supplemental Figure 1. Cell line morphologies.** Brightfield images showing the morphologies of the cell lines. Magnification is 10X.

**Supplemental Figure 2. Dose-response curves and chemosensitivity in CA19-9-expressing cell lines.** A)Representative dose-response curves for cell lines. Each cell line was incubated with the indicated drug, titrated over a range of concentrations. Cell viability was measured by Cell Titer-Glo at 72 h, and dose-response curves were plotted to determine the IC50 value for each combination. B). The IC50 values calculated from dose-response curves, grouped by marker group. The p values are based on the Mann–Whitney test.

**Supplemental Figure 3. Isogenic cell lines.** A) Development of drug-resistant sublines of L3.3. B) Immunofluorescence of sTRA and CA19-9 in the parental and sublines. Magnification is 20X. C) Comparisons of the indicated features in the parental L3.3 cell line and the sublines. D) Comparisons between the PaTu8988S and PaTu8988T cell lines. The error bars are the standard error over 3 independent replicate experiments. H&E, hematoxylin and eosin. \*indicates p < 0.05, \*\*indicates p < 0.01. RLU, relative light units.

**Supplemental Figure 4. A gene-expression classifier associated with progression free survival after adjuvant therapy.** Progression free survival curves grouped by the gene-expression classifier for sTRA-positive or sTRA-negative (applied as in Fig. 4 in the main text) in the TCGA or ICGC datasets. The p values were based on the log-rank test.

**Supplemental Figure 5. Tissue expression and outcomes.** A) Raw immunofluorescence and detected signal of sTRA and CA19-9 in representative cores from the TMAs. Magnification is 4X. B) Comparison between the CA19-9 and sTRA levels in the patient tumors. Each point is the average of the three cores for a patient. C) Comparisons of sTRA and CA19-9 immunofluorescence data between the patient groups stratified by survival. The dashed lines indicate the median values that were used as cutoffs in panel E. D) Response to adjuvant therapy in subgroups. Adj. indicates the patients who received adjuvant chemotherapy following surgery. Using the median values of either marker given in panel C, the patients were further stratified either by sTRA (left) or CA19-9 (right).

**Supplemental Figure 5. Outcomes associated with tissue staining.** For each core on the TMAs, the SignalFinder program determined the percentage of the tissue that was positive for either sTRA or CA19-9. A) Representative immunofluorescence data from the UPMC TMAs. The detected signal from SignalFinder is overlaid on the H&E images. B) SignalFinder quantified the amount of staining in each core that consisted of only sTRA, only CA19-9, or overlapping signal (referred to as dual). These values were averaged over the three cores per tumor. Each tumor was classified into one of the five indicated categories based on the relative amounts of the types of staining. The full data and quantifications are provided in Table S2.

**Supplemental Figure 7. Immunoassay correspondence between surface expression and secretions.** A) Scatter plots of CA19-9 and sTRA between cell surface and secretions over 27 2D cell lines. The Pearson correlation coefficient was used to calculate the extent of correlation with significant correlation numbers in bold text. The matrix shows the patterns of high and low values across all immunoassays using secreted media. B) Scatter plots of CA19-9 and sTRA between cell surface and secretions over 27 organoids.

**Supplemental Figure 8. Additional plasma biomarker analyses in neoadjuvant therapy.** A) Individual markers in the MCW Test Set. B) Survival curves in retrospective analyses. The data are from previously published cohorts (1) from UPMC for three immunoassays, CA19-9, sTRA (Ab1), and sTRA (Ab2). Ab1 and Ab2 refer to the capture antibodies used in the sandwich assay in combination with sTRA detection (see Fig. 6, main text). Ab1 is the CA19-9 antibody, and Ab2 is anti-MUC5AC.