Supplementary Materials for

Single-EV analysis (sEVA) of mutated proteins allows detection of stage 1 pancreatic cancer

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Modeling and Simulation
A model previously described (56) was adapted to simulate tumor-originating EV in
circulation as a function of tumor size (Table S5, Fig. S8). In order to expand the model
we sought two key parameters and their expected variability: i) the EV-shed rate of
human pancreatic tumors, and ii) the distribution of marker-panel positive EV across
human pancreatic tumors. To accomplish the first point we collected EV for each of the
7 PDX cell lines in 2D culture over a 24 hour interval, quantified the Ison-purified EV
samples via total Qubit protein assay and used a correction factor of 1E9 EV per
microgram protein to approximate total EV numbers. These were normalized to the
number of cells seeded and counted at collection in order to obtain an EV shed rate for
each cell line. It is well described in the literature that cells grown in 3D culture release
more EV than cells grown in 2D culture. We assumed tumor EV shed rates
_in vivo_ will
reflect the rate of cells growing in all spatial dimensions. To create a correction factor for
our 2D shed rates to 3D shed rates we found 5 data points in the literature where this
was done and used the average fold-increase of 5.2 and multiplied this through (68–70).
The corrected shed rates for human PDX cells spanned a similar magnitude range as
the tumor shed rates observed from the fourteen KIC mice and nine KPC spontaneous
pancreatic cancer models. The distribution of observed EV shed rates was then
checked for normality or log-normality. Across the seven patient cell lines, the data was,
as expected, more closely representative of a log-normal distribution, passing both the
Shapiro-Wilk and Kolmogorov-Smirnov test. For marker coverage observations
however, the data for all cell lines tested passed each built-in Normality test within
GraphPad Prism 8.0 and the likelihood ratio for normality (96.7%) versus log normal
(3.3%) was 29.3. Therefore, when simulating 100 representative individuals a log-
normal distribution of tEV shedding centered on 0.066 Day⁻¹ with a standard deviation of
0.063 was generated. For marker coverage, a normal distribution with a mean of 37.1%
and standard deviation of 15.65 was simulated. For the simulation, some correlation
within the lower triangular matrix was included following the observation that higher-EV
shed rates were often accompanied by higher marker coverage as well as faster growth
rate (covariance 0.35). Additional time-variant error of 20% was included in the
simulation and model outputs of tumor size and total tEV in circulation were then
plotted. All model distributions were simulated using ADAPTv5 provided by University of
Southern California Biomedical Simulations Resource. The total tEV in circulation output
by model simulations was normalized to the percentage of tEV relative to all EV (tEV +
hEV) by hEV equal to 4.2E7 and dividing by the volume of distribution, 6000 ml. The
total marker-positive tEV was computed by assigning the simulated distribution. The
observed LOD for the combined marker panel of 0.005% was then set as a threshold
and for each simulated sampling time a percentage of individuals above this threshold
was computed and this data was fit to a Hill equation.
**Fig. S1: Labeling and purification steps in sEVA.** Prior to processing, biological samples were centrifuged at 10,000g for 15 minutes to remove large debris. 1) Samples containing EV were further processed using a qEV single size exclusion column (IZON). Following 1 ml void volume, 650\(\mu\)l was collected and used for subsequent labeling. This resulted in EV ranging in size between 50-300 nm and excluded larger microvesicles. 2) EV were labeled with the amine reactive AF488-PEG-TFP dye to define all EV. Unreacted AF488 was removed using a Zeba column. 3) AF488-PEG-TFP labeled EV were co-labeled with fluorescent antibodies. Following labeling, unbound antibodies were removed with a second IZON column. 4) Labeled EV were pipetted onto hydrophobic glass sides and cover slipped. 5) EV were imaged and multicolor images were then analyzed as detailed in Fig S2. All cell line and PDX experiments were performed in triplicate using at least 10,000 EV. For the clinical samples, all images analyzed passed QC and on average several thousand vesicles were analyzed per sample (range: 3,608 - 15,051; average > 5,000).
**Fig. S2: Flowchart of image analysis.** The pipeline shows the image acquisition and analysis parameters to obtain analyzable and reproducible data. Note that following confirmation of high-quality images, all data points were obtained by automated analysis and not by “visual inspection”. Marker signal intensity was measured only on regions demarcated by TFP labeling to avoid non-specific signals not localized to EV. These measurements were analyzed in GraphPad Prism v8 to determine optimal threshold values between control samples and positive samples by maximizing the likelihood ratio of the receiver operator characteristic curve. Logic statements within Microsoft Excel were used to determine percent positive EVs for each sample, as well as co-positive EVs.
Fig. S3: Analysis of potential spectral crosstalk. A) Detection of EVs labeled with different TFP-fluorophores. EV derived from PANC1 cells were labeled with different fluorochrome (AF350, AF488, AF647) tagged TFP and then imaged by microscopy either as purified or mixed populations. B) Cross-section analysis of mixed TFP population. Note the high signal-to-noise ratio of individually labeled vesicles and specificity of each label as evidenced by the absence of signal overlap between the discretely labeled EV.
Fig. S4: Antibody specificity for KRAS mutations and cell-EV correlation. A-C) Western blot analysis of total KRAS (A), and KRAS mutation specific antibodies (B-C) using lysate of prototypical PDAC cell lines with known mutations. Note the specificity of antibodies. D) Western blot quantitation from A-C showing the general composition of cell lysates (all data are from same gel and scaled to GAPDH). E) Correlation between mutational load of parent cells and their isolated EV. Normalized cell content of G12D and G12V protein detection was correlated to the percent EV staining positive for each (AsPC-1, CAPAN-2, Mia-PaCa-2 and PANC-1). Red: KRAS$^{G12D}$ (R$^2$ 0.95); Orange KRAS$^{G12V}$ (R$^2$ 0.99). F) PANC-1 EVs stained for total KRAS (mutant + wild-type; green), and KRAS$^{G12D}$ (red). There are EV that are exclusively green (harboring predominantly wild-type KRAS), EV that are yellow (positive for KRAS$^{total}$ + KRAS$^{G12D}$) and EV that are predominantly red (KRAS$^{G12D}$ with fainter KRAS$^{total}$ signal). See Fig S4 D for bulk composition. Note that the sEVA assay only uses antibodies for mutant KRAS forms to detect cancers.
**Fig. S5 Antibody specificity for P53 mutations.** The P53\textsubscript{mut} antibody (Abcam) does not recognize P53\textsuperscript{WT} present in CAPAN-2 but does recognize mutant-P53 in BxPC3 (Y220C), MIA-PaCa-2 (R248W), and PANC-1 (R273H) cell lysate via western blot analysis. However, there was not a strong signal for P53\textsubscript{mut} cell lines AsPC-1 (C135fs\textsuperscript{*35}) or PSN-1 (K132Q) via western-blot. Clinical samples with known P53\textsubscript{mut} status included R213Ter, Y220C, Y163C, R196Ter, and R196H, all of which stained positively above levels in control plasma.
Fig. S6: Antibody validation for different biomarkers in cell lines and EV. Note the good specificity against negative and positive controls using purified protein (first two columns). The EV staining data generally matches the whole cell data with the exception of P53\textsuperscript{mut} in MIA PaCa-2 (R248W). MIA PaCa-2 appears to have more P53\textsuperscript{total} and P53\textsuperscript{mut} and compared to other PDAC cell lines but which lacks efficient incorporation into EV.
Fig. S7: ROC analysis to define threshold for detecting stage 1 PDAC
The black line represents the ROC and the grey lines are the 95% confidence intervals. The red line denotes the AUC=0 assumption. The table summarizes the numerical values of the analysis.

|                      | Full Data ROC                      | LPO Cross Validation ROC            |
|----------------------|------------------------------------|------------------------------------|
| Area under the ROC curve |                                    |                                    |
| Area                 | 0.9000                             | 0.8833                             |
| Std. Error           | 0.06862                            | 0.07987                            |
| 95% confidence interval | 0.7655 to 1.000                   | 0.7268 to 1.000                    |
| P value              | 0.0082                             | 0.0214                             |
**Fig. S8: Comparison of data to modeling.** The percent EV positive for the current marker panel along with the corresponding tumor volume from pathology are overlaid onto model simulations.
Fig. S9: Comparison of literature values of EV increases in late stage cancers compared to model used in this study. **A)** Increase in total circulating EVs in humans as a function of tumor burden is presented in fold-changes over the baseline values of EV according to model by Ferguson et al. (56). **B)** Model predicted fold-increases in EVs were compared to available clinical data. The model correlated well with clinical data from four different cancer types, including the PDAC samples from the current study (red dot; $R^2=0.96$, blue dashed line).
Fig S10. Effect of EV size on diagnostic accuracy. EV binned into small (<10 pixel area) and large (10+ pixel area). Plotted are the RFU of EV as a function of EV size (small and large) for P53mut (green), KRASmut (red) and PDACEV (blue). Note that the diagnostic information is similar across large and small EV and does not affect accuracy.
**Fig. S11 Model Equations.** Equations 1 - 5 describe tEV distribution and elimination in mass units where: $v_{asc}k_{on}$ is the first order rate constant describing association of tEV to the vascular walls, $v_{asc}k_{off}$ is the first order rate constant describing dissociation of tEV from the vascular walls back into circulation, $Renal_{EXC}$ and $Fecal_{EXC}$ are the first order rate constants of tEV eliminated into urine and feces, respectively, $RES_{uptake}$ is the first order rate constant of hepatic uptake, and $Hep_{Eli}$ is the first order rate constant of tEV eliminated in the liver (days$^{-1}$).

Equation 6 describes the tumor growth dynamics and production of tEV. $k_{Tgr}$ is the first order growth rate of the tumor (days$^{-1}$) and $Tumor_{Max}$ is the maximum tumor volume (mm$^3$). Under the case where a tumor is present, tEV input from equation 6 is modeled in equation 1 by the first order tumor-EV shed rate: $k_{EVSHED}$ (days$^{-1}$).

\[
\frac{d tEV}{dt} = -(v_{asc}K_{on} \times tEV) - (Renal_{EXC} \times tEV) - (Fecal_{EXC} \times tEV) - (RES_{uptake} \times tEV) + (v_{asc}K_{off} \times VASC) + (TUMOR \times k_{EVSHED})
\]

Eq. 1, Tumor EVs in circulation:

\[
\frac{d VASC}{dt} = (v_{asc}K_{on} \times tEV) - (v_{asc}K_{off} \times VASC)
\]

Eq. 2, Tumor EVs bound to vasculature:

\[
\frac{d LIVER}{dt} = (tEV \times RES_{uptake}) - (LIVER \times Hep_{Eli})
\]

Eq. 3, Tumor EVs cleared by the liver:

\[
\frac{d URINE}{dt} = (tEV \times Renal_{EXC})
\]

Eq. 4, Tumor EVs cleared renally:

\[
\frac{d FECES}{dt} = (tEV \times Fecal_{EXC})
\]

Eq. 5, Tumor EVs cleared fecally:

\[
\frac{d TUMOR}{dt} = k_{Tgr} \times \log \left( \frac{Tumor_{Max}}{TUMOR} \right) \times TUMOR
\]

Eq. 6, Tumor volume:
Table S1: Characteristics of PDAC cell lines used in study

EV were obtained from a total of 11 cell lines and used for single EV analysis. The commercially available cells were from ATCC and have been described(41). The PDX cell lines were created at MGH and have partially been described(9, 43).

| Cell line | Source | Sex/Age | Site   | KRAS  | P53  | MUC1 | EGFR | Comment   |
|-----------|--------|---------|--------|-------|------|------|------|-----------|
| AsPC-1    | ATCC   | F/62    | Ascites| G12D  | Mut  | +    | ++++| CRL-1682 |
| CAPAN-2   | ATCC   | M/56    | Primary| G12V  | WT   | ++++|+++++|HTB80     |
| MIA PaCa-2| ATCC   | M/65    | Primary| G12C  | Mut  | +    | ++++|CRL-1420  |
| PANC-1    | ATCC   | M/56    | Primary| G12D  | Mut  | ++++|+++++|CRL-1469  |
| 609       | MGH    | M/52    | Primary| G12D  | Mut  | ++++|++   |MGH PDX   |
| 950       | MGH    | M/57    | Primary| G12V  | Mut  | ++++|(-)  |MGH PDX   |
| 1275      | MGH    | M/70    | Primary| G12V  | Mut  | ++  |+    |MGH PDX   |
| 1309      | MGH    | M/62    | Primary| G12D  | Mut  | ++++|++   |MGH PDX   |
| 1319      | MGH    | M/66    | Primary| G12D  | Mut  | ++++|++++ |MGH PDX   |
| 1326      | MGH    | F/82    | Primary| G12D  | Mut  | ++  |+    |MGH PDX   |
| 1473      | MGH    | M/61    | Primary| G12V  | WT   | ++  |++   |MGH PDX   |
**Table S2: Frequency of biomarker positive EV compared to parental cells**

Cancer biomarkers are less common in EV when compared to parental cells. For example, in AsPC-1 cells only 37% of EV express mutant KRAS. PDAC\textsuperscript{EV} refers to EV positive for EGFR, MUC1, and/or αFG-P4OH.

| Cell line    | KRAS\textsuperscript{mut} | P53\textsuperscript{mut} | PDAC\textsuperscript{EV} | No Marker |
|--------------|-----------------------------|-----------------------------|--------------------------|-----------|
| AsPC-1       | 0.368                       | 0.046                       | 0.492                    | 0.481     |
| CAPAN-2      | 0.352                       | 0.002                       | 0.279                    | 0.501     |
| MIA PaCa-2   | 0.036                       | 0.022                       | 0.449                    | 0.519     |
| PANC-1       | 0.341                       | 0.39                        | 0.607                    | 0.243     |
| 609          | 0.349                       | 0.133                       | 0.315                    | 0.481     |
| 950          | 0.363                       | 0.047                       | 0.393                    | 0.431     |
| 1275         | 0.282                       | 0.03                        | 0.342                    | 0.487     |
| 1309         | 0.212                       | 0.217                       | 0.366                    | 0.43      |
| 1319         | 0.398                       | 0.157                       | 0.229                    | 0.456     |
| 1326         | 0.406                       | 0.055                       | 0.296                    | 0.428     |
| 1473         | 0.635                       | 0.005                       | 0.425                    | 0.314     |
### Table S3: Characteristics of patient samples

Plasma was obtained from 25 patients, including 16 patients with stage 1 PDAC, 4 patients with late stage PDAC and 5 healthy controls. SEVA was performed on 100 µL of purified plasma. *ND = not determined. KRAS and P53 status was determined by sequencing of the primary tumor.

| Patient sample | Dx       | Stage | Volume (cm³) | Age    | Sex  | KRAS   | P53               |
|----------------|----------|-------|--------------|--------|------|--------|-------------------|
| E1             | Early PDAC | I     | 0.268        | 75-79  | male | G12D   | ND                |
| E2             | Early PDAC | I     | 1.640        | 55-59  | male | G12D   | WT                |
| E3             | Early PDAC | I     | 0.014        | 65-69  | female | G12D | R213 Ter |
| E4             | Early PDAC | I     | 0.268        | 75-79  | male | G12D   | Y220C            |
| E5             | Early PDAC | I     | 3.431        | 65-69  | female | G12R | Y163C            |
| E6             | Early PDAC | I     | 4.490        | 70-74  | male | Q61K   | R196 Ter         |
| E7             | Early PDAC | I     | 0.382        | 70-74  | female | Q61H | R196H fs Ter 52; N239S |
| E8             | Early PDAC | I     | 4.490        | 75-79  | female | ND   | ND                |
| E9             | Early PDAC | I     | 0.181        | 70-74  | female | ND   | ND                |
| E10            | Early PDAC | I     | 0.105        | 75-79  | female | ND   | ND                |
| E11            | Early PDAC | I     | 0.001        | 70-74  | female | ND   | ND                |
| E12            | Early PDAC | I     | 2.827        | >80    | female | ND   | ND                |
| E13            | Early PDAC | I     | 1.115        | 75-79  | male | ND   | ND                |
| E14            | Early PDAC | I     | 0.382        | 70-74  | male | ND   | ND                |
| E15            | Early PDAC | I     | 0.034        | 75-79  | female | ND   | ND                |
| E16            | Early PDAC | I     | 0.001        | >80    | female | ND   | ND                |
| L1             | Late PDAC  | III   | 54.39        | 65-69  | male | WT    | R213W            |
| L2             | Late PDAC  | IV    | 418.88       | 55-59  | female | G12R | R273H            |
| L3             | Late PDAC  | IV    | 49.01        | 65-69  | male | G12C  | G266 Ter         |
| L4             | Late PDAC  | III   | 109.62       | 70-74  | male | G12D  | R282W            |
| C1             | Healthy    | -     | 35-39        | male   | -    | -      |                  |
| C2             | Healthy    | -     | 30-34        | male   | -    | -      |                  |
| C3             | Healthy    | -     | 25-29        | female | -    | -      |                  |
| C4             | Healthy    | -     | 20-24        | female | -    | -      |                  |
| C5             | Healthy    | -     | 30-34        | female | -    | -      |                  |
Table S4: Affinity labels used for sEVA analysis

| Target          | Reagent         | Vendor       | Catalog #   | Flurochrome | DOL | Ex/Em filters |
|-----------------|-----------------|--------------|-------------|-------------|-----|---------------|
| All EV (TFP)    | TFP             | Thermo Fisher| A37570      | AF488       | N/A | 472/520       |
| KRAS<sub>G12D</sub> | anti-KRAS<sub>G12D</sub> | Genetex    | GTX635362  | AF594       | 3.4 | 562/593       |
| KRAS<sub>G12V</sub> | anti-KRAS<sub>G12V</sub> | CellSignaling | 14412BF (Special order) | AF594 | 1.7 | 562/593       |
| P53<sub>mut</sub> | Anti-P53<sub>mut</sub> | Abcam       | ab247264    | AF488       | 4   | 472/520       |
| P53<sub>mut</sub> | Anti-P53<sub>mut</sub> | Abcam       | ab32049     | AF555       | 3.6 | 562/593       |
| MUC1            | Anti-MUC1       | Biolegend   | 355602      | AF680       | 2.8 | 628/692       |
| EGFR            | anti-EGFR       | Abcam       | Ab30        | AF680       | 3.1 | 628/692       |
| αFG-P4OH        | anti-αFG-P4OH   | Diagnocine  | KC600       | AF680       | 5.1 | 628/692       |
Table S5: Parameters for EV modeling.

| Parameter   | Description                      | Species/Cancer | Estimate (CV%) / [95% CI] | Notes                                      |
|-------------|----------------------------------|----------------|---------------------------|--------------------------------------------|
| \(V_{d-m}\) | Volume of distribution (ml)      | Mouse          | 1.53 Fix                  | Shah & Betts (2012) scaled to 25g mouse    |
| \(V_{d-h}\) | Volume of distribution (ml)      | Human          | 6,004 Fix                 | Shah & Betts (2012) scaled to 75kg male    |
| Renal\text{EXC} | Renal Elimination (day\(^{-1}\)) | Mouse/Human   | 1.932 x 10\(^{-2}\) (29.31) | Model Estimate                            |
| Fecal\text{EXC} | Fecal Elimination (day\(^{-1}\)) | Mouse/Human   | 1.031 x 10\(^{-2}\) (16)  | Model Estimate                            |
| RES\text{uptake} | Hepatic Uptake (day\(^{-1}\)) | Mouse/Human   | 0.3055 (5.516)            | Model Estimate                            |
| vas\text{Kon} | Vasculature \text{Kon} (day\(^{-1}\)) | Mouse/Human   | 0.6397 (5.418)            | Model Estimate                            |
| vas\text{Koff} | Vasculature \text{Koff} (day\(^{-1}\)) | Mouse/Human   | 2.336 x 10\(^{-2}\) (18.18) | Model Estimate                            |
| He\text{PEi} | Hepatic Elimination (day\(^{-1}\)) | Mouse/Human   | 6.264 x 10\(^{-3}\) (7.961) | Model Estimate                            |
| \(k_{Tgr}\) | Tumor Growth Rate (day\(^{-1}\)) | KPC Pancreatic | 1.424 x 10\(^{-2}\) [0.0072 - 0.0229] | Gompertz Fit                             |
| Tumor\text{Max} | Max Tumor Volume (ml) | Sharma et al (51) | 477 [401.0 - 673.8] | Gompertz Fit                             |
| \(k_{\text{EV-SHED}}\) | Shed Rate of Tumor EVs (day\(^{-1}\)) |                  | 0.2208 (5.399)            | Model Estimate                            |
| \(k_{Tgr}\) | Tumor Growth Rate (day\(^{-1}\)) | KIC Pancreatic | 0.1098 -                  | Gompertz Fit                             |
| Tumor\text{Max} | Max Tumor Volume (ml) | Sharma et al (51) | 1953 [1,629 - 2,279] | Gompertz Fit                             |
| \(k_{\text{EV-SHED}}\) | Shed Rate of Tumor EVs (day\(^{-1}\)) |                  | 5.428 x 10\(^{-2}\) (18.12) | Model Estimate                            |

| Parameter   | Description                      | Species/Cancer | Estimate | SD     | Notes                                      |
|-------------|----------------------------------|----------------|----------|--------|--------------------------------------------|
| \(k_{\text{EV-SHED}}\) | Shed Rate of Tumor EVs (day\(^{-1}\)) | Human PDX     | 0.066    | 0.0627 | Experimentally derived                     |
| \(k_{\text{EV-SHED}}\) | Shed Rate of Tumor EVs (day\(^{-1}\)) | Human         | 0.0477   | 0.0465 | Simulated trial                            |
| Marker\ (+) | % tEV positive for a tested marker | Human PDX     | 0.371    | 0.1565 | Experimentally derived                     |
| Marker\ (+) | % tEV positive for a tested marker | Human         | 0.337    | 0.1444 | Simulated trial                            |
Table S6: Comparison between SEA and sEVA methods

|                  | SEA                  | sEVA                 | Advantages                                      |
|------------------|----------------------|----------------------|-------------------------------------------------|
| Technique        | Labeling on glass    | Labeling in solution, additional purification | Faster, more reproducible, limits background    |
| Loss of EV       | >95%                 | <10%                 | Representative EV                                |
| Staining quality | Dim SNR ~3           | Bright SNR >15       | Better sensitivity for identifying (+) EV        |
| Image quality    | Borderline, spurious signal | Superb            | High confidence in signal specificity           |
| Use for rare EV  | Not suitable         | Suitable             | Early stage cancer                               |
| Reference        | ACS Nano 2018;12:494–503 | Current study       |                                                  |
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