SUPPORTING DOCUMENTS

EXTRACELLULAR VESICLE ANALYSIS ALLOWS FOR IDENTIFICATION OF INVASIVE IPMN

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Supplementary Methods

Cell lines

AsPC-1, BxPC-3, MIA PaCa-2, and Capan-2 cell lines were purchased from the American Type Culture Collection (Manassas, VA). AsPC-1 and BxPC-3 cells were cultured in RPMI-1640 medium (Thermo, 11875119). MIA Paca-2 cells were cultured in Dulbecco's modified eagle's medium (Mediatech, 10-013-CV) and Capan-2 were cultured in McCoy's 5a medium (Thermo, 16600108). All media was supplemented with 10% fetal bovine serum (Atlanta Biologicals, S12450), 100 IU penicillin, and 100 µg/mL streptomycin (Mediatech, 30-002-CI). PDAC (1531 and 1617) and IPMN (1505 and 1966) PDX cell lines were from the MGH pancreas biobank. Both PDX cell lines were maintained in a 50:50 mix of Ham's F-12 and Dulbecco's modified eagle's medium. PDAC PDX cells were supplemented as above. IPMN PDX cell lines were supplemented with 20% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Thermo, 15240062), 10 mM Nicotinamide (Sigma-Aldrich, N0636), 1X insulin-transferrin-selenium (Corning, 25-800-CR), 8.4 ng/mL cholera toxin (Sigma-Aldrich, C8052), 10 ng/mL epidermal growth factor (Sigma-Aldrich, E9644), and 10 ng/mL hepatocyte growth factor (Thermo, PHG0324).

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Antibody-bead coupling and biotinylation

Capture antibodies were coupled to Dynabeads M-270 Epoxy magnetic beads using a coupling kit (Thermo, 14311D). *All buffers used in the coupling reaction were provided in the kit (Buffers C1, C2, HB, LB, SB).* Briefly, dynabeads were weighed into Eppendorf tubes and washed once with Buffer C1. Antibody was added to beads at a ratio of 10 µg antibody/mg dynabeads. Buffer C1 was added to the antibody solution for a total volume of 50 µL/mg bead. *The total reaction volume (Buffer C1 + antibody + Buffer C2) was 100µl per mg Dynabeads (manufacturer's instructions, Thermo 14311D).* The bead-antibody mixture was incubated overnight at 37°C on a HulaMixer (35 rpm, 5° tilt, 5° rotation; all 5 sec). The antibody solution was saved to determine

coupling efficiency and beads were then washed with buffer HB and LB (containing 0.05% Tween-20), followed by two washes with buffer SB. Beads were then incubated in buffer SB for 15 min, solution was removed, and the final antibody-bead conjugate was stored at 4°C in 100 µL buffer SB/mg dynabead. After each wash, beads were incubated 1 min on a DynaMag magnet and wash buffer was discarded. Coupling efficiency was typically 20-80%, depending on the capture antibody. Detection antibodies that were not readily available as a biotinylated product, were prepared using sulfo-NHS-LC-Biotin (Thermo, A39257). Briefly, a 20-fold molar excess of biotin was calculated for 50µg antibody. 180µL ultrapure water was added to a 1 mg no-weigh vial of biotin to make a 10 mM stock solution. An appropriate volume of biotin was added to the antibody in PBS and incubated for 30 min at room temperature. Excess biotin was then removed using a 0.5 mL, 7MWCO Zeba column (Thermo, 89882), according to the manufacturers' instructions. Final biotinylated antibody concentrations were determined using a Nanodrop (Thermo, ND-1000).

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Figure S1. Effect of EV isolation on MUC5AC measurements. Plasma EV were isolated by IZON column separation or direct processing. The direct method (DEST) is a combination of immunobead enrichment, coupled with washing and lowering backgrounds by dual antibody capture (see **Fig. 1**). MUC5AC analysis in EV was done using either IZON column purification (grey bars) or the DEST method in unpurified plasma (red bars). Note the congruence of the methods. We settled on using the direct DEST method as there is no loss of EV, it is fast and does not change the make-up of EV populations.

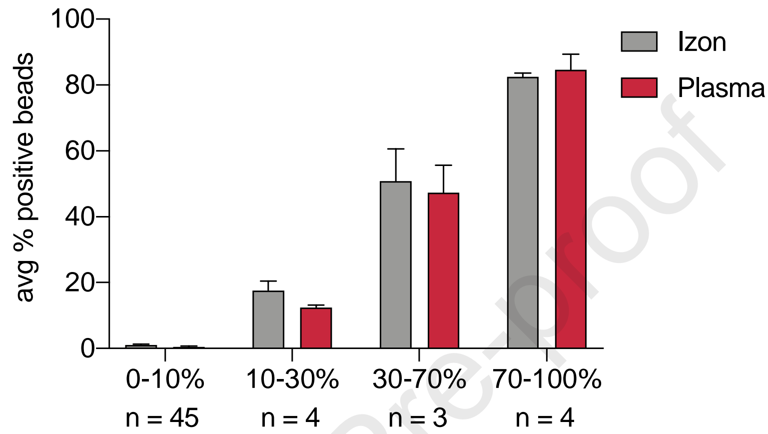

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Figure S2. Characterization of molecular targets on IPMN and PDAC cell lines and their derived EV. (*A*) Four PDAC cell lines (ASPC-1, BxPC-3, Capan-2, MIA PaCa-2), two PDAC PDX (PDAC1531, PDAC1617)[1] and two invasive HG-IPMN PDX (HGIPMN 1505, HGIPMN1966) were analyzed for the presence or absence of 16 molecular markers. Controls refer to antibody testing against isolated proteins or known positive EV lysates. (*B*) DEST analysis of EV fractions obtained from the same cell lines shows presence of certain molecular targets from parental cells. Note that EV targets were only detected when also present in parental cells. Furthermore, MUC5AC was elevated in both HG-IPMN PDX models.

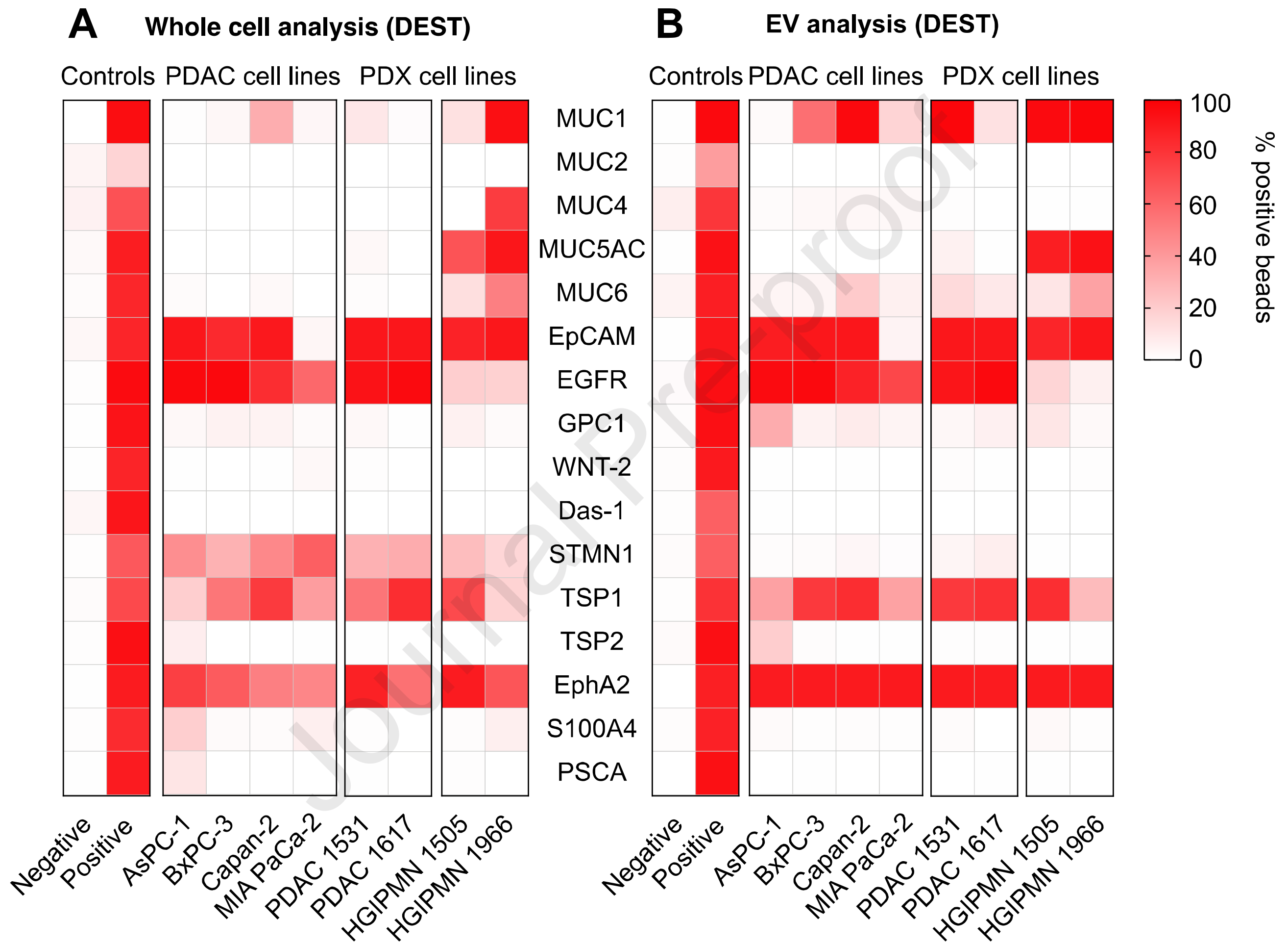

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Figure S3. ROC analysis for the different EV biomarkers in the discovery cohort. AUC (area under the curve) analysis for LG-IPMN vs HG-IPMN. Note that MUC2, MUC4, GPC1, EpCAM, Das-1, STMN1, and TSP2 are slightly better positive predictors of LG-IPMN. All other markers are positive predictors of HG-IPMN.

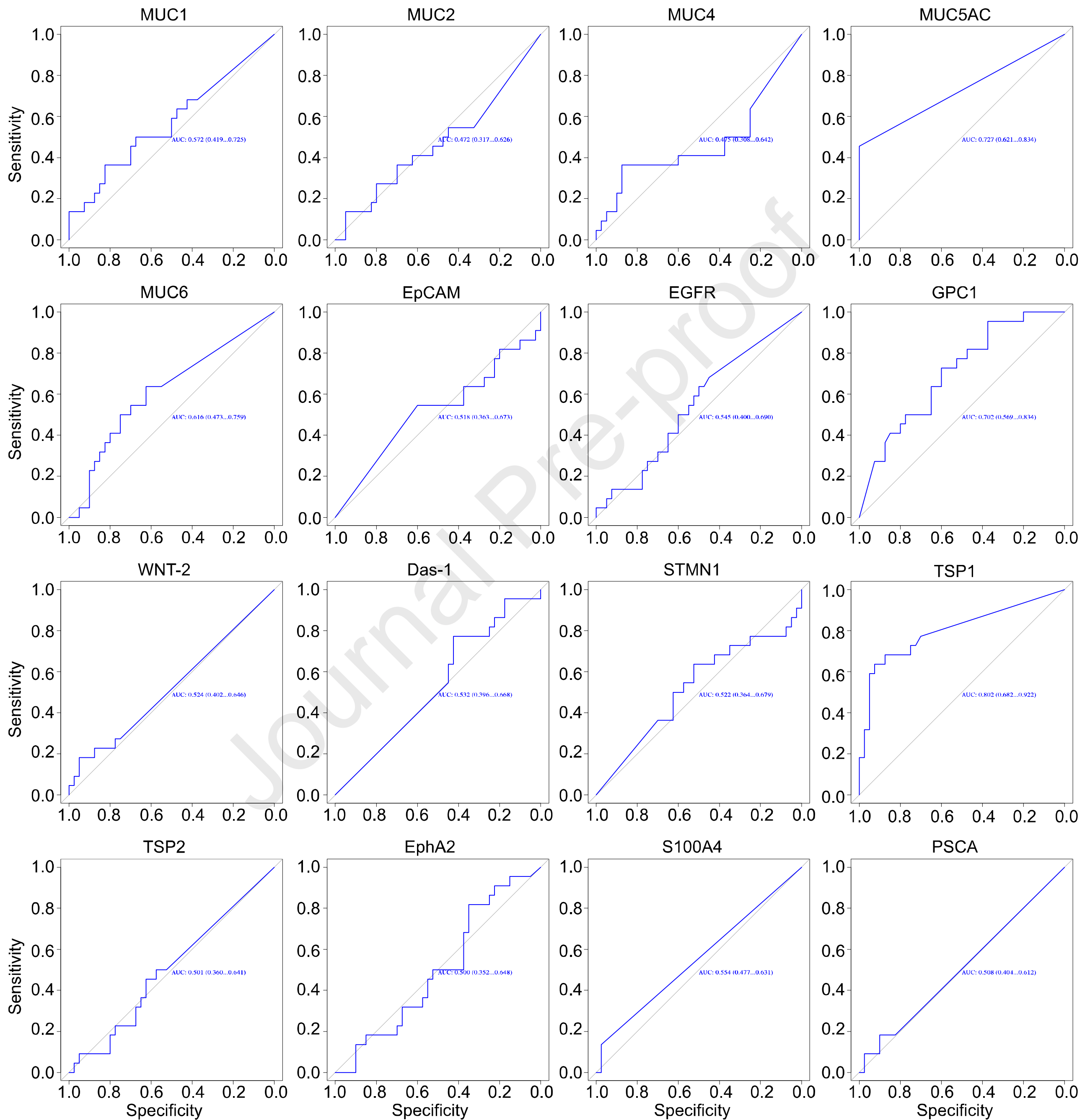

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Figure S4. Analysis of 16 prototypical biomarkers in EV from different discovery cohort patient subgroups. Each datapoint represents an EV sample from a single patient. Data shown are from healthy controls (light grey dots, n= 10); benign: age-matched control patients undergoing abdominal surgery but without evidence for any pancreatic lesions (dark grey squares, n=14 patients); low grade IPMN (blue triangles, n=40 patients); high grade IPMN (orange triangles, n=11 patients) and invasive high grade IPMN (red diamonds, n=11 patients). See **Table 1** for patient demographics. Differences between HG-IPMN and LG-IPMN are shown by asterix (**** p <0.0001, * p <0.05, ns = not statistically significant, p > 0.05; Mann-Whitney two-tailed non-parametric test). Error bars represent standard error of the mean. #TSP1 is statistically significant but the extremely low signal over background makes the results clinically unreliable.

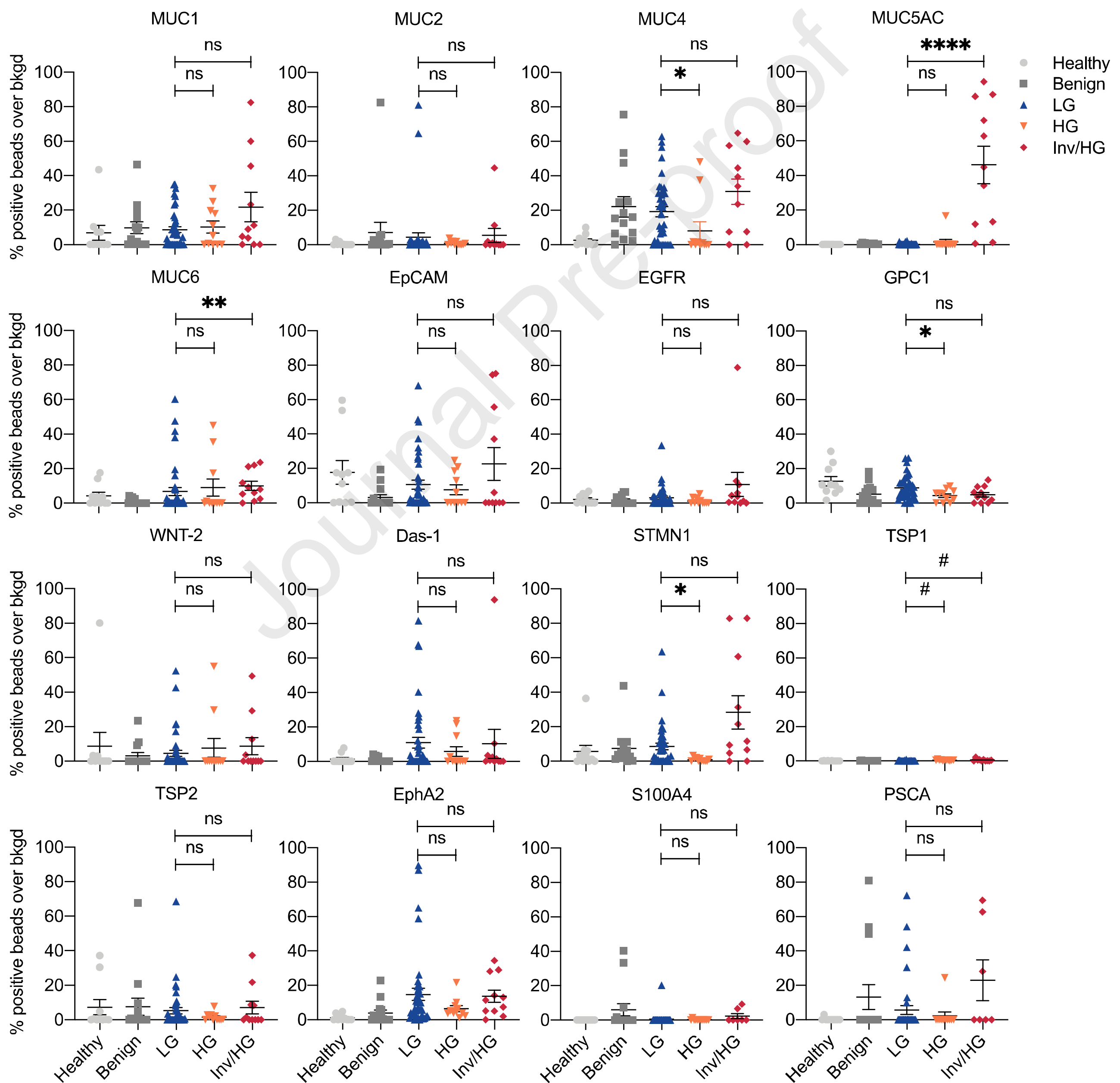

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Figure S5. Analysis of 16 biomarkers in EV from validation cohort LG and HG-IPMN lesions. Each data point represents an EV sample from a single patient. Data shown are from low grade IPMN (blue circle, n = 35) and high grade IPMN (red circle, n = 12). See **Table 1** for cohort demographics. Differences between HG-IPMN and LG-IPMN are shown by asterisk (**** p <0.0001; * p <0.05; ns = not statistically significant, p > 0.05).

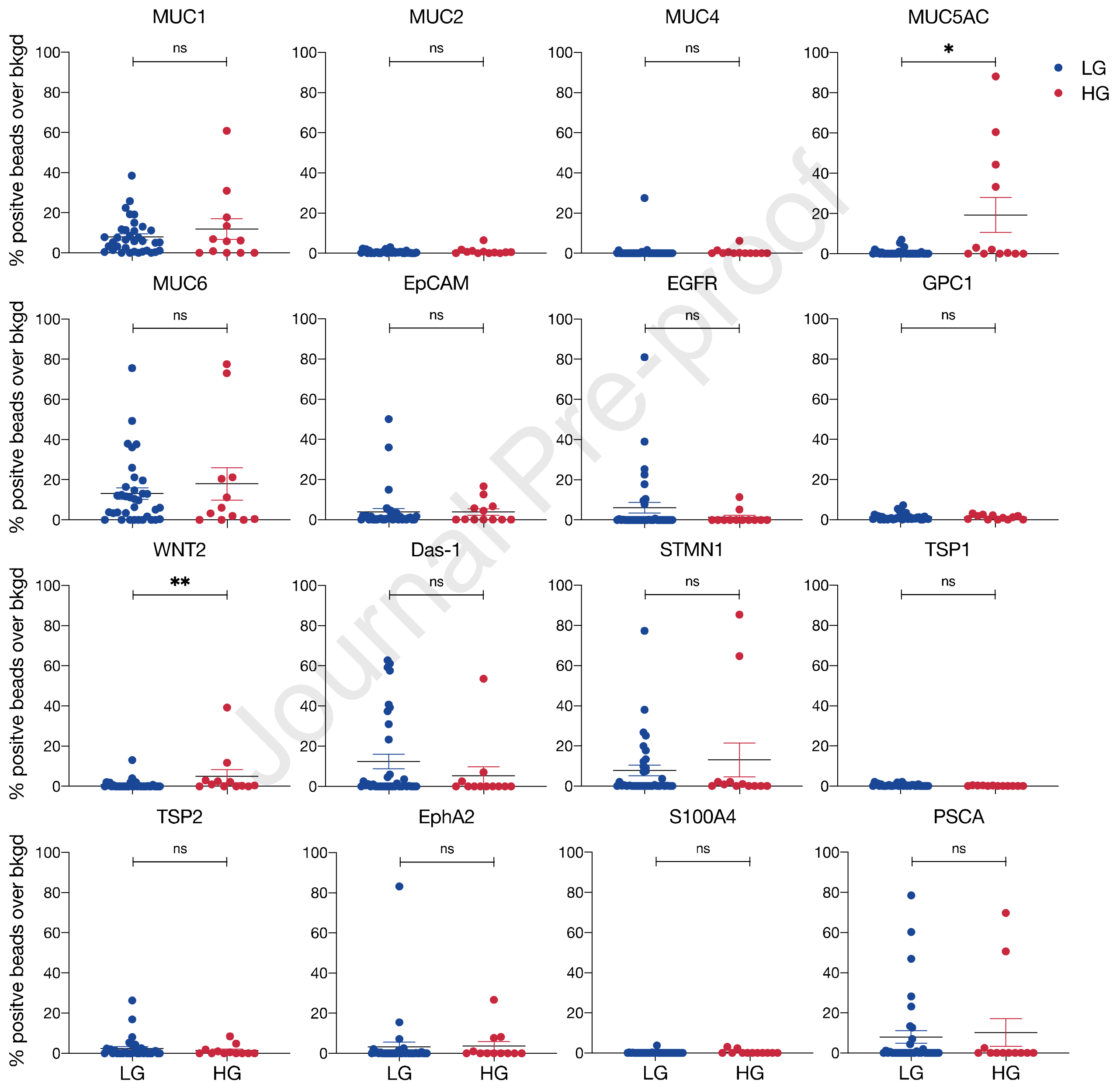

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Figure S6. Biomarkers for the decision to surgically remove HG-IPMN. In the 14 patients with inv/HG-IPMN traditional imaging and high risk stigmata alone would have missed 5 of the 14 cases requiring surgery (36% miss rate). If MUC5AC EV testing was added, all patients requiring immediate surgery would have been identified correctly.

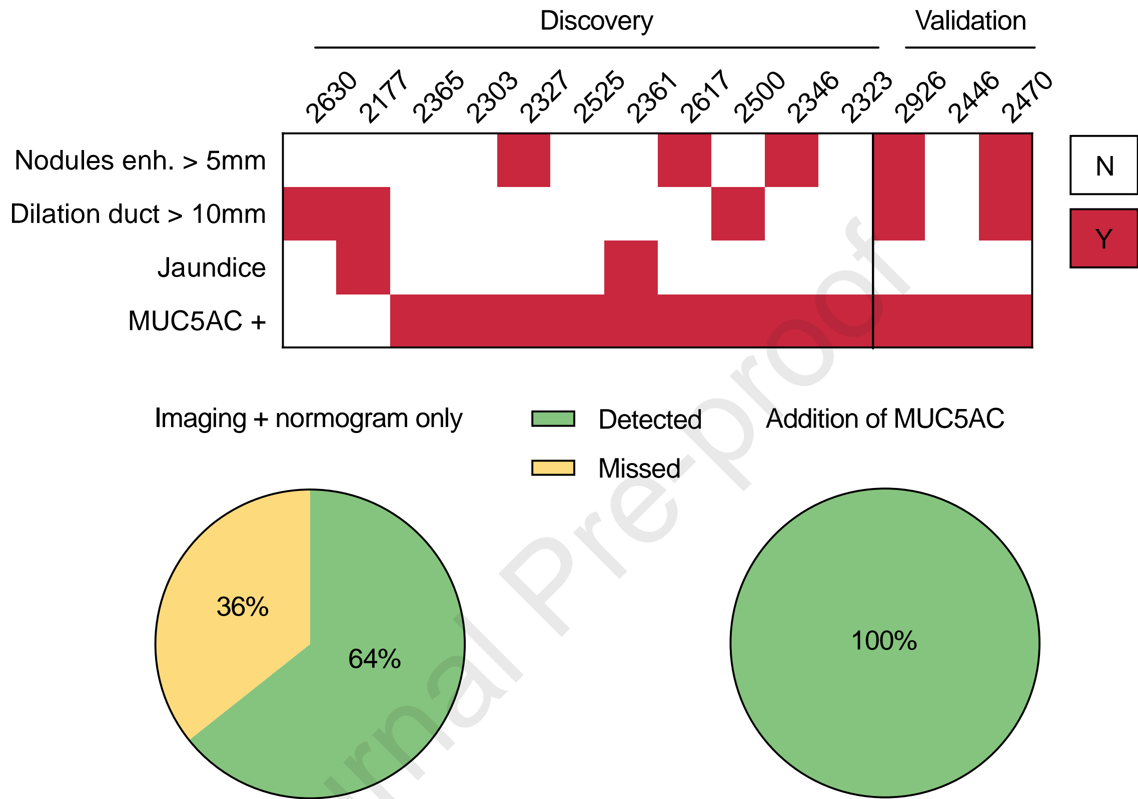

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Figure S7. Value of CA19-9 measurement in IPMN. Plasma levels of CA19-9 are not statistically different between the different groups and the AUC is 0.6, indicating poor discriminatory capabilities.

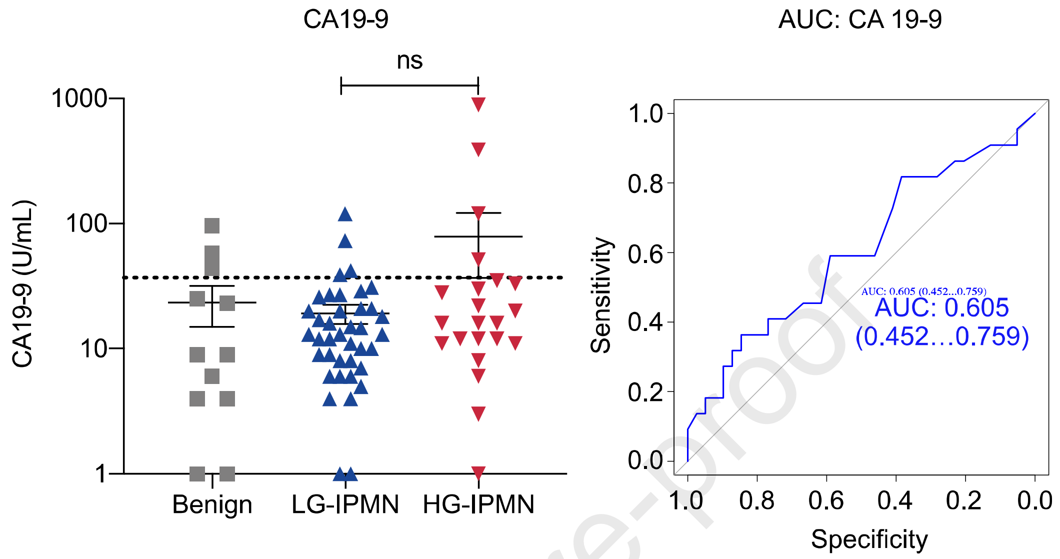

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Table S1. Experimental details of DEST assay. Each initial plasma EV sample is 1 or 10 μL. Beads are collected using a 96-well plate magnet for each wash step. Each incubation step is done on a plate shaker to maintain beads in suspension. MUC1 and MUC5AC were incubated in HAMA blocker in step 3.

Step	Reagent	Time (min)	Vol. (μL)	Conc.	Buffer
1	Blocking	30	100	1.5μl (~1 million) beads/well	Antibody dependent, see Table S3
2	Wash	4 washes	100	—	PBS + 0.1% Tween-20
3	Incubation with sample	60	100	1-10μl plasma, 250ng EVs or cell lysate controls	Blocking buffer
4	Wash	4 washes	100	—	PBS + 0.1% Tween-20
5	Detection antibody	60	50	0.5μg/mL	Blocking buffer
6	Wash	4 washes	100	—	PBS + 0.1% Tween-20
7	Streptavidin-HRP	30	100	137.5ng/mL	Blocking buffer + 0.1% Tween-20
8	Wash	4 washes	100	—	PBS + 0.1% Tween-20
9	Biotin tyramide (signal amplification)	10	100	5μg/mL	0.1M Borate buffer (pH8.5) + 0.003% H_2O_2
10	Wash	4 washes	100	—	PBS + 0.1% Tween-20
11	Brilliant violet 421 streptavidin	30	50	0.5μg/mL	Blocking buffer
12	Wash	4 washes	100	—	PBS + 0.1% Tween-20
Total		**220 minutes**			

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Table S2. List of reagents used in the DEST assay.

Reagent	Company	Catalog #	Stock conc.	Final conc.
Bovine serum albumin	Fisher Scientific	BP1605-100	—	2% w/v
UltraBlock	Bio-Rad	BUF033C	—	use neat
HAMA Blocker	Abcam	ab193969	—	use neat
PBS	Thermo Scientific	70011069	10X	1X
Tween-20	Sigma-Aldrich	P9416-100mL	100%	0.1%
Pooled normal human plasma (K2 EDTA)	Innovative Research Inc.	IPLA-N	—	1-10µl
Streptavidin-HRP	Thermo Scientific	21130	1.1mg/mL	137.5ng/mL
Biotinyl tyramide	Sigma-Aldrich	SML2135-50mg	2mg/mL (DMSO)	5µg/mL
Boric Acid	Sigma-Aldrich	B6768-500g	—	0.1M, pH8.5
Hydrogren peroxide solution	Sigma-Aldrich	H1009-100mL	30%	0.003%
Brilliant violet 421 streptavidin	BioLegend	405225	0.5mg/mL	0.5µg/mL

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Table S3. Antibodies used in this study. Polyclonal antibodies are indicated with a *, all other antibodies are monoclonal. Ms = mouse, rb = rabbit, gt = goat, sh = sheep.

Target	Capture antibody					Detection antibody				
	Vendor	Cat No.	Clone	Species	Immunogen	Vendor	Cat No.	Clone	Species	Immunogen
MUC1	Fitzgerald	10-CA15A	M201211	ms IgG2b	Hu CA15-3	Fitzgerald	10-CA15B	M2012112	ms IgG2b	Hu CA15-3
MUC2	Sigma	SAB1412598	2A9	ms IgG2ak	aa 4993-5078 (#NP_002448)	Novus	NB120-11197	996/1	ms IgG1	MUC2 tandem repeat peptide
MUC4	Sigma	WH0004585M7	5B12	ms IgG2ak	aa 79-189 (#NP_004523)	Thermo	35-4900	1G8	ms IgG1	rat ASGP-2
MUC5AC	Abcam	ab24070	1-13M1	ms IgG1	mucin from ovarian cyst fluid	Thermo	MA5-12175	45M1	ms IgG1k	M1 mucin from ovarian cyst fluid
MUC6	Novus	46760002		rb IgG*	C-terminus	Novus	46760002		ms IgG*	C-terminus
EPCAM	R&D	MAB9601	158206	ms IgG2b	Recomb. extracell. domain	R&D	BAF960		gt IgG*	aa 24-265 (#CAA32870)
EGFR	R&D	AF231		gt IgG*	aa 25-645 (#CAA25240)	Abcam	ab98133		sh IgG*	22aa near internal phos. region
GPC1	R&D	AF4519		gt IgG*	aa 24-530 (#P35052)	Sigma	SAB2700282		rb IgG*	aa 200-558 (#P35052)
WNT2	MyBio Source	2104322		rb IgG*		MyBio Source	2104322		rb IgG*	
Das-1	Millipore	MABC530	7E12H12	ms IgMk	Hu colonic protein extract	Millipore	MABC530	7E12H12	ms IgMk	Hu colonic protein extract
STMN1	Rockland	600-401-DG7		rb IgG*	20aa peptide near C-term	Rockland	600-401-DG8		rb IgG*	17aa peptide near N-term
TSP1	R&D	MAB3074	301221	ms IgG2b	aa 19-1170 (#CAA32889)	R&D	BAF3074		gt IgG	aa 19-1170 (#P07996)
TSP2	R&D	MAB16351	230927	ms IgG2a	aa 19-1172 (#P35442)	R&D	BAF1635		gt IgG*	aa 19-1172 (#P35442)
EphA2	R&D	MAB3035	371805	ms IgG2a	aa 25-534 (#P29317)	R&D	BAF3035		gt IgG	aa 25-534 (#P29317)
S100A4	MyBio Source	2089230		rb IgG		MyBio Source	2089230		rb IgG	
PSCA	Abcam	ab64919		rb IgG	proprietary	Fitzgerald	70R-19568		rb IgG	Hu PSCA
Isotype	Abcam	ab18400	MM-30	ms IgMk		n/a	n/a		n/a	
	Abcam	ab18415	MG2a-53	ms IgG2ak		n/a	n/a		n/a	
	R&D	MAB004	20116	ms IgG2b		n/a	n/a		n/a	
	R&D	MAB003	20102	ms IgG2a		n/a	n/a		n/a	
	Thermo	02-6202		gt IgG		n/a	n/a		n/a	
	Abcam	ab172730	EPR25A	rb IgG		n/a	n/a		n/a	

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Table S4. DEST antibody pair conditions and controls. Blocking buffers used for each antibody pair, plasma volume, negative and positive controls. *Control confirmed for corresponding cell line in the Human Protein Atlas database.

Target	Blocking	Vol. plasma	Negative ctrl	Positive ctrl
MUC1	2%	1µl	Daudi EV*	Capan-2 EV*
MUC2	2% BSA	10µl	Daudi EV*	1966 cell
MUC4	2% BSA	10µl	Daudi EV*	1966 cell
MUC5AC	UltraBlock	10µl	1617 EV	1505 EV
MUC6	2% BSA	10µl	Daudi EV*	1966 cell
EpCAM	2% BSA	10µl	Daudi EV*	1617 EV
EGFR	2% BSA	10µl	Daudi EV*	BxPC3 cell
GPC1	2% BSA	1µl	Daudi EV*	GPC1 protein
WNT2	2% BSA	1µl	Daudi EV*	WNT2 protein
Das-1	UltraBlock	10µl	1617 EV	LS180 EV
STMN1	2% BSA	10µl	1617 EV	Mia PaCa-2 cell
TSP1	UltraBlock	1µl	Daudi EV*	TSP1 protein
TSP2	2% BSA	1µl	1617 EV	TSP2 protein
EphA2	2% BSA	10µl	Daudi EV*	A549 EV*
S100A4	2% BSA	10µl	Daudi EV*	S100A4 protein
PSCA	2% BSA	10µl	Daudi EV*	PSCA protein

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Table S5. Additional antibodies tested, but found to be unsuitable for DEST.

Target	Alternative antibodies tested
	Vendor (Cat No.)
MUC1	Fitzgerald (10-M93A), BioLegend (355602)
MUC2	Antibodies-online (ABIN1173448), Novus (H000-4583-M02, NBP2-25221)
MUC5AC	Novus (NBP2-44452, NBP2-15196, H00004586-M07, NBP2-44458)
MUC6	Origene (TA322537), Novus (NBP2-44376)
EGFR	Sino Biological Inc. (10001-RE11, 10001-R021), R&D (BAF231)
EpCAM	BioLegend (324215), Thermo (710524), Abcam (ab20160)
WNT2	R&D (AF3464), Novus (2295002), Santa Cruz (sc-514382)
PSCA	Fitzgerald (70R-19568), Novus (H00008000-M02)
TSP1	Thermo (MA5-13395)
MUC13	Sigma (SAB4502427), Fitzgerald (70R-32134)
ZEB1	Origene (TA590279), Novus (NBP2-37329)
PLEC1	Abcam (ab32528), Santa Cruz (sc-33649), Origene (TA351536), Millipore (MAB5674)
HOOK1	Novus (H00051361-AP21, antibody pair)
PTPN6	Novus (H00005777-AP22, antibody pair)
FBN1	Millipore (MAB2499, MAB2502)

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Table S6. EV biomarker analysis in validation cohort. All numbers for sensitivity (sens), specificity (spec), F1 score and positive predictive value (PPV) are in fractions and are compared to LG-IPMN. 95% confidence intervals of this small series are shown in parentheses.

Biomarker(s)	Invasive HG-IPMN				Noninvasive HG-IPMN				Combined HG-IPMN			
	Sens	Spec	F1	PPV	Sens	Spec	F1	PPV	Sens	Spec	F1	PPV
MUC5AC	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00	1.00	0.11 (0.00, 0.33)	1.0 (1.00, 1.00)	0.20	1.00	0.33 (0.08, 0.58)	0.97 (0.91, 1.00)	0.47	0.80
Imaging alone	0.33 (0.00, 1.00)	0.94 (0.86, 1.00)	0.33	0.33	0.67 (0.33, 0.89)	0.94 (0.86, 1.00)	0.71	0.75	0.58 (0.33, 0.83)	0.94 (0.86, 1.00)	0.67	0.80
DEST + Imaging	1.00 (1.00, 1.00)	0.94 (0/86, 1.00)	1.00	0.60	0.67 (0.33, 0.89)	0.89 (0.77, 0.97)	0.63	0.60	0.75 (0.50, 1.00)	0.89 (0.77, 0.97)	0.72	0.69

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1. Pergolini, I, Morales-Oyarvide, V, Mino-Kenudson, M et al. Tumor engraftment in patient-derived xenografts of pancreatic ductal adenocarcinoma is associated with adverse clinicopathological features and poor survival. PLoS One. 2017;12:e0182855.

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