Multiple antibodies identify Glypican-1 on serum exosomes from patients with pancreatic cancer

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We previously reported on the detection of the heparin sulfate proteoglycan Glypican-1 (GPC1) on serum-derived exosomes in patients with pancreatic cancer and breast cancer[1]. GPC1 expression is elevated in several cancer types and associated with poor prognosis[2,3], including in pancreas cancer cells, mouse and human pancreatic cancer[4-9]. Informed by genetic studies, GPC1 is proposed to control fibroblast growth factor (FGF) signaling in mouse brain development[10], and may be critical for pancreatic cancer cell proliferation and VEGF-A induced pancreatic tumor angiogenic response[5,6]. The proteoglycan GPC1, GPI-anchored to the cell surface, acts as a co-receptor for several heparin binding growth factors, thereby implicating it in promoting tumor progression[6-9,11].

In patients with pancreatic cancer, we described the enrichment of GPC1$^+$ exosomes in the circulation when compared to healthy controls[1]. Exosomes are nano-sized extracellular vesicles produced by all cells and abundantly found in the circulation[12]. The renewed interest in their biology was fueled by their potential as liquid

biopsies for various pathologies, including cancer. Although there are likely distinct subpopulations of exosomes with various biological properties, exosomes are generally carriers of the nucleic acids and proteins that reflect their cell of origin[12]. As such, exosomes from the serum of patients emerged as an attractive approach to possibly detect cancerous lesions and monitor and predict outcome of cancer patients. Several studies have recently reported findings related to GPC1 detection on exosomes, including in breast cancer cells-derived exosomes, as well as colorectal and pancreas cancer cases[13-16] (**Table 1**). Although the methodologies differed in each study, GPC1 was indeed elevated in exosomes of patients with cancer.

Here we report on the use of two anti-glypican 1 antibodies for the specific detection of $GPC1^+$ exosomes in the circulation of pancreas cancer patients (n=10) compared to healthy donors (n=9) and benign pancreatic diseases (BPD, n=2) using flow cytometry as a readout. The ThermoFisher antibody used in our initial report[1] (PA5-28055) is commercially available. We also utilized antibody SAB2700282 from Sigma. Both companies described the immunogen as a recombinant fragment of human GPC1 corresponding to a region within amino acids 200 and 558. We report on the highly reproducible and strong correlation between samples evaluated with each of these antibodies (**Figure 1A-B**). Notably, all 10 patients with PDAC showed elevated circulating $GPC1^+$ exosomes bound-beads, in contrast with the 9 healthy donors and 2 BPD samples (**Figure 1A, Table 2**). We also proceeded with analyses using the ThermoFisher anti-GPC1 antibody PA5-24972, however we noted this antibody did not reproduce our initial findings (**Table 3**). Further, when exosomes were collected using isolation kits rather than the described ultracentrifugation procedure, the accuracy of

detection was lost. Therefore, ultracentrifugation for exosomes enrichment is required to evaluate GPC1 on exosomes.

In an effort to engage the scientific community in evaluating the utility of $GPC1^+$ exosomes in early detection or monitoring of pancreas cancer patients, we provide a detailed methodology, in a step-wise manner, to accompany the findings reported therein (**Table 4**).

We look forward to a productive scientific discourse on our ongoing efforts to identify GPC1 on cancer exosomes using different methods and antibodies.

Methods

We provide a detailed protocol in **Table 4** that describes the procedure for exosomes purification and flow cytometry analyses of GPC1 on exosomes. The secondary antibody used was Alexa Fluor 488 goat anti-rabbit (Invitrogen A11008). The aldehyde/sulfate beads were obtained from Invitrogen (A37304).

Serum samples were obtained from patients with pancreatic cancer. Serum samples were also obtained from patients with a benign pancreas disease and from healthy donors, who had no evidence of acute or chronic or malignant disease and had no surgery within the past 12 months. The cases were obtained under an IRB-exempt protocol of the MD Anderson Cancer Center (IRB no. PA14-0154).

Figure legends

Figure 1. A Flow cytometry analyses for the % of $GPC1^+$ circulating exosomes on beads, comparing the two anti-GPC1 antibodies listed. **B**. Linear regression analysis between % of $GPC1^+$ circulating exosomes on beads obtained using each of the listed antibodies.

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Figure 1

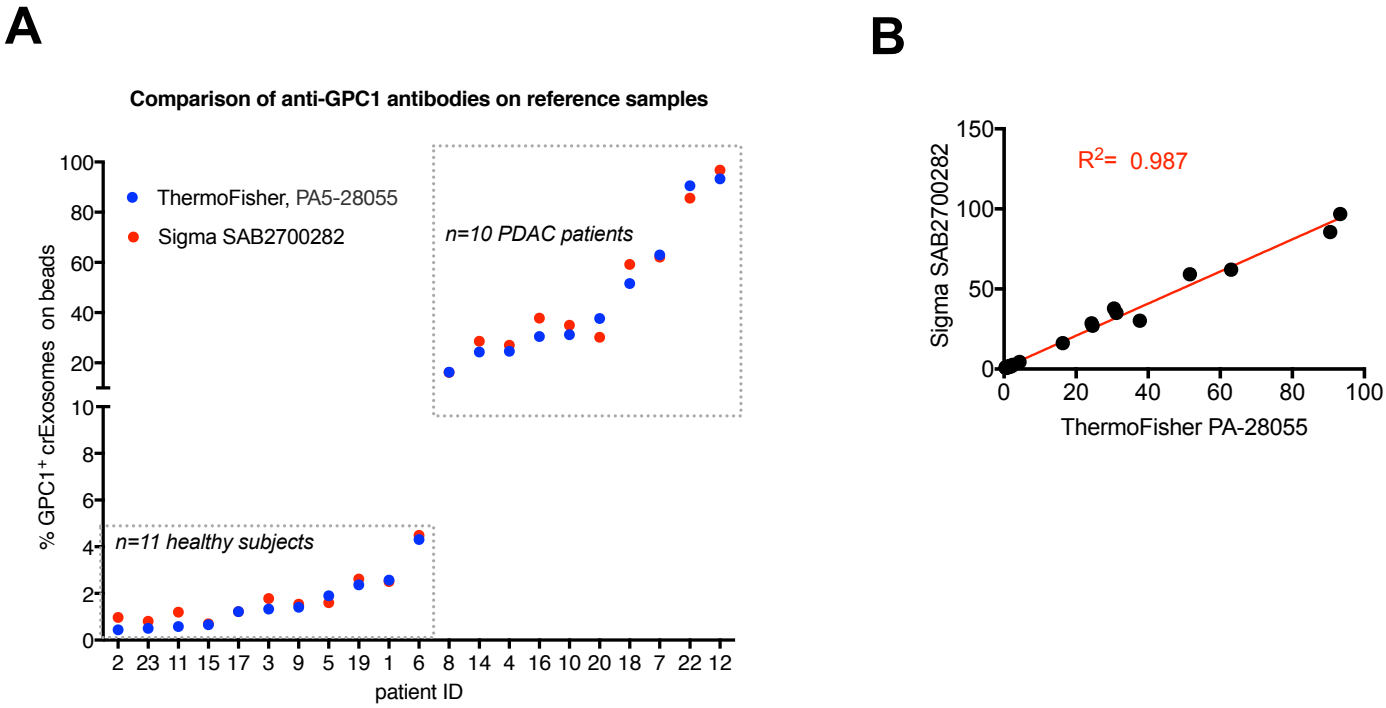
A
Comparison of anti-GPC1 antibodies on reference samples
ThermoFisher, PA5-28055
Sigma SAB2700282
n=10 PDAC patients
n=11 healthy subjects
% GPC1+ crExosomes on beads
patient ID
2 23 11 15 17 3 9 5 19 1 6 8 14 4 16 10 20 18 7 22 12
B
R2= 0.987
Sigma SAB2700282
ThermoFisher PA-28055

Table 1

Study	Link	Exosomes source	Exosomes extraction procedure	Readout	Notes and conclusions
Etayash H. et al. (2016)	https://www.ncbi.nlm.nih.gov/pubmed/27492928	Cell culture supernatant	0.2um filter and ultracentrifugation	Nanomechanical sandwish assay, EMD Millipore, Clone 4D1 anti-GPC1	GPC1 detection with excellent sensitivity
Jian Li L. et al. (2016)	https://www.ncbi.nlm.nih.gov/pubmed/28233416	Serum from 102 patients with colorectal cancer	ExoCapTM Exosomes Isolation and Enrichment kit (JSR Micro Materials Innovation)	FC with aldheyde/sulphate latex beads, SantaCruz anti-GPC1 antibody	GPC1+ circulating exosoms are sepcific markers fo the diagnosis of colorectal cancer
Lai X. et al. (2017)	https://www.ncbi.nlm.nih.gov/pubmed/28232049	Plasma from 6 normal control subjects, 3 patients with chronic pancreatitis, and 3 patients with PDAC	0.2um filter and ultracentrifugation	LC-MS/MS	Despite the low sample number and lack of normalization of exosomes concentration prior to analyses, PDAC patients show elevated GPC1+ exosomes.
Yang K.S. et al. (2017)	http://stm.sciencemag.org/content/9/391/eaal3226	Plasma from 2 cohorts of patients: Cohort 1 (Trainign cohort, n=22 PDAC, n=10 normal); Cohort 2 (Prospective cohort, n=46 PDAC, n=13 IPMN, n=12 NET, n=11 other cancer, n=21 begnin+control)	0.2um filter and ultracentrifugation	Nanoplasmonic sensors, R&D Systems BAF4519 biotinylated anti-GPC1 antibody, ThermoFisher PA5-24972 anti GPC1 antibody,	GPC1 alone showed 55% sensitivity and 60% specificity for cohort 1, 82% sensitivity and 52 specificity for cohort 2. PDAC GPC1+ exosomes signals were elevated compared to controls.

Table 2

	Secondary only (Alex 488)		Sigma SAB2700282		Secondary only (Alex 488)		ThermoFisher PA5-28												
ID	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	Size (nm)	Concentratio (E+9 exos/ml	# exos used in analysis	# events (FC)	Final volume	Voltage	Source	Diagnosis	Additional information	Sex	Age
1	98.5	0.82	98.9	2.51	98.5	0.82	98.5	2.57	120.6	1.08	3.8x10^1	10000	300 u	440	MDACC	Healthy		Unknown	Unknown
5	60.4	0.37	72.2	1.6	97.5	0.61	91.9	1.9	123.7	0.577	3.8x10^1	10000	300 u	390	MDACC	Healthy		Unknown	Unknown
9	60.3	0.79	74.4	1.54	93.6	1.24	93.6	1.41	50.3	0.928	3.8x10^1	10000	300 u	280	MDACC	Healthy		Unknown	Unknown
11	98	0.92	98.7	1.2	80.9	0.88	80.7	0.58	59.7	0.682	3.8x10^1	10000	300 u	270	MDACC	Healthy		Unknown	Unknown
15	98.4	0.32	98.6	0.7	95.2	0.93	98.6	0.66	99.4	3.39	3.8x10^1	10000	300 u	350	MDACC	Healthy		Unknown	Unknown
17	96.1	1.37	95.9	1.22	81.5	1.36	82.6	1.22	102.6	2.38	3.8x10^1	10000	300 u	245	MDACC	Healthy		Unknown	Unknown
19	92	1.04	94.6	2.62	76.3	0.59	82.2	2.37	122.2	0.496	3.8x10^1	10000	300 u	340	MDACC	Healthy		Unknown	Unknown
23	98.6	0.19	98.6	0.81	95.3	1.23	98.3	0.5	86.4	3.9	3.8x10^1	10000	300 u	311	MDACC	Healthy		Unknown	Unknown
2	97.7	1.05	97.7	0.97	95.8	1.08	98.6	0.44	98.5	4.04	3.8x10^1	10000	300 u	265	MDACC	Healthy	Serous cystadenoma	MALE	77
6	98.8	0.93	98.8	4.49	46.6	0.75	52.5	4.31	55.2	1.72	3.8x10^1	10000	300 u	265	MDACC	Healthy	Autoimmune pancreatitis	MALE	85
4	97.2	1	98.4	27	71.2	1.45	89.7	24.6	110	3.25	3.8x10^1	10000	300 u	380	MDACC	PDAC	AJCC IV	FEMALE	74
8	97.4	0.77	98.6	16.2	42.9	0.12	52.5	16.3	79.4	4.09	3.8x10^1	10000	300 u	300	MDACC	PDAC	AJCC IIB	FEMALE	64
10	60.7	0.66	61.6	35	93.7	1.34	80.3	31.2	50.7	2.57	3.8x10^1	10000	300 u	255	MDACC	PDAC	AJCC IIB	MALE	61
12	81.9	0.99	76.2	96.8	61.1	1.65	58.6	93.3	99.3	4.63	3.8x10^1	10000	300 u	395	MDACC	PDAC	AJCC IIB	MALE	66
14	97.2	0.14	98.8	28.6	98.6	0.42	98.3	24.3	61.1	5.39	3.8x10^1	10000	300 u	280	MDACC	PDAC	AJCC IIB	MALE	65
16	97.2	0.96	71.6	37.8	71.2	1.03	77.1	30.5	122	3.21	3.8x10^1	10000	300 u	400	MDACC	PDAC	AJCC IIA	MALE	69
18	60.9	0.35	72.3	59.2	72.2	1.97	68.3	51.6	102.9	0.772	3.8x10^1	10000	300 u	390	MDACC	PDAC	AJCC IIB	FEMALE	58
20	95	0.76	96.7	30.2	96.2	0.42	92.6	37.7	49.4	0.74	3.8x10^1	10000	300 u	268	MDACC	PDAC	AJCC IIB	MALE	50
22	83.1	1.07	74.7	85.6	86.5	0.6	77.3	90.5	84.6	7.36	3.8x10^1	10000	300 u	370	MDACC	PDAC	AJCC IIB	FEMALE	79
7	82.8	0.78	86.6	62.1	87.5	0.72	86.5	63	81.2	1.54	3.8x10^1	10000	300 u	375	MDACC	PDAC	AJCC IIB	FEMALE	80
3	85.9	0.82	90.6	1.78	91.8	0.96	94.9	1.33	71.3	4.76	3.8x10^1	10000	300 u	260	MDACC	Healthy		Unknown	Unknown

Table 3

																	From Melo et al *Nature* 2015		
	Secondary only (Ale		*ThermoFisher PA5-2479*														*ThermoFisher PA5-28055*		
ID	***%beads***	***%FITC+***	***%beads***	***%FITC+***	***Size (nm)***	***Concentration (E+9 exos/ml)***	***# exos used in analysis (E+10 exos)***	***# events (FC)***	***Final volume***	***Voltage***	**Source**	**Diagnosis**	**Additional information**	**Sex**	**Age**		***%FITC+ (depicting % GPC crExo beads)***	***Size (nm)***	***Concentration (E+9 exos/ml)***
1	64.8	0.54	69.5	16.2	83.9	6.39	4	10000	300 ul	255	Germany	Serous cystadenoma	Not applicable	Female	72		2.6	105	2.01
2	61.8	0.45	69.9	3.73	171.4	4.07	4	10000	300 ul	255	Germany	BPD	Not applicable	Male	58		1.1	154	1.87
3	69.3	0.57	70.8	5	88	1.27	4	10000	300 ul	260	Germany	PDAC	AJCC IIA	Female	77		17.4	40	3.71
4	67.4	0.75	71	3.97	70.6	3.42	4	10000	300 ul	265	Germany	Healthy	Anonymous				1	132	2.94

Table 4

Protocol: GPC1 FC analyses on exosomes
a. Exosomes purification
1. Thaw the serum/plasma in 37°C water by shaking it.
2. Centrifuge at 800G for 5 min, RT.
3. Centrifuge at 2000G for 10 min, RT
4. Collect supernatant and discard pellet (cells and debris).
5. Filter supernatant through a 0.2 um membrane by syringe directly into UC tube. Use ~3mL PBS to wash through the filter.
6. Add PBS to the ultracentrifuge tube for a final volume of 11mL. Centrifuge the samples at 100,000G overnight.
b. FC GPC1
6. A total of 6 samples maximum should be included per round of analyses.
7. Frozen exosomes: allow exosomes to rotate at RT for 1hr at RT.
8. Nanosight measurements: performed just prior to setting up exosomes for FC. A good exosomes prep concentration should range in the ~10^10 exo/ml concentration (for a 1:100 dilution (cells)). Nanosight measurements for serum are usually using 1:1000 dilution of the sample.
Note: Ideally, 5x10^10 exosomes for 5ul beads. For a sample with a nanosight read of 10^10 exo/ml (not accounting for dilution), the final concentration is 10^12 exo/ml or of 10^9 exo/ul and the total (usually exosomes are resuspended in 200ul PBS) is 2x10^11. For 5x10^10 exosomes: 50ul. If volume is greater than 50ul, allow exo+beads to incubate for 2hrs at RT (see below).
Note 2: if for any reason the read is poor, nanosight measurements must be repeated prior to repeating the experiment.
9. Mix beads very well. Take 5ul of beads and mix with 100ul PBS. Allow beads to rotate for 15' at RT.
10. Add exosomes (5x10^10; ~50ul. If volume of exosomes if greater than 50ul, allow exo+beads to incubate for 2hrs at RT) and mix well when adding to beads. Add PBS to volume up to 400ul. Allow to rotate at RT for at least 1 hour.
11. Add 400ul 1M glycine in PBS. Allow to rotate at RT for 1 hour.
12. Spin down 12,000rpm 2min RT, discard supernatant (pipette out) and leave ~20ul volume in tube.
13. Resuspend beads in 100ul 10% BSA/PBS. Care not to create bubbles. Allow to rotate at RT for 45'
14. Spin down 12,000rpm 2min RT, discard as much of the supernatant as possible (pipette out)
15. Resuspend beads in 40ul of 2% BSA/PBS (mix well but gently, avoid bubbles).
16. Split samples into two new fresh tubes, each containing 20ul (discard the few ul left in the original tube). Tube 1 (negative control): add nothing. Tube 2 (staining): add 1.5ul of anti-GPC1 Sigma antibody and mix very well (caution for bubbles). Allow tubes to rotate at 4C O/N
17. Spin down 12,000rpm 2min RT, discard as much of the supernatant as possible (pipette out)
18. Resuspend beads in 20ul of 2% BSA/PBS. Add 1ul of secondary ab and mix well. Allow to rotate, covered from light, at RT 30'
19. Spin down 12,000rpm 2min RT, discard as much of the supernatant as possible (pipette out)
20. Perform 3x 200ul wash of the beads using 2% BSA/PBS. Mix well, avoid bubbles.
21. Spin down 12,000rpm 2min RT, discard as much of the supernatant as possible (pipette out)
22. Resuspend in 300ul 2% BSA/PBS, mix well, avoid bubbles, and pipette through facs tube filter.
Note: the samples must be read the same day, asap post completion of the staining. If kept O/N, the beads clump and read will be poor.
23. At acquisition during FC, adjust the FITC voltage to ensure NC peak (autofluorescence detected in exosomes + beads + secondary ab only) is less than 10^3 FITC log scale and tail is ~1% into the positive signal (gate set at 103).
24. Acquire FC reads of the experimental sample with primary antibody.
25. For every each sample repeat procedure 23 and 24 to adjust for different autofluorescence in each patient sample.
26. If selected population of beads on the scatter plot corresponds to less than 60% this indicates a poor coating of the beads with exosomes or poor washing steps. Sample analysis needs to be repeatd.