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

pancreatic cancer

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Glypican-1 was found on the surface of cancer exosomes using multiple antibodies from different sources. These include anti-GPC1 antibodies from ThermoFisher (PA5-28055 and PA-5-24972)^{1,2}, Sigma (SAB270028), Abnova (MAB8351), EMD Millipore (MAB2600)³, SantaCruz⁴, and R&D Systems (BAF4519)². Here we report on the specific detection of Glypican-1 on the exosomes derived from the serum of pancreas cancer patients using multiple antibodies.

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 cancer¹. GPC1 expression is elevated in several cancer types and associated with poor prognosis^{5,6}, including in pancreas cancer cells, mouse and human pancreatic cancer⁷⁻¹². Informed by genetic studies, GPC1 is proposed to control fibroblast growth factor (FGF)

signaling in mouse brain development¹³, and may be critical for pancreatic cancer cell proliferation and VEGF-A induced pancreatic tumor angiogenic response^{8,9}. 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^{9-12,14}

In patients with pancreatic cancer, we described the enrichment of GPC1⁺ exosomes in the circulation when compared to healthy controls¹. Exosomes are nanosized extracellular vesicles produced by all cells and abundantly found in the circulation¹⁵. 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 origin¹⁵. 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^{2-4,16} (**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 three 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 report¹ (PA5-28055) is commercially available. We also utilized antibody SAB2700282 from Sigma,

and MAB8351 from Abnova. Both ThermoFisher and Sigma described the immunogen as a recombinant fragment of human GPC1 corresponding to a region within amino acids 200 and 558. Abnova describes the immunogen as recombinant protein corresponding to full length human GPC1. 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 three anti-GPC1 antibodies listed. When the Abnova antibody was used, n=6 samples were analyzed (see Table 2). **B**. Linear regression analysis between % of GPC1⁺ circulating exosomes on beads obtained using the ThermoFisher PA5-28055 and Sigma SAB2700282 antibodies.

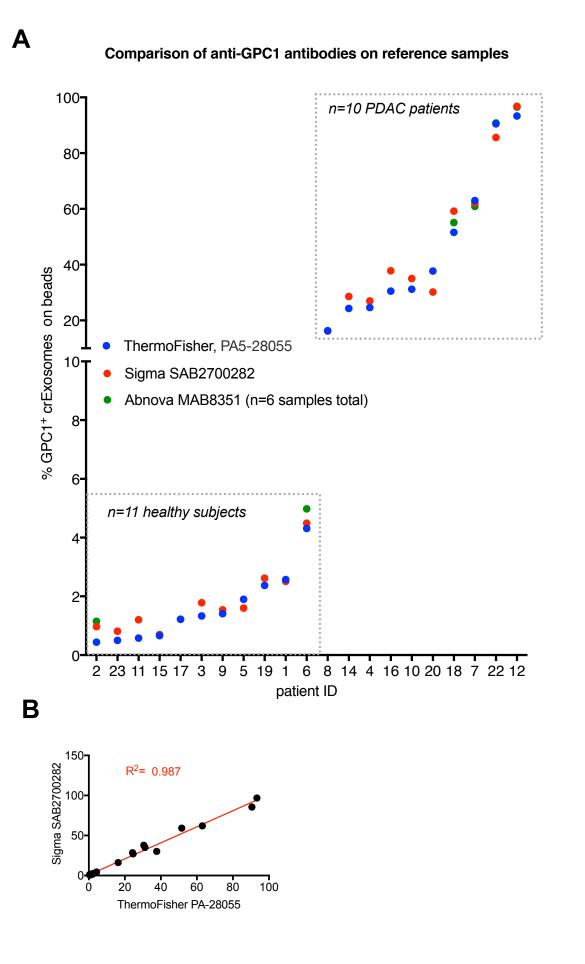
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Figure 1



Study	Link	Exosomes source	Exosomes extraction procedure	Readout	Notes and conclusions		
Etayash H. et al.	https://www.ncbi.nlm.nih.	Cell culture supernatant	0.2um filter and ultracentrifugation	Nanomechanical sandwish assay,	GPC1 detection with excellent		
(2016)	gov/pubmed/27492928	Cell culture supernatant		EMD Millipore, Clone 4D1 anti-GPC1	sensitivity		
	https://www.ncbi.nlm.nih.	Serum from 102 patients with	ExoCapTM Exosomes Isolation	FC with aldheyde/sulphate latex	GPC1+ circulating exosoms are		
Jian Li L. et al. (2016)		colorectal cancer	land Enrichment kit (ISR Micro	beads, SantaCruz anti-GPC1 antibody	sepcific markers fo the diagnosis of		
	g0v/publited/28233418		Materials Innovation)	beaus, SantaCruz anti-GFCT antibouy	colorectal cancer		
		Plasma from 6 normal control			Despite the low sample number and		
	https://www.ncbi.nlm.nih.	subjects, 3 patients with chronic			lack of normalization of exosomes		
Lai X. et al. (2017)		pancreatitis, and 3 patients with	0.2um filter and ultracentrifugation	LC-MS/MS	concentration prior to analyses,		
	gov/publited/20232049	PDAC			PDAC patients show elevated		
					GPC1+ exosomes.		
		Plasma from 2 cohorts of patients:			GPC1 alone showed 55% sensitivity		
	http://stm.sciencemag.or	Cohort 1 (Trainign cohort, n=22		Nanoplasmonic sensors, R&D	and 60% specificity for cohort 1,		
Yang K.S. et al. (2017)	g/content/9/391/eaal322	PDAC, n=10 normal); Cohort 2	0.2um filter and ultracentrifugation	Systems BAF4519 biotinylated anti-	82% sensitivity and 52 specificity for		
	6	(Prospective cohort, n=46 PDAC,		GPC1 antibody, ThermoFisher PA5-	cohort 2. PDAC GPC1+ exosomes		
	°	n=13 IPMN, n=12 NET, n=11 other		24972 anti GPC1 antibody,	signals were elevated compared to		
		cancer, n=21 begnin+control)			controls.		

	Secondary	y only (Alex			Secondar	y only (Alex			Secondar	y only (Alex													
	48	8)	Sigma S	AB2700282	48	8)	ThermoFis	her PA5-28	48	(8)	Abnova	MAB8351											
ID	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	%beads	%FITC+	Size (nm)	Concentration (E+9 exos/ml		# events (FC)	Final volume	Voltage	Source	Diagnosis	Additional information	Sex	Age
1	98.5	0.82	98.9	2.51	98.5	0.82							120.6	1.08		10000	300 ul	440	MDACC	Healthy		Unknown	Unknown
5	60.4	0.37	72.2	1.6	97.5	0.61							123.7	0.577		10000	300 ul	390	MDACC	Healthy		Unknown	Unknown
9	60.3	0.79	74.4	1.54	93.6	1.24							50.3	0.928		10000	300 ul	280	MDACC	Healthy		Unknown	Unknown
11	98	0.92	98.7	1.2	80.9	0.88							59.7	0.682		10000	300 ul	270	MDACC	Healthy		Unknown	Unknown
15	98.4	0.32	98.6	0.7	95.2	0.93							99.4	3.39		10000	300 ul	350	MDACC	Healthy		Unknown	Unknown
17	96.1	1.37	95.9		81.5	1.36							102.6	2.38		10000	300 ul	245		Healthy		Unknown	Unknown
19	92	1.04	94.6	2.62	76.3	0.59							122.2	0.496		10000	300 ul	340		Healthy		Unknown	Unknown
23	98.6	0.19	98.6	0.81	95.3	1.23							86.4	3.9	0.0/10 10	10000	300 ul	311		Healthy		Unknown	Unknown
2	97.7	1.05	97.7	0.97	95.8	1.08			98.90	1.24	98.7	1.15	98.5	4.04		10000	300 ul	265	MDACC	Healthy	Serous cystadenoma	MALE	77
6	98.8	0.93	98.8	4.49	46.6	0.75			80.80	0.19	85.1	4.98	55.2	1.72		10000	300 ul	265		Healthy	Autoimmune pancreatitis	MALE	85
4	97.2	1	98.4	27	71.2	1.45							110	3.25		10000	300 ul	380		PDAC	AJCC IV	FEMALE	74
8	97.4	0.77	98.6	16.2	42.9	0.12							79.4	4.09		10000	300 ul	300	MDACC	PDAC	AJCC IIB	FEMALE	64
10	60.7	0.66	61.6	35	93.7	1.34							50.7	2.57		10000	300 ul	255		PDAC	AJCC IIB	MALE	61
12	81.9	0.99	76.2	96.8	61.1	1.65			97.9	0.8	97.2	96.5	99.3	4.63		10000	300 ul	395		PDAC	AJCC IIB	MALE	66
14	97.2	0.14	98.8	28.6	98.6	0.42							61.1	5.39		10000	300 ul	280	MDACC	PDAC	AJCC IIB	MALE	65
16	97.2	0.96	71.6		71.2	1.03							122			10000		400	MDACC	PDAC	AJCC IIA	MALE	69
18	60.9	0.35	72.3	59.2	72.2	1.97			83.1	0.41	74.3	55.1	102.9	0.772		10000	300 ul	390	MDACC	PDAC	AJCC IIB	FEMALE	58
20	95	0.76	96.7	30.2	96.2	0.42							49.4	0.74		10000	300 ul	268		PDAC	AJCC IIB	MALE	50
22	83.1	1.07	74.7	85.6	86.5	0.6			95.3	0.28	93.9	90.8	84.6	7.36		10000	300 ul	370		PDAC	AJCC IIB	FEMALE	79
7	82.8	0.78	86.6	62.1	87.5	0.72			82.9	0.87	76.8	60.9	81.2	1.54	3.8x10^10	10000	300 ul	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	3.8x10^10	10000	300 ul	260	MDACC	Healthy		Unknown	Unknown

																From Melo et	5	
	Secondar	y only (Ale	ThermoFi	isher PA5-2479												ThermoFisher PA5-28055		
ID	%beads	%FITC+	%beads	%FITC+	Size (nm)	Concentration (E+9 exos/ml)	analysis (F+10	# ovents	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

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
 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.