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Circulating CD9-GFAP-survivin exosomes during active specific immunotherapy, a potential biomarker for glioma

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ABSTRACT

Background: We evaluated circulating exosomes isolated from the serum of malignant glioma patients enrolled in a completed phase I clinical trial of an anti-survivin vaccine (SurVaxM). Exosomes are microvesicular bodies with potential mechanisms for cell-cell communication. Identifying circulating exosomes from cancer patients as potential indicators of disease status and response to therapy is of interest. The inhibitor of apoptosis protein (IAP) survivin (SVN) promotes cancer cell proliferation and resistance to chemotherapy. Survivin is expressed in many cancer types including malignant gliomas and is a potential target for active immunotherapy. In the above mentioned clinical trial, five patients experienced early tumor progression with a mean of 2.8 months (1.9-5.4 months) from study entry, while three patients had either late (20.5-22.5 months) or no tumor progression (no evident disease at 48 months in one patient).
Methods: To determine whether changes in CD9+/GFAP+/SVN+ exosomes correlated with disease progression, patient serum was evaluated prior to treatment (study entry), 8-10 weeks after initial vaccination, and at the time of MRI-defined tumor progression. By employing ImageStream flow cytometry technology we were able to analyze multiple markers on individual exosomes, identifying highly specific populations of survivin+ exosomes which co-expressed the brain/tumor marker protein GFAP. The combination of these markers made it possible to differentiate patient exosomes from those of healthy individuals.
Results: The levels of CD9+/GFAP+, CD9+/SVN+, and CD9+/GFAP+/SVN+ (SVN+) exosomes were significantly increased in patients. Serum from three patients who experienced the longest progression-free intervals, showed 98-99% decreases in SVN+ exosomes after treatment with SurVaxM, and maintained similarly low exosome levels over several months. Five early progressing patients experienced a detectable persistence of, or increase in SVN+ exosomes at 9 weeks following initial vaccination. One patient had rising SVN+ exosomes detectable 16 weeks prior to the discovery of tumor progression by brain MRI scan. One patient with no tumor progression sustained a 99% reduction in serum SVN+ exosomes which was sustained over 48+ months.
Conclusion: This study demonstrates that increased numbers of CD9+/GFAP+/SVN+ specific exosomes appear to be associated with early tumor progression in recurrent malignant glioma patients. Larger studies are needed to determine whether the correlation between SVN+ exosome levels and disease progression is specific to SurVaxM treatment, and whether rises in SVN+ exosomes levels are predictive of generalized glioma progression. Our study suggests that levels of circulating SVN+ exosomes may be altered during anti-survivin immunotherapy and could serve as a potential biomarker for clinical trials and tumor monitoring.

BACKGROUND

- Gliomas release exosomes into the extracellular matrix. These nanobodies transport an array of proteins, lipids, and nucleic acids, suggesting a potential mechanism for cell-cell communication. Circulating exosomes in cancer patients are potential indicators of disease status.
- The inhibitor of apoptosis protein (IAP) survivin (SVN) is predominantly an intracellular molecule. It promotes proliferation, local immune suppression, resistance to chemotherapy, and its expression in gliomas is associated with a poor prognosis.
- Survivin has been observed on exosomes produced by cervical and prostatic carcinoma cells [S. Khan et al.; PLOS ONE 7(10): e46737, 2012]. Extracellular survivin is involved in immune suppression by preventing T-cell proliferation [J. Jutzy et al.; Cancer Microenviron 6(1) 57-68, 2013]. The mechanism by which survivin is localized to the external surface however remains to be clearly defined.
- We have found survivin to be expressed on exosomes in glioma patients enrolled in SurVaxM immunotherapy clinical studies.
- SurVaxM is a synthetic long peptide, reverse neo-immunogen that initiates an immune response to survivin. SurVaxM is a single 15 AA long peptide which triggers **mid-affinity** CD8+ T cell receptors to produce a m**ulti**-epitope CD8 response, specific CD4+ T cell response and B cell recognition (Antibody)
- SurVaxM patients also produce antibodies to a specific region of survivin. We have designed a murine counterpart to ascertain this mAb potential in pre-clinical therapeutic and diagnostic studies. Survivin containing exosomes may represent a potential diagnostic biomarker and/or therapeutic activity marker.
- A completed Phase I clinical study of SurVaxM in recurrent glioma patients showed safety and signs of efficacy. 7/8 patients lived > 12 months, 2 long term survivors are > 3-4 years post-vaccine.
- The Phase II study of SurVaxM in newly diagnosed glioblastoma is underway. 50% enrollment has been reached and an interim analysis has been performed. This study is continuing towards completion.

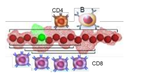
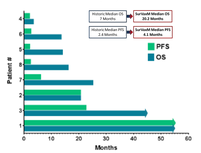
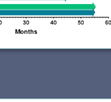


Figure 1. (above) SurVaxM structure with multiple CD8+ and CD4+ T cell epitopes highlighted. **(below)** Phase I clinical trial results for SurVaxM in recurrent glioma patients. Progression Free Survival (PFS) and Overall Survival (OS) are shown.



METHODS

- The clinical study (NCT01250470), from which blood samples were derived, was conducted in patients with survivin-positive malignant gliomas whose tumors had recurred or progressed following standard therapy. Completed results of this study are reported elsewhere.
- Patient serum samples (n=8) were collected and stored within 3 hours at -80°C. Normal (non-cancer) healthy, control individuals (n=3). Exosomes were isolated from thawed serum by differential ultracentrifugation. The supernatant was discarded and exosome pellets were re-suspended in sterile phosphate buffered saline (PBS). Exosome preparations were stored frozen at -70ºC.
- Using imaging flow cytometry, we performed direct quantitative measurements of circulating SVN+ exosomes in the serum of malignant glioma patients undergoing treatment with an anti-survivin vaccine (SurVaxM).
- Exosomes were stained with GFAP Alexa Fluor 488 antibody, CD9 PE antibody, and survivin DyLight 650 antibody for 30 minutes at 25ºC. Data were acquired on an ImageStreamX Mark II Imaging Flow Cytometer (AMNIS/Millipore, Billerica, MA). All readings were acquired at 60x magnification collected at low flow rate. Data analysis was performed using IDEAS software v6.1. Exosomes were gated upon CD9+ events versus side scatter combined with GFAP and survivin events. Exosomes were also confirmed positive for CD63 and CD81 tetraspanins (data not shown) and transmission electron microscopy for size (Figure 2D).
- Exosomes were evaluated at baseline (study entry) and an average of 9 weeks (8 – 10.3 weeks) following the first survivin vaccination out of a series of 4 vaccinations given over 6 weeks. In patients with late or no (patient #1) tumor progression, two of three individuals experienced tumor progression an average of 15 weeks after initial vaccine treatment. The remaining patient with recurrent glioblastoma has remained progression-free beyond 54 months (>4 years).



RESULTS

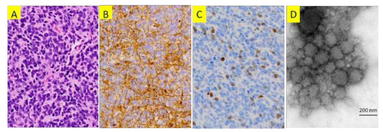


Figure2. Immunohistochemistry and exosome morphology. A) hematoxylin and eosin stain of recurrent glioblastoma from patient #1 and immunostain of sections from the same tumor for B) GFAP, and C) survivin. D) Electron microscopic image of exosomes isolated from patient serum at baseline. The image was captured at 50,000x (scale bar indicates 200nm).

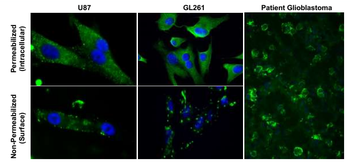


Figure3. Immunofluorescence staining of glioma cells. Representative images of surface and intracellular staining of survivin in human and murine cultured glioma cells as well as patient tumor specimen. Cell surface survivin may represent pre-release exosomes or other surface complexed survivin isoforms.

Patients were divided into 2 groups based on time to tumor progression following SurVaxM vaccination

Late Progression (LP) **Early Progression (EP)**

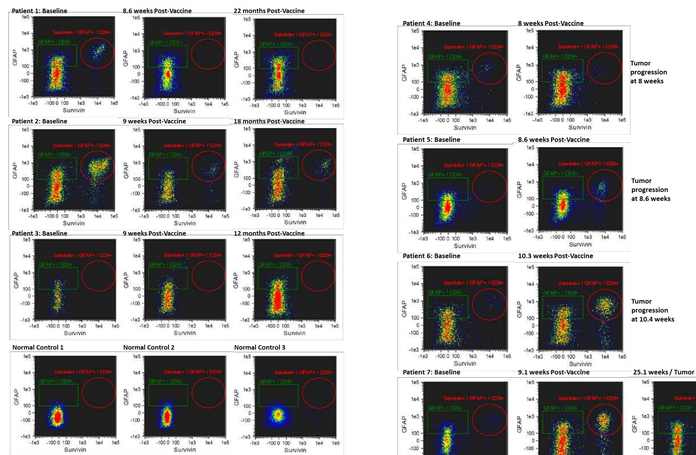


Figure 4. ImageStream flow cytometry of CD9+/GFAP+/SVN+ exosomes in patients who had late or no (12-48 months) tumor progression after initial dose of survivin vaccine. (Left column) CD9+/GFAP+/SVN+ exosomes at baseline (pre-vaccine); (Middle column) CD9+/GFAP+/SVN+ exosomes 8 weeks after receiving SurVaxM vaccine; (Right column) CD9+/GFAP+/SVN+ exosomes 12-22 months after receiving survivin vaccine. Bottom row demonstrates CD9+/GFAP+/SVN+ exosome events in 3 healthy non-cancer control individuals.

	GFAP+ (% Total CD9+ Exosomes)					Survivin+ (% Total CD9+ Exosomes)					Survivin+/GFAP+ (% Total CD9+ Exosomes)				
Patient	Base	8 Wks	Δ%	Ext	Δ%	Base	8 Wks	Δ%	Ext	Δ%	Base	8 Wks	Δ%	Ext	Δ%
1	23.7	25.9	9%	33.1	40%	12.1	0.4	-97%	0.9	-93%	10.4	0.3	-98%	0.6	-94%
2	18.2	16.9	-7%	26.3	45%	35.0	17.1	-51%	11.4	-67%	27.2	11.5	-58%	9.0	-67%
3	22.9	20.5	-10%	18.7	-18%	0.8	1.2	53%	0.3	-63%	0.6	0.4	-31%	0.2	-65%
4	28.0	30.3	21%			5.1	2.6	-61%			3.2	1.2	-63%		
5	19.8	19.4	-2%			1.6	14.1	781%			0.6	9.7	1417%		
6	24.0	19.1	-23%			6.7	31.7	373%			4.4	21.6	393%		
7	21.0	17.8	-15%	16.0	-24%	6.3	27.5	340%	46.5	644%	4.4	19.8	349%	37.6	753%
8	27.1	26.5	-2%			5.3	2.3	-57%			3.8	1.4	-63%		
C1	2.9					0.1					0.0				
C2	3.2					0.0					0.0				
C3	2.7					1.2					0.1				

Table 1. Patient exosome values as percent of total CD9+ exosomes and percent change (Δ%) from baseline (study entry).

Patient	Age	Sex	Tumor type[1]	IDH-1 (R132H)	Disease Burden[2]	Survivin Positive (%)	Doses of Vaccine	PFS (weeks)
1	38	M	G	-	-	22%	4 +14	230
2	58	M	G	+	+	1%	4 + 2	88.0
3	57	M	A	-	+	2%	4 +1	96.4
4	45	F	G	+	+++	10%	4	8.0
5	52	F	G	-	+	7%	4	8.6
6	48	F	G	-	++	8%	4	10.4
7	61	M	G	-	+	15%	4	25.1
8	54	M	G	-	+	4%	4	9.4

Table 2. Patient characteristics. [1]G, glioblastoma; A, anaplastic glioma; [2]Disease burden on MRI at first dosing: (-) no measureable contrast enhancement (C.E.); (+), measureable C.E. < 1 cm^3; (++), >1cm^3 but ≤ 5 cm^3 C.E.; (+++), >5 cm^3 C.E.; PFS: progression-free survival.

Figure 5. ImageStream flow cytometry of CD9+/GFAP+/SVN+ exosomes in patients who progressed early (2-5 months) following initial vaccination. Five patients experienced early tumor progression with a mean of 12 weeks (8 - 25.1 weeks) from study entry. Left column) CD9+/GFAP+/SVN+ exosomes at baseline (prior to first dose of vaccine at study entry), (Right column) CD9+/GFAP+/SVN+ exosomes 8-24 weeks after first dose of vaccine at early tumor progression.

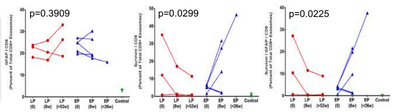


Figure 6. Comparison of late progression (LP) and early progression (EP) exosome levels over time.

Labelled exosome accumulation on surface of glioma cells

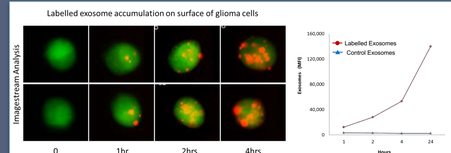


Figure 7. (above, left) ImageStream analysis of GL261 (murine glioma) derived exosomes labelled with CellIVue maroon stain. Isolated glioma exosomes accumulate over time to glioma cells growing in culture. **(above, right)** Graphical representation of accumulated exosomes.

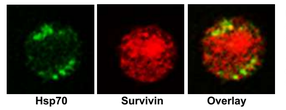


Figure 8. (left) ImageStream analysis of GI261Tumor cells showing surface and intracellular localized survivin protein. A potential mechanism of surface complex with heat shock protein (Hsp70) is shown.

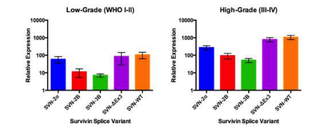
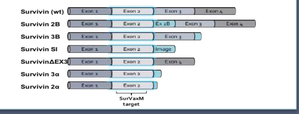


Figure 9. (above) Gliomas (both high and low grade) express several survivin splice variant isoforms as shown above (qPCR analysis). The anti-SurVaxM/survivin antibody developed by MimiVax is capable of binding to all known survivin variants **(below)** increasing its therapeutic target accessibility and diagnostic sensitivity.



CONCLUSIONS & FUTURE DIRECTIONS

- Serum from glioma patients contained abundant CD9+ exosomes with both survivin and glial fibrillary acidic protein (GFAP) on their surface. Survivin and GFAP were evaluated both independently and together as possible tumor markers on CD9+ exosomes. Patients with longer time to tumor progression (TTP) generally exhibited a decrease in circulating CD9+/SVN+ and CD9+/GFAP+/SVN+ exosomes following survivin vaccination; whereas, those with early tumor progression had either a transient decrease, or an increase in specific exosomes, despite anti-survivin therapy.
- Non-cancer healthy controls had very few CD9+/GFAP+/SVN+ exosomes in their sera, although they did have CD9+/GFAP+ exosomes. This study demonstrates that malignant glioma patients have CD9+/GFAP+/SVN+ and CD9+/SVN+ exosomes in their circulation and that reductions in their numbers following anti-survivin therapy may be associated with better disease control; whereas, increasing numbers of these specific exosomes may reflect tumor progression.
- Survivin has generally been regarded as an intracellular protein exhibiting a variety of molecular interactions. Although the intracellular functions of survivin are relatively well studied, an understanding of its role in the extracellular matrix is still in a nascent stage. Several alternately spliced survivin isoforms are expressed, reflective of a complex multi-functional mechanism, which may be related to its surface/exosomal expression pattern.
- One patient (#7), who was the latest of the patients with early progression, experienced a detectable increase in CD9+/GFAP+/SVN+ exosomes 9 weeks following initial vaccination (Figure 3), which was 16 weeks prior to the detection of tumor progression on brain MRI scanning. This patient was clinically asymptomatic at the time of that scan.
- Currently, the best way to monitor patients with malignant gliomas who are undergoing treatment is with brain MRI scans. The combined detection of CD9, survivin and GFAP markers on the surface of serum-derived exosomes from glioma patients by imaging flow cytometry may provide another tool for monitoring tumor status in patients receiving survivin-based immunotherapies.
- It might also be possible to use this, or related methods, as a biomarker to monitor glioma progression and response to other forms of anticancer therapy. The identification of additional tumor markers may enable monitoring of other tumor types, or of distinct populations within them, via sampling of either blood, urine or cerebrospinal fluid. Before utility as a biomarker can be concluded, it will be essential to assess the independent effects of surgery, radiation therapy and chemotherapy on circulating survivin-containing exosomes in larger studies.

REFERENCES & DISCLOSURES

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Disclosure Statements:

1. *MimiVax is a spin-off company of RPCI formed to develop and commercialize SurVaxM.*
2. *Michael Ciesielski & Robert Fenstermaker are co-founders and equity shareholders of MimiVax, LLC.*