Evaluating Differences in the Proteome Within Extracellular Vesicles Between Pre- Malignant and Malignant Plasma Cell Disorders

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Abstract

**Background:** Precursor plasma cell disorders such as monoclonal gammopathy of undetermined significance (MGUS) always precede the development of active malignancies such as multiple myeloma (MM). There is a need for novel biomarkers to identify those patients with such precursor plasma cell disorders who rapidly progress to MM. Plasma-derived extracellular vesicles (EVs) may serve as a reservoir of potential biomarkers that can shed light on the pathogenesis and disease biology in MM.

**Methods:** This study isolated small EVs (SEVs) and large EVs (LEVs) from the platelet-poor peripheral blood plasma of MGUS (n=9) and MM (n=12) patients using the size exclusion chromatography (SEC)-based method and evaluated their proteome using a label-free proteomics workflow.

**Results:** In total, 2,055 proteins were identified in SEVs, while 2,794 proteins were identified in LEVs. The transferrin receptor (TFRC or CD71) protein was upregulated in both populations of EVs derived from MM patients compared to MGUS patients and was of prognostic significance. Similarly, three isoforms of serum amyloid A (SAA) protein, SAA1, SAA2, and SAA4, were also highly upregulated in SEVs within MM patients relative to MGUS patients. Finally, CD40 expression was also higher within the LEVs derived from MM patients than MGUS patients.

**Conclusions:** This study demonstrates the feasibility of successfully isolating both SEVs and LEVs from the peripheral blood of patients with plasma cell disorders and quantifying protein biomarkers within these EVs that could be of prognostic and diagnostic interest.

INTRODUCTION

Multiple myeloma (MM) is a devastating clonal plasma cell disorder preceded by a pre-malignant condition termed monoclonal gammopathy of undetermined significance (MGUS) or a more advanced pre-malignant stage termed smoldering multiple myeloma (SMM). Both MGUS and SMM require continuous clinical monitoring as current methods cannot distinguish between the presence of premalignant (MGUS or SMM) or malignant clonal plasma cells (MM). As a result, some MGUS and SMM patients progress to MM in between their scheduled clinical monitoring visits, highlighting the need for novel biomarkers to identify rapid progression.

Progressive alterations in the BM microenvironment via the exchange of soluble factors, including cytokines and growth factors, are associated with the progression of MGUS to MM. Extracellular vesicles (EVs), including exosomes (small extracellular vesicles – SEVs, about 30–120 nm in diameter) and microvesicles (large extracellular vesicles – LEVs, about 150–1000 nm in diameter), have received attention for facilitating intercellular and interorgan communication and potentially involved in facilitating disease progression by mediating cross-talk between tumor cells and the surrounding microenvironment. Thus, plasma-derived EVs may serve as a reservoir of potential biomarkers that can shed light on the pathogenesis and disease biology in MM. Until recently, technical factors, including abundant plasma protein contamination and low EV recovery, have precluded useful mass spectrometry-
METHODS

MGUS and MM patient recruitment

Approval for this study was obtained from the Mayo Clinic IRB in accordance with the federal regulations and the principles of the Declaration of Helsinki. Consecutive patients with MM and MGUS were prospectively recruited in the clinic if they met the diagnostic criteria of International Myeloma Working Group (IMWG). A peripheral blood sample was collected from each of the participating patients.

Blood collection and plasma preparation

Whole blood was drawn into EDTA tubes and placed on the benchtop for 15 minutes. After 15 minutes, each tube was centrifuged at 2,000g for 10 min at room temperature (RT) to pellet red blood cells. The upper plasma fraction was then collected, transferred to a new sterile tube, and centrifuged again at RT for 10 min at 2000g. Platelet poor plasma was isolated by carefully transferring the top layer of plasma to a new tube and then frozen at -80°C in 1ml aliquots.

Isolation of plasma extracellular vesicles by size exclusion chromatography (SEC)

Extracellular vesicles were isolated and analyzed as previously described. Briefly, frozen platelet poor plasma was thawed on ice and centrifuged at 2,000xg for 10 min to pellet debris. Following centrifugation, the plasma was transferred to a new tube and placed on ice until analysis. Extracellular vesicles were isolated using a qEV 2.0 size exclusion chromatography column (Izon Science, Christchurch, New Zealand) following the manufacturer’s protocol. Briefly, SEC columns were equilibrated to room temperature and flushed with 90 ml of phosphate buffered saline (PBS) before use. Platelet poor plasma was transferred into the sample loading reservoir of the SEC column. The column cap was then removed, and the plasma sample was allowed to completely enter the column, at which point the sample loading reservoir was filled with PBS. The first 13 ml of PBS fraction was discarded and the following 8 ml of EV containing fraction were collected for each sample. The SEC columns were then flushed with 90 ml of PBS before the addition of the next sample. The 8 ml of EV containing fraction for each sample was then pelleted by ultracentrifugation at 20,000 x g to pellet LEVs. The supernatant was then transferred to a new ultracentrifugation tube and SEVs were pelleted at 100,000xg for 1.5 hours at 4°C. Following ultracentrifugation, the supernatant was discarded, and all residual PBS was removed before tryptic digestion.

Protein digestion and label free mass spectrometry analysis

Based profiling studies of plasma-derived EVs. Thus, we used a rigorously evaluated approach to avoid any plasma contamination that involved size exclusion chromatography (SEC) – followed by ultracentrifugation to isolate EVs coupled with a label-free proteomics workflow to compare protein composition and abundance of SEVs and LEVs isolated from individuals with MGUS and MM.
EV pellets were precipitated with the addition of 50µl of methanol. The methanol was then evaporated in a SpeedVac (Thermo Scientific, Waltham MA) and protein was reconstituted with 50µl of 50mM Tris pH 8.2 containing 0.002% zwittergent Z3-16 (EMD Millipore, Burlington MA) and subsequently heated at 95°C for 10 min. 5ul of 110mM DTT (5mM final) was then added to reduce disulfide bonds and the samples were heated at 70°C for 10min. Samples were then equilibrated back to room temperature over 20 minutes before alkylation with 5µl 120mM IAA (10mM final) and incubated for 30min protected from light. Following alkylation, 0.1µg of Trypsin LysC mix was added to the samples, the samples were vortexed, and then incubated at 37°C for 16hr. Finally, the digestion was terminated by adding 5µl of 5.25% TFA.

**LC-MS conditions**

Digested samples (15µl) were loaded onto a 0.33µl OptiPak trap column (Optimize Technologies) packed with Halo C18 peptide ES stationary phase. The trap was then washed with an aqueous loading buffer composed of 0.2% FA and 0.05% TFA for 4 minutes at 10µl/min. After the wash, the 10-port valve was switched, and peptides were flushed off the trap onto a 25cm x 75µm PicoFrit (New Objective) analytical column (packed with Waters BEH 1.7µm stationary phase) using a Dionex UltiMate 3000 RSLC liquid chromatography (LC) system (Thermo Scientific). The analytical gradient for peptide separation began at 2% mobile phase B (MPB) and 98% mobile phase A (MPA) for 4 min, MPB was then increased to 30% over 40min, raised to 40% MPB over 20min, increased to 95% over 10min, held for 2min, returned to 2% B in one minute and equilibrated for 15min. MPA was composed of 98:2 (water/acetonitrile) with 0.2% FA and MPB was composed of 80:10:10 (acetonitrile/isopropyl alcohol/water) with 0.2% FA. Analysis of the eluting peptides was performed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) operated in data dependent mode. Survey scans were acquired from 300-1400m/z with 120,000 resolving power and an AGC of 4e5 and maximum fill time of 50ms. MS/MS scans of selected precursor ions were performed for a maximum of 3s or until the list was exhausted and dynamic exclusion was set to 45s. Quadrupole isolation for MS/MS scans was set at 0.7 m/z followed by fragmentation in the ion trap with “Rapid” scan speed and an ion target value of 5e4 and maximum injection time of 22ms using normalized collision energy of 28% from 200–1200 m/z. The monoisotopic precursor selection was set to peptide and charge states of 1, greater than 5, or unknown were excluded.

**Data Analysis**

MS raw files were processed in MaxQuant software version 1.6.7.0. Peptides were searched using the Andromeda search engine against the human Uniprot FASTA database downloaded July 24th, 2019. Cysteine carbamidomethylation was set as a fixed modification and N-terminal acetylation and methionine oxidation were set as variable modifications. Searches were performed requiring a minimum of one unique peptide and a false discovery rate of 1% for both peptides and proteins using a target-decoy approach. Peptide length was set to at least 7 amino acids long and MS2 match tolerance was set to 0.5Da. Enzyme specificity was set to trypsin and a maximum of 2 missed cleavages were allowed. Protein data was extracted from the “proteinGroups.txt” file and differential quantitation was carried out using a generalized linear modeling method as previously described.7
Gene Ontology Over Representation Analysis

Gene ontology over representation analysis was performed using WebGestalt software. Redundancy of enriched gene sets was then reduced with the affinity propagation method using the R package apcluster and gene sets passing FDR (Benjamini-Hochberg procedure ≤ 0.05) were reported.

Survival statistical analysis

We examined the association between TFRC overexpression classification and overall survival (OS) defined by the time interval from the date of sample collection to the time of death or last contact using Kaplan-Meier curves and compared using the log-rank method. TFRC classification for patients was based on the dichotomy at their median TFRC protein expression level in LEVs to produce “low” (i.e., bottom 50% percentile) and “high” (i.e., top 50% percentile) classifiers. The publically available Depmap Portal (https://depmap.org/portal/) was queried to infer CRISPR gene knockout fitness effects of TFRC in various human MM cell lines.

RESULTS

Both SEVs and LEVs are able to be isolated for proteome assessment in patients with plasma cell disorders

We isolated SEVs and LEVs from the platelet-poor plasma of MGUS (n = 9) and MM (n = 12) patients (Supplementary Table) using the SEC-based method. We evaluated their proteome as described in the Methods section. In total, 2,055 proteins were identified in SEVs, while 2,794 proteins were identified in LEVs (Fig. 1A). While many proteins were identified commonly between the two populations of EVs, some were unique to each subtype (LEVs = 1171, SEVs = 432), indicating that these are two distinct populations of vesicles (Fig. 1B). Within the SEVs, a differential comparison of protein abundance between MGUS and MM patients identified six upregulated and two downregulated proteins in MM relative to the MGUS group (Fig. 1C). Similarly, a differential comparison within the LEVs identified three upregulated proteins in the MM group relative to the MGUS group (Fig. 1D).

Proteins within EVs can differentiate between MM from MGUS

The transferrin receptor (TFRC or CD71) protein was upregulated in both populations of EVs derived from MM patients compared to MGUS patients. When patients with MM in this study were dichotomized into TFRC “low” and “high” groups based on the level of TFRC protein in their LEVs, the median OS was significantly shorter among MM patients in the TFRC “high” (n = 5) compared to “low” (n = 7) group (21 months vs. not reached, p = 0.024) (Fig. 1E). Finally, when utilizing the publically available Depmap Portal (https://depmap.org/portal/) to infer CRISPR gene knockout fitness effects, most human MM cell lines had a 23Q2 Chronos score less than −1.0 for the TFRC gene representing moderate to strong killing of those cell lines when compared to several other non-MM cell lines (Fig. 1F). Together, these data suggest
the vital significance of TFRC to MM cells that needs to be investigated further. Similarly, three isoforms of serum amyloid A (SAA) protein, SAA1, SAA2, and SAA4, were also highly upregulated in SEVs within MM patients relative to MGUS patients (Fig. 1C). Finally, CD40 expression was also higher within the LEVs derived from MM patients relative to MGUS patients. While both populations of EVs could arise from a number of different cell types, we hypothesize that the TFRC and CD40 on malignant PCs may be shed in both SEVs and LEVs for TFRC and LEVs only for CD40 (Fig. 1G). Thus, increased levels of TFRC and CD40 in the different EVs may reflect a different disease biology between premalignant and malignant PCs or their microenvironments. Together, our data demonstrates the successful isolation of both SEVs and LEVs from the peripheral blood plasma of patients. Furthermore, extraction and identification of contained proteins shed light on the potential utilization of EVs as crucial reservoirs of proteins which could be of prognostic and diagnostic interests to understand disease etiology. However, future studies which systematically investigate and confirm these hypotheses are required.

**Proteins within EVs can differentiate between MM with standard and high risk cytogenetics**

When associations between cytogenetic abnormalities by FISH (standard risk vs. high risk) and EV protein abundance was performed, within the SEVs, a total of 73 proteins were downregulated in high cytogenetic risk relative to standard risk (Supplementary Fig. 1A). Similarly, 13 proteins were downregulated in high-risk relative to standard risk within the LEVs, while only three proteins were upregulated (Supplementary Fig. 1B). Cytogenetic risk groups also separate into distinct clusters (Supplementary Fig. 1C, D) despite displaying moderate inter-subject variability (Supplementary Fig. 1E, F). Interestingly, gene ontology over-representation analysis of the proteins downregulated in SEVs identified several enriched biological processes, including positive regulation of immune response to tumor cells, leukocyte degranulation, chemotaxis and regulation of immune system process (Supplementary Fig. 1G), suggesting that a more functional immune response may be associated with MM patients with standard compared to high cytogenetic risk. Interestingly, within the LEVs, CD40 was exclusively identified in subjects with standard cytogenetic risk and lost in subjects with high cytogenetic risk (Supplementary Fig. 1H), suggesting that adaptive immunity may be compromised in high-risk subjects.

**DISCUSSION**

This is one of the first studies to our knowledge to demonstrate the successful isolation of both SEVs and LEVs from the peripheral blood plasma of patients with precursor and malignant plasma cell disorders. Furthermore, this study demonstrates that both SEVs and LEVs contain proteins that could be of prognostic and diagnostic value in the clinic. The finding of TFRC protein levels within both SEVs and LEVs being able to differentiate between MGUS and MM is particularly interesting. TFRC is a type II transmembrane glycoprotein cell surface receptor that facilitates cellular iron uptake and is encoded by the gene *TFRC*. Iron bound by transferrin in the blood is then taken up by cells through its interaction with the TFRC receptor, internalized via receptor-mediated endocytosis and trafficked to low pH endosomes
where iron is released and TFRC rapidly recycled back to the cell membrane. This observation is expected given that proliferating MM cells, when compared to non-malignant cells, depend on an increased influx of iron, an important co-factor for multiple cellular processes, including central carbon metabolism, mitochondrial respiration, and DNA synthesis.\(^8\) The proto-oncogene c-MYC, which encodes the transcription factor c-MYC and has been associated with the pathogenesis of MM from MGUS, directly regulates the expression of TFRC via binding to a conserved E box binding site in intron 1 of TFRC.\(^9\) Furthermore, TFRC also mediates NF-κB signaling in malignant cells through the interaction with the NF-κB kinase (IKK) complex inhibitor.\(^10\) Thus, it is highly possible that the axis of c-Myc–TFRC–NF-κB could be a key mechanism of disease progression. A study utilizing the VK*MYC murine model of MM found high levels of TFRC or CD71 expression on plasma cells (PCs) and iron accumulation in BM macrophages suggesting that iron competition within the BM microenvironment is present.\(^11\)

Other interesting findings include the three isoforms of serum amyloid A (SAA) protein, SAA1, SAA2, and SAA4, that were observed to be highly upregulated in SEVs within MM patients relative to MGUS patients. SAA acute phase proteins are known to be involved in the inflammatory process and primarily have clinical utility as a marker of inflammation. They are expressed in the liver and are regulated by pro-inflammatory cytokines; however, their expression has also been observed in several primary and metastatic cancer cell lines.\(^12\) Recently, SEV-derived SAA levels have also been found to be increased in pediatric patients with anaplastic lymphoma kinase (ALK)-positive anaplastic large-cell lymphoma (ALCL) relative to healthy controls.\(^13\) Therefore, increased SAA levels in SEVs may also reflect the increased disease burden of malignant PCs and/or an inflammatory state within the bone marrow.

Finally, it was interesting to observe that CD40 expression was also higher within the LEVs derived from MM patients relative to MGUS patients. CD40 is a cell surface transmembrane glycoprotein in the tumor necrosis factor (TNF) superfamily that serves as a co-stimulatory receptor and is expressed on B cells and professional antigen-presenting cells, which is required for their activation. It is also highly expressed on the surface of tumor cells in the majority of MM patients. Binding between CD40 and CD40L induces cell proliferation and migration via PI3K and NF-κB signaling pathways, which are thought to promote tumor growth through autocrine IL-6 stimulation and induction of VEGF.\(^14\) Despite all these findings of interest whereby several proteins of biological interest were found to be differentially present in the SEVs and LEVs obtained from the peripheral blood plasma of patients with MGUS and MM, future studies investigating their clinical and diagnostic significance are warranted.

In conclusion, the results of this study demonstrate that plasma EVs may be a useful reservoir of proteins associated with the pathogenesis of MM that can serve as biomarkers. These findings may have diagnostic importance in identifying and monitoring those patients with pre-malignant disorders such as MGUS and SMM destined to progress to MM rapidly. Given the feasibility and success of this pilot study, future studies validating these findings are warranted.

**Declarations**
ACKNOWLEDGEMENTS

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DATA AVAILABILITY

Data is available upon email request to the corresponding authors.

AUTHOR CONTRIBUTIONS

P.M.V. and W.I.G. conceived the study design and drafted the manuscripts. P.M.V. did all the data analyses. All other co-authors reviewed and approved the final version of the paper.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

References


Figures

Figure 1
Differential comparison of protein composition and abundance between subjects with MM and MGUS in SEVs and LEVs. (A) Bar chart displaying the total number of proteins identified in SEVs and LEVs. (B) Venn diagram displaying the distribution of identified proteins in LEVs and SEVs. Volcano plot illustrating differentially expressed proteins in MM relative to MGUS in (C) SEVs and (D) LEVs (positive fold change indicates increased abundance in MM). (E) Kaplan-Meier curve demonstrating the difference in overall survival among patients with MM based on the “low” vs. “high” levels of TFRC in their LEVs. (F) 23Q2 Chronos score plot demonstrating the CRISPR gene knockout fitness effects of TFRC in various cancer cell lines with relationship to 20 human MM cell lines. (G) Graphical illustration of the suspected release of TFRC in SEVs and LEVs and CD40 in LEVs.

Supplementary Files

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- Figure1S.tif
- TablesupplementaryRevised.docx