Insights into the Proteome of Gastrointestinal Stromal Tumors-Derived Exosomes Reveals New Potential Diagnostic Biomarkers

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Developing tumors continuously release nano-sized vesicles that represent circulating “fingerprints” of the tumor’s identity. In gastrointestinal stromal tumor (GIST), we have previously reported that these tumors release “oncosomes” carrying the constitutively activated tyrosine kinase (TK) receptor KIT. Despite the clinical utility of TK inhibitors, such as imatinib mesylate (IM), recurrence and metastasis are clinical problems that urge the need to identify new tumor-derived molecules. To this aim, we performed the first high quality proteomic study of GIST-derived exosomes (GDEs) and identified 1,060 proteins composing the core GDE proteome (cGDEp). The cGDEp was enriched in diagnostic markers (e.g. KIT, CD34, ANO1, PROM1, PRKCQ, and ENG), as well as proteins encoded by genes previously reported expressed in GIST (e.g. DPP4, FHL1, CDH11, and KCTD12). Many of these proteins were validated using cell lines, patient-derived KIT+ exosomes, and GIST tissues. We further show that in vitro and in vivo-derived GDE carry proteins associated with IM response, such as Sprouty homolog 4 (SPRY4), surfeit 4 (SURF4), ALIX, and the cGMP-dependent 3’,5’-cyclic phosphodiesterase 2A (PDE2A). Additionally, we report that the total exosome levels and exosome-associated KIT and SPRY4 protein levels have therapeutic values. In fact, molecular characterization of in vivo-derived KIT+ exosomes indicate significant sorting of phosphotyrosine KIT+, total KIT, and SPRY4 after IM-treatment of metastatic patients as compared with the pre-IM levels. Our data suggest that analysis of circulating exosomes levels and molecular markers of IM response in GIST patients with primary and metastatic disease is suitable to develop liquid based biopsies for the diagnosis, prognosis, and monitoring of response to treatment of these tumors. In summary, these findings provide the first insight into the proteome of GIST-derived oncosomes and offers a unique opportunity to further understand their oncogenic elements which contribute to tumorigenesis and drug resistance. Data are available via ProteomeXchange with identifier PXD007997. Molecular & Cellular Proteomics 17: 10.1074/mcp.RA117.000267, 495–515, 2018.

Tumor development and progression is highly dependent on the productive and bi-directional exchange of information between tumor cells and host stromal cells (1). These interactions play critical roles at all stages of tumor development and actively establish a tumor supportive microenvironment that cellularly and molecularly “co-evolves” with tumor progression (2). During this process, tumor cells shape and educate the surrounding host stromal cells using an array of contact dependent and independent mechanisms to shape and educate surrounding host stromal cells (3).

One of these mechanisms includes the secretion of small bioactive nanovesicles (30–180 nm), known as exosomes (4, 5). Exosomes are produced by most healthy cells types under homeostatic conditions or after activation (6). In contrast, in most neoplastic cells the rate of exosome release is significantly increased and occurs constitutively (7, 8). Although the exact mechanism(s) responsible for the formation and release of exosomes is still in its preliminary stages of investigation, these vesicles are known to arise from the limiting membrane of multivesicular bodies (MVBs)1 by a mechanism of inward...
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budding, leading to the formation of intraluminal vesicles (ILVs) (9–12). The fusion of MVBs with the plasma membrane releases exosomes to the extracellular space via exocytosis (13, 14). Tumor-derived exosomes (TDEs), also called “oncosomes,” carry a specific cargo composed of an array of defined lipids, mRNA, microRNA, and biologically active proteins (6). Recent high throughput proteomic studies of exosomes isolated from various cell types and bodily fluids have catalogued thousands of vesicular proteins (15, 16). Collectively, these studies demonstrated the presence in exosomes of a common set of components and tumor-type specific cargos (17). As shown by us and others, common vesicular proteins essentially included proteins involved in vesicle structure, cargo sorting, biogenesis, and intracellular trafficking; whereas tumor-type specific cargos were often linked to the pathophysiological functions of these molecules in each tumor type (18, 19). Therefore, oncosomes carry tissue and disease specific “fingerprints” which systemically “mirrors” ongoing cellular activities and may prove to be a source of diagnostic and predictive biomarkers (19, 20).

It becomes apparent that inclusion or exclusion of cellular proteins into exosomes is not based on its cellular abundance, but rather on the controlled sorting of the cargo during biogenesis. This unique composition allows oncosomes to operate as signaling devices for short- and long-range delivery of information to specific target cells, and therefore locally and systemically modulate the primary and secondary tumor microenvironments (21, 22). Locally, oncosomes modulate the dynamic of the stromal, endothelial, inflammatory and immune cells’ functions by reprogramming these cells toward a pro-tumorigenic phenotype (6). Increasing evidence suggests that oncosomes have a vast repertoire of activities and may act as master regulators of many interactions networks which collectively influence events that drive tumorigenesis (4). These vesicles have been shown to regulate cellular processes such as inflammation, angiogenesis, extracellular matrix remodeling, tumor growth, and suppression of anti-tumor innate and adaptive immune responses (23–26). Longitudinal analysis of clinical samples reported qualitative and quantitative changes in the exosomal cargo, which was also correlated with tumor progression (22). Most importantly, the composition of the exosomal cargo reflects a unique sub-proteome of the cell of origin and identifies a distinct molecular repertoire that contains functional fingerprints that ultimately defines the various functions played by specific subpopulations of exosomes (5, 27).

Recently, the similarities between exosome biogenesis and uptake pathways with viruses and particularly their natural enrichment in tumor specific antigens further highlighted their potential use as “natural” tumor vaccines (28). In addition, the relative stability and accumulation of oncosomes in bodily fluids of cancer patients, including sera, plasma, malignant ascites, and pleural fluids (24, 29, 30); confers them an exceptional source of biomarkers for detecting and monitoring cancer. Based on the concept that exosomes contain molecular signatures of their cell of origin, large-scale proteomic studies using purified tumor-derived exosomes that aimed to systematic map their cargos are being approached with the hope that these studies may identify specific biomarkers of tumor occurrence and/or metastasis (15).

Our previous studies have shown that gastrointestinal stromal tumors (GISTs) release “oncosomes” carrying the oncogenic tyrosine kinase receptor (RTK) c-KIT, which is important for GIST invasion and disease progression (27, 31). GIST is a rare but deadly mesenchymal malignancy affecting the gastrointestinal tract (GI) (32, 33). A gain-of-function mutation in the KIT (85%) and to a lesser extend in PDGFRA (< 3%) (34–36) constitutes an important causative event of this disease (37–42). GIST is frequently asymptomatic and in many cases (50–60%) are discovered at a locally advanced stage or with distant metastasis (40). With the rapid advances in understanding of the genomic aberration driving GIST and with the successful clinical use of tyrosine kinase inhibitors (TKI), like imatinib mesylate (IM; Gleevec®) and sunitinib malate (Sutent) for the treatment of patients with inoperable or metastatic GIST; GIST has become the quintessential model for molecular targeted therapy (36, 43). However, despite the initial success of IM in the advanced setting with a clinical benefit of 80%, disease progression remains a perplexing problem, as the vast majority of patients eventually developing resistance. Importantly, once a GIST becomes metastatic, the median disease-specific survival of patients is only ~19 months with second- and third-line therapies (44, 45). Therefore, there is an urgent need for additional therapeutic strategies and/or discovery of therapeutic markers that will significantly enhance follow-up and predict response to therapy for these tumors. One possible source of biomarkers, as reported by us ad others, is the presence in the systemic circulation of GIST patients of modulatory KIT positive nanovesicles (31).

To date, most proteomic studies of exosomes have primarily focused on exosomes derived from carcinomas (16). The purpose of this study was to perform a comprehensive analysis and characterization of the vesicular proteome of highly purified GIST-derived exosomes (GDEs) and further establish

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the first prototypical proteome signature of these vesicles. Using quantitative proteomic profiling, we report insights into the selective oncopgenic cargo of GDEs, demonstrating expected enrichment of common exosome-related markers, as well as proteins involved in tumor progression, angiogenesis, kinase signaling pathways, and immune regulatory components. Importantly, our findings also provide clues to understand the roles of these vesicles in tumorigenesis, drug response, and provide a comprehensive protein signature of GIST that can be used as a resource for the discovery of new diagnostic biomarkers and therapeutic targets.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—Myometrial samples were obtained from premenopausal women undergoing hysterectomy (3). The human GIST-T1 cells were previously established from a patient with metastatic imatinib-naive GIST and display an imatinib-sensitive KIT mutation in exon 11 (V560-Y579del) (46). The imatinib-sensitive GIST882 cells were derived from a primary, imatinib-naive GIST, and exhibit a homozygous missense mutation in KIT exon 13 (K642E) (47). The KIT mutation status of the cell lines was confirmed using the TruSeq Amplicon - Cancer Panel (TSACP) from Illumina. These cells have been extensively characterized previously and are representative of the behavior, genotype, and phenotype of GIST (31). The cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen) supplemented with 100 units/ml penicillin-streptomycin and 10% exosomes-depleted FBS and maintained at 37 °C with 5% CO2. GIST cells were maintained at 80% confluence for exosome collection and the cultured were confirmed negative for mycoplasma contamination by screening biweekly using Mycoalert (Lonza).

**Patient Plasma Collection**—Plasma samples derived from healthy donors and untreated GIST patients with primary or metastatic disease were used in this study. All samples were obtained from the University of Kansas Cancer Center’s CCGS Biospecimen Repository Core Facility after approval from the internal Human Subjects Committee. In addition, paired plasma samples obtained from primary and metastatic GIST patients prior or after IM therapy, as well as tumor specimens and associated clinicopathological information were obtained from the University of Iowa. All GISTs included in this study were characterized by using classical histopathological and immunohistochemical approaches with anti-KIT, SMA, CD34, Desmin, S100, and TMEM16A (DOG-1) antibodies.

**Isolation and Purification of Exosomes**—Conditioned media derived from three different passages of in vitro cultured GIST-T1 (passages: 20, 23, 26) and GIST882 cells (passages: 18, 21, 25) was subjected to serial centrifugation to remove cells (400 x g for 10 min) and cellular debris (3000g for 15 min, followed by filtration through a 0.2 μm filter). In addition, cell free CM was subjected to centrifugation at 10,000 x g for 30 min at 4 °C to further remove cellular debris. The obtained cell free supernatants were used for downstream exosomes purification, using methodologies we have previously published (48–50), and included a combination of filtration, gel filtration, sucrose gradient and ultracentrifugation. Briefly, the CM-derived from each preparation (~200 ml) was concentrated by cell stir membrane filtration, and applied to a Sepharose 2B column equilibrated with PBS. Thirty fractions were collected and protein content monitored by absorbance at 280 nm. The void volume peak material (fractions 8 to 13) was collected, combined, and centrifuged at 100,000 x g for 1 h. The resulting vesicle pellets were then washed twice, and re-suspended in PBS. These “preisolated” vesicular pellets were further purified by a linear sucrose gradient followed by a final ultracentrifugation step. Briefly, pelleted (100 μg) TEX (GIST-T1 cell-derived exosomes) or 882 Exo (GIST882 cell-derived exosomes) were layered on top of an established continuous sucrose gradient as previously described (19). Eighteen fractions were collected from the top of each tube, washed by ultracentrifugation and analyzed by immunoblotting.

**Transmission Electron Microscopy**—Purified TEX and 882 Exo pellets or 1° SMC and GIST cells lines, were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by a post fixation in 2% osmium tetroxide. Pellets were further dehydrated through series of graded ethanol baths (from 30% to 100%), and propylene oxide. The final resulting pellets were embedded into half propylene oxide/ half embed 812 resins (Electron Microscopy Sciences, Inc.) overnight. The next day, the pellets were cured in a 60 °C oven overnight. Ultrathin sections (70 to 80 nm thick) were cut on an ultramicrotome (Leica Ultracut R, Leica Microsystems GmbH), picked up on copper thin 300 mesh grids and stained for contrast with 4% uranyl acetate and Sato’s Lead stain. Specimens were examined with a transmission electron microscope (Jeol Electric 1400; Jeol Ltd), operated at 80 kV. Digital images were captured by a Mega View III digital camera (Soft Imaging System). To determine the number of ILVs per MVs, a total of thirty individual MVs derived from individual sections obtained from three independent experiments were counted using images taken at 20,000 x magnification. In addition, total cytoplasmic area for each analyzed cell was measured from images taken at lower magnification (4000 x) using ImageJ software. The number of MVs per cellular surface was determined by subtracting the cytoplasmic area from the total section area for each sample.

**SDS-PAGE and Western Blot Analysis**—Purified exosomal protein or total cellular lysates; were separated on 10% polyacrylamide gels (Bio-Rad Laboratories) in reducing (R) or nonreducing (NR) conditions. After electrophoretic transfer to nitrocellulose membrane (Millipore), the membranes were blocked with 5% milk in Tris buffer saline (TBS 1 x) containing 0.1% Tween for 1 h. Primary antibodies were incubated overnight at 4 °C with gentle agitation. After several washes, HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) was applied for an hour. The blots were washed and developed using an ECL chemiluminescence developing kit (Amersham Biosciences). Densitometric quantification of the gels or membrane was performed using Un-scan-it software (Silk Scientific). Results are representative of at least three similar experiments. Imperial Purple protein stain (Pierce) of SDS-PAGE gels was performed for 2 h at room temperature under agitation.

**Particles Size and Concentration Analysis**—The concentration and size of the purified exosomes vesicles were analyzed by Nanoparticle Tracking analysis (NTA) or Tunable resistive pulse sensing (TRPS) using either a NanoSight LM10 instrument (NanoSight) or a qNano platform (Izon Science) respectively. NTA analysis was performed by applying a monochromatic 404 nm (blue) laser to 300 μl of pure or diluted exosomal preparation and dilution performed to be in the range of optimal detection of particles as recommended by the manufacturer’s) and measuring the Brownian movement of each particle. Video files of 30 to 60s duration with a frame rate of 25 frames/s were recorded, and analyzed using the NTA software version 2.3, as previously published by us (50). The mean, mode, and median vesicles size, as well as the concentration of particles in each preparation, was analyzed and corrected by the dilution factor when required. For tunable resistive pulse sensing (TRPS) measurements, the particles were run through an nNP150 (150 nm particles) nanopore. An aliquot of exosomes from each fraction or calibration particles was runs at a constant stretch and at two distinct pressure. Trans-membrane voltage was applied, and particles were detected in short pulses of the current (blockades). Sizes and concentrations of the particles were determined using the Ixon Control Suite (ICS v3.1) software.

**Flow Cytometric Analyses of Exosomes-Coated Beads**—GIST patients-derived exosomes (5 μg) or BSA (negative control) were incu-
bated with 4 μm aldehyde/sulfate latex beads (Invitrogen) overnight at 4 °C with agitation. The reaction was stopped by addition of 100 mM glycine, the beads were washed and incubated with anti-c-KIT (Cell Signaling) or isotype control for 30 min at 4 °C, washed, and analyzed on a FACS caliber (Becton Dickinson). At least 10,000 events were acquired from each sample and analyzed using CellQuest software (Becton Dickinson).

Magnetic Separation of KIT Positive Exosomes from Plasma of GIST Patients—Exosomes were isolated from 1 ml of archival plasma samples using similar methodologies as described above. As a final purification step, exosomes were further immuno-adsorbed to dynabeads M-450 (Invitrogen) coated with saturating amounts of monoclonal mouse anti-human KIT antibody (clone HKB-1) for 16 h at 4 °C. The dynabeads-KIT™exosome complexes were collected with the aid of a magnet (Dynal Biotech) and washed once with PBS. After washing, proteins were eluted from the beads and quantified by DC assay (Bio-Rad Laboratories).

Tryptic Digestion and LC-MS/MS Analysis—Exosomal proteins were diluted in 8 M urea in 0.1 M Tris-HCl pH 8.5 and followed by trypsin digestion according to the Filter-Aided Sample Preparation (FASP) method (51). Tryptic digests were trap cleaned using C18 PROTO™Ultra MicroSpin columns (Nest Group, Inc., Southborough, MA), lyophilized and re-dissolved into 2% acetonitrile/0.1% formic acid (UHPLC) system and an in-house ID fused silica tip packed with n-LC (Thermo) ultra-high-performance liquid chromatography (UHPLC) system and an in-house ID fused silica tip packed with Jupiter 5 μm C18 300Å material (Phenomenex, Torrance, CA). Following injection of the sample onto the column, the separation was accomplished with a 75-min linear gradient from 2% acetonitrile to 40% acetonitrile in 0.1% formic acid. The UHPLC was coupled to an LTQ-Velos-Orbitrap ELITE mass spectrometer (Thermo Fisher Scientific) and an ETD mass spectrometer. Five microliters (5 μl) of each sample were injected as part of an Elite sequence with two blanks run among each sample. A 4th Order Double Play with ETD Decision Tree method was created in Xcalibur v2.2 (54). Scan event one of the method obtained an FTMS MS1 scan (normal mass range; 60,000 resolutions, full scan type, positive polarity, profile data type) for the range 300–2000 m/z. Scan event two obtained ITMS MS2 scans (normal mass range, rapid scan rate, centroid data type) on up to ten peaks that had a minimum signal threshold of 20,000 counts from scan event one. A decision tree was used to determine whether CID or ETD activation was used. An ETD scan was triggered if any of the following held: an ion had charge state 3 and m/z less than 650, an ion had charge state 4 and m/z less than 900, an ion had charge state greater than 9; a CID scan was triggered in all other cases. The lock mass option was enabled (0% lock mass abundance) using the 371.101236 m/z poly-siloxane peak as an internal calibra. 

Data Analysis with Proteome Discoverer v1.3.0.339 and Scaffold v3.6.5—Data dependent spectra search was directed by Proteome Discoverer v1.3.0.339 (ThermoElectron) using Mascot v2.1 and Sage-N Sorcerer Sequest algorithms and the UniprotKB Homo sapiens reference proteome canonical and isoform sequences (7/10/2013 version). The HumanRef150126 file annotated with the common Repository of Adventitious Proteins (cRAP) database, which provided a total of 89,849 entries, 89,734 from Uniprot Human Ref. Proteome and 115 from the CrapOme database, was used for protein inference. Search parameters included: variable methionine oxidation, fixed cysteine carbamidomethylation, up to 2 missed cleavages, 50 ppm precursor error for MS1 Orbitrap FTMS data, 0.8 Da error for CID-based MS2 LTQ data and 1.2 Da error for ETD-based MS2 data. To estimate the false discovery rate, a decoy database was generated from this database with the program decoy.pl (from matrixscience.com). This decoy database of reverse sequences was appended to the original database. The Proteome Discoverer pipeline allowed for the extraction of MS2 scan data from the Xcalibur RAW file, separate searches of CID and ETD MS2 scans in Mascot and Sequest, and collection of the results into a single file (.msf extension).

For comparative proteomic analysis, .msf files from Proteome Discoverer were loaded into Scaffold (v3.6.5, Portland, OR). Scaffold was used to calculate the false discovery rate using the Peptide and Protein Prophet algorithms (55, 56). The results were annotated with human gene ontology information from the Gene Ontology Annotation Database (ftp.ebi.ac.uk) (57). Proteins and peptides were identified with a false discovery rate (FDR) of 0.1%. The FDR was calculated as the percentage of positive hits in the decoy database versus the target database both for proteins and peptides. Proteins of interest identified with one peptide only were manually verified. Scaffold (v3.6.5., Proteome Software Inc.) was employed to validate MS/MS-based peptide and protein identifications from database searching. Peptide identifications were accepted if they could be established at a greater than 95% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they reached greater than 99% probability and contained at least 2 identified unique peptides (supplemental Table S1 Peptide Report). Protein probabilities were assigned by the Protein Prophet algorithm (supplemental Table S2 Peptide Report). These identification criteria typically established <0.1% false discovery rate based on a decoy database search strategy at the protein level (58). Contaminants and reverse identification were excluded from further data analysis. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Experimental Design and Statistical Rationale—Mass spectrometry (MS) experiments were performed using exosomal proteins isolated from the conditioned medium of three different passages of GIST-T1 and GIST882 cell lines (biological replicates). Only proteins identified in at least two biological replicates were further considered for label-free quantification (59). To facilitate comparison of proteins among groups, common contaminants such as keratins, protein identifications related to a different species other than human (e.g. pig, horse), as well as several isotypes for a given protein were filtered out. Identified exosomal proteins were quantified and normalized across samples using the normalized intensity based absolute quantification (iBAQ) algorithm available in the Scaffold software. To enable a direct comparison of all proteins across biological and technical replicated lognormal transformation (base 2) and median central tendency algorithm were implemented. The histogram of the Logz transformed protein expression for each sample followed a bell-shaped distribution and centered on 0 (normal distribution). Imputation of missing values in all lists was performed from the normal distribution (width 0.3 and down shift 1.8), to simulate signals from low abundant proteins as previously described (60). After normalization, unsupervised hierarchical clustering analyses, such as Principal Component Analysis (PCA), Pearson’s and Spearman’s Correlation, were used to identify outlier and samples similarity. No replicates were removed from the analysis. TEX and 882 Exo proteomes were grouped preservation-method wise, and two-sided t-tests were performed to identify significantly changing proteins among groups. p values were adjusted for multiple-testing using permutation-based false-discovery rate (FDR) and q-value estimation (FDR = 0.05, q0 = 0.1, and 300 randomizations). Proteins were significantly differentially expressed between the two conditions when an adjusted p value 0.05, and a fold change <−2 or >2 were obtained. Volcano plot was used to visualize these changes. Normalized and log2 transformed values for proteins common between TEX and 882 Exo were used to generate multiple scatter plot to estimate the degree of correlation among different
samples and extend of similarity between biological and technical replicates. Hierarchical k-means clustering algorithm was used to generate a heat map of the proteome profiles of different samples after z-score normalization, using Euclidean distances. Comparison between this study and the most up to date EVpedia database was performed by importing the list of reported exosomal proteins (as recent as February 2015) into FunRich. This data set included all proteins listed in EVpedia (from various tissue, cell types, primary cells, and cell lines).

**Gene Ontology, Reactome FI, Funrich and REVIGO Enrichment Analysis**—Funrich software and Cytoscape (version 3.4.0) along with its Reactome Functional Interaction (FI) network app was used to determine statistical over-representation (enrichment) of pathways, network, and GO terms, such as biological component, cellular component, and molecular function of the identified proteins (61). Other categorical annotations were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/pathway.html). In all cases, an adjusted p value of 0.02 was used as a threshold for significance after correcting for multiple testing by Benjamini and Hochberg FDR correction to derive overrepresented components. In addition, semantic similarity measures (SimRel) to identified GO terms cluster representatives was performed using REVIGO (62). Additional pathway and network analysis were performed using IPA (Ingenuity Systems, \( p < 0.05 \), Fisher’s exact test) (63). Highly up- or downregulated pathways were cross validated by submitting the cut off data sets to the annotation enrichment tool Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) (http://david.abcc.ncifcrf.gov/). Enrichment of key terms was determined using the Fisher’s exact test and the resulting \( p \) values were corrected for multiple testing using the Benjamini-Hochberg procedure. Hierarchical clustering of identified proteins was performed on normalized log2 transformed intensities after filtration of the data to have at least two valid values (in some cases Z-score normalization was performed), using Euclidean distances among averages. Unsupervised hierarchical clustering was performed based on uncentered Pearson correlation using ClustVis 3.0. Heat map representations were generated using gplots (https://cran.r-project.org/web/packages/gplots/index.html).

**Statistical Analysis**—Statistical analysis and graphs were generated using the GraphPad Prism-5 software (version 5.01). Statistical analysis was performed using two-sided Student’s t test, Mann-Whitney U test, or analysis of variance (ANOVA) when appropriate, with \( p < 0.05 \) considered statistically significant.

**Data Deposition**—The proteomics data, including the raw data ("RAW"), peak list files (".mgf"), search result file (".xml"), and annotated search results file (".sf3") spectra for all peptides and proteins identified from Scaffold, have been uploaded in Mass Spectrometry Interactive Virtual Environment (MassIVE, ID: MSV000081625) following the ProteomeXchange (PX) consortium guidelines (64). The mass spectrometry proteomics data can be found at http://proteomexchange.org using the data set identifier PXD007997.

## RESULTS

**The Number of Intraluminal Vesicles Are Increased in the Multivesicular Bodies of GIST Cells as Compared with Primary Smooth Muscle Cells**—Multivesicular bodies (MVB) are cellular compartments characterized by the accumulation of intraluminal vesicles (ILVs) (10). Depending on the cellular state (e.g. activated or transformed), the number of ILVs per MVB may greatly vary (5). To determine whether compelling differences in the number of ILVs per MVBs existed in GIST cells, we performed transmission electron microscopy (TEM) experiments, using two GIST cell lines, i.e. GIST882 and GIST-T1 cells possess a K642E mutation in exon 13 of KIT or a 57-nucleotide (570-578) in-frame deletion in exon 11 of KIT, respectively. A nontransformed primary myometrial smooth muscle cells (1° SMC), which represent the host cells surrounding these tumors in vivo was included as a control (31). As shown in Fig. 1A and 1B, GIST882 and GIST-T1 cells displayed significantly more ILVs in their respective MVB (58.08 ± 1.107 ILVs/MBV for GIST-882 cells, \( n = 26 \) versus 18.54 ± 1.107 ILVs/MBV for GISTT1, \( n = 26 \)) when compared with 1° SMC (0.7143 ± 0.2658 ILVs/MBV, \( n = 26 \)). In addition, GIST882 cells contained >3-fold more ILVs per MVB when compared with GIST-T1 cells (Fig. 1A and 1B). No significant increase in the numbers of MVBs per cells was observed in GIST-T1 versus GIST882 cells (Fig. 1C), suggesting that the production of ILVs was enhanced under steady state conditions in GIST882 cells when compared with GIST-T1 cells. This comparison demonstrates that the number of intraluminal ILVs per MVBs is significantly increased in GIST cells when compared with primary smooth muscle cells.

**GIST Cells Continuously Produce and Release High Numbers of Exosomes**—Next, the basal levels of 1° SMC-derived exosomes (1° SMC Exo), GIST-T1 cells-derived exosomes (TEX) and GIST882-derived exosomes (882 Exo) release in the conditioned medium (CM) was investigated. TEM analysis revealed the presence of small nano-sized vesicles (Fig. 1D). Although an abundant number of vesicles were present in TEX and 882 Exo preparations, 1° SMC Exo preparations were very sparsely represented in the final TEM images (Fig. 1D, left panel). Next, nanoparticle tracking analysis (NTA) was performed to further define the size distribution profiles and concentrations of particles in all three preparations. As shown in Fig. 1E, a significant increase in the number of particles released in the CM of GIST882 cells (6.0 × 10⁸ ± 1.05 vesicles/10⁶ cells; \( n = 11 \)) was observed when compared with GIST-T1 cells (2.7 × 10⁸ ± 0.53 vesicles/10⁶ cells; \( n = 8 \)). In addition, 1° SMC cells released significantly fewer exosomes in the CM when compared with TEX (4.5-fold less) and 882 Exo (10-fold less) release, respectively (Fig. 1E). Analysis of the size distribution revealed a bell-shaped curve which suggested a homogeneous population of vesicles with a mean size of 141 ± 3 nm (\( n = 7 \)), 164 ± 11 nm (\( n = 8 \)) and 151 ± 15 nm (\( n = 11 \)) for 1° SMC Exo, TEX and 882 Exo, respectively (Fig. 1F). Analysis of total exosomal protein content in all purified preparations revealed that GIST-derived exosomes (GDE) contained significantly more total proteins, 9.1 μg ± 2.1 μg for 882 Exo (\( n = 10 \)) and 3.3 μg ± 1.0 μg for TEX (\( n = 12 \), when compared to 1° SMC Exo (0.6 μg ± 0.4 μg; \( n = 11 \)) (Fig. 1G). Molecular characterization of these preparations indicated similar expression levels of heat shock proteins HSP70 and HSP90 in 1° SMC Exo, TEX, and 882 Exo. By contrast, only GDE expressed the tetraspanins, CD9, CD81, and Annexin 1, whereas RAB7 and Flotillin proteins were differentially expressed among preparations (Fig. 1H). Altogether, our result

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supports the observation that inherent difference in the basal levels of exosome produced by GIST cell lines exists compared with nontransformed 1° SMC because of an overactive exosome production machinery.

Oncogenic KIT Receptor and Exosomal Markers are Associated with GIST-derived Exosomes—After ensuring the expression of typical exosomal markers on GDE, the cell of origin of the nanoparticle preparations was further validated by performing a continuous sucrose (0.25–2.0 M) gradient. The distribution of pKITTyr719 and total KIT, a GIST associated protein marker, and exosomes associated markers were analyzed within the collected fractions by immunoblotting. As shown in supplemental Fig. S1 and S1B, p-KITTyr719, and total KIT protein levels were mainly distributed in fraction #4 (1.142 g/ml sucrose) in TEX and fraction #2 (1.135 g/ml sucrose) in 882 Exo. Similarly, analysis of known exosomal markers in all fractions indicated an enrichment and distributions in fractions #3 to #7 with sucrose density of 1.132 and 1.196 g/ml, respectively for TEX and fractions #2 (1.135 g/ml) and #5 (1.195 g/ml) for 882 Exo; which corresponds to the density previously reported by us for exosomes (19). Taken together, our data indicated that both GIST cells lines release KIT bearing vesicles similar in size, morphology, molecular and biophysical characteristics to exosomes.

Proteomic Analysis Reveals Minor Pattern of Changes in Protein Abundance using the Whole GDE Proteome—To quantify the changes in the GDE proteome, exosomes were isolated in triplicate from the conditioned media (CM) of three different passages of GIST-T1 and GIST882 cells (noted A, B, C in supplemental Fig. S2A). The general proteomic analysis workflow of GDE is represented in supplemental Fig. S2A. Each biological replicates were run as technical duplicates, denoted 1, 2 in supplemental Fig. S2A and twelve proteomic profiles were generated (supplemental Table S3_Peptide Report) (65). Quantitative proteomic profiling identified a total of 1,606 proteins across TEX (n = 6), and 882 Exo (n = 6) (supplemental Fig. S2B). Unsupervised hierarchical clustering revealed two main clusters encompassing biological and technical replicates from each group clustered together further connected upstream supporting the concept of a common tumor origin (supplemental Fig. S2C). The reproducibility and overall features of the TEX and 882 Exo proteomes were further investigated by principal component analysis (PCA), Pearson’s and distance matrix of the Spearman rank correlation, and indicated good reproducibility across technical (>0.9) and biological (>0.7) replicates (supplemental Fig. S3 and supplemental Fig. S2A). Identified exosomal proteins were quantified and compared across sample using the nor-
malized intensity based absolute quantification (iBAQ) algorithm. The identified GDE associated proteins spread evenly across the 24 chromosomes and represented on average 9% of the genes identified on each chromosome, and the median intensity values extended over a scale of four orders of magnitude (supplemental Fig. S4).

These data were further analyzed using two approaches: The first consisted on the classification of each identified proteins into three groups (found in both, unique to TEX, or 882 Exo) (Fig. 2B) whereas the second method used statistical significance as a mean for the final classification (Fig. 2C and 2D). As shown in Fig. 2B, Venn diagram analysis of the distribution of identified proteins among each GDE population revealed a common subset of 1060 exosomal proteins out of 1282 (TEX) and 1384 unique peptides, representing an 83 and 77% overlap, respectively. These 1060 common proteins are subsequently referred to as the “core” GDE proteome (cGDEp). This initial finding suggests an inherent conservation in the exosomal proteome released by these mesenchymal tumor cells. However, it should be noted that of the total proteins (1606) identified between the two cell lines, 222 proteins (13.8%) were solely present in TEX and 324 proteins (20.2%) only found in 882 Exo (Fig. 2B). Statistical analysis based classification using variations in abundance indicate that the 1606 GDE proteins could be divided into two main clusters (59). The first group represents 25.7% (412 of the 1606) of the total proteins that were either significantly enriched (115 proteins, 7.2% of total proteins) or decreased (297 proteins, 18.5% of total proteins) in TEX as compared with 882 Exo (Fig. 2C and 2D, supplemental Table S4). The second group accounted for 1194 proteins, which constituted 74% of the identified protein that were present at similar levels in each gel.
both GDE preparations. Comparison of the cGDEp to the integrated and comprehensive Extracellular Vesicles (EV) database (http://evpedia.info, 42,226 total proteins), indicated that out of 1,606 total proteins, 924 (57.5%) and 581 proteins, were previously reported in either the vesiculopedia or the EVpedia databases, respectively (Fig. 2D). In contrast, 6.3% (101 out of 1606) of the proteins identified were previously unreported (supplemental Fig. S5, supplemental Table S5). Further analysis of the repartition of these 101 proteins indicate that 33 are shared between TEX and 882 Exo, 37 proteins are only found in TEX and 31 are exclusively detected in 882 Exo (supplemental Fig. S5A and S5B). KEGG pathways enrichment analysis indicate that these proteins are associated with “axon guidance,” “Alzheimer disease,” “VEGF signaling,” “MAPK signaling,” “Wnt signaling,” and “Neuroactive ligand-receptor interaction” (supplemental Table S6). This coverage of exosomes-associated components is consistent with other proteomic studies. Altogether, these studies for the first time identified both conserved core exosomal proteins and GIST cell specific proteins.

**Analysis of the Core GDE Proteome Highlights Common Molecular Signatures that Support Exosomal Origin and Enrichment of GIST-Related Canonical Functions**—Volcano plot analysis of the 1,060 proteins composing the cGDEp reveal that 244 proteins (23.0%) out of 1060 (p < 0.05) are differentially expressed between TEX and 882 Exo (> 2-fold, p < 0.05). In comparison, the majority of the cGDEp (816 proteins, 77.0%) are shared between the two sources of exosome (Fig. 3A, supplemental Table S7), with 662 of these proteins being previously reported in other proteomic studies of exosomes (Fig. 3B). As shown in supplemental Table S8 and supplemental Fig. S6A, the majority of these proteins originate from eight main cellular compartments, i.e. extracellular region, exosomes/vesicle, cytoplasm/cytosol, intracellular compartment, plasma membrane, endosome/vacuole, nucleus, and specialized cellular location, such as focal adhesions (p < 0.01). In addition, the predicted molecular functions and biological processes for these core GDEp included “binding” to a variety of biological molecules and involvement in many homeostatic cellular processes (supplemental Fig. S6B and S6C, p < 0.01).

To further place the identified proteins in the context of known protein-protein interactions and gain insight on the coordinated role of these proteins in specific cancer related networks and signaling pathways, we investigated the interactions among each 1060 individual exosomal proteins forming the cGDE proteome using the Reactome Functional Interaction (FI) network app in Cytoscape and overrepresentation of specific pathways in the data set was determined based on enrichment statistics. Fig. 3C and supplemental Table S9 show the top twenty signaling pathways significantly enriched in the interaction network (adjusted FDR p < 0.01). Molecules belonging to pathways such as signaling by Nerve Growth Factor (NGF), ERBB, VEGF, SCF-KIT, FGFR, and EPH, among others, are significantly enriched in the cGDEp (Fig. 3C). Most of the identified pathways are reported to be involved in tumor growth and progression including, the Wnt, PI3 kinase, FGFR signaling pathways, as well as pathways involved in angiogenesis (Fig. 3C). Further analysis of the normalized expression levels of NGFR transcript in various sarcoma tissue indicated that this receptor located in the top 8% of the overexpression gene rank (OGR) and was significantly (p > 0.001) expressed in GIST as compared with other sarcomas (Fig. 3D) (66). To further our understanding of the biological relevance of the data at the molecular level, overrepresentation analyses using the Ingenuity Pathway Analysis (IPA) identified processes previously associated with GIST biology such as “protein synthesis, energy production, and lipid metabolism,” related to digestive organ tumors (p < 0.01), translation of mRNA (p < 0.01), as well as synthesis and translation of proteins (p < 0.01). The main canonical signaling pathways significantly overrepresentation included mTOR, actin cytoskeleton, caveolar-mediated mediated endocytosis, and regulation of elf4 and p70S6K signaling (Fig. 4E), features related to GIST.

**Validation of the Mass Spectrometry Data Using Cell-lines Derived Exosomes and Cellular Lysates**—Large-scale immunoblot validation of all the proteins comprising the cGDEp was not technically feasible, therefore we focused on a subset of previously reported GIST related proteins, selected exosomal markers, and others signaling components. Shown in Fig. 4A are immunoblot analyses using similar amount of proteins derived from exosomes or cellular lysates preparations under reducing and nonreducing conditions. A notable enrichment of well-established GIST-associated proteins markers such as KIT, p-KITTyr719, hypoxia inducible factor 1 α (HIF-1α), CD34, anoctamin-1 (ANO1), prominin 1 (PROM1), the protein kinase C theta (PRKCO), succinate dehydrogenase B (SDHB), and endoglin (ENG) was observed in GDE (Fig. 4A, supplemental Table S4). In addition, two recently identified proteins important for GIST development (67), i.e. ETS variant 1 (ETV1) protein and the E3 ubiquitin ligase, COP1, which regulates ETV1 protein stability, were also found enriched in GDE (68). As expected, several key proteins associated with exosome biogenesis (including Alix, TSG101, VPS26A/B, VPS29, constituents of the ESCRT sorting system, and flotillins), trafficking and fusion (e.g. tetraspanins, ADP-ribosylation factors, clathrin/coatomer subunits, and variety of small Rab GTPases), along with proteins involved in vesicle release (e.g. synaptotagmin 1/2, dynamin 1/2, and VAMPs), were identified in cGDE further supporting the endosomal origin of extracellular vesicles. Selected proteins which included exosomal markers such as CD63, CD81, Heat Shock Protein (HSP) 90, HSP70, HSP60, Tumor Susceptibility Gene 101 (TSG101), Annexin 1, ALG-2-interacting protein X (Alix), and Flotilin were validated by immunoblotting in exosomes and the corresponding cellular lysates (denoted Exo on Fig. 4A). Because of the mesenchymal nature of these tumors, the presence of mesenchymal markers, such as vi-
mentin (VIM) and fibronectin 1 (FN1) was similarly found in GDE (Fig. 4A). Other proteins validated by immunoblotting included, proteins with receptor function and/or targeting of recipient cells for uptake, such as β1 Integrin, Intercellular Adhesion Molecule 1 (ICAM1), and CD44; proteins involved in intravesicular trafficking (RAB1, RAB7); as well as proteins with immunoregulatory activities (e.g., human leukocyte antigen I (HLA-I) and FAS). Surprisingly, GDE also displayed a specific enrichment of markers of autophagy (MAP1 light chain 3-like protein A and B (LC3A/B)) which represent a survival mechanism used by GIST, further corroborating the origin of these vesicles. Finally, molecules involved in intracellular signal transduction, such as Extracellular signal-Regulated Kinases 1/2 (ERK1/2), and Signal Transducers and Activators of Transcription factor 1 (STAT1), and Apolipoprotein A1 (ApoA1), a protein involved in the lipid metabolism was highly enriched in both exosomes preparation as compared with cellular lysate, under both reducing and nonreducing conditions (Fig. 4A).

Evaluation of Selected GDE-Associated Proteins using Immuno-Isolated KIT Positive Exosomes-Derived from Clinical GIST Patients Plasma Samples—To further verify and validate our findings using clinical specimens, total exosomes were isolated from GIST plasma samples by differential ultracentrifugation. The number of particles as determined by nanoparticle tracking analysis (NTA) was enriched by 2-fold in the

![Figure 3](image-url)
plasma of GIST patients (GPE) \((2.3 \times 10^{10} \pm 1.3 \text{ particle/ml of initial plasma} (n = 18 \text{ samples}))\) when compared with age matched healthy donors (HPE) \((1.0 \times 10^{10} \pm 0.5 \text{ particle/ml of initial plasma} (n = 12 \text{ samples})\) (Fig. 4B). Given that majority of human GIST have gain-of-function KIT mutations and we have previously reported that these tumors release oncogenic KIT protein in association with exosomes (31), KIT + exosomes were purified with anti-KIT-coated dynabeads (beads-KIT). IgG coated beads (beads-IC) were used as control for the immunoaffinity separations (Fig. 4C). The purified KIT + vesicles were further compared with vesicles isolated from healthy donors. As shown in Fig. 4D; subpopulation of KIT + exosomes were enriched in plasma from GIST patients as compared with healthy controls. Next, expression of KIT was confirmed to be only present in the KIT + immuno-isolated fractions as compared with beads-IC (Fig. 4D) with a 3-fold selective enrichment in KIT + vesicles (Fig. 4D). We further validated these results by flow cytometry. As shown in Fig. 4E and F, KIT was nearly undetectable on the surface of exosome preparations derived from HPE, whereas a significant enrichment of KIT + vesicles was detected after KIT immunoeisolation in GIST patient samples. Next, KIT expression was evaluated on isolated KIT + vesicles isolated from GIST patients \((n = 9)\) and healthy donors control \((n = 9)\) by immuno-
blotting (Fig. 4G and 4H). As expected, KIT⁺ vesicles from HPE were negative for p-KIT Tyr719 and total KIT (Fig. 4G), whereas KIT⁺ vesicles isolated from GIST patients expressed the receptor and its constitutively activated form (Fig. 4H). Immunoblotting also demonstrated the presence of several proteins found by mass spectrometry, e.g., ApoA1, Claudin1, Flotilin, FN1, HIF1-α, HLA-I, ICAM-1, RAB1, RAB7, SDHB, and STAT1 (Fig. 4H and 4F). We also noted variable levels of expression of heat shock protein HSP60, tetraspanins, CD9 and CD81, Fas Ligand (Fasl), and ANO1, likely reflective of inter-patient variability (Fig. 4F). These data confirm that a number of potential biomarker candidates with different prognostic utility in GIST can be tracked in circulating extracellular vesicles.

Identification and Validation of Novel cGDEp-associated Kinases, Phosphatases, and Tumor Associated Antigens—As previously mentioned most GIST are driven by oncogenic KIT; however, our proteomic study identified a number of serine/threonine and tyrosine kinases previously unreported in GIST biology (Fig. 5A and supplemental Table S3). In addition, various receptor-type tyrosine or serine/threonine-protein phosphatases were present in GIST-derived exosomes, including members of the LP family (phosphatidic acid, inositol monophosphate, or inositol polyphosphatases), phosphatases with protein tyrosine phosphatase (PTPs) domains, and serine/threonine specific protein phosphatases (PPPs) (Fig. 5B). Network enrichment analysis indicated that the top five enriched terms enriched in these kinases and phosphatases included, signaling by NGF, PDGF, insulin receptor, EGFR, and axon guidance (Fig. 5C). The expression of selected kinases such as the receptor tyrosine-like orphan receptor ROR2, frizzled receptor 7 (FZD7), phosphor-JNK, total JNK, and MST1R in paired exomes and cellular lysates, and KIT⁺ plasma-derived exosomes and the corresponding tumor lysate (matching TL).

![Fig. 5. cGDE are enriched in protein kinases and phosphatases and tumor-associated antigens.](image)

**A**. List of protein kinases and phosphatases identified by proteomic in the cGDE. C. Network analysis of the proteins kinases and phosphatases present in cGDE indicate enrichment of tumor associated signaling pathway. D. Immunoblot analysis of ROR2, Frizzled receptor 7 (FZD7), phosphor-JNK, total JNK, and MST1R in paired exosomes and cellular lysates. E. ROR2 transcript expression value in fibrosarcoma (FS), GIST, leiomyosarcomas (LMS), and malignant fibrous histiocytoma (MFH) as reported in GSE3443. OGR and FC indicate the overexpression gene rank and fold change respectively. F. Analysis of the expression of selected kinases and phosphatases in paired cell line-derived exosomes and cellular lysate, and KIT⁺ plasma-derived exosomes and the corresponding tumor lysate (matching TL). G. Immunoblot analysis of tumor associated antigens, MAGED2, GPC4, and ephrins receptors (EPHA3, A4), as well as immune regulatory proteins, in in paired cell line-derived exosomes and cellular lysate, and in KIT⁺ plasma-derived exosomes and the corresponding tumor lysate (matching TL).
Supplemental Fig. S8

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a 2.0-fold increase in circulating exosomes was observed in the peripheral blood after the patient progressed on the second round of IM (blood sample "4," 12.0 $\times 10^{13}$ particles/ml as compared with initial diagnosis of progressive disease from primary tumor to metastasis blood sample "3," 5.8 $\times 10^{13}$ particles/ml). Most representative of the diagnostic value of circulating exosome levels relative to tumor burden, the concentration of exosomes increased by 129-fold from NED (sample #2) to PD (sample #3) and by 267-fold from NED (#2) to metastatic disease (#4) (Fig. 7B–7D).

**GIST-derived Exosomes Carry Proteins with Therapeutic Value**—To determine the therapeutic value of p-KIT$^{Y719}$, total KIT, and SPRY4 levels, KIT$^{+/}$ exosomes (Fig. 4G and 4H), were purified from primary and metastatic GIST plasma samples, and were evaluated by electron microscopy (Supplemental Fig. S9A). Immunoblotting analysis of the expression of p-KIT$^{Y719}$, KIT, and SPRY4 indicated that KIT$^{+}$ exosomes derived from a metastatic GIST patient, which initially displayed partial response (PR) to IM treatment (patient #15 in Fig. 7E), carry significantly more p-KIT$^{Y719}$, total KIT, and SPRY4 after IM-treatment as compared with the pre-IM levels, which suggests that the presence of these markers on TDEs could potentially indicate resistance to therapy. In contrast, in an IM responsive, patient #3 (demonstrating no evidence of disease (NED)), p-KIT$^{Y719}$, total KIT, and SPRY4 were significantly decrease in circulating exosomes isolated from post-IM samples (Fig. 7E and supplemental Fig. S9B). Furthermore, we found that patient #8, who was diagnosed with progressive disease (PD), all three proteins were significantly elevated in post-IM samples as compared with pre-IM levels. In addition, quantitative analysis indicates that compared with primary GIST, the levels of KIT and SPRY4 in KIT$^{+/}$ TDEs are significantly increased in metastatic patients (Fig. 7F–7G and supplemental Fig. S9B). In contrast, only KIT protein is significantly elevated ($p < 0.0001$) in exosomes isolated from IM-treated metastatic GIST patients (Fig. 7F and supplemental Fig. S9B). Importantly, in IM responsive primary GIST, both KIT and SPRY4 levels are significantly decreased in isolated KIT$^{+}$ exosomes following IM treatment (Fig. 7F and 7G), when compared with patients with metastatic disease. To further verify SPRY4 expression in tumor tissue, immunohistochemistry staining was performed using tumor tissues.

Fig. 6. **cGDE carry proteins involved in imatinib response.** A, Western blot validation of PDE2A, PROM1, SPRY4, vATPase H, Surfeit4, and exosome-associated markers, Alix (a.k.a PDCD6IP), HSP90, Flotillin, CD9, and a GIST-associated marker, COP1 in GIST cells lines and exosomes; and (B) GIST plasma-derived immunoisolated KIT$^{+}$ vesicles. C, Immunohistochemical analysis of the tissue distributions of KIT, SPRY4, PROM, and PDE2A in normal stomach, leiomyosarcoma, and GISTs-tumors. D, Normalized median centered expression value extracted from GSE13861 indicating the distribution values for SPRY4, PDE2A, PROM1 transcripts in NAGT (normal adjacent gastric tissue), GIST, DGA (diffuse gastric adenocarcinoma), GA (gastric adenocarcinoma), GITA (gastric interstitial type adenocarcinoma), and GMA (gastric mixed adenocarcinoma).
derived from primary and metastatic GIST. As shown in supplemental Fig. S9C, SPRY4 is significantly expressed in metastatic GIST tissue as compared with resected primary GIST. Collectively, these results show that the SPRY4 and KIT content on circulating KIT+/H11001 exosomes represent markers of tumor progression that might be useful to predict IM response in GIST patients.

**DISCUSSION**

Intercellular communication is crucial to maintain homeostasis or, in certain instances, promote tumorigenesis (1, 2). As shown by our group and others, exosomes create environments that are favorable to tumor progression, invasion, angiogenesis, metastasis, and drug response (31, 70, 71). Exosomes are present and readily accessible in many bodily fluids, including blood, saliva, urine, cerebrospinal, and ascites fluids (72–74). Studies aiming to determine the potential of exosomes as biomarkers for cancer diagnosis, prognosis, and monitoring mainly focus on determining the qualitative and quantitative changes in their proteome, microRNA and mRNA content (75, 76). The proteome of tumor-derived exosomes included many common exosomal proteins; but also tumor antigens and tumor-associated proteins that were reflective of the tumors type they derived from (77–79), such as the tumor antigens melanoma antigen recognized by T cells 1 (MelanA/Mart-1) and glycoprotein 100 (GP100), carcinoem-
bryogenic antigen (CEA), and human epidermal growth factor receptor 2 (HER2) enriched in melanoma-derived exosomes (70, 80). To date all of these studies provided shared and tumor specific genomic proteomic signatures and included many cancer types, such as bladder cancer, breast cancer, colorectal cancer, glioblastoma, ovarian cancer, medulloblastoma, melanoma, and mesothelioma (18, 81, 82). For example, exosomes isolated from pancreatic cell carried the onco-
gen full-length epidermal growth factor (EGFR) (83). In contrast, exosomes associated Let-7 miRNA, which correlated with poor survival in lung cancer patients (84), was also identified in exosomes isolated from metastatic gastric cells (85). In addition, Rab-5b and CD63 or caveolin-1 bearing exosomes were shown to be significantly increased in the systemic circulation of melanoma patients when compared with healthy donors derived plasma samples (86). Further, the use of glypican-1 expression on pancreatic cancer-derived exosomes presented a superior prognostic value for early detection compared with CA19–9, the only clinical biomarker routinely used to manage pancreatic cancer (87–90). Similarly, phosphatidylserine-positive exosomes detected in the per-
ipheral circulation of ovarian cancer patients displayed a higher diagnostic power (91). Similarly, Claudin-4-bearing exosomes have been reported to be released into the peripheral circulation of ovarian cancer patients (92). In addition, circulating levels of let-7b and miRNA-18a was used as pos-
itive prognostic markers to identify high risk patients and predict survival rates in multiple myeloma (93). Similar results have been found in multiple types of cancers, including nons-
mall cell lung cancer, esophageal squamous cell carcinoma, colorectal cancer, and hepatocellular carcinoma (84, 94–96). Finally, EGFRvIII splice variant bearing exosomes were iden-
tified in serum samples isolated from glioblastoma multiforme patients and were used as predictor of treatment response to tyrosine kinase inhibitors (97). Altogether these studies sup-
port the use of patients-derived exosomes as noninvasive tools for the early diagnosis and monitoring of cancer. How-
ever, more studies are needed to evaluate rigor and repro-
ducibility in the detection of these exosomes associated ana-
lytes and their use as diagnostic or therapeutic markers. Therefore, a deeper understanding of the changes in the qualitative and quantitative composition of these vesicles is crucial to understand the biology of many diseases (98–101).

Given the recent advances in proteomics technologies, we present here the first comprehensive proteomic profiling of GIST-derived exosomes and provide a unique insight into their content, potential biologic roles, and therapeutic value. We report that these KIT+ vesicles are enriched in the MVBS of GIST cell lines when compared with primary smooth muscle cells, demonstrating for the first time that GIST have an inherent overactive exosomes production machinery which leads to their release and accumulation. However, the molec-
ular mechanism responsible for this output is still unknown and requires further studies. Our proteomic analysis further revealed that exosomes-derived from GIST cells carry several common exosomal proteins, tumor specific markers, and previously unreported proteins indicating that GDE display shared proteomic features with exosomes-derived from other cells types, while conserving tumor identity. Many proteomic studies, aiming to identify tumor specific markers, took ad-
vantage of the homogeneous distribution profiles of exo-
somes produced by various cultured cell types (102–107). Yet, translation and validation of these findings using in vivo-
derived exosomes are still very challenging. This is because of the heterogeneous nature of biological fluids which contain an exceptionally complex variety of vesicles derived from various cells types, and other constituents that contaminate the final exosomes isolate, and therefore hinders the identification of tumor specific markers (108). For example, plasma specimens contain a mixture of ectosomes, microvesicles, and exo-
somes that may derived from heterogeneous cell populations such as immune cells, platelets, or endothelial cells (108). Therefore, unbiased identification of tumor markers on tumor-
derived exosomes remains a major challenge in the field (109, 110). We rationalized that to specifically isolate GIST derived exosomes from plasma samples, the implementation of pre-
parative KIT based immunosolation approaches will further enrich for tumor specific markers and eliminate the contribu-

tion of high abundance proteins such as albumin, immuno-
globulin, or complement components in our preparations. Importantly, our findings also highlight that circulating levels of KIT+ exosomes in GIST patients correlate with tumor bur-

den. We observed that the accumulation of circulating exo-
somes is enhanced in the peripheral blood of patients with metastatic GIST as compared with primary disease. This find-
ing suggests that quantitative changes in the levels of circu-
lating exosomes might indicate response to therapy and/or malignant capabilities, such as recurrence or metastasis to secondary sites. Furthermore, we show that evaluation of p-KIT Tyr719, total KIT, and SPRY4 profiles on circulating exo-
somes, pre- and post-IM therapy, could potentially be used to indicate disease state and likely response to therapy. This observation supports our previous study which identified ge-
ettomic response markers associated with therapeutic response to IM in GIST biopsy specimens, and found that down-regu-
lation of Sprouty 4A (SPRY4A), Frizzled Class Receptor 8 (FZD8), and Phosphodiesterase 2A (PDE2A) were highly reli-
able predictors of immediate response to IM (111). In this current offering, we found that EVs isolated from GIST cells are enriched in SPRY4 protein, a negative regulators of re-
ceptor tyrosine kinase-mediated signaling (112–114). This ob-
ervation is compatible with enhanced KIT activation in GIST as compared with other cancers, and suggest that GIST might sort this protein to avoid negative feedback loop mediated interference with downstream signaling pathways such as ERK activation (115). Recently, SPRY4 mRNA stability and transcription was shown to be enhanced by a growth factor-
independent regulatory mechanism that included hypoxia and
desferrioxamine (DFO), an iron chelator (116). Our data demonstrate that SPRY4 levels are increased in metastatic GIST as compared with circulating primary tumor-derived exosomes and tumor tissue. Because increased SPRY4 levels were not always accompanied by diminished levels of KIT in exosomes isolated from patients treated with IM, SPRY4 sorting in metastatic patients might be enhanced by other regulatory mechanisms such as increased MAPK signaling, or enhanced hypoxia in metastatic tumors as compared with primary GIST. HIF-1α plays an important role in aggressive behavior and tumor angiogenesis in GIST (117–119), as high expression of HIF-1α was significantly correlated with tumor recurrence/distant metastasis in GIST (120). Evaluation of the underlying regulatory mechanisms responsible for SPRY4 modulation in GIST are in progress. Collectively, our findings support larger validation studies; however, to further assess these circulating tumor exosome-associated proteins as potential diagnostic/predictive biomarkers, collaborative studies will be required to expand the number of highly annotated clinical samples for this orphan disease. By profiling these proteins and additional candidate, we hope to further identify and validate exosomes associated proteins signatures, and determine whether these signatures have therapeutic values to define subgroups of patients, either individually or in clusters, to help predict response to therapy.

As shown previously by us and other groups, tumorigenic cells release higher levels of exosomes in the peripheral circulation of cancer patients than is found in individuals without cancer (31, 50). However, we found variability in the methodology used to evaluate particle concentrations. As we report, the number of exosomes in plasma of GIST patients was generally higher using the TRPS method (10^13 particles per milliliters) versus the more common NTA platform (10^10 particles per milliliters). These observed discrepancies in particle concentration might be explained by the more concentrated sample used by TRPS (300 μl of diluted sample for NTA versus 70 μl of a smaller dilution for TRPS). Another explanation for the differences in particle concentration might be related in the principle behind the measure, as NTA tracks Brownian motion of particles in solution for a given time lapse (121–124), whereas TRPS performs resistive pulse sensing and requires to run calibrated beads at two pressure prior to running the samples of interest (125–129). To differentiate among these, further studies using both methodologies on exosomes samples isolated from patient samples are ongoing. Although we have previously examined total exosomes present in the plasma of GIST patients (31), there are no studies to date specifically describing KIT positive (KIT+) exosomes isolated from the plasma of GIST patients. A thorough examination of the proteome of isolated KIT+ exosomes will be informative; however, this is outside of the scope of this report. Nevertheless, we present our initial efforts to ascertain the feasibility of these type of analyses using KIT+ exosomes purified from the plasma of GIST patients using a combination of ultracentrifugation, and immunocapture methodologies. KIT signaling regulates normal cellular processes in several cell types (ICC, mast cells, melanocytes, gonads, stem cells) (130, 131) and, we show that the contribution of KIT positive vesicles to the pool of circulating vesicles in healthy donor plasma is negligible, suggesting that majority of KIT+ vesicles in patients derived from the tumor. Furthermore, the decrease of KIT+ exosomes in the circulation of patients responding to therapy further support this concept. Based on these results, the detection of c-KIT positive exosomes isolated from biological fluids, appears to be an interesting method for therapeutic monitoring of GIST.

Finally, several in vitro and in vivo studies, in both animal models and GIST patients, have demonstrated that GIST rely on their transcription, translation, and secretory machinery to thrive and communicate with host cells in the tumor microenvironment (TME) (47, 132, 133). GIST develops in the walls of the GI tract and presents as submucosal tumors surrounded by smooth muscle cells and a matrix rich in collagen (134, 135). The clinical course of GIST spans a wide spectrum ranging from a curable disorder to a highly malignant disease (36, 136). Treatment of GIST patients harboring KIT or PDG-FRA mutations, with tyrosine kinase inhibitors such as IM or sunitinib, have proven to be effective in many cases of inoperable or metastatic disease (39). As adjuvant IM treatment significantly improves recurrence-free survival, risk stratification for IM therapy has become an important step in the care of GIST patients (33). Three common clinical features of primary GIST are used for risk assessment: size, mitotic index, and location; however, a subset of GIST patients classified as low-risk have metastasis (36). Several recent studies have aimed to identify prognostic biomarkers for GIST and have reported that high prominin 1 (PROM1) expression in tissue is associated with poor prognosis (137–139). In this study, we report for the first time, that the proteome of GIST-derived exosomes consists of prognostic markers of GIST, included CDKN2A, EPHA4, FHL2, DPP4, EZR (140), HIF1A (117, 120), and KCTD12 (also known as Pfetin) (141, 142). We also report the presence in exosomes of proteins commonly associated with GIST, such as KIT (CD177) (143), CD34, anoctamin 1 (ANO1) (144–146), and DOG1 (Discovered on GIST 1) (147). Furthermore, others markers such as CA2 (carbonic anhydrase II), expressed in 95% of GIST (148), PRKCCQ (149–152), and CD34 (153), were identified in GDE by our proteomic approach, supporting the GIST origin of these vesicles.

Taken together, our comprehensive proteomic analysis of exosomes secreted by GIST cells has revealed a large number of candidate and clinically-relevant extracellular molecules known to be involved in the regulation of tumor progression, metastasis, angiogenesis, immunoregulation, and drug response, and for some, represent potential diagnostic and/or therapeutic targets for this disease. The biological significance of our findings is highlighted by the oncogenic effect of exosomes on directly influencing cells in the tumor.
microenvironment (31). Further functional insights and clinical correlation of these enriched exosomal cargo components in biofluids will extend our understanding of the development of GIST. Tumor-derived exosomes and their cargo, represent exciting circulating biomarkers of cancer and other diseases. Our studies further extend the growing field of exosomes and other extracellular vesicles as biomarkers of tumor burden and response to therapy.

In summary, we report the first high quality proteomic study of GIST-derived exosomes and identify several promising diagnostic and therapeutic exosome associated protein biomarkers, such as SPRY4. Follow-up investigations, based on this report, are now ongoing and aim to validate additional GIST exosome-associated markers in the blood of patients with the goal of improving currently methods used to diagnosis and monitoring disease.

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DATA AVAILABILITY

The proteomics data, including the raw data (".RAW"), peak list files (".mgf"), search result file "mzID," and annotated search results file "s3f" spectra for all peptides and proteins identified from Scaffold, have been uploaded in Mass Spectrometry Interactive Virtual Environment (MassIVE, ID: MSV00081625) following the ProteomeXchange (PX) consortium guidelines (64). The mass spectrometry proteomics data can be found at http://proteomecentral.proteomexchange.org using the data set identifier PXD007997.

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REFERENCES

1. Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., Gong, Z., Zhang, S., Zhou, J., Cao, K., Li, X., Xiong, W., Li, G., Zeng, Z., and Guo, C. (2017) Role of tumor microenvironment in tumorigenesis. J. Cancer 8, 761–773
2. Littipeage, L. E., Egebald, M., and Werb, Z. (2005) Coevolution of cancer and stromal cellular responses. Cancer Cell 7, 499–500
3. Quail, D. F., and Joyce, J. A. (2013) Microenvironmental regulation of tumor progression and metastasis. Nature Med. 19, 1423–1437
4. Colombo, M., Raposo, G., and Thery, C. (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Ann. Rev. Cell Developmental Biol. 30, 255–289
5. Kowal, J., Tkach, M., and Thery, C. (2014) Biogenesis and secretion of exosomes. Curr. Opin. Cell Biol. 29, 116–125
6. Thery, C. (2011) Exosomes: secreted vesicles and intercellular communications. F1000 Biol. Reports 3, 15
7. Valenti, R., Huber, V., Iero, M., Filippazzi, P., Parmiani, G., and Rivoltini, L. (2007) Tumor-released microvesicles as vehicles of immunosuppression. Cancer Res. 67, 2912–2915
8. Andreola, G., Rivoltini, L., Castelli, G., Huber, V., Perego, P., Deho, P., Squarzina, P., Accornero, P., Lozupone, F., Lugini, L., Stringaro, A., Molinari, A., Arancia, G., Gentile, M., Parmiani, G., and Fais, S. (2002) Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J. Exp. Med. 195, 1303–1316
9. Johnstone, R. M., Mathew, A., Mason, A. B., and Teng, K. (1991) Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. J. Cell. Physiol. 147, 27–36
10. Kanemoto, S., Nitani, R., Murakami, T., Kaneko, M., Asada, R., Matsuhisa, K., Safo, A., and Imaizumi, K. (2016) Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. Biochem. Biophys. Res. Commun. 460, 166–172
11. Huebner, A. R., Cheng, L., Somporn, P., Knepper, M. A., Fenton, R. A., and Pisitkun, T. (2016) Deubiquitylation of Protein Cargo Is Not an Essential Step in Exosome Formation. Mol. Cell. Proteomics 15, 1556–1571
12. Yuyama, K., Yamamoto, N., and Yanagisawa, K. (2008) Accelerated release of exosome-associated GM1 ganglioside (GM1) by endocytic pathway abnormality: another putative pathway for GM1-induced amyloid fibril formation. J. Neurochem. 105, 217–224
13. Valadi, H., Ekstrom, K., Bossios, A., Sjostrom, M., Lee, J. J., and Lotvall, J. O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell. Biol. 9, 654–659
14. Mathivanan, S., Ji, H., and Simpson, R. J. (2010) Exosomes: extracellular organelles important in intercellular communication. J. Proteomics 73, 1907–1920
15. Simpson, R. J., Lim, J. W., Moritz, R. L., and Mathivanan, S. (2009) Exosomes: proteomic insights and diagnostic potential. Expert Rev. Proteomics 6, 267–275
16. Simpson, R. J., Jensen, S. S., and Lim, J. W. (2008) Proteomic profiling of exosomes: current perspectives. Proteomics 8, 4083–4099
17. Greening, D. W., Xu, R., Gopal, S. K., Rai, A., and Simpson, R. J. (2017) Proteomic insights into extracellular vesicle biology - defining exosomes and shed microvesicles. Expert Rev. Proteomics 14, 89–95
18. Henderson, M. C., and Azorsa, D. O. (2012) The genomic and proteomic content of cancer cell derived exosomes. Front. Oncol. 2, 38
19. Atay, S., Gercel-Taylor, C., Kesimer, M., and Taylor, D. D. (2011) Morphologic and proteomic characterization of exosomes released by cultured extravillous trophoblast cells. Exp. Cell Res. 317, 1192–1202
20. Javeed, N., and Mukhopadhyay, D. (2016) Exosomes and their role in the micro-/maco-environment: a comprehensive review. J. Biomed. Res. 30
21. Taylor, D. D., and Black, P. H. (1986) Shedding of plasma membrane fragments. Neoplastic and developmental importance. Dev. Biol. 3, 33–57
22. Taylor, D. D., Lyons, K. S., and Gercel-Taylor, C. (2002) Shed membrane fragment-associated markers for endometrial and ovarian cancers. Gynecol. Oncol. 84, 443–448
23. Cocucci, E., Racchetti, G., and Meloedesi, J. (2009) Shedding microvesicles: artefacts no more. Trends Cell Biol. 19, 43–51
24. Andre, F., Scharzt, N. E., Movassagh, M., Flament, C., Pautier, P., Morice, P., Pomel, C., Lhomme, C., Escudier, B., Le Chevalier, T., Tursz, T., Amigorena, S., Raposo, G., Angevin, E., and Zitvogel, L. (2002) Malignant effusions and immunogenic tumour-derived exosomes. Lancet 360, 295–305
25. Chaput, N., Scharzt, N. E., Andre, F., and Zitvogel, L. (2003) Exosomes for immunotherapy of cancer. Adv. Exp. Med. Biol. 532, 215–221
26. Whiteside, T. L. (2005) Tumour-derived microvesicles or microvesicles: another mechanism of tumour escape from the host immune system? Br. J. Cancer 92, 209–211
27. Atay, S., Banskota, S., Crow, J., Sethi, G., Rink, L., and Godwin, A. K. (2014) Oncogenic KIT-containing exosomes increase gastrointestinal stromal tumor cell invasion. Proc. Natl. Acad. Sci. U.S.A. 111, 711–716
28. Meckes, D. G., Jr, Shair, K. H., Marquitz, A. R., Kung, C. P., Edwards, R. H., and Raab-Traub, N. (2010) Human tumor virus utilizes exosomes for intercellular communication. Proc. Natl. Acad. Sci. U.S.A. 107, 20370–20375.

29. Huber, V., Fais, S., Iero, M., Lugini, L., Canese, P., Squarcina, P., Zaccheddu, A., Colone, M., Arancia, G., Gentile, M., Seregni, E., Valenti, R., Ballabio, G., Belli, F., Leo, E., Parmiani, G., and Rivoltini, L. (2005) Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology 128, 1796–1804.

30. Bard, M. P., Hegmans, J. P., Hemmes, A., Luider, T. M., Willemsen, R., Severijnen, L. A., van Meerbeeck, J. P., Burgers, S. A., Hoogsteden, H. C., and Lambrecht, B. N. (2004) Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am. J. Respir. Cell Mol. Biol. 30, 751–757.

31. Atay, S., Tetik, A., Bozk Cetintas, Y., Yakar Tuluce, S., Tuluce, K., Kayikcioglu, M., and Eroglu, Z. (2014) Beta-myosin heavy-chain mutations R403QLW, V606M, K615N and R663H in patients with hypertrophic cardiomyopathy. Anatolu Kardiyol Derg 14, 244–250.

32. Goetttsch, W. G., Bos, S. D., Breekveldt-Postma, N., Casparie, M., Herings, R. M., and Hogendoorn, P. C. (2005) Incidence of gastrointestinal stromal tumours is underestimated: results of a nation-wide study. Eur. J. Cancer 41, 2858–2872.

33. Nilsson, B., Bumming, P., Meis-Kindblom, J. M., Oden, A., Dortok, A., Gustavsson, B., Sableska, K., and Kindblom, L. G. (2005) Gastrointestinal stromal tumours: the incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era–a population-based study in western Sweden. Cancer 103, 821–829.

34. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, T., Kadoya, I., and Sassa, K. (2002) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors (GISTs) with KIT and PDGFRA mutations have distinct gene expression profiles. Oncogene 21, 577–580.

35. Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C. J., Joseph, N., Singer, S., Griffin, D. J., Haley, A., Town, A., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2003) PDGFRα activating mutations in gastrointestinal stromal trophic cardiomyopathy. Anadolu Kardiyol Derg 14, 14333–14338.

36. Jenesu, H., and Kindblom, L. G. (2004) Gastrointestinal stromal tumour–a review. Acta Orthopaedica Scand. Supplement. 75, 62–71.

37. Lasota, J., Wozniak, A., Sarlomo-Rikala, M., Rask, J., Kordek, R., Nasser, A., Sobin, L. H., and Miettinen, M. (2000) Mutations in exons 9 and 13 of KIT gene are rare events in gastrointestinal stromal tumours. A study of 200 cases. Am. J. Pathol. 157, 1091–1095.

38. Kondo, T., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, T., Kadoya, I., and Sassa, K. (2002) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors (GISTs) with KIT and PDGFRA mutations have distinct gene expression profiles. Oncogene 23, 7780–7790.

39. Tarn, C., Mertel, E., Canutescu, A. A., Shen, W., Skorobogaty, Y., Heslin, M. J., Eisenberg, B., Birbe, R., Patchefsky, A., Dunbrack, R., Amolett, J. P., von Mehren, M., and Godvin, A. K. (2005) Analysis of KIT mutations in sporadic and familial gastrointestinal stromal tumours: therapeutic implications through protein modeling. Clin. Cancer Res. 11, 3668–3677.

40. Corless, C. L., Schroeder, A., Griffin, D., Town, A., McGreevey, L., Harrell, P., Shiraga, S., Bainbridge, T., Morich, J., and Heinrich, M. C. (2005) PDGFRα mutations in gastrointestinal stromal tumours: frequency, spectrum and in vitro sensitivity to imatinib. J. Clin. Oncol. 23, 5357–5364.

41. Antonescu, C. R., Besmer, P., Guo, T., Arkin, K., Hom, G., Korytowski, B., Leverha, M. A., Jeffrey, P. D., Desantis, D., Singer, S., Brennan, M. F., Maki, R. G., and DeMatteo, R. P. (2005) Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. Clin. Cancer Res. 11, 4182–4190.

42. Godwin, A. K. (2011) Bench to bedside and back again: personalizing treatment for patients with GIST. Mol. Cancer Ther. 10, 2026–2027.

43. Patel, S. (2012) Managing progressive disease in patients with GIST: factors to consider besides acquired secondary tyrosine kinase inhibitor resistance. Cancer Treatment Rev. 38, 467–472.

44. Jayanthi, C. N. (2010) Germline mutations in the tyrosine kinase domain of the c-kit proto-oncogene in patients with familial GIST. Clin. Genet. 78, 293–298.
Proteome of GIST-derived Exosomes

Hermjakob, H., and Vizcaino, J. A. (2017) The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res.* **45**, D1100–D1106

Mischak, H., Crites, E., Hanash, S., Gallagher, W. M., Vlahou, A., and Ioannidis, J. P. (2015) Epidemiologic design and analysis for proteomic studies: a primer on -omic technologies. *Am. J. Epidemiol.* **181**, 635–647

Jinn, S. C., West, R. B., Polack, J. R., Zhu, S., Hernandez-Bousard, T., Nielsen, T. O., Rubin, B. P., Patel, R., Goldblum, J. R., Siegmund, D., Botstein, D., Brown, P. O., Gilks, C. B., and van de Rijn, M. (2003) Gene expression patterns and gene copy number changes in dermatofibrosarcoma protubersans. *Am. J. Pathol.* **163**, 2383–2395

Chi, P., Chen, Y., Zhang, L., Guo, X., Wongvipat, J., Shamu, T., Fletcher, J. A., Dewell, S., Maki, R. G., Zheng, D., Antonescu, C. R., Allis, C. D., and Meyerson, M. (2010) ETVI is a lineage survival factor that cooperates with Kit in gastrointestinal stromal tumors. *Nature* **467**, 849–853

Birner, P., Beer, A., Vinatzer, U., Stary, S., Hofbauer, R., Nrtl, N., Wrba, F., Streubel, B., and Schoppmann, S. F. (2012) MAPKAP kinase 2 overexpression influences prognosis in gastrointestinal stromal tumors and associates with copy number variations on chromosome 1 and expression of p38 MAP kinase and ETVI. *Clin. Cancer Res.* **18**, 1879–1887

Frolow, A., Chahwan, S., Ochs, M., Arnoletti, J. P., Pan, Z. Z., Favorova, O., Fletcher, J., von Mehren, M., Eisenberg, B., and Godwin, A. K. (2003) Response markers and the molecular mechanisms of action of Gleevec in gastrointestinal stromal tumors. *Mol. Cancer Therapeut.* **2**, 699–709

Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Bron, J., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajari, C., Thery, C., Zitvogel, L., and Amigorena, S. (2002) Exosomes: composition, biogenesis and function. *Nat. Rev. Mol. Cell Biol.* **3**, 569–579

80. Somasundaram, R., and Herlyn, M. (2012) Melanoma exosomes: messengers of metastasis. *Nat. Med.* **18**, 853–854

81. Fontana, S., Saieva, L., Taverna, S., and Alessandro, R. (2013) Contribution of proteomics to understanding the role of tumor-derived exosomes in cancer progression: state of the art and new perspectives. *Proteomics* **13**, 1581–1594

82. Crow, J., Atay, S., Banskota, S., Artaie, B., Schmitt, S., and Godwin, A. K. (2017) Exosomes as mediators of platinum resistance in ovarian cancer. *Oncotarget* **8**, 11917–11936

83. Adamczyk, K. A., Klein-Scory, S., Tehrani, M. M., Warnken, U., Schmiegel, W., Schnizler, M., and Schwarte-Waldhoff, I. (2011) Characterization of soluble and exosomal forms of the EGFR released from pancreatic cancer cells. *Life Sci.* **89**, 304–312

84. Liu, Q., Yu, Z., Yuan, S., Xie, W., Li, H., Zuo, X., Yuan, S., Wu, N., Wu, L., Bai, L., and Li, Y. (2017) Circulating exosomal circRNA-As as prognostic biomarkers for non-small-cell lung cancer. *Oncotarget* **8**, 13048–13058

85. Ohshima, K., Inoue, K., Fujimura, H., Hatakeyama, K., Kanto, K., Watanebe, Y., Muramatsu, K., Fukuda, Y., Ogura, S., Yamaguchi, K., and Mochizuki, T. (2010) Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 13247

86. Logozzi, M., De Milito, A., Lugini, L., Borghi, M., Calabro, L., Spada, M., Perdiccio, M., Marino, M. L., Federici, C., Iessi, E., Brambilla, D., Venturi, G., Lozupone, F., Santinami, M., Huber, V., Maio, M., Rivoltini, L., and Fais, S. (2009) High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PloS One* **4**, e5219

87. Lai, X., Wang, M., McElyea, S. D., Sherman, S., House, M., and Korc, M. (2015) A microRNA signature in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer. *Cancer Lett.* **393**, 86–93

88. Lorenzon, L., and Blandino, G. (2016) Glypican-1 exosomes: do they initiate a new era for early pancreatic cancer diagnosis? *Transl. Gastroenterol. Hepatol.* **1**, 8

89. Herreross-Villanueva, M., and Bujanda, L. (2016) Glypicin-1 in exosomes as biomarker for early detection of pancreatic cancer. *Ann. Transl. Med.* **4**, 88

90. Melo, S. A., Luecke, B. L., Kahler, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBlue, V. S., Mittendorf, E. A., Weltz, J., Rahbari, N., Reissfelder, C., Pilar-Cisneros, F., Margala-Wilks, D., and Kalluri, R. (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523**, 177–182

91. Lea, J., Sharma, R., Yang, F., Zhu, H., Ward, E. S., and Schroit, A. J. (2017) Detection of phosphatidylserine-positive exosomes as a diagnostic marker for ovarian malignant ovaries: a proof of concept study. *Oncotarget* **8**, 14395–14407

92. Li, J., Sherman-Baust, C. A., Tsai-Turton, M., Bristow, R. E., Roden, R. B., and Morin, P. J. (2009) Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. *BMC Cancer* **9**, 244

93. Manier, S., Liu, C. J., Avet-Loloeau, H., Park, J., Shi, J., Campigotto, F., Salem, K. Z., Huynh, D., Glavey, S. V., Rivotto, B., Sacco, A., Roccaro, A. M., Bouyssou, J., Minville, S., Moreau, P., Facon, T., Leleu, X., Weller, Trippa, L., and Ghobrial, I. M. (2017) Prognostic role of circulating exosomal miRNAs in multiple myeloma. *Blood* **129**, 2429–2436

94. Matsumoto, Y., Kano, M., Akutsu, Y., Hanari, N., Hoshino, I., Murakami, K., Usui, A., Suito, H., Takashahi, M., Otsuka, R., Xin, H., Komatsu, A., Iida, K., and Matsubara, H. (2016) Quantification of plasma exosome is a potential prognostic marker for esophageal squamous cell carcinoma. *Oncol. Reports* **36**, 2535–2543

95. Sandfield-Paulsen, B., Aggerholm-Pedersen, N., Baek, R., Jakobsen, K. R., Meldgaard, P., Folker, B. H., Rasmussen, T. R., Varming, K., Jorgensen, M. M., and Sorensen, B. S. (2016) Exosomal proteins as prognostic biomarkers in non-small-cell lung cancer. *Mol. Oncol.* **10**, 1595–1602

96. Sandfield-Paulsen, B., Jakobsen, K. R., Baek, R., Folker, B. H., Sorensen, T. R., Meldgaard, P., Varming, K., Jorgensen, M. M., and Sorensen, B. S. (2016) Exosomal proteins as diagnostic biomarkers in lung cancer. *J. Thorac. Oncol.* **11**, 1701–1710

97. Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., and Morin, P. J. (2008) Glioblastoma microvesicles transport RNA and...
Proteome of GIST-derived Exosomes

proteins that promote tumour growth and provide diagnostic biomarkers. Nat. Cell Biol. 10, 1470–1476
98. Garnier, D., Magnus, N., Meehan, B., Kislinger, T., and Rak, J. (2013) Qualitative changes in the proteome of extracellular vesicles accompanying cancer cell transition to mesenchymal state. Exp. Cell Res. 319, 2747–2757
99. Pearson, L. J., Klahr, I. Y., Thongsonwatt, B., Manuprasert, W., Saejew, T., Sompras, P., Chunsangsri, P., Krunjakubul, T., and Pitsutkun, T. (2017) Multiple extracellular vesicle types in peritoneal dialysis effluent are prominent and contain known biomarkers. PLoS ONE 12, e0176801
100. Tanase, C. P., Codrici, E., Popescu, I. D., Mihai, S., Enciu, A. M., Necula, L. G., Preda, A., Ismail, G., and Albucescu, R. (2017) Prostate cancer proteomics: Current trends and future perspectives for biomarker discovery. Oncotarget 8, 18497–18512
101. Shek, K. L., Luther, J. M., and Rose, K. L. (2015) Proteomics characterization of exosome cargo. Methods 87, 75–82
102. Colletti, M., Petretto, A., Galardi, A., Di Paolo, V., Tomao, L., Lavarello, C., Inglese, E., Bruschi, M., Lopez, A. A., Pascucci, L., Geiger, B., Pei, nado, H., Locatelli, F., and Di Giannatale, A. (2017) Proteomic analysis of neuroblastoma-derived exosomes: new insights into a metastatic signature. Proteomics
103. Wittke, D., Chaputa, D., Khan, H., Stevens, S. M., Jr., and Kang, D. (2017) Isolation and Proteome Analysis of Microvesicles and Exosomes from HT22 Cells and Primary Neurons. Methods Mol. Biol. 1598, 255–267
104. Jerez, S., Araya, H., Thaler, R., Charlesworth, M. C., Lopez-Solis, R., Kalerig, A. E., Cespedes, P. F., Dukdakov, A., Stein, G. S., von Wijnen, A. J., and Galindo, M. (2017) Proteome analysis of exosomes and exosome-free conditioned media from human osteosarcoma cell lines reveals secretion of proteins related to tumor progression. J. Cell. Biochem. 118, 351–360
105. Duijvesz, D., Burnum-Johnson, K. E., Gritsenko, M. A., Hoogland, A. M., Vredenbregt-van den Berg, M. S., Williamsen, R., Luider, T., Pasa-Tolic, L., and Jenster, G. (2013) Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer. PLoS ONE 8, e62589
106. Meese, R., Craven, R. A., Hanrahan, S., Totty, N., Upton, C., Young, S. L., Patel, P., Selby, P. J., and Banks, R. E. (2004) Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics 4, 4019–4031
107. Hegmans, J. P., Bard, M. P., Hemmes, A., Luider, T. M., Kleijmeer, M. J., de Vries, S. Z., van Wijnen, A. J., and Gross, I. (2004) Prostate cancer stem cells: characterization and therapeutic implications. J. Cell. Biochem. 86, 1185–1193
108. Schipper, T., Zillikens, D., Grootenhuis, G. A., van der Graaf, P., Majoor, A. A., and van der Meulen, J. G. (2009) Response markers and the molecular mechanisms of action of Gleevec in gastrointestinal stromal tumors. J. Cell. Biochem. 10, 259–269
109. Fang, Q., Hanrahan, S., Totty, N., Upton, C., Young, S. L., Patel, P., Selby, P. J., and Banks, R. E. (2004) Proteomic analysis of extracellular vesicles measured using NanoSight nanoparticle tracking analysis. Placenta 25, 307–312
110. Sivakumaran, M., and Platt, M. (2016) Tunable resistive pulse sensing: Potential applications in nanomedicine. Nanomedicine 11, 2197–2214
111. Weatherall, E., Hauer, P., Vogel, R., and Willmott, G. R. (2016) Pulse size distributions in tunable resistive pulse sensing. Anal. Chem. 88, 8648–8656
112. Hassan, H. T. (2009) c-KIT expression in human normal and malignant stem cells: prognostic and therapeutic implications. Leuk. Res. 33, 5–10
113. Fukushima, T., Kamishima, T., Tsuchiya, M., Suzuki, Y., Kakinouchi, T., Kato, M., Hashimoto, K., Suda, T., and Nishio, H. (2003) Expression of the c-kit gene product in normal and neoplastic mast cells but not in neoplastic basophil/mast cell precursors from chronic myelogenous leukemia. J. Pathol. 207, 139–146
114. Tarn, C., Skorobogatko, Y. V., Taguchi, T., Eisenberg, B., von Mehren, M., and Godwin, A. K. (2006) Therapeutic effect of imatinib in gastrointestinal stromal tumors. Oncol. Rep. 10, 797–802
115. Haigl, B., Mayer, C. E., Siegwart, G., and Sutterluty, H. (2010) Sprouty4 expression and angiogenesis in gastrointestinal stromal tumor of the stomach. Oncol. Rep. 27, 99–131
116. Takahashi, R., Tanaka, S., Hiyama, T., Ito, M., Kitadai, Y., Sumii, M., Haruma, K., and Chayama, K. (2003) Hypoxia-inducible factor-1alpha expression and angiogenesis in gastrointestinal stromal tumor of the stomach. Oncol. Rep. 10, 797–802
117. Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat. Cell Biol. 5, 427–432
118. Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat. Cell Biol. 5, 427–432
119. Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat. Cell Biol. 5, 427–432
120. Chen, W. T., Huang, C. J., Wu, M. T., Yang, S. F., Su, Y. C., and Chai, C. Y. (2005) Hypoxia-inducible factor-1alpha is associated with risk of aggressive behavior and tumor angiogenesis in gastrointestinal stromal tumor. J. Clin. Oncol. 35, 207–213
121. Filipe, P., Hawe, A., and Jiskoot, W. (2010) Critical evaluation of Nanoparticle Tracking Analysis (NTA) for the measurement of nanoparticles and protein aggregates. Pharm. Res. 27, 796–810
122. Heider, S., Muzard, J., Zaruba, M., and Metzner, C. (2016) Tunable resistive pulse sensing: Potential applications in nanomedicine. Nanomedicine 11, 2197–2214
123. Weatherall, E., Hauer, P., Vogel, R., and Willmott, G. R. (2016) Pulse size distributions in tunable resistive pulse sensing. Anal. Chem. 88, 8648–8656
124. Hassan, H. T. (2009) c-KIT expression in human normal and malignant stem cells: prognostic and therapeutic implications. Leuk. Res. 33, 5–10
125. Fukushima, T., Kamishima, T., Tsuchiya, M., Suzuki, Y., Kakinouchi, T., Kato, M., Hashimoto, K., Suda, T., and Nishio, H. (2003) Expression of the c-kit gene product in normal and neoplastic mast cells but not in neoplastic basophil/mast cell precursors from chronic myelogenous leukemia. J. Pathol. 207, 139–146
126. Tarn, C., Skorobogatko, Y. V., Taguchi, T., Eisenberg, B., von Mehren, M., and Godwin, A. K. (2006) Therapeutic effect of imatinib in gastrointestinal stromal tumors: AKT signaling dependent and independent mechanisms. Cancer Res. 66, 5477–5486
127. Koh, J. S., Trent, J., Chen, L., El-Naggar, A., Hunt, K., Pollock, R., and Zhang, W. (2004) Gastrointestinal stromal tumors: overview of patho-logic features, molecular biology, and therapy with imatinib mesylate. Histol. Histopathol. 19, 565–574
128. Wong, N. A., Wingate, J., and Colling, R. (2014) A study of alpha5 chain of collagen IV, caldesmon, placentation alkaline phosphatase and smoothelin

514
Molecular & Cellular Proteomics 17.3
as immunohistochemical markers of gastrointestinal smooth muscle neoplasms. J. Clin. Pathol. 67, 105–111

135. Reichardt, P., Hogendoorn, P. C., Tamborini, E., Lodì, M., Gronchi, A., Poveda, A., and Schoffski, P. (2009) Gastrointestinal stromal tumors I: pathology, pathobiology, primary therapy, and surgical issues. Semin. Oncol. 36, 290–301

136. Gill, S., Thomas, R. R., and Goldberg, R. M. (2003) New targeted therapies in gastrointestinal cancers. Curr. Treat. Options Oncol. 4, 393–403

137. Lu, C., Liu, L., Wu, X., and Xu, W. (2013) CD133 and Ki-67 expression is associated with gastrointestinal stromal tumor prognosis. Oncol. Lett. 6, 1289–1294

138. Bozzi, F., Tamborini, E., and Pilotti, S. (2012) The CD133 expression levels and its role as potential cancer stem cells marker in gastrointestinal stromal tumor. Int. J. Cancer 131, E849–E850

139. Bozzi, F., Conca, E., Manenti, G., Negri, T., Brich, S., Gronchi, A., Pierotti, M. A., Tamborini, E., and Pilotti, S. (2011) High CD133 expression levels in gastrointestinal stromal tumors. Cytometry. 80, 238–247

140. Patil, D. T., and Rubin, B. P. (2011) Gastrointestinal stromal tumor: advances in diagnosis and management. Arch. Pathol. Lab. Med. 135, 1298–1310

141. Kubota, D., Orita, H., Yoshida, A., Gotoh, M., Kanda, T., Tsuda, H., Hasegawa, T., Katai, H., Shimada, Y., Kaneko, K., Kawai, A., and Kondo, T. (2011) Pten as a prognostic biomarker for gastrointestinal stromal tumor: validation study in multiple clinical facilities. Jap. J. Clin. Oncol. 41, 1194–1202

142. Kikuta, K., Gotoh, M., Kanda, T., Tochigi, N., Shimoda, T., Hasegawa, T., Katai, H., Shimada, Y., Kawai, A., Hirohashi, S., and Kondo, T. (2010) Pfetin as a prognostic biomarker in gastrointestinal stromal tumor: novel monoclonal antibody and external validation study in multiple clinical facilities. Jap. J. Clin. Oncol. 40, 60–72

143. Hornick, J. L., and Fletcher, C. D. (2002) Immunohistochemical staining for KIT (CD117) in soft tissue sarcomas is very limited in distribution. Am. J. Clin. Pathol. 117, 188–193

144. Sozutek, D., Yanik, S., Akkoca, A. N., Sozutek, A., Ozdemir, Z. T., Avsar, C. U., Gunaldi, M., Sahin, B., and Doron, F. (2014) Diagnostic and prognostic roles of DOG1 and Ki-67, in GIST patients with localized or advanced/metastatic disease. Int. J. Clin. Exp. Med. 7, 1914–1922

145. Lopes, L. F., West, R. B., Bacchi, L. M., van de Rijn, M., and Bacchi, C. E. (2011) DOG1 for the diagnosis of gastrointestinal stromal tumor (GIST): Comparison between 2 different antibodies. Appl. Immunohistochem. Mol. Morphol. 18, 333–337

146. Lee, C. H., Liang, C. W., and Espinoso, I. (2010) The utility of discovered on gastrointestinal stromal tumor 1 (DOG1) antibody in surgical pathology-the GIST of it. Adv. Anat. Pathol. 17, 222–232

147. Abdel-Hadi, M., Bessa, S. S., and Hamam, S. M. (2009) Evaluation of the novel monoclonal antibody against DOG1 as a diagnostic marker for gastrointestinal stromal tumors. J. Egyptian Natl. Cancer Inst. 21, 237–247

148. Parkkila, S., Lasota, J., Fletcher, J. A., Ou, W. B., Kivelä, J. A., Nuova, K., Parkkila, A. K., Ollikainen, J., Sly, W. S., Waheed, A., Pastorekova, S., Pastorek, J., Isola, J., and Miettinen, M. (2010) Carbonic anhydrase II. A novel biomarker for gastrointestinal stromal tumors. Modern Pathol. 23, 743–750

149. Ou, W. B., Zhu, M. J., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2008) Protein kinase C-theta regulates KIT expression and proliferation in gastrointestinal stromal tumors. Oncogene 27, 5626–5634

150. Lee, H. E., Kim, M. A., Lee, H. S., Lee, B. L., and Kim, W. H. (2008) Characteristics of KIT-negative gastrointestinal stromal tumours and diagnostic utility of protein kinase C theta immunostaining. J. Clin. Pathol. 61, 722–729

151. Duensing, A., Joseph, N. E., Medeiros, F., Smith, F., Hornick, J. L., Heinrich, M. C., Corless, C. L., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2004) Protein Kinase C theta (PKCtheta) expression and constitutive activation in gastrointestinal stromal tumors (GISTs). Cancer Res. 64, 5127–5131

152. Blay, P., Astudillo, A., Buesa, J. M., Campo, E., Abad, M., Garcia-Garcia, J., Miquel, R., Marco, V., Siera, M., Losa, R., Lacave, A., Brana, A., Balbin, M., and Freije, J. M. (2004) Protein kinase C theta is highly expressed in gastrointestinal stromal tumors but not in other mesenchymal neoplasias. Clin. Cancer Res. 10, 4089–4095

153. Miettinen, M., and Lasota, J. (2006) Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. Arch. Pathol. Lab. Med. 130, 1468–1478