A robust strategy for proteomic identification of biomarkers of invasive phenotype complexed with extracellular heat shock proteins

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Abstract

As an extension of their orchestration of intracellular pathways, secretion of extracellular heat shock proteins (HSPs) is an emerging paradigm of homeostasis imperative to multicellular organization. Extracellular HSP is axiomatic to the survival of cells during tumorigenesis; proportional representation of specific HSP family members is indicative of invasive potential and prognosis. Further significance has been added by the knowledge that all cancer-derived exosomes have surface-exposed HSPs that reflect the membrane topology of cells that secrete them. Extracellular HSPs are also characteristic of chronic inflammation and sepsis. Accordingly, interrogation of extracellular HSPs secreted from cell culture models may represent a facile means of identifying translational biomarker signatures for targeting in situ. In the current study, we evaluated a simple peptide-based multivalent HSP affinity approach using the Vn96 peptide for low speed pelleting of HSP complexes from bioreactor cultures of cell lines with varying invasive phenotype in xenotransplant models: U87 (glioblastoma multiforme; invasive); HELA (choriocarcinoma; minimally invasive); HEK293T (virally transformed immortalized; embryonic). Proteomic profiling by bottom-up mass spectrometry revealed a comprehensive range of candidate biomarkers including primary HSP ligands. HSP complexes were associated with additional chaperones of prognostic significance such as protein disulfide isomerases, as well as pleiotropic metabolic enzymes, established as proportionally reflective of invasive phenotype. Biomarkers of inflammatory and mechanotransductive phenotype were restricted to the most invasive cell model U87, including chitinase CHI3L1, lamin C, amyloid derivatives, and histone isoforms.

Keywords Extracellular heat shock proteins · Exosomes · Vn96 · Proteomics · Mass spectrometry · Biomarkers

Introduction

Chaperones, including heat shock proteins (HSPs), transport, stabilize, and ultimately decommission client proteins at all levels of biochemical complexity from viral to multicellular systems. HSPs and chaperones also organize into multimers

and arrays called chaperomes that provide epigenetic scaffolding for intersecting pathways that fluctuate substantially between normal and diseased physiology (Wang et al. 2006, 2018; Palotai et al. 2007). During cellular proliferation, repair, or dysregulation, HSPs relocate to the cell surface and are secreted. HSP70 for example is essentially a universal surface feature of exosomes released by cancer cells, as demonstrated by several studies (Gastpar et al. 2005; Gobbo et al. 2016; Castillo et al. 2018). HSP secretion also contributes to an exported stress response for intercellular triage and repair (Bhatia et al. 2016; Rodvold et al. 2017). Chronic inflammatory diseases also appear driven by dysregulated secretion of extracellular HSP, which in turn may influence tumorigenesis (Calderwood et al. 2012; Khandia et al. 2017; Edkins et al. 2018). There is also growing evidence associating HSP70 and HSP90 in the pathophysiology, clinical course, and outcome of sepsis and SIRS. Extracellular HSP is increased in sepsis associated with organ failure and mortality whereas intracellular

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HSP is repressed associated with acute inflammatory metabolic stress, immune-paralysis, hormonal dysregulation, and repressed bioenergetics and metabolism across all age groups (Briassoulis et al. 2019; Vardas et al. 2017; Papadopoulos et al. 2017; Fitrolaki et al. 2016). Accordingly, due to comprehensive relevance to pathology, an effective strategy for extracellular HSP capture and profiling would be valuable in many areas of clinical management.

In prior work, a class of peptides referred as Venceremins (Vn) were identified as high avidity ligands for bacterial HSP70 (Griffiths et al. 2011). When extended with sequences of tumor-associated antigens, hybrid Vn peptides exhibited multivalent affinity for vertebrate HSP70, HSP60, and HSP90, successfully precipitating HSP complexes directly from cell lysates, media, and tissue fluids (Knol et al. 2016; Bijnsdorp et al. 2017). We previously demonstrated that proteome profiling of Vn96-precipitated HSP complexes in the form of small extracellular vesicles could clearly distinguish breast cancer phenotype (Griffiths et al. 2017). In the current study, we extended Vn96 for direct functional proteomic analysis of extracellular HSP complexes secreted by three commonly used xenotransplant models of invasive cell phenotype: U87, a glioblastoma multiforme considered immediately invasive (Zhao et al. 2010); HELA, a cervical carcinoma with limited invasiveness (Chatterjee and Chatterjee 2001); and HEK293T, virally transformed human embryonic kidney, only tumorigenic following successive transplantations (Debeb et al. 2010). Profiling of extracellular HSP complexes following Vn96 capture revealed a comprehensive protein biomarker signature of invasive potential that may be extended to routine analysis of other cell cultures for translational development.

Methods

Culture

HELA (ATCC® ccl-2™), U87 (ATCC® HTB14™), and HEK293T (ATCC® CRL-1573™) cells were grown at 37 °C in a 5% CO₂ atmosphere using Integra CELLline Bioreactors (Hudson, NH). The 500-mL upper chamber of the bioreactor is separated by 10-kDa MWCO filters and replenished with Eagle's minimal essential medium (EMEM), as supplied by ATCC Cat# 30-2003, and supplemented with 10% fetal bovine serum. Prior to the introduction of cells into the bioreactor, the bioreactor medium was also supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. The cultures used were between 4 and 5 months old and were monitored for continued robust health. Cell growth was established in the 15-mL lower chamber containing exosome free fetal bovine serum (Exo-FBS Systems Biosciences, Mountain View CA), with supernatant samples containing the HSP material recovered on a weekly basis.

Harvest of HSP affinity complexes

The cell-free culture supernatants were first centrifuged at 14,500g. Equivalent volumes (1.8 mL) of cell culture supernatant from each of the three cell lines were individually incubated overnight at 4 °C, following addition of 50 µg Vn96 peptide (PSQGKGRGLSLRFSWGALTGEFLKL), dissolved in extraction buffer 1 ("EB1") of the ProteoExtract Subcellular Proteome Extraction kit (S-PEK; Millipore Sigma, Burlington, MA). The mixtures were then centrifuged at 4500g and the pellet washed by resuspending in 1 mL of PBS with 10 µL of protease inhibitor cocktail III (Millipore Sigma) followed by pelleting using the conditions described above. The supernatant was discarded and the recovered extracellular HSP pellet was subject to immediate analysis as described below.

Electrophoresis and blotting conditions

The HSP pellet was resuspended in 200 µL of urea sample buffer (Wubbolts et al. 2003), consisting of SDS gel loading buffer supplemented with 4 M urea and 10 µL protease inhibitor cocktail III. USB-suspended pellets were incubated at 95 °C for 5 min, briefly vortexed, and centrifuged to retain the clarified solution. Next, 25 µL of each HSP pellet were applied to 10% XT Bis-Tris Criterion precast midi gels in XT-MES running buffer (Bio-Rad, Hercules CA). Gels were run in Criterion modules (Bio-Rad) for approximately 55 min at 150 V. Following the runs, the gels were rinsed in Towbin transfer buffer (Bio-Rad) and layered onto supported nitrocellulose, blotting pads, and paper (Bio-Rad), for insertion in the Criterion blotting module. Blotting was run at 90 V for 30 min. The blot was processed with the Pierce Reversible Protein Stain Kit (ThermoFisher Scientific, Mississauga, Canada) and imaged using a Bio-Rad Chemi-Doc. The blots were next processed with Pierce Destaining Reagent (ThermoFisher Scientific). Following 30 min blocking with 5% skim milk powder, PBS with 0.075% Tween 20 (TPBS), the membranes were probed with the following antibodies: CD63 (MX-49), GAPDH (H-12), PKM (C-11), and PARP-1 (5A5) (Santa Cruz Biotechnology, Dallas TX); HMGB1 (Cell Signalling Technology, Danvers MA); PARP-1 (C2-10) (Trevigen, Gaithersburg, MD); γH2AX (Millipore Sigma, Toronto, Canada). All secondary antibodies labeled with HRP were matched with primary Ig isotype (Santa Cruz Biotechnology). Imaging was achieved with Pierce Super Signal West Dura HRP substrate (ThermoFisher Scientific) and the Chemi-Doc system (Bio-Rad).

Successive detergent extraction of HSP complexes

Differential detergent fractionation was achieved with the ProteoExtract Subcellular Proteome Extraction Kit (S-PEK),

available from Millipore Sigma (Ramsby and Makowski 1999). The extracellular HSP pellet was resuspended in 100 μ L "Extraction Buffer I" (EBI) of the S-PEK kit. EBI is a mild detergent buffer formulated to permeabilize cell membranes and solubilize cytosolic proteins. Following 1-h incubation (4 °C), the sample was centrifuged (3000g, 5 min), and the soluble portion was collected. The HSP pellet was then successively re-extracted with increasingly stringent buffers EBII through EBIV of the S-PEK kit. All steps were conducted with protease cocktail III added at 5 μ L per 100 μ L. The soluble proteins from each of the detergent extraction buffers were precipitated with acetone and, together with the remaining HSP pellet, were solubilized by heating at 95 °C in equal volumes of SDS/USB electrophoresis buffer (with or without 25 mM tris(2-carboxyethyl)phosphine, TCEP, as reducing agent). Samples were separated by SDS PAGE as described above and transferred to nitrocellulose for staining of total protein, then destained and probed with antibodies to CD63 or GAPDH.

In-gel digestion and mass spectrometry

Equivalent volumes (30 μ L) of the USB-solubilized HSP pellets from each of the three cell lines were loaded in triplicate onto 1 mm, 10% T SDS PAGE gels and resolved at 120 V for 10 min. The unstained protein bands, constituting the top ~ 1 cm of the resolving gel, were subject to in-gel tryptic digestion according to standard protocols (Shevchenko et al. 2006). The recovered peptides from each cell line were pooled and subject to LC/UV cleanup (Orton and Doucette 2013), which also quantifies recovered peptides relative to a standard curve of digested BSA protein.

Bottom-up LC-MS/MS analysis was performed by duplicate injection of each sample (0.5 μ g total protein per injection) onto a monolithic C18 column (0.1 $\times$ 150 mm, Torrance, CA), coupled to a 10- μ m New Objective PicoTip Emitter Tip (Woburn, MA). An Dionex Ultimate 3000 LC nanosystem (Bannockburn, IL) delivered a 2-h linear gradient from water + 0.1% formic acid to 35% acetonitrile into an Orbitrap Velos Pro (ThermoFisher Scientific), operating in MS mode at a resolution of 30,000 FWHM, scanning in rapid mode for MS² (66,666 Da s⁻¹, at < 0.6 Da FWHM). The Orbitrap was set to data-dependent mode (MS followed by MS/MS of the top ten peaks).

Peptides were identified using the Proteome Discoverer software, searching the human database and allowing modifications of oxidized methionine or carbamidomethylation at cysteine. Mass tolerance was set to 20 ppm (MS mode) and 0.8 Da (MS/MS mode) with two missed cleavages at a peptide false positive rate of 1%. The identified protein lists are provided as [supplementary tables](#), with the requirement of one unique peptide per protein.

Results

Western blot of extracellular HSP from equivalent volumes of bioreactor was probed with antibodies to CD63, GAPDH, and pyruvate kinase (PKM2). HSP complexes have previously been identified to contain the late endosome membrane protein CD63, as have glycolytic enzymes which are well-established markers of the secretome (Ucker et al. 2012; Østergaard et al. 2017). As seen in Fig. 1a, the CD63 signal was strong in the U87 affinity complexes, as compared with equivalent volumes from HELA and 293 T. However, equivalent signal intensities were observed for both PKM2 (Fig. 1b) and GAPDH (Fig. 1c), indicating that comparable level of HSP affinity complexes had been pelleted by Vn96 and sufficient for direct comparative proteomic analysis. These results were confirmed from proteome profiling of the samples; when equivalent volumes of the HSP pellet were loaded to SDS PAGE, in-gel digestion recovered similar quantities of peptides (U87, 10 μ g; HELA, 10 μ g; HEK293T, 7 μ g). These results therefore indicate that the Vn96 protocol recovered comparable quantities of protein from the three cell lines.

Fig. 1 Western blot analysis of equal volumes of cell-free media precipitated by Vn96 from bioreactor cultures prior to proteomic analysis. Lane 1: MW ladder; lane 2: 10% fetal bovine serum (control); lane 3: U87; lane 4: HELA; lane 5: HEK293T. **a** Western blot showing recovery of CD63 (~43 kDa) from the HSP pellet of each cell line. **b**, **c** The relative expression of PKM2 (~58 kDa) and of GAPDH (~37 kDa) are approximately constant in the three cell lines

From prior work, we observed extracellular complexes pelleted by the Vn96 affinity peptide to be resistant to detergent buffers, with key antigens retained despite washing, pelleting, and resuspension in sequentially stronger buffers (Griffiths et al. 2017). In the current study, HSP complexes were similarly assessed by successive resuspension and pelleting with buffers of increasing detergent strength. The solubilized portion of the HSP complexes were precipitated with acetone and again resolubilized with equal volumes of electrophoresis buffer. The blot was reversibly stained for total protein followed by incubation with antibodies to CD63 or GAPDH (Fig. 2). A gel image showing total proteins isolated from the bioreactor cell culture extract, including background proteins (10% fetal bovine serum), is provided as supplemental Fig. S1, which also reveals minimal cross reactivity of the CD63 antibody. Total protein stain of Fig. 2 indicated that the majority of HSP affinity complex material remained intact after resuspension in Extraction Buffer II of the subcellular protein extraction kit, a formulation intended to solubilize membrane-associated protein (lane 2 of each gel in Fig. 2). Interestingly after resuspension in buffer III (intended to solubilize nucleus-associated protein), all complexes yielded 3 strong bands of ~ 15 kDa, visible in lane 3 (Fig. 2a). These

Fig. 2 Western blot analysis of fractions from differential detergent fractionation of precipitated Vn96 HSP affinity complexes pelleted from cell-free bioreactor media of U87 (left), HELA (center), and HEK293T (right). After an initial wash in PBS, HSP pellets were extracted with Extraction Buffer I through IV (S-PEK, Millipore Sigma), and the solubilized proteins from each successive extraction are shown in lanes 1 through 4 of the blots. Lane 5 corresponds to the protein remaining in the HSP pellet, as solubilized by heating in USB buffer. **a** Shown is the total protein stain. **b** Probing with antibodies to CD63 following destaining of the blot. **c** Western blot as probed for GAPDH

bands are likely associated with histones. CD63 was also retained until the third extraction buffer in U87, while some GAPDH signal continued to be associated with the HSP pellet until solubilization in SDS/Urea buffer at 95 °C (Fig. 2c, lane 5, for all three cell lines). Though the bulk of GAPDH is released when washed with extraction buffer IV (lane 4 of Fig. 2), a smaller, though variable proportion is also released into buffer III. Appearance of GAPDH in various sub-fractions may reflect different levels of protein interaction within the extracellular HSP complex, reflecting varied moon-lighting functions of this and potentially other glycolytic enzymes, particularly during tumorigenesis (Sirover 2018).

Extracellular release of HSPs, glycolytic enzymes, and histones may be due to non-specific cell lysis rather than a regulated pathway (Parseghian and Luhrs 2006; De Maio and Vazquez 2013). Therefore, to address the potential contribution of necrosis or apoptotic activity to proteins co-isolated with HSP complexes during proteomic analysis, we analyzed Western blots of whole cell lysates from bioreactor cultures as well as HSP complexes for PARP-1 (Fig. 3) as an indicator of cell death (Duriez and Shah 1997; Chaitanya et al. 2010; De Maio and Vazquez 2013). Cleavage of PARP-1 by caspases is a hallmark of apoptosis. All parent cells expressed full length PARP-1 (~ 120 kDa band in Fig. 3a, b) with some evidence of C- and N-terminal proteolysis observed below the major 120 kDa band in HEK293T (Fig. 3, lane 2). Full length PARP-1 and N-terminal 24 kDa fragments were detected in HSP affinity complexes prepared from 293 T and HELA (Fig. 3a, lanes 5 and 6); HELA was also associated with the C-terminal 87 kDa fragment (Fig. 3b, lane 6). However, neither full length PARP-1 nor proteolytic fragments were detectable at the same exposure in U87 extracellular HSP complexes (Lane 7, Fig 3a, b). The ability to detect the 24-kDa fragment in complex with extracellular HSP is intriguing. The 87 and 24-kDa fragments are generated by caspases 3 and 7. The 24-kDa N-terminal apoptotic fragment attenuates necrotic cell death; there may be some functional significance for export. In the current context, while it was possible that non-specific sources contributed to HSP and client protein content, the most invasive phenotype U87 appeared free of such contributions and further, extracellular HSP profiling had the potential to define specific mechanisms of cell death, innate or induced. Specific content was further determined by mass spectrometry analysis.

As alternative indicators of non-specific cell lysis contributing to the extracellular HSP proteome, HSP complexes were also examined for γ H2AX and high mobility group protein 1, HMGB1 (Plesca et al. 2008; Sharma et al. 2012), with Western blots shown in Fig. 4a and b, respectively. Phosphorylation of H2AX, at Serine 139, serves as the nucleation site for the accumulation and retention of the central components of the signaling cascade initiated by DNA damage. HMGB1 is released during apoptosis, triggered by nucleosomal DNA fragmentation. HEK293T provided the strongest signal for γ H2AX and HMGB1 in association with

Fig. 3 Western blot analysis of equal volumes of whole cell lysate (cells) or Vn96 precipitated material (HSP affinity) from bioreactor cultures for evidence of apoptosis and necrosis. Lane 1 + 4, U87; lane 2 + 5, HELA; lane 3 + 6, HEK293T. Antibodies are directed to N terminus by **a** PARP antibody 5A5 or C terminus by **b** PARP antibody C2-10

extracellular HSP (Fig. 4a, b lane 3), perhaps reflective of viral transformation and genetic instability with weaker signal observed in HELA. However, neither marker was observed with extracellular HSP complexes from U87 further corroborating the absence of necrotic or apoptotic activity contributing to the subsequent proteome recovered in this cell line.

Proteomic analysis

Proteomic analysis was performed on the pelleted HSP complexes employing equivalent volumes of the three cell lines. A total of 254 unique proteins were identified from U87, which was slightly lower than the 305 proteins observed from the

HSP pellet of HEK293T, and 313 proteins for the HELA pellet. Together, these account for 590 unique proteins across the three cell lines. A Venn diagram summarizes the identified proteins common to the three cell lines (Fig. 4). Only 14% of the total identified proteins were shared across all three systems. Comparable percentages ranging from 19 to 24% were uniquely observed from each phenotype (Fig. 5).

Fig. 4 Western blot analysis of equal volumes Vn96 precipitated HSP complexes from bioreactor cultures for evidence of apoptosis and necrosis. γ -H2AX, gamma H2AX; HMGB1, high-mobility group (non-histone chromosomal)

Fig. 5 Venn diagram summarizes the proteins identified from bottom-up MS analysis of HSP complex material recovered from Vn96 affinity precipitation. 313 proteins were recovered from the HELA cell line preparation, while 303 proteins were identified from HEK293 and 254 protein from the HELA preparation. Full details of the identified proteins are provided as supplementary Tables S1, S2, and S3

Relative protein abundance across the three test samples was ranked by normalized peptide spectral matches, PSMs (Liu et al. 2004). Total PSM counts were within 18% across the three test samples (U87 = 3875; HELA = 4459; HEK293T = 3775). The normalized PSM count for a given protein is obtained by multiplying the raw PSM score by the maximum total PSM observed (i.e., 4459, for HELA), divided by the total PSM count for the respective cell line. Confirming our observations made with breast cancer cell models (Griffiths et al. 2017), casual appraisal of the most abundant proteins co-isolated in the extracellular HSP complex confirms the presence of several clinical biomarkers of established or emerging significance in tumorigenesis and malignancy (see supplemental Table S4). Detailed listings of all proteins and peptides identified across the three cell lines, together with their respective PSM counts, are tabulated and provided (Online Resource Table S1, U87; S2, HELA; S3, HEK293T). Some differences in the identified proteins are reflected of different tissue origin. A specific example is provided as Supplemental Fig. S2, demonstrating the variable abundance of Tenascin C across the three different cell lines.

As primary targets of the Vn96 affinity peptide, numerous HSPs were prominently detected by bottom-up MS in the extracellular HSP complex pelleted from each cell line (Table 1). This is reflective of increased expression and export of HSPs for enhanced cell survival during dysregulation (Niforou et al. 2014; Rodvold et al. 2017). Analysis of relative expression of HSP content and isoforms may be informative in prognosis (Kakkar et al. 2014). As shown in Table 1, the most abundant canonical HSPs included HSP90 isoforms α and β , as well as HSP70-5 (GRP78). Cell surface and extracellular HSP90 α is associated with tissue migration and malignancy (O'Brien et al. 2014; Crowe et al. 2017). Interestingly, the virally transformed HEK293T was most prominent in extracellular HSP90 α . Prominent representation by GRP78 in extracellular HSP complexes is significant with regard to a multi-modal influence on tumorigenesis and as the

hub of the translational ER stress response (Ghaderi et al. 2018; Steiner et al. 2017; Thomaidou et al. 2018). Interestingly, the clearest differentiation of HSP content was seen for distribution of inducible HSP70-1/2; while abundant in HEK293T, PSMs were not detected in equivalent eHSP of U87. Differentiation in HSP70 isoform representation may reflect specific features of tumorigenic phenotype and genetic stability, as recently determined by Chiosis group (Wang et al. 2019). HSP60 was prominent in U87 and choriocarcinoma HELA and may be ratiometric of invasive phenotype (Caruso Bavisotto et al. 2018) and inflammatory response (Kato and Svensson 2015). HSP60 is also significant by association with extrachromosomal histones, also prominently co-abundant in the extracellular HSP complexes as indicated below (Khan et al. 1998; Kobiyama et al. 2013).

Other chaperones were prominent in extracellular HSP complexes (Table 2). All three sources contained abundant endoplasmic reticulum ATPase p97 (VCP) central to invasive phenotype (Cui et al. 2015; Fu et al. 2016) and modulation of proteotoxicity (Vekaria et al. 2016; Gugliotta et al. 2017). Protein disulfide isomerases P4HB and PDIA3 as well as peptidyl prolyl isomerases were also significant as exported chaperone components (Perrucci et al. 2015; Lee and Lee 2017; Zou et al. 2017; Stifani 2018).

HSP complexes were also richly associated with glycolytic proteins, significant in the current context as an exported index of tumor progression and prognosis (Ziegler et al. 2016). The comparable distribution suggests universal advantage of exporting enzymes with secondary functions such as cell adhesion and manipulation of extracellular matrices (Snaebjornsson and Schulze 2018). Comparable abundance was noted for pyruvate kinase isoform 2 (PKM2; Dong et al. 2016), fructose-bisphosphate aldolase (Klinke et al. 2014; Grandjean et al. 2016), lactate dehydrogenase (Jurisic et al. 2015), Fructose 1, 6 bisphosphatase (Dai et al. 2017), GAPDH (Sirover 2018), enolase (Principe et al. 2015; Capello et al. 2016), and phosphoglycerate kinase (Wang et al. 2010) (Table 3).

Extracellular HSP complexes were notably rich in histone content (suspect bands in total protein stain of Fig. 2a), significant in differential diagnosis of cancer and inflammatory disease (Chen et al. 2014; Szatmary et al. 2018). Significantly histone H2AX was only associated with HEK293T, corroborating Western blot results (Fig. 3). H2AX isoforms are used for staging and drug response (Kuo and Yang 2008; Yu et al. 2006; Palla et al. 2017). Extracellular H2A and H2B are believed to be the source of circulating tumor DNA (Marsman et al. 2016) which suggests genetic material may be associated with HSP affinity complexes (Table 4).

Other discrete features were noted among protein categories such as singular representation by Lamins, amyloid, and extracellular matrix proteins, all well as established extracellular biomarkers of prognostic value (supplemental Table S4)

Table 1 Relative abundance of canonical heat shock proteins identified from HSP pellets

Protein name	ID	Normalized PSM count per cell type		
		U87	HELA	HEK293T
HSP70-5	16507237	49	26	46
HSP60	31542947	41	31	17
HSP 90- α	154146191	16	35	61
HSP90B	4507677	9	13	26
HSP70-8	5729877	8	17	0
HSP 90- β	20149594	6	37	47
HSP70-2	13676857	0	0	12
HSP70-1/2	167466173	0	10	48

Table 2 Relative abundance of chaperone proteins identified from HSP pellets

Protein name	ID	Normalized PSM count per cell type		
		U87	HELA	HEK293T
Endoplasmic reticulum ATPase	6005942	55	49	70
Protein disulfide-isomerase P4HB	20070125	21	17	22
Protein disulfide-isomerase A3	397487867	18	18	0
Peptidyl-prolyl isomerase A	10863927	17	20	31
Peptidyl-prolyl isomerase B	4758950	17	43	19
Calmodulin	4502549	16	4	0
Protein disulfide-isomerase A6	544186040	7	15	14
Stress-induced-phosphoprotein 1	544063449	3	12	12
Cdc37 HSP90 co-chaperone	5901922	2	2	5

Discussion

Functional proteome analysis of extracellular heat shock protein (HSP) complexes reveals a substantial number of protein markers indicative of tissue origin and with potential clinical relevance. While some content may originate from non-specific cell lysis, profiling of PARP-1 fragments and γ H2AX indicated that extracellular release of HSPs in complex with histones can occur in the absence of necrosis or apoptotic activity. Further, ability to simultaneously profile specific biomarkers permits contextual relevance.

Histones were abundant in HSP complexes. The capability to capture and analyze extracellular histone is clinically significant in tumor progression and inflammatory disease (Marsman et al. 2016; Jiang et al. 2017). Histone and HSP60 association may provide a mechanism for plasma membrane translocation of chaperone export without endosomal involvement in an ATP-independent manner (Hariton-Gazal et al. 2003; Zannikou et al. 2016). Cells in proliferative and inflammatory phenotypes engage in metabolic pathways favoring biosynthesis, with certain glycolytic enzymes universally bundled with extracellular HSP complexes, may also export non-enzymatic “moonlighting

functions” including cell adhesion and extracellular matrix modification (Min et al. 2016; Jeffery 2018).

As the most invasive phenotype of the three studied cell models examined, extracellular HSP complex of U87 glioblastoma cell line was notable for association with chitinase 3 like protein (CHI3L1 also known as YKL-40). CHI3L1 has emerged as a significant biomarker of invasive phenotype (Jeet et al. 2014; Hamilton et al. 2015; Chiang et al. 2015; Chen et al. 2017a, b; Gandhi et al. 2018; Libreros and Iragavarapu-Charyulu 2015; Cavassani et al. 2018; Cohen et al. 2017). It is also a circulatory marker for staging inflammatory and neurodegenerative disease (Ściborski et al. 2018; Wang et al. 2018; Llorens et al. 2017; Hall et al. 2018). Due to potential for comorbidity, ability to detect CHI3L1 in context with other biomarkers described here may be useful in differential diagnosis for a variety of critical and clinical care scenarios. Potentially significant in this regard is singular representation of Lamin C in U87. Lamin A/C isoforms are biomarkers of mechanophenotype providing end-point signatures of disease onset or progression (González-Cruz et al. 2018). High-abundance extracellular lamins are clearly and discretely isolated by extracellular HSP complexes secreted by glioblastoma model U87 (Supplemental Table S4). In parallel to

Table 3 Relative abundance of metabolic enzymes identified from HSP pellets

Protein name	ID	Normalized PSM count per cell type		
		U87	HELA	HEK293T
Pyruvate kinase isozymes M1/M2	33286418	67	44	41
Glyceraldehyde-3-phosphate DH	7669492	53	73	64
Fructose-bisphosphate aldolase A	4557305	31	24	25
Enolase alpha	4503571	30	47	93
Phosphoglycerate kinase 1	4505763	28	51	58
Triosephosphate isomerase	4507645	21	18	27
Lactate dehydrogenase A	5031857	13	15	17
Lactate dehydrogenase B	4557032	9	9	11
Transketolase	4507521	0	47	0

Table 4 Histones and high mobility group proteins identified in the HSP pellets, along with their relative abundance

Protein name	ID	Normalized PSM count per cell type		
		U87	HELA	HEK293T
Histone H2B type 1	4504257	104	54	105
Histone H3	4504281	59	21	54
Histone cluster 1 (H2aj)	10800144	43	0	79
Histone H2A type 1	4504245	40	15	77
Histone H2A type 2	28195394	21	5	37
Histone H4	4504301	20	12	31
High mobility group HMG-I/HMG-Ya	22208967	18	6	0
Histone H1.2	4885375	17	12	30
High mobility group HMG-I/HMG-Yb	4504433	16	6	12
High mobility group HMGI-C	4504431	16	0	13
Histone H2A.Z	4504255	14	9	46
Histone H1.5	4885381	13	4	0
Histone macro-H2A.1	20336746	13	8	0
High mobility group protein B1 X6	530402322	0	9	0
Histone H2A.x	4504253	0	0	58
Histone H2B type 2	4504277	0	0	104
Histone H2B type 3	28173554	0	0	78
Histone H2B type 1	4504257	104	54	105

CHI3L1, extracellular HSP complexes of glioblastoma U87 were also prominently associated with amyloid proteins, which are of prognostic importance in glioblastoma (Pandey et al. 2016; Moir et al. 2018; Chen et al. 2018; Lim et al. 2014). Amyloid protein derivatives are emerging as significant factors of chronic neurodegenerative inflammation (Moir et al. 2018), possibly due to pathogenic involvement such as HSV, which also express constitutively extracellular HSPs (Gober et al. 2005) and are associated with malignant glioma (Strojanik et al. 2017). Context-dependent identification of extracellular HSP may provide further inputs for prognosis and treatment options.

With consideration to the potential significance of the HSP affinity platform, secreted material is reflective of phenotype in situ (Chen et al. 2008; Klinka 2016). Therefore, any opportunity to collect multiple secreted biomarkers afforded by robust HSP affinity enrichment is significant for translation to targeted clinical assays (Diamandis 2012). With regard to cumulative significance of extracellular HSP, in a survey of over 200 cancer cell cultures and primary isolates, Chiosis' group found that genetic stress rather than acute toxin treatment induced the most dramatic change in HSP profile as a stress-specific fingerprint (Wang et al. 2019). For example, under toxic stress, HSP60 was recruited to the HSP90 chaperome network while genetic stress enlisted HSP70 isoforms. These observations may have been reflected in our current comparative proteomic analysis of invasive inflammatory phenotype of U87 and the genetic instability of HEK293T caused by viral transformation. The inducible isoform HSP70-1/2 is abundant in HEK293T, but absent in

equivalent material from U87 (Table 1). Genetic instability may be further reflected by H2AX prominence.

Extracellular HSP profiling may be of critical importance in oncology. During tumorigenesis for example, HSPs may form stable overlapping high affinity complexes that Chiosis has identified as the epichaperome (Tai et al. 2016). Type 1 cancers with codependent chaperomes were identified as vulnerable to single HSP inhibitors as a central lethality regardless of tissue origin or stage. In contrast, type 2 cancers were determined to mitigate inhibition of one HSP node by functional substitution within another, perhaps explaining variable drug response in otherwise similar clinical presentations. It was further reported that integrated HSP chaperomes are typical of other pathologies such as neurodegenerative disease (Brehme et al. 2014). Extracellular HSP categorization may thus provide realistic examples of precision medicine where target, biomarker, and therapies can be correlated and integrated.

We report here that *exported* chaperome material may be captured for functional proteomic analysis using multivalent high avidity HSP affinity peptides. Our approach not only reveals exported chaperome components, but also a broad range of clinically relevant biomarkers in a detergent resistant matrix. The multiplexed biomarkers indicate additional functionality in perpetuating inflammatory/invasive phenotype or survival during genotoxic stress with potential for differential diagnosis. In this approach, extracellular HSP complexes are collected by direct incubation, low speed centrifugation, and an option for stringent washing to remove non-specific

material. The method is robust and rapid, and requires no ultracentrifugation. Although overlap with HSP surface-associated exosomes is anticipated, the approach does not rely on specific categorization of the collected material as being extracellular vesicles, with overlap in nomenclature (Zijlstra and Di Vizio 2018). Extracellular HSP complexes will also reflect cell surface presentation and thus targets for therapy that minimize collateral damage. Drugs that target cell surface HSPs are likely to become a common feature in oncology (Crouch et al. 2017; Shevtsov et al. 2018). HSP inhibitors are recognized as having substantial potential (Wang et al. 2019; Neckers et al. 2018). Furthermore, extracellular HSP profiles will allow differential diagnosis of other critical pathologies such as trauma, hyperthermia, and sepsis (Briassoulis et al. 2014, 2015; Briassoulis et al. 2014; Vardas et al. 2014). Candidates for therapy might be identified and then monitored by methods described here, possibly completing a loop in precision medicine.

Although HSP affinity complexes will include vesicular material suggested by CD63 in U87, canonical markers of EVs are variable with overlap in nomenclature (Zijlstra and Di Vizio 2018). Traditional TEM of HSP affinity material reveals amorphous structures with little resolution (Ghosh et al. 2014). However, when sectioned in resin, diameters are generally smaller than EVs with discrete particles between 10 and 50 nm in diameter (Griffiths et al. 2017). Recently, particles within this size range have been reported as being clinically significant and possibly overlooked due to challenges in purification (Zhang et al. 2018, 2019). HSP affinity proteomes may also reflect cell surface presentation and thus targets for therapy that minimize collateral damage. Indeed, drugs that target cell surface HSPs are likely to become a common feature in oncology (Crouch et al. 2017; Shevtsov et al. 2018). Accordingly, HSP affinity may be of value in staging and response that inform therapeutic options (Hadizadeh Esfahani et al. 2018) and reduce unnecessary treatment.

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