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Extracellular heat shock proteins and cancer: New perspectives

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ABSTRACT

Heat shock proteins (HSPs) are a large family of molecular chaperones aberrantly expressed in cancer. The expression of HSPs in tumor cells has been shown to be implicated in the regulation of apoptosis, immune responses, angiogenesis and metastasis. Given that extracellular vesicles (EVs) can serve as potential source for the discovery of clinically useful biomarkers and therapeutic targets, it is of particular interest to study proteomic profiling of HSPs in EVs derived from various biological fluids of cancer patients. Furthermore, a divergent expression of circulating microRNAs (miRNAs) in patient samples has opened new opportunities in exploiting miRNAs as diagnostic tools. Herein, we address the current literature on the expression of extracellular HSPs with particular interest in HSPs in EVs derived from various biological fluids of cancer patients and different types of immune cells as promising targets for identification of clinical biomarkers of cancer. We also discuss the emerging role of miRNAs in HSP regulation for the discovery of blood-based biomarkers of cancer. We outline the importance of understanding relationships between various HSP networks and co-chaperones and propose the model for identification of HSP signatures in cancer. Elucidating the role of HSPs in EVs from the proteomic and miRNAs perspectives may provide new opportunities for the discovery of novel biomarkers of cancer.

Introduction

Heat shock proteins (HSPs) are evolutionally conserved and ubiquitously expressed molecular chaperones abundantly present in cancer [1–3]. Mammalian HSPs are divided into families, namely HSP70/HSPA, HSP90/HSPC, HSP40/DNAJ, HSPB, HSP110/HSPH and chaperonins [4]. Several research groups showed that naturally occurring antitumor agents such as geldanamycin and radicicol exert their action by inhibiting ATPase activity of HSP90 chaperone [5–8]. This finding has led to the development of various HSP90 inhibitors (Table 1). Subsequently, the ability of HSP70 to promote immune responses stimulated the development of HSP-based cancer vaccines [9,10]. Currently, various HSP-based therapies and several HSP-based biomarkers are assessed in clinical trials (Table 1).

Researchers studying the role of HSPs in cancer proposed that malignant cells are "addicted to chaperones", particularly emphasizing the importance of three well-studied HSPs such as HSP70, HSP27 and HSP90 in tumor development [11,12]. Indeed, overexpression of HSPs provides selective advantage to malignant cells by inhibiting apoptosis, promoting tumor metastasis and regulating immune responses [1,13–16]. Considering critical role of HSPs in cancer, several studies have proposed HSPs as potential biomarkers for cancer diagnosis [17,18]. Controversially, researchers studying the HSP expression in various types of cancers have observed that HSPs are overexpressed in various types of tumors [1]. Thus, they concluded that HSPs might not be informative as tissue- restricted molecular markers, but rather can be used to identify the degree of differentiation and aggressiveness in some cancers [1]. Recent studies using gene expression have shown that HSPs can be used

Abbreviations: HSP, heat shock proteins; miRNA, microRNA; MS, mass spectrometry; NSCLC, non-small cell lung cancer; AML, acute myeloid leukemia; ESCC, esophageal squamous cell carcinoma; CTC, circulating tumor cells; SCCNH, squamous cell carcinoma of the head and neck; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; DICS, ductal carcinoma *in situ*; CUSA, cavitron ultrasonic surgical aspirator; CHIP, C-terminus of HSP70 interacting protein; HOP, HSP70-HSP90 organizing protein; BAG, BCL2-associated athanogene; ccRCC, clear cell renal cell carcinoma; HL, Hodgkin lymphoma; DLBCL, Diffuse large B-cell lymphoma.

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Table 1
HSPs in cancer clinical trials.

Intervention	HSPs	Type of cancer	Study	Refs.
HSP inhibitors				
AUY922 (Luminespib)	HSP90	• Advanced or metastatic adenocarcinoma of the stomach or gastroesophageal junction	Phase Ib	[23]
		• Myeloproliferative Neoplasms	Phase II	[24]
		• Advanced solid tumors	Phase I	[25]
		• Lymphoma	Phase II	[26]
		• Non-small cell lung cancer (NSCLC)	PhaseI/II	[27,28]
		• Gastrointestinal Stromal Tumor	Phase II	[29]
		• Advanced Anaplastic lymphoma kinase (ALK)-positive NSCLC	Phase II	[30]
		• NSCLC	Phase II	[31]
		• Breast cancer	Phase I/II	[32]
		• Multiple myeloma	Phase I/II	[33]
		• Metastatic colorectal cancer	Phase I	[34]
HS-201	HSP90	Solid tumor	Phase I	[35]
HS-196	HSP90	Solid tumor	Phase I	[36]
STA-9090 (Ganetespib)	HSP90	• SCLC	Phase II	[37]
		• Acute Myeloid Leukemia, Acute Lymphoblastic Leukemia and Blast-phase Chronic Myelogenous Leukemia	Phase I	[38]
		• Acute Myeloid Leukemia; High Risk Myelodysplastic syndrome	Phase I/II	[39]
		• Ocular melanoma	Phase II	[40]
		• Prostate cancer	Phase II	[41]
		• NSCLC	Phase II	[42]
		• Solid tumors	Phase I	[43,44]
		• Hematologic malignancies	Phase I	[45]
		• Ovarian cancer	Phase II	[46]
		• Platinum-resistant ovarian cancer	Phase I/II	[47]
		• Melanoma	Phase II	[48]
		• Metastatic human epidermal growth factor receptor 2-positive breast cancer	Phase I	[49]
		• Breast cancer	Phase II	[50]
		• Advanced Gastrointestinal Carcinomas; Non-Squamous NSCLC; Urothelial Carcinomas; Sarcomas	Phase I	[51]
		• Malignant Peripheral Nerve Sheath Tumors	Phase I/II	[52]
HSP990	HSP90	Advanced solid tumors	Phase I	[53,54]
TAS-116	HSP90	Advanced solid tumors	Phase I	[55]
KOS-953 (Tanespimycin)	HSP90	Multiple myeloma	Phase III	[56]
CNF2024 (BIIB021)	HSP90	B-cell Chronic Lymphocytic Leukemia	Phase I	[57]
NVP-BEP800	HSP90	Acute T Lymphoblastic Leukemia; Acute B Lymphoblastic Leukemia	Pilot	[58]
AT13387 (Onalespib)	HSP90	• Prostate cancer	Phase I/II	[59]
		• Advanced solid tumor; Recurrent ovarian, fallopian tube, primary peritoneal or triple negative breast cancer	Phase I	[60]
		• Solid tumors	Phase I	[61]
		• Non-small cell lung cancer	Phase I/II	[62]
		• Advanced triple negative breast cancer	Phase I	[63]
		• Solid tumors	Phase I	[64]
IPI-504 (Retaspimycin)	HSP90	• Non-small cell lung cancer	Phase II	[65]
		• Metastatic melanoma	Phase II	[66]
		• Advanced breast cancer	Phase I/II	[67]
Alvespimycin	HSP90	• Relapsed chronic lymphocytic leukemia; small lymphocytic lymphoma; B-cell prolymphocytic leukemia	Phase I	[68]
		• Lymphoma; Small Intestine Cancer; Solid tumors	Phase I	[69]
Debio 0932	HSP90	• Advanced solid tumors; Lymphoma	Phase I	[70]
		• Non-small cell lung cancer	Phase I	[71]
IPI-493	HSP90	Hematologic malignancies	Phase I	[72]
PU-H71	HSP90	Solid tumors; Non-Hodgkin's Lymphoma	Phase I	[73]
DS-2248	HSP90	Advanced solid tumors	Phase I	[74]
MPC-3100	HSP90	Refractory or relapsed cancer	Phase I	[75]
CNF1010	HSP90	B-cell positive chronic lymphocytic leukemia	Phase I	[76]
SNX-5422	HSP90	• Refractory solid tumor; Non-Hodgkin's lymphoma	Phase I	[77]
		• Hematologic malignancies	Phase I	[78]
		• Neuroendocrine tumors	Phase I	[79]
		• Lung adenocarcinoma	Phase I	[80]
		• Chronic lymphocytic leukemia	Phase I	[81,82]
Minnelide	HSP70	• Acute Myeloid Leukemia	Phase I	[83]
OGX-427	HSP27	• Prostate cancer; Ovarian cancer; Breast cancer; bladder cancer; NSCLC	Phase I	[84,85]
		• Bladder cancer	Phase I	[86]
		• Castration resistant prostate cancer	Phase II	[87]

(*continued on next page*)

Table 1 (*continued*)

Intervention	HSPs	Type of cancer	Study	Refs.
HSP-based vaccines				
HSP70 vaccine	HSP70	Chronic Myelogenous Leukemia	Phase I	[88]
HSP70 vaccine	HSP70	Breast Neoplasms	Phase I/II	[89]
HSPPC-96 vaccine (Vitespen)	HSP90B1 (gp96)	• High-grade glioma	Phase I	[90]
		• Recurrent or progressive high-grade glioma	Phase I/II	[91]
		• Malignant melanoma	Phase III	[92]
		• Recurrent glioblastoma	Phase II	[93]
		• Lymphoma	Phase II	[94]
		• Pancreatic cancer	Phase I	[95]
		• Recurrent soft tissue sarcoma	Phase II	[96]
		• Liver cancer	Phase II/III	[97]
		• Glioblastoma	Phase II	[98,101]
		• Liver cancer; Pancreatic adenocarcinoma	Phase I/II	[99]
		• Gastric carcinoma	Phase I	[100]
HSP110-gp100 chaperone complex vaccine	HSP110	Advanced melanoma	Phase I	[102]
HspE7 (SGN-00101)	HSP65*	Cervical intraepithelial neoplasia	Phase II	[103]
pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine	HSP70	Cervical cancer	Phase I/II	[104]
Gp100 fused with OVA BiP and HSP70	HSP70	Melanoma	Phase I	[105]
HSP-based prognostic biomarkers				
HSP110DE9	HSP110	Colorectal cancer	Pilot	[106]
HSP-based predictive biomarkers				
HSP27	HSP27	Androgen-independent prostate cancer	Pilot	[107]
HSP90	HSP90	Peritoneal carcinomatosis	Pilot	[108]
HSP70	HSP70	Melanoma	Phase I	[109]

* derived from *Mycobacterium bovis* Bacillus Calmette-Guerin.

as prognostic markers to predict clinical outcome of breast cancer patients [19]. Furthermore, the discovery of the role of extracellular vesicles (EVs) in transferring proteomic and genetic information (mRNAs, microRNAs and DNA) and identification of HSPs in EVs have opened new opportunities and challenges for determining clinical biomarkers of cancer [20].

EVs are phospholipid bilayer- encapsulated particles released by many types of cells [21]. Advances in mass spectrometry (MS) provided the opportunity to identify and quantify thousands of EV cargo proteins, allowing for the search of potential biomarkers in different types of cancer (reviewed in [22]).

The main focus of this review is extracellular HSPs and microRNAs regulating HSPs in EVs as potential biomarkers for cancer diagnosis. We discuss expression of various HSP members in EVs derived from different biological fluids and patient samples and their use as biomarkers for cancer diagnosis. We will also provide novel perspective of using HSP profiling of EVs released by immune cells as clinical biomarkers and potential targets for investigational therapies. This review may help to better understand the role of HSP chaperones in EVs and their clinical importance for the identification of new cancer biomarkers and therapeutic targets.

Membrane-bound and secreted forms of HSPs in cancer

Plasma membrane plays an important role in HSP expression and localization [110]. Several studies demonstrated that heat stress or membrane fluidizers such as benzyl alcohol and heptanol induce changes in membrane fluidity and microdomain reorganization leading to subsequent activation of heat shock genes [111–113]. Noticeably, heat stress resulted in membrane hyperfluidization and membrane rearrangements in leukemia cells [114]. Dempsey and co-workers showed that membrane fluidizers affect the HSPs localization causing decrease in intracellular and increase in surface HSP60 and HSP70 [115]. In addition, HSP70-membrane positive tumors showed to contain globotriaoslyceramide allowing for the anchorage of HSP70 in plasma membrane [116]. HSP70-membrane interaction is also showed to be mediated by HSP70 binding to phosphatidylserine [117]. Since HSP70 released into extracellular space via endolysosomal system, elevated level HSP70 in lysosomes showed to stabilize lysosomal membranes of cancer cells by regulating sphingolipid catabolism [110,118–122].

Surface expression of HSPs often indicates highly aggressive tumors [14,110,123]. In this regard, many members of HSP family have been found in extracellular space or on the cell membranes- collectively called extracellular HSPs [124–131]. High expression of extracellular HSPs showed to correlate with increased cell proliferation, cancer stage and poor clinical outcome suggesting potential use of HSP expression in cancer diagnosis [110,123,132]. Studies assessing extracellular HSPs as cancer biomarkers are summarized in Table 2. A clinical prospective pilot study conducted by Gobbo and colleagues showed that concentration of HSP70-positive exosomes in plasma of lung and breast cancer patients was significantly higher compared to healthy volunteers [133]. Moreover, plasma-derived HSP70-positive exosomes showed to be better in discriminating metastatic from non-metastatic patients than circulating tumor cells (CTC) [133]. Campanella and co-workers showed that HSP60 level in plasma-derived exosomes was significantly higher in patients with colorectal adenocarcinoma than in healthy controls[134]. Elevated expression of HSP70 was found in plasma-derived exosomes of stage IV melanoma patients [135]. Several studies showed that HSPs expression in blood could discriminate between patients with early and late stage of cancer, suggesting the potential use of HSPs in early detection of cancer (Table 2) [136–139].

HSPs in extracellular vesicles of cancer patients

EVs have been shown to be implicated in various stages of cancer progression including immune suppression, epithelial-mesenchymal transition, angiogenesis, proliferation and metastasis [21]. Several researchers have used MS proteomic analysis to determine and characterize EV proteome in various cancer tissues and biofluids [174–179]. However, no previous study, has analyzed the HSP profile in EVs derived from clinical samples of cancer patients. For this review, we closely looked at the expression of HSPs in EVs from different biological fluids obtained from various MS studies, which have been summarized in

Table 2
Selection of studies assessing extracellular HSPs in patient samples.

HSPs	Cancer type	Sample	Findings	Refs
HSP70	NSCLC	Serum	Significantly higher expression of serum HSP70 compared to healthy volunteers. Positive correlation between serum HSP70 expression and tumor volume	[131]
			Elevated level of antibodies to HSP70 in cancer patients than in healthy controls	[140]
	SCLC		Serum HSP70 was significantly higher in SCLC patients than in healthy individuals and correlated with poor clinical outcome	[141]
			High level of serum HSP70 compared to controls. Serum HSP70 showed to correlate with stage of the disease	[142]
	Lung cancer		High expression of HSP70 in serum increased the risk of developing lung cancer in Japanese males	[143]
	Colorectal cancer		High level of soluble HSP70 associated with poor survival	[144]
			Significant increase in serum HSP70 with the stage of the disease. High baseline serum HSP70 associated with poor survival	[145]
	Breast cancer		sHSP70 was significantly higher in breast cancer patients than in healthy individuals. HSP70 level of >2.41 ng/ml cutoff was used to predict breast cancer with >90% specificity and sensitivity	[146]
	Breast, colon, colorectal cancers; GIST		Significantly higher serum HSP70 level than in normal samples	[147]
	Pancreatic cancer		sHSP70 level was significantly higher in patients with pancreatic cancer compared to patients with chronic pancreatitis and healthy volunteers. The sensitivity and specificity for discriminating cancer patients from healthy controls were 74% and 90%, respectively	[148]
	HCC		Significantly higher level of serum HSP70 in HCC patients than in control groups. High level of HSP70 was found in cohorts of patients with liver cirrhosis, who eventually developed HCC	[149]
	ESCC		Significantly higher level of serum HSP70 autoantibody in patients with ESCC than in colon, gastric cancer patients and healthy controls	[150]
	Head and neck cancer; Lung cancer; Colorectal cancer; Pancreatic cancer; Glioblastoma; Hematologic malignancies		Significantly higher serum HSP70 in cancer patients compared to healthy volunteers	
	NSCLC	Plasma	High level of post-therapeutic circulating HSP70 associated with improved response to radiochemotherapy.	[151]
	AML; MDS; ALL		High level of circulating HSP70 associated with significantly shorter overall survival	[152]
	Breast cancer; NSCLC	Plasma-derived exosomes	HSP70-positive tumor-derived exosomes showed to be better at predicting cancer dissemination than CTC and showed to correlate with the disease status and response to therapy.	**[133]**
	Melanoma		Statistically higher level of HSP70 in exosomes isolated from melanoma subjects with stage IV melanoma compared to healthy controls	[135]
	Colon cancer; Lower rectal cancer; Gastric cancer; LSCC	Patient tissue	mHSP70 expression showed to be associated with poor outcome in patients with lower rectal and squamous cell carcinoma of the lungs and positive clinical outcome in patients with colon and gastric cancers	[132]
	Colorectal cancer; AML; Lung cancer; Neuronal tumors; Pancreatic cancer		mHSP70 expression was found in leukemic blasts of AML patients and freshly isolated biopsies from patients with colorectal, lung, neuronal and pancreatic tumors. No surface HSP70 expression was found on normal tissues and bone marrow of healthy donors.	[153]
	Melanoma		HSP70-membrane positive phenotype was found in melanoma metastases	[154]
	SCCHN	Tumor biopsy and serum	Significantly higher expression of mHSP70 in tumor biopsy than in reference tissue. Significantly higher expression of soluble HSP70 in SCCHN than in healthy controls. Elevated level of mHSP70 on tumors associated with high soluble HSP70 in patients' serum. Prior therapy, soluble HSP70 showed to correlate with tumor volume.	[155]
	AML	Aspirated bone marrow cells	Elevated HSP70-membrane expression associated with poor patient prognosis	[156]
	Breast and pulmonary cancers	Urine-derived exosomes	Elevated concentration of HSP70-positive exosomes in cancer patients compared to healthy volunteers	[157]
HSP90	NSCLC	Serum	No significant difference was found between serum antibodies to HSP90 of cancer patients compared to healthy controls	[140]
	SCLC; LAC; LSCC		Serum HSP90AB1/HSP90β was significantly increased in patients compared to healthy controls and significantly associated with grade and stage of cancer	[158, 159]
	Melanoma		Serum HSP90 significantly higher than in healthy controls. No correlation was found between the level of serum HSP90, patient survival and response to chemotherapy	[160]
	HCC		Significantly elevated level of serum HSP90 in cancer patients compared to healthy individuals	[161]
	Colorectal cancer		Serum HSP90AA1/HSP90α expression significantly higher in cancer patients compared to healthy volunteers	[162]
	AML		Significantly higher serum HSP90AA1/HSP90α expression compared to healthy controls.	[163]
	Ductal and lobular breast tumors		No significant association was found between the level of serum HSP90 and the severity of the lesion in patients with ductal and lobular breast tumors	[164]
	NSCLC; SCLC	Plasma	Plasma HSP90AA1/HSP90α level was significantly higher in cancer patients than in healthy volunteers. Patients with advanced stages had higher plasma HSP90 expression than patients in early stages. Significant difference was found in the level of plasma HSP90 before and after surgery.	[138]

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Table 2 (*continued*)

HSPs	Cancer type	Sample	Findings	Refs
	Liver cancer		Level of plasma HSP90AA1/HSP90α could discriminate between patients with liver cancer and control groups, diagnose early-stage liver cancer with >90% sensitivity and specificity	[137]
	Colorectal cancer		Significantly elevated level of plasma HSP90AA1/HSP90α in patients with colorectal cancer compared to healthy volunteers. Significantly higher level of plasma HSP90α in late stage than in early stage of cancer	[139]
	Melanoma	Plasma-derived exosomes	HSP90 isoform was found in exosomes of 70% patients with stage IV melanoma	[135]
HSPB	Gynecologic cancers	Serum	Significantly higher number of patients had serum antibodies to HSPB1/HSP27 compared to patients with benign lesions	[165]
	Ovarian cancer		Significantly elevated level of anti-HSP27/HSPB1 antibodies in patients than in healthy women. Higher level of anti-HSP27 antibodies associated with less advanced stage of cancer	[166]
	Breast cancer		HSPB1/HSP27 level was up-regulated in breast cancer patients	[167]
			Significantly higher level of serum HSPB1/HSP27 in breast cancer patients than in healthy volunteers	[168]
	Epithelial ovarian cancer		Elevated sHSP27 level in patients with peritoneal metastases. The serum level of HSP27 significantly decreased following chemotherapy in patients with peritoneal metastases.	[169]
	Gynecologic cancers	Serum and serum-derived exosomes	Significantly higher level of sHSPB5/CRYAB in endometrial cancer patients compared to endometriosis patients. No significant difference in HSPB5, HSPB6/HSP20, HSPB8/HSP22 expression was found in serum-derived exosomes	[170]
HSP40	SCLC	Serum	Significantly higher level of autoantibodies to HSP40 in serum was found in lung cancer patients than in healthy controls	[171]
Chaperonins	Ovarian cancer	Serum	No significant difference in the level of antibodies to HSPD1/HSP60 was found between patients with ovarian cancer and healthy women. Significantly higher concentration of antibodies to HSPD1/HSP60 was found in early stages of cancer than in control groups	[172]
	Colorectal cancer	Serum	Elevated level of sHSP60 in colorectal cancer patients compared to controls. HSP60 level in serum showed to be more specific for late-stage colorectal cancer	[136]
	Breast cancer; DCIS	Serum	Autoantibodies to HSP60 were found in patients with early breast cancer and DCIS compared to healthy individuals.	[173]
	Colorectal adenocarcinoma	Plasma-derived exosomes	The level of HSP60 in exosomes was significantly higher in patients before surgery than in patients after surgery and in healthy controls	[134]

NSCLC, non-small cell lung cancer; mHSP70, membrane HSP70; sHSP70, serum HSP70; AML, acute myeloid leukemia; MSD, Myelodysplastic syndrome; ALL, Acute lymphoblastic leukemia; SCLC, small cell lung cancer; ESCC, esophageal squamous cell carcinoma; CTC, circulating tumor cells; SCCHN, squamous cell carcinoma of the head and neck; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; DCIS, ductal carcinoma *in situ;* LSCC, Lung squamous cell carcinoma; LAC, adenocarcinoma of the lung

Table 3. We have also included in the analysis co-chaperones involved in HSP70 and HSP90 functional cycles. HSP profile in EVs seems to vary in different types of cancer and biological fluids. Urine-derived EVs showed to contain members from different HSP families as well as various HSP70 and HSP90 co-chaperones and might be a promising liquid biopsy for the identification of noninvasive HSP-based biomarkers. However, it is important to note that data generated by MS has a lot of missing values and, therefore, the absence of the protein expression in a sample may not reflect the actual HSP proteome composition in EVs and may occur due to the challenges in analyzing different types of liquid biopsies by MS-based proteomics and differences in EV isolation methods discussed later in this section (reviewed in [180])[181].

In search of HSP-based biomarkers in EVs of cancer patients, it is important to understand relationships between different HSP networks as HSP families seem to be closely linked with each other. For example, DNA vaccines coding for HSP70 or HSP90 showed to affect HSP60-specific T cell response [182]. Along this line, HSP90 inhibitors showed to upregulate expression of HSP27 and HSP70 [183–185]. Furthermore, HSP70 and HSP90 networks interact via HSP70-HSP90 organizing protein (HOP/STIP1) while members of HSP40/DNAJ and HSP110/HSPH families act as co-chaperones for HSP70 [186,187]. In addition, HSP90 was found to be constitutively associated with chaperonin member CCT2/CCT-β [188]. These data strongly suggest that there is a cross-talk between various HSP networks.

HSP co-chaperone profile varies between EVs derived from different sample sources (Table 3). EV cargos derived from melanoma tissue contain major HSP90 co-chaperones and all five types of molecular co-chaperones critical for regulation of HSP70 functional cycle: DNAJ/HSP40 molecules deliver client proteins to HSP70, nucleotide-exchange factors (HSPH1, HSPBP1, SIL1, GRPEL1, BAG) stabilize open conformation of HSP70, HSP70 interacting protein (HIP/ST13) delays peptide release from HSP70, STIP1/ HOP co-chaperone transfers client proteins from HSP70 to HSP90 while C-terminus of HSP70 interacting protein (CHIP/STUB1) co-chaperone mediates degradation of HSP70 client proteins (Table 3) [189–196]. Interestingly, EVs isolated from plasma of melanoma patients contained two HSP70 co-chaperones HSPA4/HSPH2 and ST13/HIP, and no HSP90 co-chaperones were observed, whereas EVs derived from lymphatic drainage of the matched patients showed to carry more members of HSP70 network (Table 3) [174]. It is important to note that the relationships of HSPs with its co-chaperones define the fate of the client peptides and functional state of HSPs. For example, low concentration of DNAJ is required to stimulate HSP70 folding function while increase in DNAJ concentration results in reduced refolding of client proteins [189,191]. Therefore, understanding relationships between HSP families as well as understanding inter-relationships of chaperones with co-chaperone networks in EVs may form the basis for the identification of multiple biomarkers.

Even though analysis of EV proteome may open new perspectives for HSP-based biomarker discovery, however, it is important to point out that technical challenges exist in isolation of EVs from cell culture conditioned media and various biological fluids [197]. Separation of EVs from non-EVs soluble proteins and lipid particles is critical step for further assessment of EV content for biomarker discovery and validation [181] There are several methods for EV isolation and the most commonly used is differential ultracentrifugation (UC), while other methods include density gradients (DG), immunoaffinity, precipitation, size-exclusion chromatography (SEC), ultrafiltration and microfluidics [197].In order to achieve better specificity, combination of different methods (ultrafiltration, DG and SEC) can be used following primary step of EV isolation [197,198]. UC showed to be widely

Table 3
Heat shock proteins in extracellular vesicles of cancer patients.

Cancer type	HSP family	Heat Shock Proteins	Sample	Isolation method	Refs.
Melanoma	HSP70 network	HSPA5;HSPA9;HSPA8;HSPA2;HSPA1A;HSPA12A;HSPA12B; HSPA14; HSPA13; **Co-chaperones**:DNAJC10;DNAJB11;DNAJC3;DNAJA1;DNAJC7;DNAJA2; DNAJC16;DNAJC11;DNAJB1;DNAJC9;DNAJC19;DNAJA3;DNAJB14;DNAJA4; DNAJB4;DNAJB2;DNAJC5; DNAJB6; DNAJB12; DNAJC25; DNAJB9; DNAJC30; DNAJC8; DNAJC14; DNAJC15; DNAJC13; HSPA4L; HSPA4; HSPH1; HSPBP1; SIL1; GRPEL1;BAG1;BAG2,BAG6;BAG3; BAG5; STIP1; STUB1; ST13	Tissue	UC	[176]
	HSP90 network	HSP90B1; HSP90AB1;HSP90AA1; TRAP1; **Co-chaperones:**Cdc37;TSC1;AHSA1; FKBP4;FKBP5; FKBP8; PPP5C; PPID; PPIF; STIP1			
	HSPB family	HSPB1;HSPB5			
	Chaperonins	HSPD1;HSPE1;CCT3;CCT4;CCT2; CCT6A; CCT5;CCT8; CCT7; CCT1			
Melanoma	HSP70 network	HSPA1A/HSPA1B; HSPA8;HSPA5; **Co-chaperones:** ST13; HSPA4	Plasma	UC	[174]
	HSP90 network	HSP90AA1; HSP90AB1;HSP90B1;			
	HSPB family	HSPB1			
	Chaperonins	HSPD1;HSPE1;CCT2;CCT7;CCT4; CCT6A; CCT1			
	HSP70 network	HSPA1A/HSPA1B;HSPA5;HSPA8;HSPA2; HSPA6/HSPA7; **Co-chaperones:**DNAJA4;DNAJC5; ST13;DNAJB11;DNAJA2;DNAJC13; DNAJB2; STIP1; HSPA4	Seroma		
	HSP90 network	HSP90AA1; HSP90AB1; HSP90B1 **Co-chaperones:** STIP1			
	HSPB family	HSPB1; HSPB6;			
	Chaperonins	HSPD1;CCT5;CCT3;CCT8;CCT4;CCT2;CCT7; CCT6A; CCT1			
Glioblastoma	HSP70 network	HSPA12A;HSPA13;HSPA2;HSPA5;HSPA8; HSPA9; **Co-chaperones:**HSPH1;STIP1;STUB1;ST13;BAG2;BAG6;GRPEL1;DNAJA1; DNAJA2;DNAJA3;DNAJB1;DNAJB11; DNAJB2; DNAJC10; DNAJC11; DNAJC13; DNAJC19; DNAJC3; DNAJC5; HSPA4	CUSA fluid	UC	[175]
	HSP90 network	HSP90AA1; TRAP1; HSP90AB1;HSP90B1; **Co-chaperones:**AHSA1;STIP1;FKBP4;FKBP5; FKBP8; PPIF;			
	HSPB family	HSPB1; HSPB6; HSPB5			
	Chaperonins	HSPD1;HSPE1;CCT2;CCT3;CCT4;CCT5;CCT6A;CCT7;CCT8; CCT1			
	HSP70 network	HSPA8; HSPA5; HSPA1A/HSPA1B;			
Pancreatic cancer		**Co-chaperones:** DNAJB11; ST13;	Serum	UC	[179]
	HSP90 network	HSP90AA1; HSP90AB1;HSP90B1;			
	HSPB family	HSPB1			
	Chaperonins	–			
Prostate cancer	HSP70 network	HSPA12A; HSPA2; HSPA5; HSPA8; **Co-chaperones:**HSPH1;DNAJA1;HSPA4;DNAJA2;DNAJB1;DNAJB2;DNAJB6; ST13; DNAJC13; DNAJC5; STIP1; STUB1; BAG2; BAG5;	Urine	DGC	[177]
	HSP90 network	HSP90AA1;HSP90B1;HSP90AB1;HSP90AB2P; **Co-chaperones:**Cdc37;STIP1;AHSA1; FKBP4; PPP5C			
	HSPB family	HSPB5; HSPB1;			
	Chaperonins	CCT2; CCT3; CCT4; CCT5; CCT6A; CCT7; CCT8; CCT1			
	HSP70 network	HSPA1A; HSPA5;HSPA8; **Co-chaperones:**DNAJA2;DNAJB6;DNAJB1;DNAJB2;DNAJA1;DNAJC5; ST13; HSPH1; STIP1; BAG2; HSPA4;	Tissue		
	HSP90 network	HSP90AB1; HSP90AA1; HSP90B1; **Co-chaperones:** Cdc37; STIP1; FKBP4; FKBP5;			
	HSPB family	HSPB1; HSPB5			
	Chaperonins	CCT1;CCT5; CCT8; CCT2; CCT7; CCT6A; CCT4;CCT3			
Bladder cancer	HSP70 network	HSPA1A; HSPA8; HSPA2; HSPA5; **Co-chaperones:** DNAJA1; ST13; DNAJB1; DNAJA2;STIP1	Urine	UC	[208]
	HSP90 network	HSP90AB1; HSP90AA1; **Co-chaperones**: STIP1; FKBP4;			
	HSPB family	HSPB1			
	Chaperonins	CCT7; CCT2;CCT3;CCT6A;CCT4;CCT8; CCT1;			
Lung cancer	HSP70 network	**Co-chaperones**: DNAJC3;HSPA4;	Saliva		[209]
	HSP90 network	HSP90AB1;HSP90AA1; **Co-chaperones:** SIL1		UC	
	HSPB family	HSPB1;			
	Chaperonins	CCT4;			
Papillary thyroid cancer	HSP70 network	HSPA8; HSPA5;HSPA1B; **Co-chaperones:** STIP1; HSPA4;DNAJC5;DNAJA2; DNAJA1;DNAJC7; STUB1; ST13	Serum	UC	[210]
	HSP90 network	HSP90B1;HSP90AA1;HSP90AB1; **Co-chaperones:** STIP1;			
	HSPB family	HSPB1;			
	Chaperonins	HSPD1; CCT2; CCT5; CCT6A;CCT3;CCT4;CCT7;CCT8; CCT1			

CUSA, Cavitron ultrasonic surgical aspirator; UC, ultracentrifugation; DGC, density gradient centrifugation

accepted and cost-efficient method as the first step in purification of EVs [199]. However, the selection of EV isolations methods largely depends on the sample type and starting volume [199]. For example, SEC showed to be better at removing plasma proteins than UC and effective in isolating intact and functional active EVs when combined with ultrafiltration [200–203]. A recent study of Brennan and colleagues have compared the most commonly used isolation techniques such as UC, ExoQuick kit, SEC using IZON qEV columns and UC coupled with iodixanol density gradient for isolation of EVs from 200 µl of human serum [204]. All of these methods showed to successfully isolate EVs with the size ranging from 61 nm to 150 nm, though particle size, the yield of EVs and protein content showed to differ based on isolation methods used [204]. Furthermore, SEC, DG centrifugation and UC showed the greatest depletion of lipoproteins while at the same time decreased EV yield [204]. Moreover, several studies showed that miRNA profile in EVs also vary depending on isolation methods [205,206]. International Society for Extracellular Vesicles (ISEV) formulated guidelines for Minimal Information for Studies of Extracellular Vesicles (MISEV) highlighting importance for standardization of sample collection, EVs isolation, characterization and storage [198]. Moreover, EV-TRACK database that has been launched recently involves reporting of experimental parameters related to EV isolation and characterization, which may further improve transparency and reproducibility of results [207]. Nevertheless, EVs derived from various biofluids carry specific molecular messages and, despite the challenges posed by isolation methods, EVs hold promise for prediction, diagnosis and therapy response for clinical cancer research [197].

HSPs in extracellular vesicles released by immune cells

Increasing evidence has shown that extracellular HSPs play an important role in tumor immunity. The ability of HSP70 to bind antigenic peptides and elicit CD8+ T cell response led to the entrance of HSP70-based anti-tumor vaccines into clinical trial timeline [211–215]. Furthermore, extracellular HSP70 activates T regulatory cells and myeloid-derived suppressor cells (MDSC), leading to the production of anti-inflammatory cytokines [216,217]. These suppressive effects of HSP70 on tumor microenvironment (TME) further results in decreased T cell response and reduced stimulatory capacity of myeloid-derived dendritic cells [218,219]. Additionally, HSPs showed to play important role in innate and adaptive immune responses (reviewed in [220]) [221,222]. Considering critical role of HSPs in tumor immunity, we were particularly interested in the HSP profiling of EVs released by immune cells. Table 4 summarizes the data obtained from MS experiments on the expression of HSPs in EVs released by different types of immune cells from healthy donors.

HSPs and natural killer cells

Lugini and collegues have shown that natural killer (NK) cells secrete exosomes in both resting and activated states [223]. Moreover, NKEVs derived from activated NK cells undergo uptake by tumor cells and not by resting peripheral blood mononuclear cells (PBMCs) [223]. Federici and co-workers have demonstrated that NK cells of healthy individuals have higher proliferation rate compared to NK cells isolated from melanoma patients [224]. Furthermore, lower amount of NKEVs was detected in 1 ml of plasma of melanoma patients compared to healthy controls [224]. Cancer patients showed altered phenotype of peripheral blood NK cells represented by low degranulation and interferon-γ production [225]. Since cancer causes defects in NK cell activity, it will be of interest to study the role of HSPs in NKEVs which may further help in the discovery of specific biomarkers of cancer.

Multhoff and colleagues showed that stress-inducible member of HSP70 family (HSPA1A) serves on the surface of tumor cells as a recognition structure for NK cells [226]. Furthermore, NK cell proliferation and cytolytic activity are enhanced with an addition of stress-inducible member of HSP70 (HSPA1A) or HSP70-derived TKD peptide in combination with a low dose of interleukin-2 (IL-2) or IL-15, but not when incubated with its constitutive analogue HSC70 (HSPA8) [227,228]. Along this line, expression of HSP70 and BCL2-associated athanogene 4 (BAG4) on the surface of tumor-derived exosomes showed to stimulate migration of NK cells towards HSP70-positive tumors [229,230]. Interestingly, MS proteomic analysis of EVs of healthy donors showed that NKEVs contain HSPA1A and all other major HSP members and co-chaperones (Table 4). Notably, only NK cells and DCs derived EVs showed to contain BAG6 (Table 4). Exosomes released from DCs with BAG6-surface positive expression showed to activate NK cell receptor NKp30 [231]. Given that BAG6 is a co-chaperone for HSP70, it can be further speculated that expression of HSP70-BAG6 complex on the surface of DCs –derived exosomes promotes NK cell activation.

HSP90 inhibitor geldanamycin showed to downregulate cytotoxic activity of NK cells in cancer cell lines, suggesting important role of HSP90 in functional activity of NK cells [232,233]. NKEVs-derived from healthy donors contain all major members of HSP90 family, including its stress-inducible HSP90α (HSPAA1) and constitutive HSP90β (HSP90AB1) forms as well as its mitochondrial isoform TNF receptor associated protein 1 (TRAP1) (Table 4). Further studies should be performed to assess the differences in HSP90 profiling of NKEVs in cancer patients.

Overall, NK cell-derived EVs have the widest HSP profiling compared to other immune cell types (T cells, DC cells, neutrophils, platelets), suggesting that HSP profiling of NKEVs obtained from peripheral blood of cancer patients may be useful for detection of clinical biomarkers of cancer. More importantly, understanding the cross-talk between DCs and NK cells in the form of HSPs in EVs may lead to the development of more effective HSP-based immunotherapy. The important factor that should be considered when utilizing EVs in comparative studies is that amount of EVs should be normalized and this is particular true for NKEVs [198,224]. Amount of EVs can be normalized by either particle number or total EV protein or multiple normalization strategies can be applied [198,200]. Several studies have used number of donor-secreting cells for normalization of EV amount [234,235]. As an example, Tkach and colleagues when compared pellets derived from DC-secreting cells of different centrifugation speeds (2k,10k and 100 k pellets) observed that using either protein amount or particle numbers for normalization of EVs may lead to the biased results and,thus, they chose to normalize by amount of EV pellet obtained from a given number of secreting cells [234]. Therefore, the choice of normalization strategies largely depends on the scientific question [198].

HSP profiling of EVs secreted by antigen presenting cells and t lymphocytes

Weng and colleagues demonstrated that HSP70-peptide complex extracted from fusion of DCs and tumor cells resulted in enhanced cytotoxic T lymphocyte (CTL) response [212]. HSPs can present tumor-associated antigens to antigen presenting cells (APCs) through MHC class I and class II pathways, leading to CD8+ and CD4+ T cell response [211,236–238]. EVs released by monocyte-derived DCs (mDCs) and T lymphocytes of healthy donors contain different HSP members (Table 4). It is interesting to point out that HSPB family members were not detected in EVs derived from T cells, whereas mDCs showed to release one member of chaperonin family HSPD1/HSP60, which was also not detected in EVs derived from T cells (Table 4). Chaperonin member HSPD1/HSP60 showed to have dual effects on an immune system, depending on the HSP60 concentration, site of HSP60 action and HSP60 epitope (reviewed in [239]). Pro-inflammatory HSP60 resulted in maturation of monocytes for the production of pro-inflammatory cytokines and activation of B cells for the production of anti-HSP60-autoantibodies and IL-10 [239–241]. By contrast, anti-inflammatory HSP60 acted via anti-ergotypic regulatory T cells [239,242]. These data suggest that each type of immune cells has specific profile of HSP members released in EVs which may reflect its functional activity in immune responses. Further

Table 4
HSP profiling of extracellular vesicles secreted by immune cells of healthy donors.

Immune cells	HSP family	Heat shock proteins	Isolation method	Refs.
NK cells	HSP70 network	HSPA13;HSPA14;HSPA9;HSPA1A/HSPA1B; HSPA1L;HSPA2;HSPA5;HSPA8; **Co-chaperones:** DNAJA1; DNAJA2; DNAJA4; DNAJB1; DNAJB11; DNAJB4; DNAJB6; DNAJC13;DNAJC3;DNAJC5; DNAJC7; DNAJC9; HSPA4L;HSPBP1; HSPH1; STIP1; STUB1; ST13; BAG2; BAG6; BAG5; HSPA4;	UC	[224]
	HSP90 network	HSP90AA1;HSP90AA4P;HSP90AB1;HSP90AB4P; HSP90B1; TRAP1 **Co-chaperones:** Cdc37;AHSA1; STIP1; FKBP4; FKBP5; PPP5C; PPID		
	HSPB family	HSPB1;HSPB11;		
	Chaperonins	HSPD1; TCP1/CCT1; CCT2; CCT3; CCT4; CCT5; CCT8; CCT6A; CCT6B; CCT7		
T cells	HSP70 network	HSPA8; HSPA1L; HSPA2; HSPA5; **Co-chaperones:** HSPH1; DNAJA1; DNAJA2; ST13	UC	[255]
	HSP90 network	HSP90AA1;HSP90AB1;HSP90AB3P; HSP90AB2P; HSP90AA2P; HSP90B1; **Co-chaperones:** Cdc37;FKBP4;		
	HSPB family	–		
	Chaperonins	CCT5;CCT3; CCT4; CCT2;CCT6A;		
mDC cells	HSP70 network	HSPA1L; HSPA1A/HSPA1B; HSPA6;HSPA8; **Co-chaperones:** HSPH1; HSPA4L; DNAJC13; DNAJA1; DNAJA2; DNAJC5; DNAJC7;STIP1; BAG6; HSPA4;	UC	[256]
	HSP90 network	HSP90AA1; HSP90AB1; TRAP1; HSP90B1 **Co-chaperones:** STIP1; Cdc37; FKBP4; FKBP5;		
	HSPB family	HSPB1;		
	Chaperonins	HSPD1;		
Platelets	HSP70 network	HSPA8; HSPA5; HSPA6; HSPA1L; HSPA1B; HSPA9; HSPA2; **Co-chaperones:** STIP1; DNAJB11; ST13; HSPA4	UC	[257]
	HSP90 network	HSP90AA1; HSP90AB1; HSP90B1; TRAP1 **Co-chaperones:** STIP1;		
	HSPB family	HSPB1;		
	Chaperonins	HSPD1; CCT1; CCT2; CCT3; CCT7;CCT5;CCT6A; CCT8		
Neutrophils	HSP70 network	HSPA1A; HSPA8; **Co-chaperones:** ST13;	UC	[258]
	HSP90 network	HSP90AA1; HSP90AB1; HSP90B1 **Co-chaperones:** -		
	HSPB family	–		
	Chaperonins	HSPD1; CCT8;		

studies should be performed to analyze the abundance of HSP members exported by different types of immune cells in EVs in different types of cancer.

HSPs and other types of immune cells

Neutrophils of healthy individuals showed to export in their EV cargos only two members of HSP70 network, such as major stress-inducible (HSPA1A) and its constitutive isoform HSC70 (HSPA8) as well as ST13/HIP co-chaperone that stabilizes high-affinity state of HSP70 by slowing substrate release from HSP70 and, thus, preventing protein aggregation (Table 4) [187,194]. Two members of chaperonin family HSP60/HSPD1 and CCT8 and no HSPB family members were found in EV cargos derived from neutrophils (Table 4). Recent studies have shown that HSPD1/HSP60 modulates neutrophil activation, whereas HSPB1/HSP27 regulates apoptosis, chemotaxis and exocytosis in neutrophils [243–245]. Interestingly, no HSP90 co-chaperones were detected in EVs derived from neutrophils of healthy donors, although HSP90 co-chaperones are required for HSP90 functional cycle and HSP90-HSP70 interaction (Table 4) [196,246]. Moreover, mitochondrial HSP90 isoform TRAP1 has not been detected in EVs released by T cells and neutrophils (Table 4). Several studies demonstrated important role of TRAP1 in tumor cell metabolism, therefore, further studies should be performed to assess expression of TRAP1 and HSP90 co-chaperones in EVs derived from neutrophils of cancer patients [247,248].

EVs released by platelets constitute 70–90% of all EVs present in peripheral blood [249,250]. Several studies showed that activation of platelets by thrombin leads to HSP27 phosphorylation, whereas HSC70-HSP90 complex showed to be involved in platelet adhesion (reviewed in [251]) [252,253]. In addition, HSP70 inhibition showed to prevent platelet aggregate formation and granule secretion [254]. Strikingly, EVs derived from platelets of healthy donors contain all major members of HSP70 and HSP90 networks as well as HSPB1/HSP27, HSPD1/HSP60 and CCT chaperonins. However, not many HSP co-chaperones showed to be detected in platelet- derived EVs (Table 4).

HSPs and microRNAs

Another class of potential biomarkers abundantly present in circulation are small noncoding microRNAs (miRNAs) [259]. MiRNAs play important role in the regulation of gene expression [260,261]. Specifically, complementary base-pairing of miRNA with target messenger RNA (mRNA) or partial binding of miRNA to mRNA results in mRNA cleavage and translation repression, respectively [260,262,263]. Several studies reported altered expression of miRNAs in serum and plasma of cancer patients, making them potentially useful for identification of blood-based biomarkers [259,264–266]. Along this line, several studies have used specific miRNA signatures to identify tissue of origin of metastatic cancer and distinguish patients with different subtypes of cancer. For example, Feraccin and co-workers used tissue samples from primary and metastatic tumors to identify cancer-type specific 47-miRNA signature for predicting primary sites of metastatic cancers [267]. In another study, Yousseff and colleagues have used specific miRNA patterns to distinguish patients with different subtypes of renal cell carcinoma [268]. Furthermore, aberrant expression of certain miRNAs showed to indicate an early event in the development of pancreatic ductal adenocarcinoma (PDAC) precursor lesions, suggesting the use of miRNAs in early diagnosis [269]. In addition to the use of miRNAs in diagnosis, several studies used miRNAs as prognostic biomark-

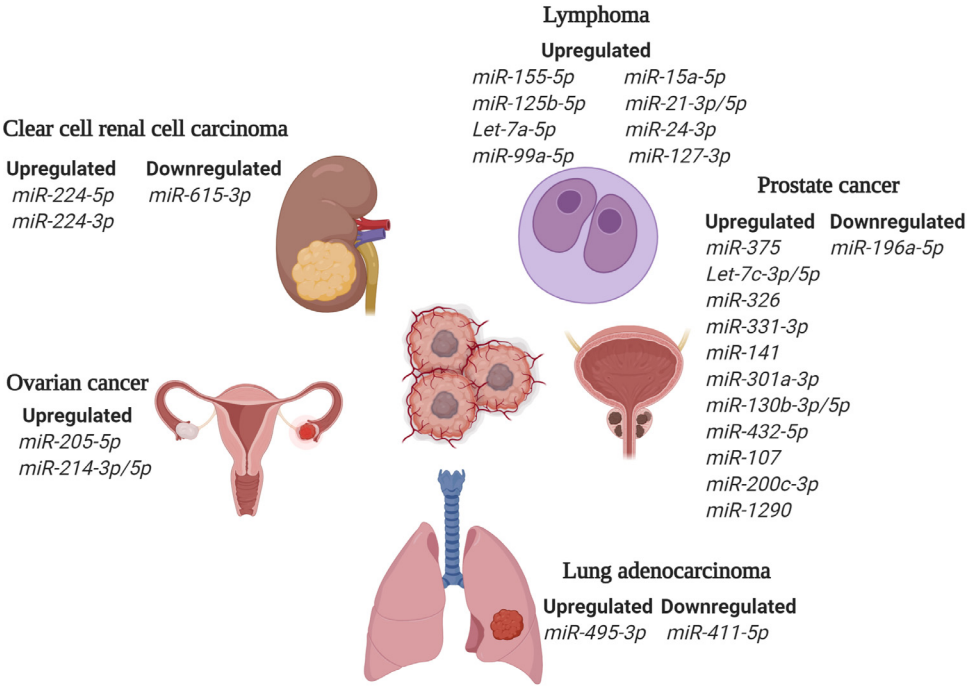

Fig. 1. Deregulated miRNAs targeting HSPs in cancer. Graphical representation of deregulated miRNAs in various types of cancer. MicroRNAs regulate the expression of major members of HSP70 and HSP90 networks (Table 5).

ers of cancer. As an example, high expression of members of *miR-183* family in tumor and serum showed to associate with poor outcome in patients with lung cancer [270]. Promisingly, it was also observed that miRNAs can be used to predict response to therapy in cancer patients [271]. As an example, highly expressed *miR-21* associated with shorter overall survival in PDAC patients treated with gemcitabine [272]. Additionally, potential use of circulating miRNA as biomarkers showed to be particularly relevant for lymphoma patients (reviewed in [259]). Taking into account that miRNAs may deregulate HSPs and serve as potential predictive and prognostic biomarkers, it is important to identify miRNAs that target HSP networks [273]. Deregulated miRNAs in EVs obtained from clinical samples of cancer patients and their experimentally validated HSP targets are summarized in Table 5 (Fig. 1) [274].

MicroRNAs are protected from degradation by external RNAses when either encapsulated into EVs, bound to argonaute 2 (Ago2) or transported by high-density lipoproteins (HDL) [259,275–277]. Notably, during translational stress HSP90 together with its co-chaperones Cdc37, p23, Aha1 and STIP1/HOP showed to recruit Ago2 to stress granules [278]. Inhibition of HSP90 affects miRNA-gene silencing function of Argonaute [278]. Importantly, HSP90 are required for stable binding of Argonaute to Dicer, enzyme responsible for processing of small interfering RNAs and miRNAs in the cytoplasm for their further loading on RNA-induced silencing complex (RISC) [279–281]. HSP70 also showed to be associated with Ago2 following T-cell receptor (TCR) activation for the regulation of T-helper 17 (Th17) cells via miRNA expression in EL-4 murine lymphoma cells [282].

miR-21 showed to modulate resistance of cholangiocarcinoma cells to HSP90 inhibitors via DNAJB5 [283]. Along this line, *miR-361* binds and regulates expression of HSP90α/HSP90AA1, leading to the suppression of epithelial-mesenchymal transition in cervical cancer lines [283,284]. *miR-27a* downregulates the expression of HSP90, resulting in further degradation of HSP90 client proteins in esophageal squamous cell carcinoma cell lines [285]. Stope and co-workers showed that HSP27/HSPB1 inhibits *miR-1* in prostate cancer cells [286]. Interestingly, HSP70 inhibitor triptolide showed to upregulate expression of *miR-142–3p*, consequently inhibiting proliferation of pancreatic ductal adenocarcinoma cells [287]. Additionally, inhibition of *miR-29a* induces apoptosis by increasing expression of HSP60 and decreasing HSP90, HSP70, HSP27 and HSP40 levels in breast cancer cells [288]. Conclusively, miRNAs play important role in HSP regulation, therefore, elucidating the effects of miRNAs on HSPs may open new perspectives for diagnosis and treatment of cancer patients.

HSP profiling of EVs: future perspectives

Quantitative MS-based proteomics coupled with machine learning (ML) statistics is a novel and promising approach for identification of suitable biomarkers in EVs derived from biological fluids of cancer patients [22,305]. Unlike classical statistics, which identifies only differentially expressed proteins, ML performs simultaneous analysis of all proteins in a sample as well as analyzes relationships of these proteins across different patients, capturing all changes in a protein level. Based on our analysis of HSP profiling, we propose the following scheme for the identification of potential HSP candidates (Fig. 2).Extracellular vesicles derived from urine and NK cells showed to be the most abundant source of HSPs and co-chaperones and, therefore, can be a good source for identifying HSP signatures. Regarding a class of EVs, exosomes can be the most useful source for identification of cancer-specific biomarkers as they are formed by inward budding of endosomal membranes and, thus, reflect the protein composition of plasma membranes of the cells they were derived from [229].

ML algorithms can be used to classify patients by the type of cancer in which specific proteins may contribute more or less to the cancer prediction model. Various types of cancers may have specific patterns of upregulated and downregulated HSPs that may distinguish them from healthy individuals and from patients with other non-cancerous conditions or even discriminate one type of cancer from another. Since proteins that constitute HSP networks may behave differently in different types of cancer and control groups, ML may be used to identify critical relationships between HSP networks and whether one network contribute more to the prediction of cancer than the other network. Furthermore, monitoring the changes in the level of HSPs in response to therapies may provide a further clues on how different types of therapies affect HSP release and how this is related to the outcome of the patient, which may provide further improvement towards the more personalized approach in treating cancer patients. It will be also of interest to determine specific threshold level of specific protein so that elevated

Table 5
Micro-RNAs and their HSP targets in EVs derived from cancer patients.

MicroRNA	Target gene	Sample	Cancer	Refs.
↑ *miR-205–5p*	*HSPA8; DNAJA1*	Serum EV	Ovarian	[289]
↑ *miR-214–3p*	*HSP90AB1;HSPD1*	Serum EV	Ovarian	[289]
↑ *miR-214–5p*	*HSPA2;HSPA14; BAG2*	Serum EV	Ovarian	[289]
↑ *miR-495–3p*	*HSPA5; HSPA1B; HSP90AA1; DNAJC21*	Plasma EV	Lung	[290]
↓ *miR-411–5p*	*DNAJB9;DNAJC10;ST13*	Plasma EV	Lung	[290]
↓ *miR-615–3p*	*HSPA1B;HSPA4;CCT8;HSPA8;HSPA9;CCT1;HSP90AB1;DNAJA2;DNAJC9;DNAJC10; PPIF;CDC37;TRAP1;BAG6;BAG5;CCT7*	Plasma EV	ccRCC	[291]
↑ *miR-224–5p*	*HSP90AA1*	Serum EV	ccRCC	[292]
↑ *miR-224–3p*	*DNAJB4; DNAJC28*	Serum EV	ccRCC	[292]
↑ *miR-375*	*HSP90AA1;DNAJC8; DNAJC2*	Urine EV	Prostate	[293, 294]
↑ *Let-7c-5p*	*HSPA4;DNAJC6;DNAJC16; DNAJC28*	Urine EV	Prostate	[294]
↑ *Let-7c-3p*	*DNAJB6; BAG4*	Urine EV	Prostate	[294]
↓ *miR-196a-5p*	*HSPA4L; TRAP1*	Urine EV	Prostate	[295]
↑ *miR-326*	*HSPA1B*	Plasma EV	Prostate	[296]
↑ *miR-331–3p*	*HSPA1B; HSPA8;CCT1; HSPD1;TRAP1;BAG6*	Plasma EV	Prostate	[296]
↑ *miR-141*	*HSPA4;DNAJC28;HSPA2;HSP90AA1; DNAJB2*	Plasma EV; Serum EV	Prostate	[296, 297]
↑ *miR-301a-3p*	*HSPA8;CCT6A;CCT8*	Plasma EV	Prostate	[296]
↑ *miR-130b-3p*	*HSPA8;HSP90B1;CCT6A*	Plasma EV	Prostate	[296]
↑ *miR-130b-5p*	*HSPA1B; HSPA6; HSPA8;BAG2*	Plasma EV	Prostate	[296]
↑ *miR-1233–5p*	*HSP90AB1;DNAJC8;DNAJC24; DNAJC10;*	Serum EV	ccRCC	[298]
↑ *miR-432–5p*	*DNAJB6*	Plasma EV	Prostate	[296]
↑ *miR-107*	*DNAJA1;DNAJC10*	Plasma EV	Prostate	[296]
↑ *miR-200c-3p*	*DNAJB9;DNAJC3;DNAJB6;FKBP5;BAG4*	Plasma EV	Prostate	[299]
↑ *miR-1290*	*DNAJB9*	Plasma EV	Prostate	[300]
↑ *miR-375*	*HSP90AA1;DNAJC8; DNAJC2*	Plasma EV	Prostate	[299, 300]
↑ *miR-155–5p*	*HSPA4L; HSPB11; DNAJC2;DNAJC19; CCT2; DNAJB1; BAG5;CDC37*	Plasma EV	HL	[301]
↑ *miR-15a-5p*	*HSPA1A; HSPA1B; HSPA8; HSP90B1; DNAJA1; DNAJC9;DNAJC10;BAG4;CCT6B*	Serum EV	DLBCL	[302]
↑ *miR-125b-5p*	*HSPA1B;HSPD1;HSPBP1*	Serum EV	DLBCL	[303]
↑ *miR-21–3p*	*DNAJC10;ST13*	Serum EV	DLBCL	[304]
↑ *miR-21–5p*	*DNAJC16;PPIF; FKBP5;STUB1*	Plasma EV	HL	[301]
↑ *Let-7a-5p*	*DNAJC6; DNAJC28; FKBP8*	Plasma EV	HL	[301]
↑ *miR-24–3p*	*DNAJC3; DNAJC10; DNAJB12*	Plasma EV	HL	[301]
↑ *miR-99a-5p*	*DNAJA3; FKBP5*	Serum EV	DLBCL	[303]
↑ *miR-127–3p*	*BAG5*	Plasma EV	HL	[301]

ccRCC, Clear cell renal cell carcinoma; HL, Hodgkin lymphoma; DLBCL, Diffuse large B-cell lymphoma.

or reduced level of one or multiple proteins is better at predicting cancer. The use of clinical data is advantageous as it may provide a great insight into the understanding of the role of HSPs in cancer. Use of ML that can analyze big data and find certain patterns opens new possibilities for the discovery of specific signatures of cancer and development of more efficient anti-cancer therapies.

Conclusion

Heat shock protein family constitutes a large network of molecular chaperones classified into several families with aberrant expression in cancer. Profiling of HSPs in EVs derived from various biological fluids may serve useful for diagnosis, prediction and treatment of cancer pa-

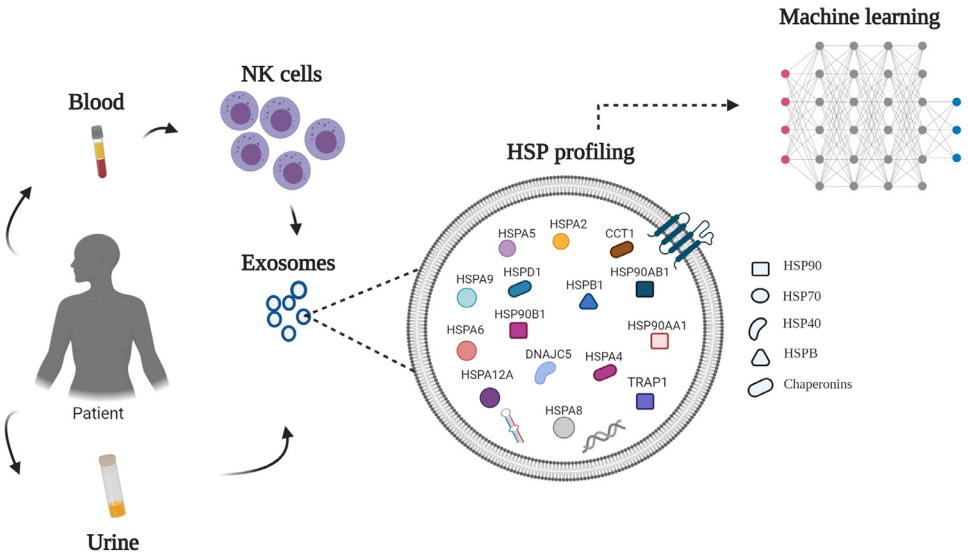

Fig. 2. HSP profiling of exosomes for identification of potential HSP-based biomarker candidates. The quantitative MS-based proteomics coupled with machine learning might be used to detect all changes in the level of expression in a sample and between cohorts of patients for identification of clinically useful HSP- based biomarkers in urine and/or NK- derived exosomes of cancer patients.

tients. More importantly, understanding relationships between different HSP networks and co-chaperones may form the basis for identification of multiple HSP-based biomarkers. In this review, we summarize mass spectrometry studies of EVs derived from various liquid biopsies and different types of immune cells with particular interest in HSP profiling. In addition, we have emphasized emerging role of circulating miRNAs in regulation of HSPs in the context of cancer. Further elucidating role of HSPs in EVs from proteomic and miRNAs perspectives may provide new opportunities for the discovery of clinically useful biomarkers and novel targets for cancer therapies.

Authors contributions

Zarema Albakova: Conceptualization, Formal analysis, Investigation, Data Curation, Writing – Original draft preparation, Writing - Reviewing & Editing, Visualization. **Mohammad Kawsar Sharif Siam & Pradeep Kumar Sacitharan:** Writing - Reviewing & Editing. **Rustam H. Ziganshin, Dmitriy Y. Ryazantsev, Alexander M. Sapozhnikov**: Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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