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Review

Roles of Extracellular Vesicles (EVs) carrying HSPs in Cancer Biomarkers, Immune Surveillance, and Immune Evasion

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Abstract: Extracellular vesicles (EV) released by tumor cells are a major aspect of the resistance-associated secretory phenotype (RASP), by which immune evasion can be established. Heat shock proteins (HSPs) are an evolutionarily conserved family of molecular chaperones, which stabilize proteins, minimize protein misfolding and aggregation within the cell, besides facilitating protein translocation, refolding and degradation. (i) Releases of extracellular HSPs (ex-HSP) and EV-associated HSPs (EV-HSP) are essential in RASP, by which molecular cotransfer of HSPs with oncogenic factors into recipient cells can promote cancer progression and resistance against stress such as hypoxia, radiation, chemicals, and immune system. (ii) RASP of tumor cells can eject anticancer drugs, molecularly targeted therapeutics, and immune checkpoint inhibitors with EVs. (iii) Cytotoxic lipids can be also released from tumor cells as RASP. Nevertheless, ex-HSP and EV-HSP can play immunostimulatory and immunosuppressive roles by binding to receptors such as LRP1/CD91/A2MR, scavenger receptors, and toll-like receptors expressed on recipient cells. Liquid biopsy of HSPs in body fluids may be useful in diagnosis, prognosis, and treatment in cancer. Regarding HSP90-targeted therapeutics, we summarize the pros, cons, and problem solutions in this review. Although production of HSPs are canonically induced by heat shock factor 1 (HSF1) and hypoxia-inducible factor 1 (HIF-1), recent studies discovered that production of HSPs is also regulated by matrix metalloproteinase 3 (MMP3) and heterochromatin protein 1 (HP1) and production of cochaperone CDC37 is reciprocally regulated by myeloid zinc finger 1 (MZF1) and SCAN-D1.

Keywords: heat shock protein (HSP); extracellular vesicle (EV); exosome; oncosome; immune evasion; resistance-associated secretory phenotype (RASP); EMT; hypoxia; biomarker; liquid biopsy

1. Introduction

1.1. Intracellular HSPs and extracellular HSPs

Tumor cells are often exposed to stress such as hypoxic stress, immune and inflammatory stress, microbial stimuli, and therapeutic stress. These stresses often induce heat shock proteins (HSPs),

stress-resistant cytoprotective proteins bearing anti-apoptotic activity. Intracellular HSPs are molecular chaperones essential for protein folding and balancing between proteostasis and proteolysis and play anti-apoptotic roles in cancer [1-4]. HSPs are one of the highly conserved and most abundant chaperones playing a fundamental role in maintaining cellular proteostasis under physiological and stress conditions. HSPs are able to interact with various intracellular proteins to promote proper protein folding.

Meanwhile, extracellular HSP (ex-HSP) plays key roles in cell-cell communication in cancer and immunology. Ex-HSPs are released from cells by passive release e.g. by damaged, stressed or dead cells and active release, including secretion of HSP-containing exosomes. Proteomics of EVs discovered that EVs were enriched with HSP90 members, HSP70 members, HSP105, and chaperonin [5] (see section 6). Recent studies have discovered two types of ex-HSPs: extracellular vesicle (EV)-associated HSPs (EV-Hsp) and EV-free ex-Hsp. Ex-Hsp and EV-Hsp can bind to cell surface receptors such as CD91 also known as low-density lipoprotein receptor-related protein (LRP1) or alpha 2 macroglobulin receptor (A2MR), scavenger receptors, and toll-like receptors (TLRs) leading to activation of intracellular signaling pathway and endocytosis (see section 4). Ex-Hsp and EV-Hsp can promote cancer progression by promoting epithelial-mesenchymal transition (EMT), migration, invasion, heterogeneity, metastasis, cell stem cell (CSC) properties, and drug resistance in cancer cells and angiogenesis [6-12]. Proteomics of EVs revealed that several members of the HSP family are carried within EVs; such as HSP90 homologs, large HSP members, and HSP70 family members [5]. HSPs and oncoproteins within EVs could be a resistant-associated secretory phenotype (RASP), cotransferred to recipient cells leading to cancer expansion and malignant conversion of tumor microenvironment (Figure 1). Several aspects and proof-of-concept (POC) of RASP are summarized in section 2.

1.2. HSP family and subfamilies

Members of the HSP family are classified into two types: an inducible type of HSPs and a constitutively expressed type of HSPs. The inducible HSPs are expressed when cells are exposed to stress such as heat and hypoxia [3,13]. Nevertheless, a number of studies have reported that both inducible and constitutive types of HSPs are often overexpressed in malignant tumors and associated with the incidence as well as the progression of the disease and lymph node metastatic rate [14-16]. Genetic amplification of *HSP* genes found in particular types of cancer can cause high expression of HSPs [1], while genetic mutations in *HSP* genes have barely found. According to their structural homologies, the Hsp family members are classified into subfamilies composed of;

- HSP70 family [1]
- HSP90 family (Sections 1.3, 5, 6, and 7)
- Small HSP (HSP27 / HspB) family
- Large HSP (HSP110) family
- HSP60 family
- HSP40/DnaJ (cochaperone of Hsp70)
- HSP47 family (collagen-specific molecular chaperones)
- HSP10 (chaperonin)

1.3. HSP90 family

HSP90 homologs are the major intracellular chaperones that ensure the correct folding and function of proteins by interacting with various intracellular proteins [2,3,17,18]. HSP90 has been implicated in promoting the tumor growth and metastasis of breast cancer, leukemia, pancreatic cancer, and ovarian cancer [19-21]. Four homologs of HSP90 are localized in different cellular compartments. HSP90α, an inducible type of HSP, and HSP90β, a constitutively expressed type of HSP, are found in the cytoplasm. Glycoprotein 96 (GP96) also known as glucose-regulated protein 94kD (GRP94), HSP90B1, tumor rejection antigen (TRA) or endoplasmin is present in the endoplasmic reticulum (ER). Tumor necrosis factor (TNF) receptor-associated protein 1 (TRAP1) exists in the mitochondria. TRAP1 is a homolog of HSP90, although its molecular weight is 75kD.

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1.4. Extracellular vesicle-associated HSP (EV-HSP)

It has been shown that HSP90α is highly expressed in cancer cells and secreted to extracellular space as a soluble protein [4] and/or as a cargo of EVs [5]. Additionally, HSP90β, TRAP1, and some members of HSP70 are often packaged in EVs derived from cancer cells [5]. However, the mechanism by which HSPs are incorporated within the EVs and their biological significance are still unknown. We here propose two models of EV-HSPs: (i) intra-vesicular packaged HSPs and (ii) EV-associated HSPs bound with membrane proteins on the outer surface of EVs (Figure 1).

Ex-Hsp and EV-Hsp can bind to cell surface receptors for stimulation of intracellular signaling pathways leading to transportation by endocytosis/transcytosis or be molecularly transferred to recipient cells (see sections 1 and 4). Such recipient cells include cancer cells, epithelial cells [22], fibroblasts such as cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSC), vascular endothelial cells, lymphatic endothelial cells, and various types of immune cells. A number of studies showed that tumor-derived exosomes promote cancer progression by transferring oncogenic factors, including oncoproteins and oncogenic miRNA (oncomiR), to recipient cells in the tumor microenvironment and in the pre-metastatic niche. Recipient immune cells mediate immunostimulatory and immunosuppressive roles of EVs, depending on types of immune cells (see section 3).

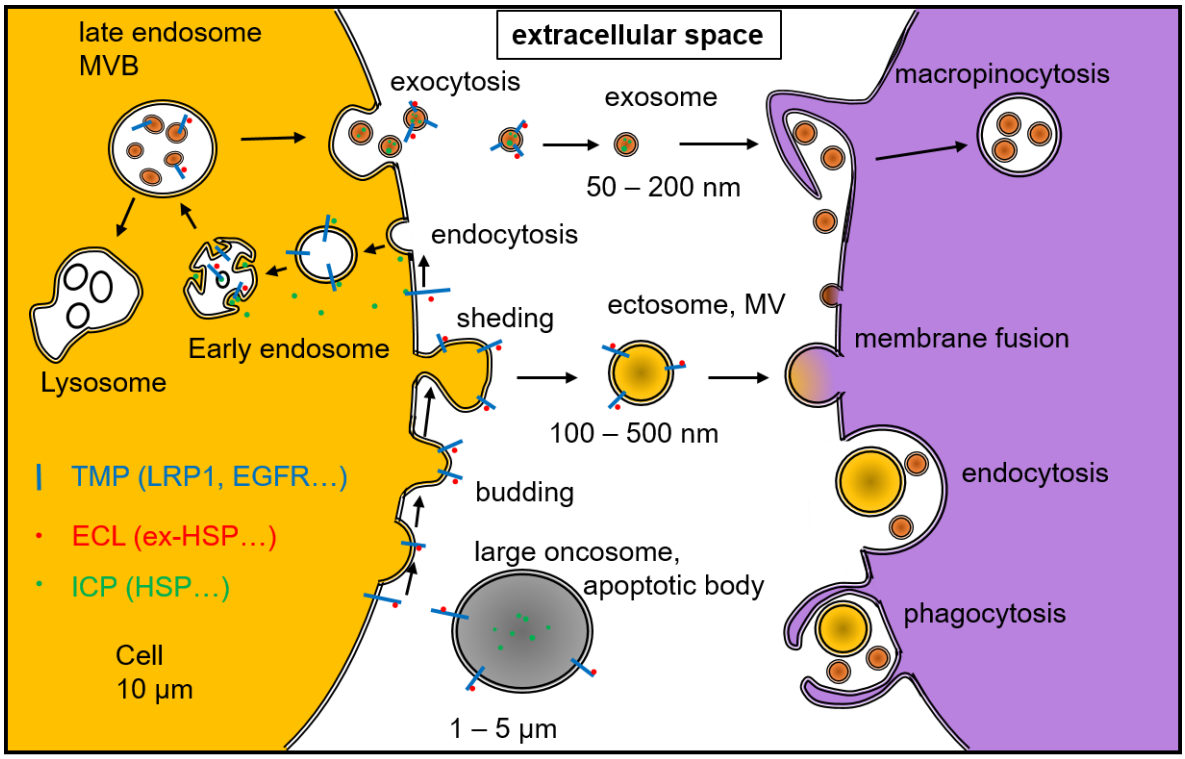

Figure 1. Mechanisms of secretion and uptake of EVs and HSPs. EVs are often a heterogeneous mixture of exosomes, microvesicles (MVs), oncosomes, large oncosomes, and apoptotic bodies. Exosomes are secreted via exocytosis of late endosomes also known as multi-vesicular bodies (MVBs) that contains intra-luminal vesicles (ILVs) (top left). Distinctively, budding and shedding of plasma membrane generate MVs (center). EV-free ex-HSPs can be released from cells upon cell damage and stress. Transmembrane proteins (TMP: blue bars) such as LRP1/CD91/A2MR can localize on the surface of EVs and keep binding of ex-HSPs on the EV surface. Extracellular ligands (ECL: red dots) such as ex-HSPs can bind to the extracellular domain of TMP on the surface of EVs. Intracellular proteins (ICP: green dots) such as HSPs can be kept bound to the intracellular domains of the TMPs on the cells and EVs. EVs are often taken up by recipient cells in a variety of ways such as endocytosis, macropinocytosis, membrane fusion, and phagocytosis (right).

1.5. Transcription factors that regulate chaperone and co-chaperone genes

Heat shock factor 1 (HSF1) is a canonical transcription factor that mediates cell stress and induces production of HSPs, although several additional transcription factors recently identified can be involved in cancer progression and resistance. To target intracellular HSP90, a number of HSP90 inhibitors have been developed and tested, although advantages and drawbacks of HSP90 inhibitors have been concerned. The potential reasons why HSP90 inhibitors have not been approved for clinical application are discussed below. One canonical reason has been considered to be a feedback system of HSP90 complexing with HSF1 [17,23-25] (see sections 7 and 9).

In the hypoxic condition in cancer and wound healing, hypoxia-inducible transcription factor-1 (HIF-1) induces ex-HSP90 that promotes cancer progression as well as skin wound healing (see sections 5 and 9). Moreover, recent studies have discovered that intracellular matrix metalloproteinase-3 (MMP-3) and heterochromatin protein 1 (HP1) also known as chromobox proteins (CBX) activate *HSP* genes [26]. Notably, two SCAN-type transcription factors- myeloid zinc-finger 1 (MZF1) and SCAN-D1 were shown to reciprocally regulate cell division control 37 (CDC37), a kinome co-chaperone of HSP90 [27,28]. These transcription factors regulate genes encoding molecular chaperones and co-chaperones and thus crucial in cancer progression and resistance. The transcriptional mechanisms by which HSPs are produced are summarized (in section 9).

1.6. Table of contents

2. Resistance-Associated Secretory Phenotype (RASP)

2.1. HSP-rich EVs and oncoprotein-rich EVs

HSPs are often carried by EVs, e.g. exosomes, oncosomes, and microvesicles (MVs) as EV cargos and/or are associated on the surface of EVs [4,5] (Figure 2). EV-mediated molecular transfer of oncoproteins such as mutant epidermal growth factor receptor (EGFR) and amplified HSPs [1] can enhance carcinogenesis and resistance in surrounding recipient cells such as cancer cells themselves, normal epithelial cells, fibroblasts, adipocytes, endothelial cells, macrophages, and other immune cells [5,22,29]. As EV-free HSPs do, HSPs associated on the surface of EVs could activate the receptors such as CD91 and promote cancer cell EMT, migration, invasion, heterogeneity, angiogenesis, metastasis, and drug resistance. Thus, EV-HSP and ex-HSP are major aspects of the RASP.

2.2. Ejection of drugs and antibodies with Hsp-EVs

The RASP is also important in drug resistance inasmuch as cancer cells are able to eject molecularly targeted drugs with EVs. Particularly, molecularly targeted anti-EGFR antibody drug cetuximab is able to bind to EGFR and inhibit EMT, a key step in cancer progression [22]; however, oral cancer cells ejected cetuximab with EGFR-containing EVs in response to administration of cetuximab, indicating a novel EV-mediated mechanism of drug resistance, a POC of RASP [30]. The antibody drugs can recruit Fc receptor (FcR)-expressed immune cells leading to phagocytosis by macrophages and/or cytolysis by cytotoxic T lymphocytes (CTLs) and by natural killer (NK) cells, although these anti-cancer immune cells can be released with EVs from cancer cells. The EV-mediated ejection of drugs is a new manner of drug resistance in cancer cells as well as a novel aspect of RASP.

Anticancer drugs can cause the release of exosomes with HSPs, consistent with the concept of RASP. As another POC, anticancer drugs caused the release of exosomes with HSPs from human

hepatocellular carcinoma cells, although the released HSP-exosomes elicit effective NK cell antitumor responses in vitro [31], suggesting an immunostimulatory role of EV-Hsp.

2.3. Release of redundant toxic lipids

Lipid efflux is the other aspect of RASP. Redundant lipids are released from cells through the release of lipid-layered EVs and lipid cholesterol efflux pump proteins. One of such pumps overexpressed in metastatic cancer cells was adenosine triphosphate (ATP)-binding cassette G1 (ABCG1) [32]. Targeted silencing of ABCG1 resulted in accumulation of EV lipid and triggered cell death in tumors, suggesting that cancer cells can often release redundant toxic lipid whereas loss of the ABCG1 pump could trigger the accumulation of redundant, toxic lipids. Thus, the release of redundant toxic EV lipid can be the other aspect of RASP, whereas accumulation of the redundant lipid could be toxic to tumor cells, suggesting a conceptually and substantially novel therapeutic approach.

3. Immunomodulatory roles of ex-HSP

3.1. Immunogenic immunostimulatory roles of ex-HSP

A number of studies reported antitumor immunostimulatory roles of Hsp peptides complex vaccines. Vaccination with HSP-peptide complexes elicits protective immunity against tumors or other cells used as the source of HSPs and suggest that HSP-peptide complexes can be suitable as vaccines against cancers and infectious diseases [33]. From the aspect of the immune surveillance system, ex-HSP released from damaged cells can stimulate professional antigen-presenting cells (APCs), followed by cytokine release and expression of cell surface molecules [34-36]. In addition to such activity stimulating innate immunity, ex-HSPs can promote the cross-presentation of HSP-bound peptide antigens to major histocompatibility complex (MHC) class I molecules in dendritic cells (DCs), leading to efficient induction of antigen-specific CTLs. The roles of HSPs stimulating both innate immunity and adaptive immunity can explain at least in part the molecular mechanism by which thermal stress bolsters the host immune system [37]. Use of HSP peptide complexes as vaccination has been evident to induce antigen cross-presentation by APCs such as DCs and macrophages [38], thereby elicits Hsp-cross-primed antigen-specific CD8+ CTLs [39-42]. The HSP peptides vaccines have been examined in cancer [43], infectious diseases [44]. Immunogenicities of Gp96 [45], Hsp90 [46], Hsp70 [38,47], and Grp170 also known as oxygen-regulated protein 150 (Orp150) [48] have been examined. Intratumor vaccination with a recombinant oncolytic adenovirus overexpressing the HSP70 protein eradicated primary tumors, as well as inhibit the growth of established metastatic tumor in mice [43]. Anticancer drugs caused the release of exosomes with HSPs from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro [31].

There were over 150 medical centers worldwide enrolling patients in randomized, controlled Phase III clinical trials testing autologous cancer-derived HSP-peptide complexes for the treatment of renal cell carcinoma and melanoma in 2003 [39]. Autologous HSP-peptide complexes had been tested in Phase I and II trials of chronic myelogenous leukemia, lymphoma and pancreatic, gastric and colorectal cancers.

ER chaperones such as binding immunoglobulin protein (BiP; also known as HSPA5/Hsp70), endoplasmin (also known as GRP94 or HSP90B1), calreticulin, and isomerases perform a multitude of functions within the ER, although many of these chaperones can translocate to the cytosol and eventually the surface of cells, particularly during ER stress induced by e.g., drugs, UV irradiation, and microbial stimuli [49]. On the cell surface or in the extracellular milieu, the ER chaperones can take on immunogenic characteristics in the context of cancer, appearing as damage-associated molecular patterns (DAMPs) recognized by the immune system targeting tumor cells for cell death. Notably, BiP / HspA5 was found in HNSCC cells-derived EVs, although decreased in the high metastatic EVs compared low metastatic ones [5]. The release of chaperones can also exacerbate autoimmune conditions such as rheumatoid arthritis and multiple sclerosis.

3.2. Anti-inflammatory immunosuppressive roles of ex-HSP

Immunization with HSPs has protective effects in models of induced arthritis [50]. Immune reactivity to Hsp has been found to result from inflammation in various disease models and human chronic inflammatory conditions, such as rheumatoid arthritis (RA), type 1 diabetes, and atherosclerosis [51]. Incubation with microbial Hsp70 induced tolerogenic DCs and promoted a suppressive phenotype in myeloid-derived suppressor cells (MDSCs) and monocytes [52]. Potent regulatory T cells (Tregs) known as anti-inflammatory immunosuppressive T cells recognized HSP70 self-antigens, enabling selective targeting of such Tregs to inflamed tissues [53]. Therefore, HSPs are attractive candidates for therapeutic intervention in chronic autoimmune diseases, with the ultimate goal of inducing long-lasting immune tolerance [35,54].

4. Receptors for ex-Hsp and EV-Hsp

Cell surface receptors known to be bound with ex-Hsp90 are CD91/LRP1/A2MR, toll-like receptors (TLRs), and scavenger receptors such as SREC-1. These receptors can be involved in the activation of intracellular signaling pathways and endocytosis. In the case that these receptors are found on the surface of EVs, these can hold ex-Hsp90 on the surface of EVs.

4.1. CD91 / LRP1 / A2MR

Several receptors of ex-HSPs have been reported. It was first shown that HSP60 (mitochondrial molecular chaperone) was a putative endogenous ligand of the TLR4 complex [55]. However, Hsp60 binding to macrophages occurred in the absence of surface TLR4, although no cytokine response was induced by Hsp60 in TLR4-deficient macrophages [56]. Distinctively, HSP70, HSP90, and GP96 share the alpha(2)-macroglobulin receptor (A2MR) also known as CD91 and LRP1 as a binding site in macrophages [56]. CD91 also known as LRP1 has been identified as a key receptor of ex-HSP90 [57]. Ex-HSP90 binds to the subdomain II of LRP1 and the intracellular NPVY motif is essential for activation of Akt1/2 signaling [7]. It was recently re-demonstrated that establishment of tumor-associated immunity requires the interaction of HSPs with CD91 [58].

CD91/LRP1/A2MR is expressed in hypoxic stress and plays a key role in endocytosis and transcytosis [59], thereby this macro-molecule can be also crucial in endocytosis of EVs and ex-Hsp90 in a hypoxic microenvironment.

4.2. Toll-like receptors (TLRs)

In addition, endogenous HSP70 activated the Toll / IL-1 receptor signal pathway similar to HSP60 and pathogen-associated molecular patterns (PAMP) [60]. In this study, HSP70 induced interleukin-12 (IL-12) and activated a promoter of endothelial cell-leukocyte adhesion molecule-1 (ELAM-1: also known as E-selection or CD62E) in macrophages and MyD88-deficient DCs did not respond to HSP70 with proinflammatory cytokine production. In the same journal, it was reported that HSP70-induced proinflammatory cytokine production is mediated via the MyD88/IRAK/NF-κB signal transduction pathway and that HSP70 utilizes both TLR2 (receptor for Gram-positive bacteria) and TLR4 (receptor for Gram-negative bacteria) to transduce its proinflammatory signal in a CD14-dependent fashion [61].

These studies indicated that (i) CD91/LRP1/A2MR can be a receptor of ex-HSPs and (ii) TLR2/4 can be receptors of ex-HSPs as well. In the latter case, any pathogen such as lipopolysaccharide (LPS), any other PAMP or DAMP can be possibly contaminated within recombinant HSP fractions purified from bacteria inasmuch as diminishing contamination of pathogens may be difficult methodologically and technically. It has also been suggested that HSPs augment the ability of associated innate ligands such as LPS to stimulate cytokine production and DC maturation [62]. Nevertheless, co-factors, co-receptors, co-stimulatory factors or adaptor proteins might be of interest on the cell surface.

4.3. SREC-1 and scavenger receptors

T cell activation by HSP70 vaccine requires TLR signaling and scavenger receptor expressed by endothelial cells-1 (SREC-1) [63,64]. HSP70 peptide complex isolated from tumor-dendritic cell fusions (HSP70.PC-F) induces potent antitumor immunity and prevents the growth of such tumors. In this study, antitumor immunity induced by HSP70.PC-F depended on intact TLR signaling in immunized animals, and mice in which the *Tlr2* and *Tlr4* genes were both inactivated did not respond to the vaccine. Notably, TLR-dependent, tumor cell killing was suppressed by SREC-1 knockdown in DC, suggesting a significant role for SREC-1 in HSP70.PC-F-mediated tumor immunity [63,65].

SREC-1 plays a role in Hsp90-mediated efficient antigen cross-presentation [66]. Hsp90-OVA peptide complexes bound to the scavenger receptor on the surface of APCs. SREC-1 mediated internalization of Hsp90-OVA polypeptide complexes through a Cdc42-regulated, dynamin-independent endocytic pathway known as the GPI-anchored protein-enriched early endosomal compartment to recycling endosomes. SREC-1 plays a primary role in Hsp90-peptide complexes antigen uptake both through cross-priming of MHC class I molecules and entry into the class II pathway [67].

In spite of ex-HSP, SREC-1 modulates the function of TLRs with essential roles in innate immunity. SREC-1 promoted double-stranded RNA-mediated TLR3 activation in human monocytes [68]. SREC-1 mediated entry of TLR4 into lipid microdomains and triggered inflammatory cytokine release in RAW264.7 macrophages upon LPS activation [69]. SREC-1 stimulated double-stranded RNA / CpG DNA-mediated TLR3/TLR9 activation of the innate immune response by triggering signaling through the NF-κB, IRF3, and MAPK pathways leading to transcription of cytokine genes [70].

HSP70 can bind to additional scavenger receptors. HSP70 can bind to LOX-1, a member of both the c-type lectin receptors and scavenger receptors, with the c-type lectin binding domain as well as the scavenger receptor family members SREC-I and FEEL-1/CLEVER-1/STABILIN-1, which have arrays of EGF-like repeats in their extracellular domains [71].

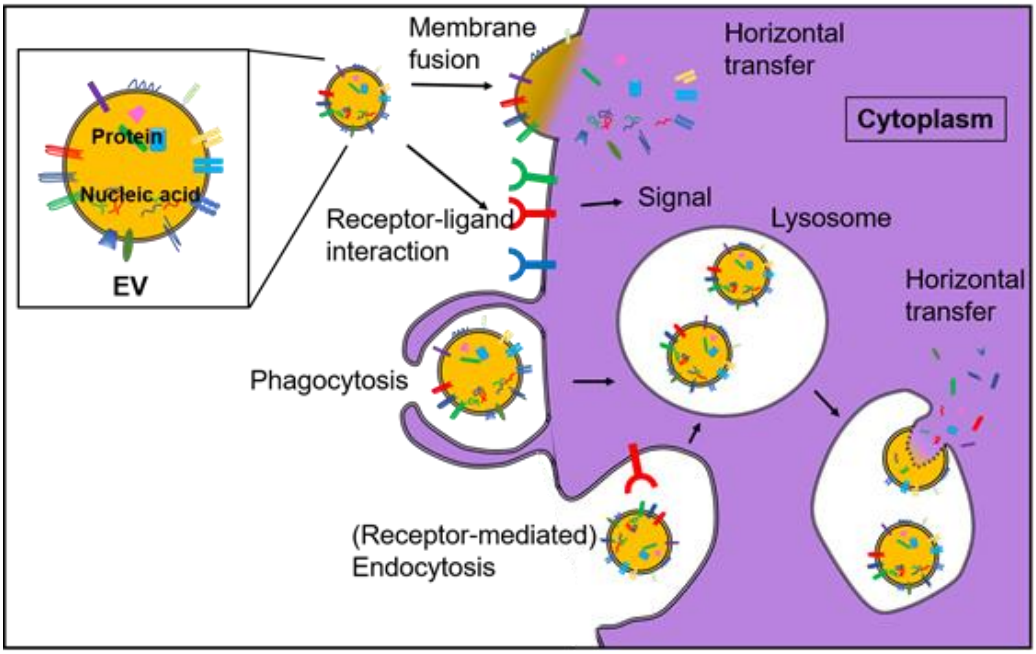

Figure 2. The multiple actions of EVs on/to the cells. The actions of EVs on cells are classified to (i) horizontal transfer of EV cargos, (ii) EV-surface molecules-mediated activation of cell surface receptors and subsequent signal transduction in the recipient cells, and (iii) The activation can also trigger membrane fusion, phagocytosis, macropinocytosis or endocytosis. After the uptake, EV cargos can be processed in lysosomes, horizontally transferred into the cytoplasm or recycled in recycling endosomes. These actions can be involved in the ex-HSP on the surface of EVs and receptors such as LRP1/CD91/A2MR on the surface of recipient cells and EVs.

5. Hypoxia-inducible HSP90

The hypoxic environment in tumors and wound healing is essential for the production of ex-Hsp90. Tumor hypoxia is a distinguishing feature of solid tumors resulting from inadequate oxygen delivery of the abnormal blood vessels supplying the tumor which cannot meet the demands of the rapidly proliferating cancer cells [72-74]. For example, molecularly targeting of C-X-C (Cysteine-X-Cysteine) motif chemokine receptor 4 (CXCR4) on vascular endothelial cells induced tumor angiogenic inhibition triggered necrosis (TAITN) in oral cancer, although HIF-1α was induced in the hypoxic and necrotic tumor tissue [75]. Intratumor hypoxic stress induces HIF-1 that trans-activates a number of target genes, including *HSP90AA1* gene encoding HSP90α [4,76,77], ATP-binding cassette (ABC) transporter genes such as *ABCG1* and *ABCG2* [32], *MMP* genes and connective tissue growth factor (*CTGF)/CCN2* gene [78,79]. Secreted ex-HSP90α and ex-HSP90β were found in the conditioned media of breast cancer cell lines, in which HIF-1α is constitutively active [6]. In breast cancer MDA-MB-231 cells, the secreted ex-HSP90 increased cancer cell survival in a hostile hypoxic environment via CD91-mediated activation of Akt, a kinase mediating cell survival. The three-dimensional (3D) tumor organoid (tumoroid) culture system enabled to reproduce intratumor hypoxia with CSC properties from which ex-HSP90 was abundantly released, although not from 2D-cultured normoxic cells [4,80].

HIF-1 signaling stimulates the migration of human dermal fibroblast (HDF) by inducing the HSP90α secretion into the extracellular environment [77,81]. The secreted ex-HSP90α, in turn, promotes the hypoxia cell motility signaling. Interestingly, recombinant Hsp90α treatment greatly doubled the hypoxia effect on HDFs. On the other hand, antibody blockade of ex-HSP90α completely abrogates the hypoxia–HIF-1 pathway-stimulated HDF migration [57,82]. Transforming growth factor-alpha (TGFα), a member of the EGF ligand family, also stimulates secretion of HSP90α [57]. CD91 also known as LRP1 has been identified as a key receptor of ex-HSP90 [57]. Ex-HSP90 binds to the subdomain II, a ligand-binding repeat, of LRP1 and the intracellular NPVY motif (Asparagine-Proline-Valine-Tyrosine motif) was essential for activation of Akt1/2 signaling [7]. It is thought that EV-HSP90 could also bind to and stimulate LRP1 on the surface of recipient cells (Figure 2).

Therefore, tumor hypoxia induces LRP1 and HSP90 expression and LRP1-HSP90 interaction on the surfaces of cells and EVs could promote tumor growth.

6. Cancer liquid biopsies

6.1. Potentials of HSPs as diagnostic and prognostic biomarkers.

HSPs can be released from tissues into body fluids upon cellular/tissue stress, damage, cell death, hypoxia in cancer progression and exist as forms of free proteins, protein complex, ribonucleoprotein (RNP) complex [1], EV-HSPs or cargos packaged in EVs or exosomes [127] (Figure 3). EV-Hsp and/or ex-Hsp can be attractive biomarkers for diagnosis and prognosis in cancer, including HNSCC [5] and prostate cancer [4]. EVs secreted by high-metastatic HNSCC cells contained high amounts of TRAP1, Hsp90β, Hsp90α, Hsp105/HspH1, and Hsp72/HspA1A, compared to low-metastatic HNSCC cells [5]. Indeed, patients harboring TRAP1-high or HSP90β-high tumors are correlated with poor prognosis compared to low-expression patients groups. In HNSCC patients cases, high expression of TRAP1 and HSP105 were found over the stages (I to IV), while HSP90α/β-high expression cases were increased in later stages (stage II to IV) compared to stage I cases [5].

Ex-HSP90α was abundantly secreted by enlarged 3D hypoxic tumoroids formed with castration-resistant prostate cancer (CRPC) cell line PC-3, although neither by smaller tumoroids nor by 2D-cultured cells [4]. In this model, Ex-HSP90α was abundantly released, while EV-HSP90α was barely detected.

Besides, HSPs belong to tumor-associated antigens (TAAs) overexpressed in various human cancers. Elevated HSP can stimulate the immune system to produce anti-HSP autoantibodies (AAbs). AAbs against HSPs have been identified in the circulation of various cancer patients [128]. Because of their specificity and stability in the sera, AAbs against HSPs can be also attractive biomarkers for the development of less invasive serological tests for the diagnosis and prognosis of cancer.

6.2. Cancer liquid biopsies

Tissue biopsies are commonly used for diagnostic, prognostic and treatment purposes. This surgical procedure has a lot of limitations such as its invasive nature, failure to reflect the tumor heterogeneity, and the most important thing is the discomfort suffered by the patient. Liquid biopsies are considered as a non-invasive alternative to tissue biopsies and can provide advanced diagnostic information compared to tissue biopsies. Liquid biopsy can derive numerous genetic and proteomic information of primary and metastatic cancer, estimate the cellular and molecular characteristics of cancer-associated cells, and monitor the response to different anticancer therapies [129]. It is worth mentioning that liquid biopsies can be performed by using blood [130,131], saliva [132-134], urine [135-137], stool [138,139], semen, sweat, tear, nasal mucus, milk, and cerebrospinal fluid [140-142]. However, blood is more universal and can be used to detect all cancers [143]. Blood analytes are composed of circulating cell-free DNA (cfDNA), circulating cell-free RNA (cfRNA) including small and mRNA, circulating tumor DNA (ctDNA), EVs such as exosomes, circulating tumor cells (CTCs), tumor-educated blood platelets (TEPs), proteins, and metabolites [129,144-148].

In contrast to the normal cells, cancer cells are often characterized by a rapid cellular turnover as a consequence higher numbers of necrotic and apoptotic cells are detected in cancer patients compared to healthy individuals. Interestingly, the circulating cfDNA levels are higher in cancer patients than healthy individuals [149]. In addition, if the size of these fragments is between 180-200 bp, this means that the majority of cfDNA in the circulation is generated from apoptotic cells. But, if the size of the fragments is large of thousands of base pairs, this indicates the necrotic origin. Higher concentrations of the circulating cfDNAs were detected in the blood of lymphoma, lungs, ovaries, uterus, and cervix tumors [150]. Additionally, one-third of cancer patients exhibited a greater quantity of cfDNAs, whereas not detected in the healthy people [151].

EVs existing in liquids such as exosomes, MVs, and oncosomes serve as a promising "liquid biopsy" candidate inasmuch as their cargo contents including protein, RNA, DNA, and lipids reflect their parental cell and might indicate the pathophysiological features of the tumor in real-time status. Moreover, exosomes are exchanged between cells allowing intercellular communications and are involved in cancer progression and resistance. More importantly, exosomes can be easily isolated and characterized in almost all body fluids [152]. Numerous studies showed the significant correlations between exosomal miR-21 [153], miR-195 [154], miR-484/191 [134] and the stage of tumorigenesis. The increased concentration of miR-21 and miR-1246 in plasma exosomes was demonstrated in breast cancer patients compared with those of the healthy donors [155].

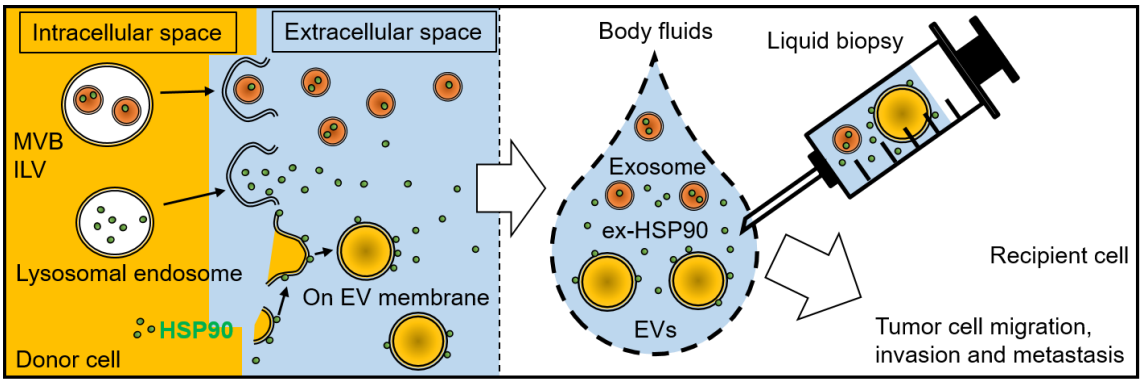

Figure 3. Liquid biopsies for diagnosis, prognosis, and treatment of diseases. Liquid biopsies can be performed by using blood, saliva, urine, stool, semen, sweat, tear, nasal mucus, milk, and cerebrospinal fluid. Blood analytes are composed of circulating cfDNA, cfRNA, ctDNA, EVs such as exosomes, CTCs, TEPs, proteins, and metabolites. HSPs can be released from tissues upon cellular/tissue stress, damage, cell death, hypoxia and exist in body fluids as forms of free proteins, protein complex, RNP complex or EV-HSPs. HSPs belong to TAAs stimulating the immune system to produce anti-HSP autoantibodies and released HSPs can alter secondary tumor niche as well as host immune system. HSPs in body fluids may be a diagnostic or prognostic value as a cancer biomarker. Depletion of HSPs in the blood may prevent cancer progression and resistance.

7. HSP90-targeted therapies: pros, cons, and problem solutions

7.1. Discovery and clinical trials of HSP90 inhibitors

The first discovered HSP90 inhibitor is geldanamycin (GA), belonging to the benzoquinone ansamycin antibiotics [83]. GA was found to arrest the tumor proliferation by inhibiting the Src tyrosine kinase activity, although unable to directly inhibit the activity of purified Src kinase [84,85]. Further studies revealed that the anti-proliferative effect of GA resulted from its binding to the ATP binding pocket of HSP90. Consequently, GA inhibits the binding of the client proteins to HSP90 and leads to the proteasomal degradation of these proteins. These results proved that the efficacy of HSP90 inhibitors is closely related to their binding ability with HSP90.

In order to reduce the hepatotoxicity and increase water solubility, the structure of GA was modified to generate 17-allylamino-17-demethoxygeldanamycin (17-AAG) also known as tanespomycin. The 17-AAG was the first HSP90 inhibitor used in human clinical trials [86]. Although the 17-AAG is still insoluble in water, a considerable effect was observed in clinical phase I trials. In addition, the phase II trials were performed on patients with metastatic breast cancer and melanoma and side effects such as tiredness, nausea, diarrhea, and liver damage were reported, by which the use of 17-AAG was stopped [87]. Thereafter new HSP90 inhibitors have been developed, although did not produce expected results [87].

A number of HSP90 inhibitors inhibit ATP hydrolyzing activity by binding to the ATP-binding site of HSP90 and suppress its chaperone function required for client proteins conformation changes. Such an effect of HSP90 inhibitors decreases the binding affinity of the client proteins to the HSP90, resulting in their dissociation from HSP90. The client proteins became structurally unstable, ubiquitinated, and degraded by the proteasome. The reduction of client oncoproteins prevents the growth of cancer cells. The most surprising finding with HSP90 inhibitors is their higher affinity and selectivity towards the tumor cells and not to the normal cells [88].

7.2. HSP90 inhibitor combination therapies

To overcome this drawback, combining the HSP90 inhibitors with other drugs [89,90] and/or radiation [91,92] have been investigated, but they still under investigation. Most recently, an HSP90 inhibitor XL888 in combination with a BRAF inhibitor vemurafenib has clinical activity in patients with advanced BRAF-V600-mutant melanoma, with a tolerable side-effect profile [93], while it was indicated that HSP90 inhibitors warrant further evaluation in combination with current standard-of-care BRAF plus MEK inhibitors in BRAF-V600-mutant melanoma.

7.3. Limitations of Hsp90 inhibitors

7.3.1. ATP-independent activities of HSP90

Although the most HSP90 inhibitors target the ATP binding site, chaperokine activities of ex-HSP90 and EV-HSP90 are not dependent on the ATP hydrolyzing activity. EV-HSP90 incorporated within the EVs could be propagated in tumor microenvironment and in body fluids and not easily targeted by the small molecule chemical inhibitors. EV-mediated RASP could promote the release of HSP90 inhibitors with EVs.

7.3.2. The physiological necessity of HSP90 and target cell selectivity

HSP90 is required for homeostasis of normal, non-cancerous cells. Without cancer cell-targeted drug delivery system (DDS), HSP90 inhibitors could be harmful and toxic to normal cells leading to unfavorable side effects. Notably, HSP90β is a housekeeping protein whose activities are essential in all cells. Besides, HSP90α is an inducible protein essential for physiological stress response also in normal cells.

7.3.3. HSP90/HSF1 feedback system

HSP90 binds to and keep inactivated status of HSF1, whereas HSP90 inhibitors trigger the release of HSF1 from the HSP90/HSF1 complex and subsequent trans-activation of *HSP* genes and other numerous genes, which induce a stress response and resistance of cancer cells. HSF1 is a stress-

responsive transcription factor and has been reported as a multi-faceted modulator of tumorigenesis [14,94-98]. In response to heat shock stress [26,96,99,100], intracellular accumulation of misfolded proteins [2,101-106] or tumor-promoting signaling such as phosphatidylinositol 3-kinases (PI3K)-Akt-mTOR signaling [94,107], HSF1 is activated and translocated into the nucleus where it binds to HSP genes promoters and fosters their transcription. HSF1 transcriptional activity can be regulated through feedback inhibition by HSP90 [17,23-25]. Therefore, HSP inhibitors could trigger the release of HSF1 from the HSP90/HSF1 complex and de-repress HSF1, which is then able to trans-activate a number of HSP genes and oncogenes [23,24]. Importantly, these stress-responsive genes and the up-regulation of oncogenes will enable tumor cells to respond to a variety of stresses and allow them to thrive unfavorable growth conditions. Thus, the HSP90/HSF1 feedback system could counteract the cell-killing (cytotoxic) effect of HSP90 inhibitors.

7.4. HSP90 mRNA-targeted RNAi therapy

HSP90-rich EVs are released by metastatic cancer cells, whereas small interfering RNA (siRNA) double-targeting HSP90α and HSP90β mRNAs efficiently decreased cancer cell viability, indicating a novel concept of HSP90 mRNA-targeted oligonucleotide therapeutics [5].

8. CDC37 is a key co-chaperone for HSP90 in cancer progression and resistance

A number of studies have reported pathophysiological roles of HSP90 in various diseases, including bacterial and viral infection [108-111], autoimmune diseases [112-116], cerebrovascular diseases [117-119], and cancer. It is worth noting that the up-regulation of HSP90 in cancer is due to the fact that cancer cells are constantly under stressful conditions such as acidosis, hypoxia, metabolic, and nutrient deficiency [4,15,120]. High expression of HSP90 has been reported in various cancer types, including lung cancer, breast cancer, colon cancer, and blood cancer and correlates with poor prognosis [17,121]. HSP90 is involved in the maturation and stabilization of a wide range of oncogenic client proteins crucial for oncogenesis and malignant progression, such as signal transduction molecules SRC and RAF1, cyclin-dependent kinase-4 (CDK4), steroid hormone receptors, nitric-oxide synthase (NOS), Akt, PI3K, mutant p53 [122,123] , ERBB2 (also known as HER2) [124], and HIF-1α. The stabilities of these client proteins depend on co-chaperones of HSP90, which are composed of more than 10 types [18]. CDC37 plays a crucial role as a co-chaperone of HSP90 in the stabilization of the most kinases, including SRC, RAF1, and CDK4, and steroid hormone receptors [27,125,126]. Therefore, HSP90 and/or CDC37 are attractive therapeutic targets against various cancers inasmuch as HSP90 and CDC37 are involved in the functionalization of oncogenic proteins in many signaling pathways important for tumor progression, survival, and resistance.

9. Transcription factors that regulate the production of chaperones and cochaperones: MMP3, HP1, MZF1, and SCAN-D1

Induction of HSPs upon cell stress is primarily mediated by HSF1 and by HIF-1 under hypoxic stress, although recent studies have discovered additional transcriptional factors that control HSP expression. Intracellular MMP-3 and HP1 also known as CBX activate *HSP70* gene [26]. HSP90 co-works with more than 10 types of co-chaperones including CDC37 [125]. CDC37 transcription and expression are reciprocally regulated by two SCAN-type transcription factors- MZF1 and SCAN-D1, the former activates and the later repress *CDC37* gene [27,28].

Molecularly targeted therapeutics for HSPs, HSF1, and HIF-1 have been developed. However, co-chaperones, including CDC37, other transcriptional regulators such as MMP3, HP1, and MZF1, their cross-talks and feedback are potentially important in cancer progression and resistance.

10. Conclusions

EV released by tumor cells are a major aspect of the resistance-associated secretory phenotype (RASP), by which immune evasion can be established. (i) Releases of ex-HSPs and EV-HSPs are essential in RASP, by which molecular cotransfer of HSPs with oncogenic factors into recipient cells can promote cancer progression and resistance against stress such as hypoxia, radiation, chemicals,

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and immune system. (ii) RASP of tumor cells can eject anticancer drugs, molecularly targeted therapeutics, and immune checkpoint inhibitors with EVs. (iii) Cytotoxic lipids can be also released from tumor cells as RASP. Nevertheless, ex-HSP and EV-HSP can play immunostimulatory and immunosuppressive roles by binding to a few types of receptors expressed on recipient cells. Liquid biopsy of HSPs in body fluids may be useful in diagnosis, prognosis, and treatment in cancer. Regarding HSP90-targeted therapeutics, there have been pros, cons, and problem solutions. Although production of HSPs is canonically induced by HSF1 and HIF-1, recent studies discovered that production of HSPs is also regulated by MMP3 and HP1/CBXs and production of cochaperone CDC37 is reciprocally regulated by MZF1 and SCAN-D1.

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Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
A2MR	Alpha 2 macroglobulin receptor
AAb	Autoantibody
ABC	ATP-binding cassette
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BiP	Binding immunoglobulin protein
CAF	Cancer-associated fibroblast
CBX	Chromobox protein
CDC37	Cell division control 37
CDK	Cyclin-dependent kinase
cfDNA	Cell-free DNA
cfRNA	Cell-free RNA
CIC	Cancer-initiating cell
CRPC	Castration-resistant prostate cancer
CSC	Cancer stem cell
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
CTGF	Connective tissue growth factor
CTL	Cytotoxic T-lymphocyte
CXC	Cysteine-X-cysteine motif
DAMP	Damage-associated molecular pattern, danger-associated molecular pattern
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
EV	Extracellular vesicle
EV-Hsp	Extracellular vesicle-associated heat shock protein
ex-Hsp	Extracellular HSP
FcR	Fragment-crystallizable receptor
GP96	Glycoprotein 96
GRP	Glucose-regulated protein

HIF	Hypoxia-inducible factor
HP1	Heterochromatin protein 1
HSF	Heat shock factor
HSP	Heat shock protein
ILV	Intra-luminal vesicle
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein receptor-related protein 1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MV	Microvesicle
MVB	Multi-vesicular body
Myd88	Myeloid differentiation 88
MZF1	Myeloid zinc finger 1
NK	Natural killer
OncomiR	Oncogenic microRNA
OSCC	Oral squamous cell carcinoma
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
PI3K	Phosphatidylinositol 3-kinases
POC	Proof of concept
RA	Rheumatoid arthritis
RASP	Resistance-associated secretory phenotype
RTK	Receptor tyrosine kinase
SCAN	SREZBP-CTfin51-AW1-Number 18 cDNA
SREC	Scavenger receptor expressed by endothelial cells-1
TAA	Tumor-associated antigen
TAITN	Tumor angiogenic inhibition triggered necrosis
TEP	Tumor-educated blood platelet
TLR	Toll-like receptor
TRAP-1	TNF receptor-associated protein-1
Treg	Regulatory T cells
Tumoroid	Tumor organoid

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