Heat Shock Proteins 21
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Heat Shock Proteins in Human Diseases

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Heat Shock Proteins

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Heat Shock Proteins offers rapid publication of novel and outstanding research on all aspects of Heat Shock Protein research, the Heat Shock Response, and Cell Stress. In addition, as cells possess additional responses to Hypoxia, Genomic Stress, Oxidative Stress and related pathways that may involve molecular chaperones as well as unique molecular responses, these topics are welcomed. Degeneration of HSP expression and the multiple stress responses underlies many human, animal and plant diseases and this forms a major component of our content. This range of subjects have great topicality as the stresses associated with climate change advance rapidly in the earth's environment. In addition, the potential to escape from the eroding earthbound environment, such as with space travel will impose novel challenges to the human system that we will cover in the series.

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Preface

The heat shock protein family consists of several proteins, which are synthesized in response to a variety of stressors including hyperthermia, ischemia, infarct, lesions, and seizures or following less drastic metabolic changes as those produced by physical exercise or psychological stress. HSP is present in all living organisms. HSP regulates the stability, activation, and degradation of a diverse array of proteins associated with growth, proliferation, and survival. Thus, it is core to regulation of protein stability and protein-degradation pathways and modulating transcription factors, signaling transduction networks, and kinases. It facilitates the survival of cells during stress response and exhibits a pronounced anti-apoptotic and stabilization effect. HSP performs various functions in the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix and has been linked to diabetes, stress response, cancer, and certain types of immunological disorders.

The book *Heat Shock Proteins in Human Diseases* provides the most comprehensive review on contemporary knowledge on the role of HSP in various human diseases. Using an integrative approach to the understanding of HSP structure, function, and immunobiology, the contributors provide a synopsis of novel mechanisms by which HSP is involved in the regulation of various human diseases.

Key basic and clinical research laboratories from major universities, academic medical hospitals, biotechnology, and pharmaceutical laboratories around the world have contributed chapters that review present research activity and importantly project the field into the future. The book is a must read for graduate students. Medical students, basic science researchers, and postdoctoral scholars in the fields of Translational Medicine, Clinical Research, Human Physiology, Biotechnology, Natural Products, Cell & Molecular Medicine, Pharmaceutical Scientists and Researchers involved in Drug Discovery.

Toledo, OH, USA

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About the Editors

Prof. Dr. Alexzander A. A. Asea is a highly innovative and accomplished world renowned clinical and basic research scientist and visionary executive leader who has exceptional experience spearheading clinical and basic science research, training, education, and commercialization initiatives within top-ranked academic biomedical institutes. Prof. Dr. Asea's initial findings studying the effects of Hsp72 on human monocytes lead to the proposal of a novel paradigm that Hsp72, previously known to be an intracellular molecular chaperone, can be found in the extracellular milieu where it has regulatory effects on immunocompetent cells—a term now called chaperokine. Prof. Asea has authored over 320 scientific publications including peer-reviewed articles, reviews, books, book chapters, editorials, and news headliners in a wide range of biomedical-related disciplines. Prof. Asea is the Editor-in-Chief of the widely successful book series *Heat Shock Proteins* (Springer Nature Publishing) and is an editorial board member of numerous scientific peer-reviewed journals. Prof. Dr. Asea is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Dr. Punit Kaur is an expert in onco-proteogenomics, with extensive training and experience in quantitative mass spectrometry imaging, protein chemistry, and biomarker discovery. Dr. Kaur's main research focus is on the use of heat-induced nanotechnology in combination with radiotherapy and chemotherapy in the cancer stem cell therapy. Dr. Kaur has published more than 70 scientific articles, book chapters, and reviews, and currently serves as editorial board member for the *European Journal of Cancer Prevention* and the *Journal of Proteomics and Bioinformatics*. Dr. Kaur is the Associate Editor of the highly successful *Heat Shock Proteins* book series by Springer Nature Publishers. Currently, Dr. Kaur is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Inter-Relationship Between the Inflammation and Heat Shock Protein in Cancer Development: A Possible Target for Diagnosis and Cancer Immunotherapy

Prathap Somu and Subhankar Paul

Abstract

Introduction Inflammation has been considered an essential, albeit insufficient factor in Tumour induction, progression, and metastasis. The multiple components of both innate and adaptive immune systems are involved in The development of various stages of tumorigeneses such as tumor angiogenesis, metastatic properties of neoplasms, and tumor tolerance. However, the role of Heat-shock proteins (HSP) found to act as an inflammatory mediator and providing a link for the infection-mediated chronic inflammation and subsequently leading to cancer pathogenesis. Heat-shock proteins (HSP), highly conserved stress protein across, have any different other than its primary chaperone activity, such as autophagy, apoptosis, and immunity. The HSP has found to have strong influence modulating innate and adaptive immunity due to its capability interacting with professional antigen-presenting cells in the immune surveillance mechanism to prevent cancer. This chapter aims at the discussion and exploration of the relationship between inflammation and HSP in infection-induced carcinogenesis by inflammatory mediators non-chaperone activity of HSP as well as its in the cancer diagnosis and the discovering of new therapeutic targets and strategies.

Methods The authors have reviewed most of the relevant papers regarding the relationship of HSP and inflammation in cancer development and progression as well as cancer prevention by simulation of a specific antitumor immune response.

Results The duality in the HSP activity is well reported, i.e., an immunostimulatory and immunosuppressive activity. These unique immunological attributes of HSP have opened new anticancer regimens HSP-based immunotherapeutic approaches where HSP has been used as an antigen and an adjuvant for specific antitumor immune response. Further HSP based vaccines have been developed for cancer

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prevention. For example, Vitepsin-Gp96-based vaccine that is under phase III clinical trials has shown promising results against melanoma, glioma, and ovarian cancer.

Conclusions HSP-based cancer immunotherapy has become one of the therapeutic options for cancer treatment. Nevertheless, the more effective HSP based immunotherapeutic strategies can be developed by studying the underlying mechanism of HSPs in the modulation of the tumor microenvironment in eliciting an antitumor immune response. Moreover, HSP based immunotherapy might also be used in combination with conventional treatments such as chemotherapy and radiotherapy for sensitizing cancer cells for better therapeutic intervention. However, in the future, it is anticipated that HSP-based immunotherapy remains as a critical focus area in cancer therapeutic with the hope for more discoveries in terms of diagnosis, therapy as well as in clinical management of cancer patients.

Keywords Chronic inflammation · Heat shock proteins · Immunotherapy · Nanomaterials · Tumorigenesis · Tumour immune surveillance

Abbreviations

APCs	antigen-presenting cells
APE1	apurinic/apyrimidinic endonuclease
BCG	Bacille Calmette–Guérin
BER	base excision repair
CHKI	checkpoint kinase 1
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T cell responses
CTLs	cytotoxic T-lymphocytes
DCs	dendritic cells
DDR	DNA damage response
EBV	Epstein-Barr virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPV	Human Papillomavirus
MAPK	mitogen-activated protein kinase
MGMT	O6-meG-DNA methyltransferase
MIBC	muscle-invasive bladder cancer
MIF	macrophage-derived migration inhibitory- factor
NMIBC	nonmuscle-invasive bladder cancer
RANTES	regulated on activation normal T cell expressed and secreted
SCC	squamous cell carcinoma
SMAC	second mitochondrial-derived activator of caspase
TAM	tumor-associated macrophages

TGF-β	transforming growth factor-β
TLR2	toll-like receptors-2
TNF-alpha	tumour necrosis factor-alpha
UDG	uracil DNA glycosylase

1 Introduction

Cancer has been identified as the second major cause of death by the WHO as nearly 1 out of 6 deaths by cancer, and also, almost 10 million new cases are registered every year [1]. Even though there are multiple therapeutic techniques for cancer treatment, but their faces significant threat due to genetic plasticity ability adoptive cellular machinery like molecular chaperones of cancer to adoption efficient against the therapeutic agent such as multi-drug resistance (MDR) in case of chemotherapy [2, 3]. Hence, the best possible strategy for treating cancer is to target the very cellular-machinery that allows adapting successfully to stress applied via therapy and survive [4]. Heat shock proteins (HSP) or Molecular chaperones such as one such essential cellular-machinery in cancer cells that allows them to adapt therapeutic stress such as oxidative damage, high temperature, and hypoxia [3] and there are found to be synthesized in elevated levels in the cancer cells to protect itself against such stress induced by various therapy.

Molecular chaperones or Heat shock proteins (HSP) are an essential group of ubiquitous polypeptides-proteins molecules which are highly conserved during evolution across prokaryotes as well as eukaryotes [5, 6]. HSP expression is a stress-dependent an adaptive mechanism for maintaining cellular homeostasis in both optimal and detrimental growth conditions through cytoprotective mechanisms, thereby enhancing the survival of such stressed cells [7]. HSP maintains cellular homeostasis by being actively involved in protein folding and re-folding as well as assembly of multiunit protein structure and their translocation, further directing the defective or irreversibly misfolded protein to degradation in a broad range of cellular processes in healthy cellular growth and development [8, 9]. Moreover, Molecular chaperones or Heat shock proteins (HSP) also possess extra-chaperone activity such as regulating the immune system [6, 10], cell senescence and differentiation [11–13], signal transduction [14], gene expression [14], apoptosis [15], as well as have a negative effect designated to favor cancer progression, development, and metastasis [16–18].

Recently due to the identification of new tumor-associated antigens as the target for immunotherapy along with a better understanding of the HSP roles in tumor immunity as tumor-rejection antigens have provided an excellent opportunity for cancer treatment [19]. The immunoregulatory activity of HSP has been grouped into three tenets: Firstly, Molecular chaperones or HSP ability to bind to tumor-associated peptides/protein myriad. Secondly is the presence of specific surface-receptors over antigen-presenting cells (APCs), allowing for the efficient uptake of

peptide/protein–chaperones complexes. This process of cellular-uptake of peptide/protein–chaperones complexes leads to the exogenous antigens cross-presentation shuttled by chaperones, thereby subsequently resulting in antigen-specific cytotoxic T cell (CTL) responses. Third and finally, the initiation of adaptive immune responses due to interaction between molecular chaperones or HSP with innate immune-components like NK or APC cells) resulting in the activation of CD8+ CTL and CD4+ T helper cells [20, 21]. Thus, the discovery of chaperone preparation from autologous tumors used for cancer treatment will open up a new-domain in immunotherapy. Human clinical trials have been conducted used tumor-derived chaperones and have shown promising results. However, novel strategies for cancer treatment using chaperone-based-vaccination are under clinical trials. This book chapter summarizes the immunomodulating functions of molecular chaperones in cancer development as well as its potential application as a therapeutic target in cancer immunotherapy.

2 Cancer, Inflammation and HSP

2.1 Role of Inflammation in Cancer Development

Although, there are multiple significant factors for causing cancer such as genetic mutations, environmental factors (e.g., ionizing radiation, cancer-causing), lifestyle (e.g., excess alcohol consumption, smoking, imbalanced diet and) and hormonal imbalance, but inflammation has been considered as a one of albeit insufficient cause for tumorigenesis in some cancers which date back to early work of Rudolf Ludwig Karl Virchow [22, 23]. Inflammation forms the non-specific part of the immune system, which acts as the first line of defence against to all kinds of cellular damages such as infections due to viral and bacterial, toxins and chemicals, thereby helps in clearing cellular damage by initiating the cellular repair and healing process [24]. The Inflammatory response has been broadly classified into two types, namely acute and chronic inflammation. Acute inflammation is a self-limiting short-term response and considered to be advantageous to the body when it is short-lived, but when it develops into an uncontrolled and long-termed, it becomes chronic inflammation. Chronic inflammation characterized by activation of the inflammatory signaling pathway, acute-phase reactants, increased production of both inflammatory mediators, and abnormal cytokines [25]. This chronic inflammation will destroy several healthy cells and tissues, which may also lead to organ failure or even mortality in some instances in cancer as well as in HIDs [26].

The prolonged chronic inflammation due to viral and bacterial infections such as *human papillomavirus (HPV)* and *Helicobacter pylori* have been considered to contribute immensely to the development of several types of cancer [22]. However, it is also estimated that about 25% of cases of cancer are due to chronic inflammation caused by infectious or Physico-chemical agents, For example, persistence *Helicobacter pylori* and hepatitis (Type A and C) endorses the high risk of stomach

cancer and hepatocellular carcinoma, respectively [27]. The development of cancer due to chronic inflammation might also be as a result of improper regulation and stimulation of cytokines as well as immune cells, causing DNA damage and mutation of oncogenes leading to cancer development. The proper stimulation of inflammatory response leads to immediate clearance of inflammatory cytokines (e.g., interleukins) and immune cell apparatus through phagocytosis as well as apoptosis after acute inflammation [29]. The engulfing of the apoptotic factors by phagocytic cells helps anti-apoptotic action by promoting the anti-apoptotic transforming growth factor-β (TGF-β) production. The binding of TGF-β-threonine/serine kinase complex to TGF-β receptors triggers the cascade of signaling pathways that helps to regulate cell proliferation, cell differentiation, chemotaxis, and several immune cell activators leading to inhibition of cancer development [30]. Moreover, inadequate and improper regulation of inflammatory response causing chronic inflammation due to massive cellular destruction as well as neoplastic transformation may result in cancer progression and other inflammatory diseases [28]. As both Inflammation and cancer are complicated pathologic processes that are driven by multiple factors rather than one, thus Fig. 1 illustrates the underlying mechanisms in the involvement of inflammation in cancer development as well as its role in drug resistance. Chronic inflammation caused due to external factors (viral infection, prolonged exposure environmental irritants, etc.), as well as intrinsic factors (oncogene activation), thereby precedes the mechanisms for tumor initiation, development, invasion, and metastasis. Chronic inflammation results in the activation of transcription factors leading to production of cytokine and chemokine production in the tumor microenvironment which in turn help in the recruitment of various innate immune cells (including mast cells, macrophages, neutrophils, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) as well as adaptive immune cells (T and B lymphocytes). These immune cells communicate with each other through cytokine and chemokine balance toward tumor-supporting inflammation and inhibits anti-tumor immunity by inducing genomic instability, oncogenic mutations, early tumor promotion, as well as increased angiogenesis. Further, the activation of the inflammatory response alters the therapeutic response to hormones during cancer therapy, thereby helps in accruing tumor re-emergence and resistance to therapy.

2.2 Role of Heat Shock Proteins (HSP) in Cancer Development

Heat shock protein (HSP) are recognized over the last quarter of a century as stress protein selectively expressed by the organisms in response to various stress conditions. Major HSP of mammalian cells has been classified into six major families based on their molecular weight, which includes hsp20–30, hsp50–60, hsp70, hsp90, and hsp110 families. The HSP of different families has minimal homology in their

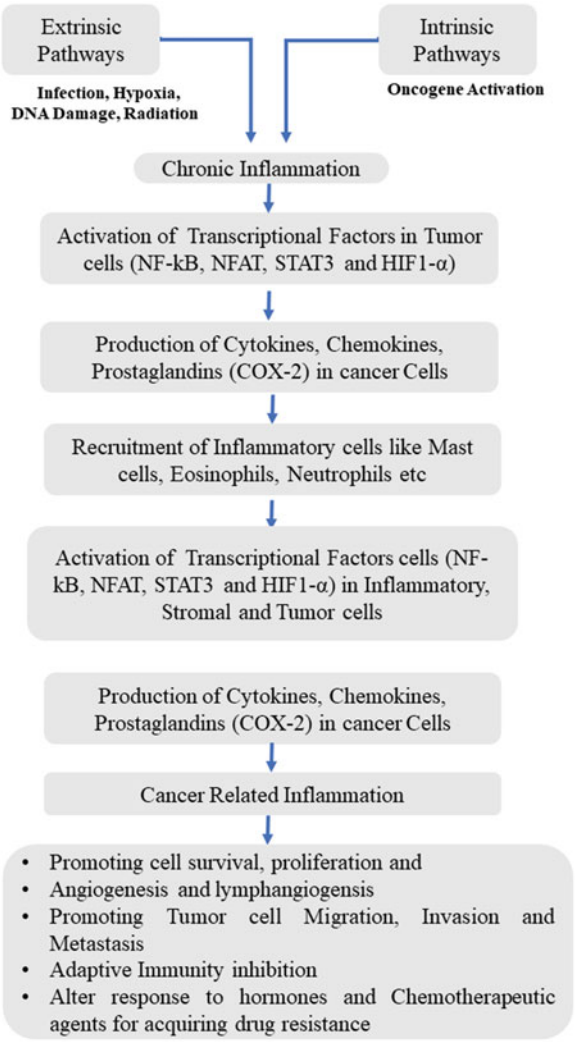

Fig. 1 The schematic representation illustrating the underlying mechanism of various inflammatory components involved in tumor development, progression and metastasis as well as The development of drug resistance. Chronic inflammation caused due to external factors (viral infection, prolonged exposure environmental irritants, etc.), as well as intrinsic factors (oncogene activation), thereby precedes the mechanisms for tumor initiation, development, invasion, and metastasis. Chronic inflammation results in the activation of transcription factors leading to production of cytokine and chemokine production in the tumor microenvironment which in turn help in the recruitment of various innate immune cells (including mast cells, macrophages, neutrophils, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) as well as adaptive immune cells (T and B lymphocytes). These immune cells communicate with each other through cytokine and chemokine balance toward tumor-supporting inflammation and inhibits anti-tumor immunity by inducing genomic instability, oncogenic mutations, early tumor promotion, as well as increased angiogenesis. Further, the activation of the inflammatory response alters the therapeutic response to hormones during cancer therapy, thereby helps in accruing tumor re-emergence and resistance to therapy

amino acid sequence, whereas members of the same family show were quite highly similar. The primary responsibility of HSP is to acts as molecular chaperones to maintain cellular homeostasis. The chaperone activity of HSP is to provide support proper three-dimensional folding to an active conformational form of newly formed proteins, refolding of misfolded/damaged proteins as well as protein translocation and elimination of old and broken protein by guiding to proteasome thereby acts a key regulator for cell growth, proliferation, and survival [8, 31].

It has been reported about HSP are especially activated in cancer cells with its role in tumor prognosis, development, invasion, and metastasis. As during oncogenesis, the essential functions of HSP are undermined, such that to make malignant transformation possible by facilitating continued protein translation and cellular proliferation, even undergrowth factors-deprived conditions [32]. For example, it has been identified that the HSP derived from tumor cells is acetylated, further found to be having directly no relationship with HSP obtained from the normal or microbial cell [33]. As gp96, an endoplasmic reticulum chaperone isolated from tumor cells found to have profoundly different glycosylation patterns when compared to its native form of the host cell [34]. Hence, these elucidate the immune surveillance deficiencies in the tumor as gp96 plays a vital role in tumor immune surveillance [34].

Tumor-derived HSP90 present entirely as multi-chaperone complexes showed high ATPase activity compared to non-tumor HSP90, which was able to rescue both wild type proteins and unstable mutant proteins [35]. For instance, ZAP-70+ lymphocytes of chronic lymphocytic leukemia (CLL) found to express the activated HSP90 binding to ZAP-70 as well as stabilizing ZAP-70 with several HSP co-chaperones [36]. The family of HSP90 consists of mitochondrial TRAP1/hsp75, cytoplasmic inducible α-form, HSP90β, and GRP94/gp96. HSP90 found to be assisting the multiple significant tumorigenic proteins like Bcr-Abl, Akt, Raf-1, HER-2/ErbB2, and v-Src [37, 38]. Further, it was also found that overexpression of HSP90 in the solid-tumor and hematologic-tumor was responsible for poor prognosis and therapeutic failure [39]. The expression of GRP94/gp96 (glucose-regulated HSP belongs to HSP90) found to been significantly associated with intrahepatic metastasis and vascular invasion [40]. HSP90 also found to be promoting metastases by NF-kB-dependent MMP-2 chaperone activity [41]. It has found that forced overexpression of intracellular HSP27 or HSP70 in the cultured cells, as well as, in transgenic mice resulted in tumor formation by cellular transformation [42, 43].

HSP70 belonging to the group of inducible HSP proteins has also to regularly expressed over the plasma membrane of various cancer metastases such as breast, pancreas, lung, and colon [44]. Since HSP70 is an inducible HSP protein and its expression might be induced by a wide range of stimuli, including chemotherapy. HSP70 also found to have intense anti-apoptotic activity by preventing the caspase activation, thereby reducing mitochondrial damage and nuclear fragmentation [45]. HSP70 can inhibit apoptosis by blocking Bax translocation and leading to the outer mitochondrial membrane stabilization [46].

HSP27, which also belongs inducible HSP family, has shown to infer the drug resistance to multiple myeloma cells against dexamethasone by inhibiting the release of Second Mitochondrial-derived Activator of Caspase (SMAC) from mitochondrial

which acts as a master regulator of apoptosis [47]. Cornford et al. (2000) have reported that there is a high correlation between the HSP27 expression level and the Gleason score in prostate cancer [48]. Further, there are multiple reports for the elevated expression of HSP40, HSP60, and HSP70 in response to the development of intraepithelial neoplasia and cervical cancer [47]. These above examples may provide insight into the complicated relationship between HSP and cancer formation.

2.3 Relationship Between HSP and Inflammation in Cancer Development

The evidence currently available does not sufficiently explain the role of chronic inflammation in cancer development, which can be partly reconciled by determining unique HSP present in and around the tumor site. Recently, HSP has been drawn up as biochemical components having both oncogenesis activity [49] and anti-tumor immunity [21]. In the immune system, the role of HSP depends on its presentation to antigen and innate receptors expression as well as for the extracellular functions in autoimmunity and tumor immune surveillance [50]. The extracellular activity of HSP depends on its potential effects on the immune response in both tumor stimulatory and regulatory roles [50]. The numerous studies reported the dual function of HSP, which depends on their cellular location. Intracellular HSP shows cytoprotective function by various mechanisms. HSP 70 or HSP 27 directly involved in the programmed cell death by interacting with various cellular machinery playing a vital role in programmed cell death. Whereas extracellular or membrane-bound HSP found to mediated different immunological functions by the modulating, both innate as well as adaptive immune systems [44]. As described above, it has been identified that gp96, an endoplasmic reticulum chaperone isolated from tumor cells, found to have profoundly different glycosylation patterns when compared to its native form of the host cell [34]. Hence, these elucidate the immune surveillance deficiencies in the tumor as gp96 plays a vital role in tumor immune surveillance [34].

Moreover, Paget (1887) has believed that microbial parasites or their product, essential found relation to cancer and cancerous disease [51]. Dr. Johannes Fibiger demonstrated the induction of several cancer types in the stomach of rats by feeding nematode collected from cockroach [52]. In 1851, Theodor Bilharz reported that Schistosoma a parasitic trematode causing chronic local inflammation, thereby increasing the chance of developing squamous cell bladder cancer [53]. However, each year around 27% of bladder cancers patients in Egypt has caused by schistosomiasis [54]. The Adult schistosome trematodes are predominantly found around venous plexus of the urinary bladder, and they release eggs that can traverse the bladder wall, causing hematuria. During the early stages of schistosomiasis infection, a T_H1 profile has been found as a result of immune responses as antigens

directed against of schistosomula. However, T_H2 response replacing T_H1 responses profile vigorous with the egg-laying as the immune-response is redirected towards egg antigens, thereby resulting in tissue granuloma surrounding eggs with an infiltrate of T_H2 cells, macrophage, eosinophils, and a dense collagen-rich matrix with fibroblast cells. However, Schistosome-induced macrophages and neutrophils serve as one of the significant sources for endogenous single let oxygen or hydroxyl radicals, which in turn may lead to the production of *N*-nitrosamines, a well-known carcinogen [55].

Further, the inflammatory cells induced due to schistosomiasis may also contribute in the procarcinogens activation like aromatic amines and polycyclic aromatic hydrocarbons, resulting in the generation carcinogenic metabolites [56], thereby leading to genotoxic effects, such as sister chromatid exchanges, mutations, as well as single and double-strand DNA breaks. Furthermore, the increase in the number of inflammatory cells around the urinary bladder of schistosomal patients may increase the potential of these carcinogenic agents by enhancing their activation rate. Moreover, Tumor-associated macrophages (TAM) are attracted to the bladder in the patients with S. *haematobium* and bladder cancer, found to produce Tumour Necrosis Factor-alpha (TNF alpha) which a vital component of inflammation that is upregulated by HSP60 and HSP90. Maresca et al. (1992) have reported the production of HSP by a wide variety of parasitic organisms [57]. In S. *mansoni*, the production of HSP27, HSP58, HSP60, HSP70, and HSP86 have been reported. In superficial transitional cell bladder cancer, it was found poor prognosis might be due to the loss of expression of tumor-derived surface HSP60 and HSP90 as T-cells and NK cells not able to recognize these tumor cells due to the absence surface HSP60 and HSP90 [58].

Further, there are also reports on bacterial infection as a cause of cancer such as Salomon in 1896 and Krienitz in 1906, and a similar finding was related to gastric cancer in a human patient [59]. The most vital invention described related to bacterial infection-induced cancer is the identification of *Helicobacter pylori* in 1983 as one of the primary causes of gastric cancer and gastric lymphoma [60, 61]. In gastric mucosa, *Helicobacter pylori*-associated with infiltration by mononuclear and neutrophils cells, *often* attracted by RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) and granulocyte-macrophage colony-stimulating factor. Subsequently, monocytes and macrophages cells respond to *Helicobacter pylori* presence through toll-like receptors-2 (TLR2) leads to the activation of NF-κB and the early release of proinflammatory cytokines, such as IL-1β. MIF (macrophage-derived migration inhibitory- factor) expressed by *Helicobacter pylori* is a potent cytokine that has the ability overrules tumor suppressor p53 activity by overwhelming its transcriptional activity, thereby leading to the increased DNA damage by inflammatory cells [62]. In the presence of lipopolysaccharide, the disinhibition of iNOS due to H. *pylori* infection results in significantly reducing the HSP70 and HSP27 expression [63]. The chronic infection of gastric mucosa by H. *pylori results* in the increased expression of iNOS, which subsequently leads to oxidative damage [64]. Further, the increase in expression of various cytokines, including IL-1β, IL-6, IL-8, and TNF-α, have been reported [65].

Moreover, multiple reports are stating that the up-regulation of IL-8 in the host cell due to its infection with H. *pylori* [66]. Also, the high vascularity in human gastric carcinomas has been correlated with the NF-κB-dependent expression of IL-8 [67]. Takenaka et al. (2004) have reported that induction of IL-8 production and secretion in KATO III human gastric epithelial cells through TLR-2 and TLR-4 pathways by the activation of NF-κB and mitogen-activated protein kinase (MAPK) by H. *pylori*-derived HSP60 [68]. HSP62, which is the homolog to the HSP of H. *pylori, also known as* GroEL, found to participate in the extracellular assembly of urease (virulence factor) derived from *H. pylori* [69]. These mechanisms offer insight into the relationship between tumorigenesis, chronic inflammation, HSP, and H. *pylori* infection.

Parasites and bacterial infection not only causes cancer. Dr. Peyton Rous first reported that sarcomas in chickens is caused by RNA retrovirus and received the Nobel Prize for the same in 1966 [70]. Since after that, several human cancers have been considered to be caused due to viral infections, but the exact mechanism involved not let illuminated.

Hepatitis B virus (HBV) have been cause hepatocellular carcinoma (HCC) in humans by Blumberg in 1963. The activation of CD8+ cytotoxic T lymphocytes due to the surface expression of viral HBsAg and HBcAg in association with MHC class I molecules leads to the production of IFN-gamma. Hepatic GRP94/gp96, molecular chaperones associated with an endoplasmic reticulum member and belongs to the HSP90 family found to be associated with Hepatitis B virus DNA and core antigen protein in hepatocellular carcinoma biopsies [71]. Further, it has been found that the expression of Hepatic gp96 is correlated with the tumor size and degree of tumor differentiation [71]. Interestingly, the expression of the Second Mitochondrial-derived Activator of Caspase (SMAC), a well-known HSP27 inhibitor, has been to correlate with disease-free, prognosis, and overall survival rate of patients with HBV-associated hepatocellular carcinoma [72].

Epstein has reported in Central Africa Epstein-Barr virus (EBV) is responsible for causing endemic Burkitt's lymphoma, which is a highly aggressive but curable form of nasopharyngeal carcinoma, as well as non-Hodgkin lymphoma. EBV virus binding to CD21 on B-cells results in the HSP induction as well as the transformation of some B-cells in becoming independent regulatory factors, including T cells. Cheung et al. (1993) have reported the influence of EBV infection in the induction of HSP70 and HSP90 at mRNA and protein levels. HSP Induction, as well as B-cell transformation, are dependent on *trans*-membrane Ca2+ currents induced by EBV infection but not dependent on gene products of Epstein-Barr virus (EBV). However, it was found that blockade of the induction of HSP found to prevent transformation [73], thereby providing possible evidence to prove the essential requirement for HSP induction and deciphering its role in tumorigenesis.

3 Role of the Immune System and HSP in Cancer Diagnosis and Prevention

3.1 Ingenious Tumour Immune Surveillance Mechanism to Prevent Cancer

The immune system plays three primary roles in cancer or tumor prevention. Firstly, the prevention of virus-induced tumors by protecting the host from viral infections. Secondly, the prevention of the formation of an inflammatory environment favorable to tumorigenesis by timely and regular eliminating or suppressing pathogens. Thirdly, the most important mechanism of the immune system known as tumor immune surveillance involving identifying tumor cells/cancerous or precancerous cells as well as eliminate them basis on their tumor-specific antigens or molecules expression induced by cellular stress. Despite the presence of functional tumor immune surveillance ability of an immune system, there is a development of tumors. Therefore, it has led to the new and updated concept of tumor immunoediting for providing an extensive explanation for the role of the immune system in tumor development and prevention [74, 75], as described in Fig. 2.

Tumor immunosurveillance ability of the immune system for tumor suppression. Tumor or cancer immunoediting process is extrinsic tumor suppressor mechanisms by the immune system against the transformed cells that have escaped the intrinsic-cellular system to detect and eliminate developing tumors before becoming clinically apparent. Cancer immunoediting process of the immune system protects the host body from tumor development is the 3 Es of cancer immunoediting: cancer immune surveillance, or elimination phase; Secondly, the equilibrium phase in which tumor remains dormancy where the immune system and tumor cells attain a dynamic equilibrium; and Finally, escape phase, the immune system is no longer capable preventing tumor progression leading to tumor cells displaying the reduced immunogenicities or gaining of possible immunosuppressive mechanisms to reduce antitumor immune responses leading the progression of tumor growth. IDO, indoleamine 2,3-dioxygenase; MICA/B, MHC class I chain-related antigens A and B; sMICA/B, soluble MICA/B.

The concept of tumor immunoediting has been divided into three phases, namely, elimination, equilibrium, and escape [76]. The elimination process involves the same method as that of tumor immune surveillance, where the immune system identification and removal of tumor cells that have developed due to the failure of intrinsic-cellular-tumor-suppressor mechanisms. The elimination phase can be complete or incomplete when all the tumor cells are eliminated, or a part is removed, respectively. The second phase of tumor immunoediting equilibrium is observed in the case of partial or incomplete tumor elimination, where a temporary equilibrium state is attained between the developing tumor and the immune system.

Moreover, during this equilibrium phase, tumor cells either remain inactive or continue to evolve by further changes such as gene mutations or changes in gene expression that modulates expression of the tumor-specific antigens and stress-

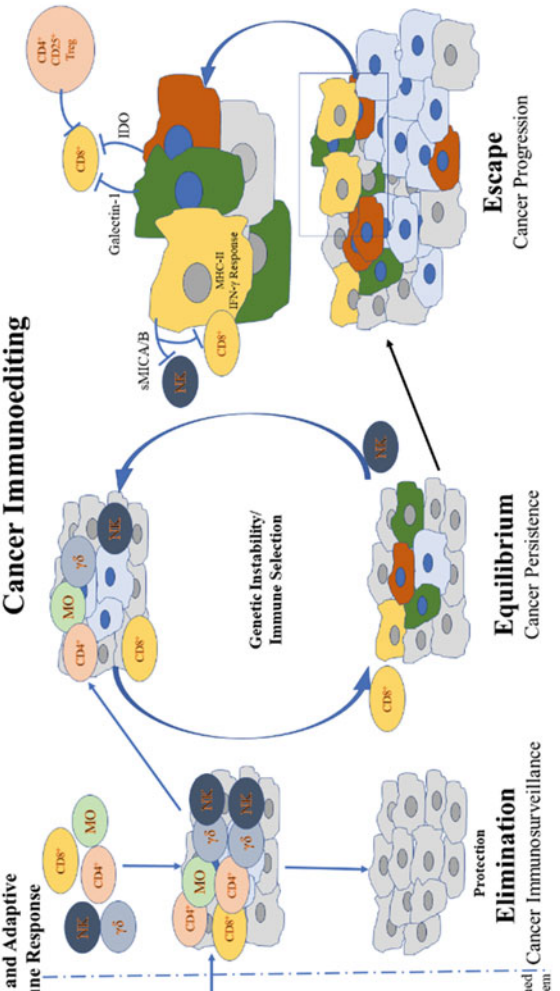

Fig. 2 Tumor immunosurveillance ability of the immune system for tumor suppression. Tumor or cancer immunoediting process is extrinsic tumor suppressor mechanisms by the immune system against the transformed cells that have escaped the intrinsic-cellular system to detect and eliminate developing tumors before becoming clinically apparent. Cancer immunoediting process of the immune system protects the host body from tumor development is the 3 Es of cancer immunoediting: cancer immune surveillance, or elimination phase; Secondly, the equilibrium phase in which tumor remains dormancy where the immune system and tumor cells attain a dynamic equilibrium; and Finally, escape phase, the immune system is no longer capable preventing tumor progression leading to tumor cells displaying the reduced immunogenicities or gaining of possible immunosuppressive mechanisms to reduce antitumor immune responses leading the progression of tumor growth. *IDO* indoleamine 2,3-dioxygenase, *MICA/B* MHC class I chain-related antigens A and B, *sMICA/B* soluble MICA/B

induced antigens. But as the tumor immunoediting process continues in the equilibrium phase, selective pressure is exerted by the immune system for eliminating susceptible tumor clones where it is likely possible. Although, the immune system applies the selective pressure in the equilibrium phase might be sufficient to control tumor development, but still, the immune system may fail to eliminate the tumor resulting in the selection of tumor cell variants that is applicable suppress or resist against the antitumor immune response, leading to the escape phase. In the escape phase of the immunoediting process, the immune system is no longer capable of preventing tumor progression.

3.2 HSP as a Biomarker for Cancer Diagnosis

The elevated expression levels of various HSP have been reported in patients suffering from infectious diseases and providing possible application as promising biomarkers for diagnosis [77]. The biomarkers used for the cancer diagnosis is commonly grouped into three classes, namely prognostic, predictive, and pharmacodynamic markers. However, noninvasive methods are most preferred for both diagnosis and also evaluate the efficacy of anticancer therapies; thus, tumor biomarkers of blood play an essential role in both cancer diagnosis and treatment [78]. In the tumor cells, HSP are found overexpressed primarily, Hsp70, that is actively released in exosome. The assessment of free Hsp70 in serum and plasma can be carried out using ELISA, providing a powerful tool for the cancer diagnosis and its monitoring of the outcome of therapy during treatment [79].

Further, soluble HSP could provide an easy tool for detecting cancer in the early stages. HSP70 validation as a tumor-specific biomarker has been investigated for monitoring the therapeutic result of radiation therapy in both tumor mouse models and cancer patients [80]. Abe et al. (2004) have reported that the application of Hsp70 as a biomarker in the case of prostate cancer [81]. Even though the Hsp70 level in plasma is not efficient than prostate-specific antigen (PSA) for the diagnosis prostate cancer patients, but Hsp70, along with prostate-specific antigen allows for determining the patients even in the early stages of prostate cancer which might be not possible only with PSA [81]. Further, glucose-regulated proteins (Grp78 and Grp75), HSP70 protein 1 (Hsp70.1), and heat shock cognate protein (HSC70) have also found essential play a role in the pathogenesis of hepatitis C virus (HCV) -related Hepatocellular carcinoma (HCC) and might be used as a biomarker for diagnosis as well as a therapeutic target for treatment [82]. Feng et al. (2005) have reported that HSP27 might also be used as a potential biomarker in Hepatocellular carcinoma diagnosis [83].

Moreover, the identification of new and novel tumor-related antigens and autoantibodies will allow the early-stage detection of malignant tumors and also can improve the efficacy of the immunotherapies. Concerning these, an autoantibody against HSP70 in the serum of multiple esophageal squamous cell carcinoma (SCC)

patients allowed for the determination of new and novel serum markers having clinical application against cancer [84].

Syrigos et al. (2003) have shown the frequent overexpression of Hsp70 in bladder cancer cells and its possible use as a biomarker for the diagnosis of bladder cancer patients [85]. In the study conducted by Margel et al. (2011) have reported that the patients with muscle-invasive bladder cancer (MIBC) can be easily distinguished from those with nonmuscle-invasive bladder cancer (NMIBC) using the increased levels of Hsp60, Hsp70, and Hsp90 expression, while the same is not possible by evaluating Hsp60 and IL-13 levels in most of the cases [86]. Hsp90 expression n malignant cells have found to enhanced by nearly two- to tenfold compared to that of healthy cells stating the importance of high expression level of Hsp90 in tumor growth and survival, thereby providing an opportunity to use HSP90 as a therapeutic target [87].

Circulating HSP27 present in that plasma has immunomodulatory and anti-inflammatory functions [88]. Hsp27 plays an essential role as a cytoskeleton regulator dynamic intracellular trafficking during autophagy and mitophagy [89]. Hence, the HSP27 could to used as a biomarker in tracking the outcome during the chemotherapy inducing autophagy in osteosarcoma in response to treatment, where the enhanced expression of p-Hsp27 indicated autophagy was inhibited due to the high sensitivity to anticancer drugs. Hence, the results obtained from monitoring the p-Hsp27 as a biomarker might provide useful information above the overall survival of patients for combination therapy of autophagy modulators and chemotherapeutic drugs [90]. Rappa et al. (2016) have demonstrated that the use of Hsp10 and Hsp60 as a promising biomarker for the diagnosis in initial stages of tubular adenoma in both epithelium and lamina propria, as well as during its differentiation from more advanced malignant lesions [91]. Rappa et al. (2016) have also stated the application of both Hsp10 and Hsp60 as potential targets for therapy [91].

In an investigation, the correlation of Hsp60 serum level with HBcAg-specific IL-10-secreting Treg cells was found in patients with chronic hepatitis B virus (HBV and found that Hsp60, serum level significantly higher in chronic HBV patients than the chronic HCV patients. Moreover, the preincubation of recombinant-HSP60, along with CD4+ CD25+ cells, found to increase the frequency of HBcAg-specific-IL10-secreting Tregs [92]. In study conduction by Shekhawat et al. (2016) found that both host Hsp70 and Hsp90 have the capability with the excellent sensitivity and specificity for the tuberculosis meningitis disease diagnosis [93]. Further, Shekhawat et al. (2016) also stated that the combined application of all the HSP, especially Hsp25, Hsp60, Hsp70, and Hsp90, would help the effective differentiation of tuberculosis meningitis patients from its controls [195] [93]. The above investigation suggests that the potential application of HSP as a biomarker for diagnosis, a therapeutic target for treatment, and also a molecular marker for tracking the outcome during the corrective procedure for various cancer as well as infectious diseases.

3.3 Targeting the HSP-Inflammation Cooperative System as a Target for Cancer Diagnosis Therapeutics

As described earlier, heat shock proteins are one of the critical factors in carcinogenesis and found highly regulated under stress conditions such as inflammation and the onset of tumors. As a part of an immune-inflammatory complex, HSP in response to cellular damage, as well as DNA breaks. The frequent damage to the DNA and, if not correctly-repaired, may lead to genome instability, thereby the risk of carcinogenesis. It had earlier reported by Nunes et al. (1993), and Matsumoto et al. (1995) have reported that elevated expression of heat shock proteins in reducing mutation frequency like in glioblastoma cell lines, the high expression of HSP70 and its interaction with p53 induced the functioning of p53 such as cell cycle control, apoptosis, and DNA repair processes [94, 95].

Furthermore, there are reports on the HSP70 and HSP27 involvement DNA repair mechanisms in association base excision repair (BER) enzymes such as uracil DNA glycosylase (UDG) and human AP endonuclease (APE1). Hence, HSP mediated DNA repair mechanisms may be utilized to increase cancer therapeutics efficacy [96, 97]. Interestingly, there is scientific evidence for HSP playing a vital role in DNA damage response (DDR) mechanisms via interaction with its client protein where DDR involves various pathways such as activation of signaling networks, cell cycle checkpoints, DNA damage detection, and induction of DNA repair or apoptosis [98]. DNA damage response (DDR) ability to respond to DNA damage has been controlled by maintaining the balance between the synthesis of client protein and their degradation by HSP. For instance, the binding of HSP90 results in preventing DDR proteins from ubiquitination and degradation mediated by E3 ubiquitin-CHIP (chaperoning) [96]. DDR proteins include checkpoint kinase 1 (CHKI), Apurinic/Apyrimidinic Endonuclease (APE1), O6-meG-DNA methyltransferase (MGMT), MLH1 and MLH2, and among others.

As there are reports on the inability of cells to repair the DNA damage formed as a result of stress may lead to loss of genomic integrity and increase the risk of carcinogenesis [99]. The role of HSP in genetic stability, DNA damage response (DDR) mechanisms, and tumor progression have been well reported [100]. Moreover, the role of the HSP in inflammation cascade, causing cancer, is well illuminated. Ikwegbue and co-workers have reported that cytoprotection activity exerted by HSP in contrast to an inflammatory response by inflammation cascades modulation resulting in pro-inflammatory cytokines activation (e.g., TNF-α), and attenuating chronic inflammation [101]. However, based on the above findings, targeting HSP, especially HSP90 and their inhibitors involved in inflammation-mediated cancer might provide novel mechanistic insight as a possible target to cancer therapeutics efficacy, as reported in earlier reports [39].

3.4 HSP Based Cancer Immunotherapy

The following unique immunostimulatory properties of molecular chaperones that make possible candidates by using as physiological adjuvants for cancer immunotherapy are as follows

1. Ensuring the proper sensitivity and specificity of antigen targeting via receptor-mediated uptake of molecular chaperones by the antigen-presenting cells (APCs)
2. The ability of molecular chaperones to serve as "danger signals," thereby activating innate immune components which play a vital role in the development of an active immune response.

Hence, utilizing these unique immunostimulatory properties of molecular chaperones or HSP enables them to be used as physiological adjuvants in developing immunotherapy for cancer treatment by different immunotherapeutic approaches as follows.

3.4.1 Vaccines Based on HSP

Vaccines based on using HSP or molecular chaperones are evolving as a novel therapeutic approach in cancer treatment. The substantial properties possessed by the HSP might be used targeting dendritic cells (DCs) such as the ability to act as a natural adjuvant, their ability to delivering the multiple antigens, thereby inducing the adaptive immune responses, and finally they serve as the safe components of existing vaccines [102]. Due to the chaperone activity of HSP, under stress or immunological danger signaling conditions, the release of Hsp-bound tumoral peptides in the extracellular medium could take place. The interaction of HSP with antigen-presenting cells (APCs) takes place through various receptors such as CD40, CD91, and LOX-1. After the Hsp-peptide complexes endocytosis, these complexes of Hsp-peptide are were degraded and resulting in the cross-presentation of the tumoral peptide to CD8+ T cells via major histocompatibility complex I (MHC-I) molecules [103].

Multiple studies have stated that the determination of HSP optimal doses is critical. An autologous cancer vaccine, namely Vitespen, which is derived from tumor-specific Gp96, has shown promising results for kidney cancer and melanoma in Phase III clinical trials [104]. Further, some studies have reported that the cytokine effects of HSP are probably because of the contamination of LPS and its associated molecules and serving as an essential point before using HSP as therapeutic agents [105]. Further, HSP induced immune responses using the purified HSP obtained from tumors depend on HSP-bound peptides maintenance. Hence, finding the peptide dose associated with HSP is also very crucial and becomes problematic in the application of HSP-peptide complexes in clinical trials. For instance, oncophage such as HSPPC-96 or vitespen, used as an adjuvant treatment for renal cell carcinoma patients which is prepared using the patient's tumor containing Hsp–peptide

complex, where immune response have found to be tumor/patient-specific for vitespen but to be dependent on vitespen peptide component leading to the rejection of its approval (http://www.ema.europa.eu/docs/en_GB/document_library/Application_withdrawal_assessment_report/2010/03/WC500075459.pdf).

Presently, HSP has also been used as antigens or adjuvants in the preparation of DNA and peptide-based vaccines for cancer as well as for various infectious diseases [106]. Several vaccines prepared based on HSP are under different stages of preclinical and clinical trials. For instance, the promising results have been obtained in the mouse Mycobacterium tuberculosis aerosol challenge model for a tuberculosis vaccine containing HSP complex obtained from Bacille Calmette–Guérin (BCG) also known as (T-BioVax) [107, 108]. A vaccine consisting of complexed noncovalently between 32 synthetic HSV-2 peptides and the recombinant human Hsp70 protein (HerpV) have found to be safe and well-tolerated in humans. The first vaccine based using HSP, HerpV, has shown as provoke significantly CD4+ and CD8+ T-cell immune responses for HSV-2 antigens. Supplementary 2 illustrates the use of HSP in preclinical and clinical trials. Generally, the immunogenicity of HSP results from two different properties: peptide-dependent ability to chaperone and elicit adaptive CTL responses against antigenic peptides, and peptide-independent immunomodulatory potency. The studies showed that specific HSP, including Hsp70 and Gp96, are highly active carrier molecules for cross-presentation [109]. We have summarized some of vaccine-based HSP under clinical trials for cancer treatment.

3.4.2 Immune Responses Stimulation Using HSP as an Antigen

The non- chaperonin activity of HSP might be used as an antigen for stimulation of the immune system for cancer treatment. For instance, promising results were obtained for Hsp70 intratumoral injection or upregulation inside the tumor in preclinical trials and be used as a possible therapeutic strategy [116]. Li et al. (2009) also have reported that tumor growth can be reduced or suppressed by Hsp70-mediated immune responses by intratumoral vaccination using Hsp70 overexpressing recombinant oncolytic type-2 adenovirus [117]. In the xenograft mouse model with myeloma can be effectively treated using HSP peptide-specific Cytotoxic T-lymphocytes (CTLs). Indeed, the application of dendritic cells pulsed with Hsp27 and Hsp90 peptides in myeloma patients found to stimulate peripheral blood mononuclear cells, thereby leading to the generation Hsp peptide-specific Cytotoxic T-lymphocytes [118]. The chaperone-based vaccine resulted from Hsp70-peptide complexes purification obtained from the fusion of dendritic cells and tumor (Hsp70.PC-F) found to induce significantly immune responses against tumor cells in animal models via C maturation and Cytotoxic T-lymphocytes (CTL) activity [119]. M-Hsp70-PCs, Hsp70-peptide complexes were obtained from various human melanoma cell lines such as A375, SK-HEL-1, M21, WM-35, and M14. The results further showed that the induction of IFN-γ secretion and CTL responses

in patients when matured dendritic cells pulsed with M-Hsp70-PCs compared to that of autologous Hsp70-PCs [110].

In a study Brown et al., (2001) have reported a novel small HSP of molecular weight around 20 kDa have been conserved hemoprotozoan parasite *Babesia botis* and *Babesia bigemina* capable of stimulating the memory CD4+ T-lymphocyte responses thereby causing the production of IFN-γ in *Babesia botis* immune cattle [120]. Similarly, He et al., (2014) have demonstrated that the recombinant Hsp20 (rBoHsp20) obtained from B. orientalis might be acting as an immunodominant antigen and also serve as a potential vaccine candidate and also a diagnostic reagent for detecting the antibodies for B. orientalis in water buffalo [121]. However, it has been found that significant humoral responses developed in around 62% of the Leishmania-infected animals against the Hsp20. Whereas the serum collected from few leishmaniasis patients have seen, show a positive reactivity for recombinant Hsp20. Hence, suggesting that recombinant Hsp20 has poorly antigenic against the human immune system [122]. In the merozoites, Hsp20 also found to be expressed intracellularly. In a study, when truncated rHsp20 proteins and overlapping peptides were tested in the immune cattle to stimulate T cells, it was found that both the N-terminal (1-105 amino acids) and C-terminal (48-177 amino acids) region were immunogenic and stimulating proliferation as well as production in IFN-γ for the majority of cattle [123]. A recombinant Modified Vaccinia Ankara (MVA) strain developed was studied as a candidate vaccine in homologous and heterologous prime-boost immunizations which is capable of expressing a chimeric antigen containing B and T-cell epitopes from three antigenic proteins of Babesia botis having HSP20, RAP-1, and merozoite surface protein-2c (MSA-2c).

Ortiz et al. (2014) have reported that the best vaccination strategy for protecting against *bovine babesiosis* might be achieved by using as a prime of protein cocktail along with a boost of the recombinant virus capable of inducing the high levels IFN-γ and specific IgG antibodies as well as able to a high degree of activation of IFN-γ + CD4+ and CD8 + -specific T cells [124]. Ortiz et al., (2014) also have demonstrated bacterial HSP role in vaccine design for various infectious diseases for an example vaccination with HSP complexes such as GroEL/S (58 kDa belonging to HSP60 or HSPB class and 13 kDa belonging to HSPA class, respectively) and the DNAJ/DNAK (also known Hsp40 and Hsp70, respectively) have induced protection against *H pylori* in mice without the application of any exogenous adjuvant [125]. Chionh et al. (2014) have reported that vaccination with Helicobacter pylori HspC provokes the expression of antibodies and cytokines at high levels, thereby providing a significant level of protection against the next for this pathogen without any severe inflammatory response induction [125]. On the contrary, Hsp70 of *Trichinella spiralis (Ts)* found to activating Dendritic cells (DCs) and inducing protective immunity in BALB/c mice and also accusing DC maturation by the secretion of TNF-α, IL-1β, IL-6, and, IL-12p70 as well as the increased expression of CD40, CD80, CD86, and MHC-II. Dendritic cells (DCs) activated using *Trichinella spiralis* heat shock protein 70 (Ts-Hsp70) found to induce both Th1 (IFN-γ and IL-2) and Th2 (IL- 4 and IL-6) cytokines secretion in CD4+ T cells from mice infected with *Trichinella spiralis*. However, it was found that 38.4% reduction

in muscle larvae in the group vaccinated with rTs-Hsp70-activated DCs compared to the group treated with only DCs [126]. Moreover, immunization with a Mycobacterium leprae Hsp65-based DNA vaccine (GroEL2) in BALB/c mice found to induce both the production of IFN-γ as well as potent antibody responses and protective efficacy for Buruli ulcer [127].

3.4.3 The Application of HSP as an Adjuvant for the Immune System Stimulation

Adjuvants are the substances or molecules that are capable of stimulating innate immune responses as well as modify the adaptive immune response quantity, and quality, which depending on the type of innate responses activated [128]. Heat shock proteins have the potential to be used as adjuvants for the immune system stimulation, such as activation of cytotoxic T lymphocytes (CTLs) response against various cancer as well as for infectious diseases. There have reports stating the immune activities of HSP be located in its N- or C-terminal fragments. Hence it is possible to use part or small pieces of HSP as an adjuvant in vaccine development and also in cancer immunotherapy [128]. Various reports are stating the HSP as whole or in fragment used as adjuvants, for instance, Gp96, as well as its N-terminal piece (NT-gp96), have been used as potential adjuvants for enhancing the specific cytotoxic T lymphocytes (CTLs) responses in mice against hepatocellular carcinoma and hepatitis B virus (HBV) infection [128, 129]. Li et al. (2005) also have demonstrated that the application of NT-gp96 as an adjuvant, significantly enhancing the humoral immune response via HBsAg [130]. In another report by Chen et al. (2013) have demonstrated that the application of NT-gp96 as an effective immunoadjuvant for antigen-specific humoral immune responses induction by B-cell epitopes of porcine reproductive and respiratory syndrome virus in swine [131] (Table 1).

Moreover, the fusion of the Gp96 N-terminal domain with the C-terminal end of a polytope (PT) HCV DNA vaccine found to have better adjuvant activity and also more capable in immune response induction. The investigation by Pishraft-Sabet et al. (2015) have shown that the significantly IFN-γ induction and TNF-α secretion, as well as antibody responses (IgG2a followed by IgG1) in CB6F1 mice when immunized with NT-gp96 fused PT DNA vaccine compared to that of mice treated with PT DNA alone [132]. NT-gp96 also has shown its capability to enhance the immunity potency of recombinant HCV NS3 protein inducing proinflammatory cytokines production [133]. In a study conducted by Bolhassani et al. (2008) have demonstrated that the codelivery of human papillomavirus (HPV) E7 with the Gp96 with the complete sequence as a DNA/DNA or with CT-gp96, Gp96C-terminal fragment as DNA/protein were able to stimulate E7-specific immune responses in C57BL/6mice [134]. The immunization of mice using the recombinant p24-N336 fusion protein was able to induce antibody responses specific to p24 as well as peptide-specific CTL responses [135].

A study investigated by Nasiri et al. (2016) has reported that protective efficacy against visceral leishmaniasis in BALB/cmice for the recombinant *Leishmania*

Table 1 List of HSP based vaccines for cancer immunotherapy and their current clinical status

HSP based vaccine	Target cancer type	Current clinical Status	References
HSP. 70PC-HSP70-based vaccine	Breast cancer	Phase I clinical study	[110]
HPV16oE7-HSP70-based vaccine	Cervical cancer	Under preclinical study	[111]
Vitepsin-Gp96-based vaccine	Several-liver, ovarian, glioma, melanoma, etc.	Phases II and III clinical trials have been approved	[112]
HSP.PC 96-Gp96-based vaccine	Renal carcinoma	Under phases II and III clinical trials	[113]
HSP70 activated NK cells	Colon cancer and NSCLC	Under phases I and II clinical trials	[114, 115]

tarentolae expressing KMP11-NT (gp96)-GFP fusion as a live engineered-recombinant-vaccine [136]. Moreover, Hosseinzadeh et al. (2013) also demonstrated that subcutaneous administration of recombinant *Leishmania tarentolae* expressing E7-CT (gp96) fusion could increase IFN-γ expression level along with significant protective effects to that mice vaccinated only with *L.tar*-E7 [137]. Furthermore, Daemi et al. (2012) have reported that the efficiency of DNA vaccines against tumors can effectively be increased by generating effective immune responses by providing electroporation following E7 DNA fused to CT (gp96) subcutaneous injection [138]. The protein vaccination of fused adjuvant-free E7-NT-gp96 can provide enhanced protective antitumor immunity by direct immune responses toward Th1 immunity compared to that when treated alone with E7 protein [139].

Zhang and Huang (2006) had evaluated antitumor responses for the fusion proteins of Hsp70 along with tumor-associated antigen and found the HSP70 C-terminal peptide-binding domain is vital for inducing antitumor response as well as activation of NK cell against mela tumor antigen-expressing B16 tumor-bearing murine [140]. On the contrary, the HSP70 N-terminal fragment fusion with HPV E7 to induced E7-specific CTL response as well as protected against tumor challenge in the mice where CD4+ T cells and NK cells had no significant role. The investigation also demonstrated that the importance of the peptide-binding domain of Hsp70 for in ensuring the E7-Hsp70DNA vaccine potency. Further, it also stated the clinical significance of the orientation of linking between HPV E7 and Hsp70 in the Hsp70-based DNA vaccines optimization as well as that of the Hsp70 functional domain [141]. The elevated induction was observed in BALB/c mice for TNF-α, IgG, IFN-γ, IL-4, IL-5, IL-6, and IL-12 under nonprogressive pneumonia using elongation factor *Tu* and Hsp70 are membrane-associated proteins from *Mycoplasma ovipneumoniae, where* the recombinant Hsp70 acted as a Th1 cytokine-like adjuvant [141]. Hsp70 with thew trophoblastic peptides as a complex served as a novel contraceptive vaccine by activating the T cells specifically toward Th1 causing of trophoblast cytolysis resulting in the termination of pregnancy in a mouse model [141]. The fusion of murine Hsp70 with the endotoxin-minimized HIV-1 p24 elicited p24-specific Th1 response in mice [142].

Similarly, there are also multiple studies where Small HSP like HSP27 has been used as an effective adjuvant for enhancement of HIV-1Nef antigen-specific immunity. For example, the protein of HIV-1Nef and Hsp27 (Hsp27-Nef fusion) demonstrated a significant Nef-specific T-cell response by eliciting the enhanced expression of IgG2a and IFN-γ toward Th1 responses and causing the secretion of Granzyme B. Hence, and this study shows that Hsp27 has significant immunostimulatory properties in different immunization strategies than Freund's adjuvant thereby suggesting that the application of Hsp27 in protein-based vaccines can improve the HIV-1 Nef-specific B- and T-cell immune responses [143] as well as a carrier for DNA vaccine design against HIV-1 infections [144]. There are also investigations were the HSP derived bacteria have used for vaccine development. For instance, Th1-type cellular responses have been enhanced by the co-delivery of DNA vaccine encoding Hsp65 of *Mycobacterium tuberculosis along* with the human IL-2 fusion (HSP65-IL-2 DNA vaccine) by inducing the high leavel IFN-γ and IL-2 production with high antigen-specific cytotoxicity activity and the greater titer of antigen-specific-anti-Hsp65-IgG2a [145]. HSP65-E7 antigen fusion protein immunization of mice showed a remarkably changed E7 response from the production of IL-5 to IFN-γ, indicating a shift in response to a type-1 [145]. In another investigation, it has been found that HSPX of Mycobacterium tuberculosis significantly enhanced CD86 and major histocompatibility complex (MHC) class-II type expression, as well as the secretion of pro-inflammatory cytokine in dendritic cells via Myeloid differentiation primary response 88 (MYD88) and TRIF-dependent pathways. Indeed, the significant suppression of tumor growth can be observed by HspX-E7-DCs systemic administration in the mouse model. Hence, Mycobacterium tuberculosis HSPX might be used as an effective immunoadjuvant in the cased dendritic cells, i.e., DC-based tumor immunotherapy [146].

4 Conclusions

Heat-shock proteins (HSP), apart from having chaperone activity, also possess different functions such as regulating the immune system, cell senescence, and differentiation, apoptosis, autophagy. The immunogenicity of HSP, i.e., the potential of HSP to stimulate the innate immune responses through Toll-like receptors and scavenger receptors. The ability to HSP enhance the antigens processing and presentation via the endocytic pathway in the adaptive immune system when HSP mediates HSP mediates the phagocytosis of antigens. These immunogenicity potentials of HSP can be as therapeutic targets for immunotherapy by developing of prophylactic-therapeutic vaccines for cancer. Further, HSP has found to have the ability to acts as a carrier, i.e., as adjuvants for antigen presentation, or as targets for the innate immune system for tumor-derived immunogenic peptides for eliciting antitumor immune responses. Moreover, the relationship between HSP and drug resistance can also be used as a molecular marker to monitor cancer patients during the course of treatment. Thus, the chaperone or HSP-based immunotherapy could be

considered to provide a novel promising approach for both cancer therapy and management as a therapeutics target as well as molecular marker. Hence, The broad applicable of HSP as an adjuvant to conventional cancer therapies provide significant benefits to patients and expected that translation of HSP-based vaccine approaches into a clinically viable approach in cancer immunotherapy in the near future in terms of diagnosis, therapy as well as in management.

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Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

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Chaperonin Hsp60 and Cancer Therapies

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Abstract

Introduction The heat shock protein 60 (Hsp60) is a chaperonin belonging to the chaperoning (chaperone) system that typically contributes to protein homeostasis inside mitochondria, but also plays various non-canonical roles unrelated to protein quality control beyond the organelle. Chaperonopathies are disorders in which chaperones play an etiologic-pathogenic role and contribute to the onset/progression of disease. Hsp60 chaperonopathies by mistake are diseases in which the chaperonin is apparently normal (as far as it can be determined with current methodologies) but it actively contributes to pathology, for example in certain types of cancer, and autoimmune and chronic inflammatory disorders. In certain cancers, Hsp60 is associated with the onset of malignancy and metastasization, although the mechanisms are poorly understood. In this chapter, we summarize findings on Hsp60 quantitative changes and distribution alterations in cells and tissues accompanying

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tumor initiation and progression. We also discuss the potential of HSP60-based anticancer therapies that are currently being investigated.

Methods Journals and data bases were surveyed, and pertinent works were chosen for discussion.

Results The data have stimulated experimental and clinical studies aiming at establishing the usefulness of Hsp60 as biomarker for diagnosis, and for assessing prognosis and response to treatment. Likewise, investigations are ongoing on the possible use of Hsp60 as a therapeutic agent or target. The reported results indicate that in neoplasms, Hsp60 migrates outside its canonical location, the mitochondrion, augments in the cytoplasm and plasma-cell membrane, and exits the cell via lipid rafts-exosomes. Exosomal Hsp60 occurs in extracellular fluids such as blood, through which it reaches target cells near and far, normal or cancerous. With these target cells, exosomes carrying Hsp60 and other molecules interact and, thereby, modify their functions. Thus, detection of exosomal Hsp60 in body fluids appears as a promising variety of liquid biopsy applicable to monitoring cancers already diagnosed and to screen for malignancies before they are clinically manifest. We also discuss Hsp60-based vaccines as a novel means of eliminating cancer cells with cytotoxic T lymphocytes (CTL). Tumor-derived Hsp60 associated with a tumor-derived antigen activates CD8+ T cells and induces an antitumor immune response. It is highly probable that soon there will be implementation of clinical trials, involving the use of Hsp60 alone or in various combinations and complexes to prevent cancer progression and treat patients.

Conclusions Hsp60 is actively involved in tumor development and progression. Its presence in extracellular fluids renders it a potential non-invasive biomarker. Also, considering the antitumor activities of Hsp60 observed in some types of cancer one can foresee a bright future for Hsp60-based therapies.

Keywords Anticancer chaperonotherapy · Biomarker · Cancer · Chaperonin · Chaperoning (chaperone) system · Chaperonopathies · Exosomes · Heat shock proteins · Hsp60 · Immune response · Therapy · Tumor · Vaccine

Abbreviations

BCG	bacillus Calmette-Guérin
Cpn60	chaperonin 60
CTL	cytotoxic T lymphocytes
DC	dendritic cells
EMT	epithelial-mesenchyme transition
EV	extracellular vesicle
EX	exosomes
HPV	human papillomavirus
Hsp	heat shock proteins

ILV	intraluminal vesicle
MHC	major histocompatibility complex
mM-CSF	macrophage colony-stimulating factor
MVB	multivesicular bodies

1 Introduction

The emerging roles of the chaperoning (chaperone) system in a range of physiological and pathological situations, including carcinogenesis, and considering the complexity of that system, make it necessary for us to focus on only one of its components, the chaperonin Hsp60, to discuss possible application in therapeutics, specifically for cancer. However, before we can discuss Hsp60 in cancer therapies it is necessary to clarify several aspects of this multifaceted molecule pertinent to its potential in therapeutics. Therefore, we first present various Sections on the role of Hsp60 in physiological and pathological areas directly relevant to its possible pro-tumor and anti-tumor activities, and then we discuss the potential and future applications of the chaperonin in cancer therapies. Since human Hsp60 and its bacterial orthologues are very similar in structure and mode of action for certain functions pertaining to protein homeostasis, and because much of what is known about the structure-function of the human molecule has been inferred from experimental data obtained with the bacterial counterpart, we also present a brief description of the similarities and differences between the two.

Many of the roles that Hsp60 plays in tumorigenesis implicate the immune system; therefore, we also discuss aspects of their interactions, and the resulting immune responses prior to considering the potential applications of the chaperonin in anti-cancer therapeutics. Hsp60 displays a range of different functions depending on its location, inside the mitochondria or outside it in the cytosol, the plasma-cell membrane, the intercellular space and in the biological fluids, such as blood and cerebrospinal fluid. Thus, Hsp60 roles are closely related to its environment, extra or intracellular; consequently, to understand its possible applications in anti-cancer therapy we have to briefly survey the various locales in which the chaperonin resides and what it does in each. In its physiological and pathological odysseys, Hsp60 travels by itself, or associated with various types of particles, including exosomes. Because of this, we have also included Sections on these microvesicles to help explain how the society Hsp60-exosomes might be very promising for developing novel anti-cancer therapies. Lastly, Hsp60 is typically considered cytoprotective, namely it defends the cell and the organism against aggressors of all kinds, living (e.g., microbes, malignant cells) and non-living (e.g., pH and temperature changes), but it can also be pathogenic by itself. When this happens, Hsp60 is the etiopathogenic agent that causes disease, a chaperonopathy. Hsp60 chaperonopathies can be genetic or acquired and at least a superficial knowledge of them is necessary to fully appreciate the advantages and difficulties of using the chaperonin for anti-cancer treatment. This is the reason why we discuss some aspects of chaperonopathies in certain parts of this article.

2 Human Hsp60 and Its Bacterial Orthologues

2.1 Hsp60 vs. GroEL

Heat shock protein 60 (Hsp60) is a prominent member of the chaperoning system [82]. As indicated by the name, heat shock proteins (Hsp) are the product of genes induced by heat shock, and several among them are chaperones. The terms Hsp and molecular chaperone (chaperone in short) are used as synonyms although not all chaperones are Hsp. Hsp60 (also named Cpn60 or chaperonin 60) functions as a molecular chaperone under physiological conditions inside mitochondria and chloroplasts, facilitating protein folding and transport, and preventing aggregation of misfolded proteins.

Hsp60 belongs to group I chaperonins, namely GroEL in bacteria and some archaea, and Hsp60 in the eukaryotic mitochondria and chloroplasts. Typically, group I chaperonins form functional ring-shaped teams of seven monomers (heptamers) which associate end-to-end to build tetradecamers with an inside cavity [49, 69]. This double-ring structure associates with a GroES (Hsp10) heptamer, which functions as a lid to close the aperture of the central cavity of the Hsp60 tetradecamer during folding of client polypeptides. The mechanism of action of GroEL consists of entrapping the unfolded client polypeptide inside the double ring structure, isolating it from the intracellular environment and allowing its proper folding within a protected environment. The client polypeptide, i.e., the substrate, initially binds to the hydrophobic apical domain of the monomers in the open end of the tetradecamer, and then ATP binds inducing conformational changes in GroEL that allow GroES binding in what is called the cis stage of the chaperoning complex, with encapsulation of the client polyeptide for folding. Simultaneously, at the opposite GroEL ring, ADP and GroES dissociate from the trans complex, releasing the previous client polypeptide already folded. The internalized client polypeptide folds during ATP hydrolysis in the cis complex. The process is terminated by binding of ATP and GroES to the trans complex, resulting in the consequent opening of the cis complex [49, 69].

2.2 Hsp60 and Autoimmune Diseases

Human Hsp60, usually referred to as HSP60 or HSPD1, shares immunogenic and antigenic epitopes with the bacterial GroEL, therefore, making the human chaperone susceptible to autoimmune reactions and consequent pathogenesis. This phenomenon is called chaperonopathy by mistake, in which the chaperone is seemingly normal but participates in pathology rather than in cytoprotection. The pathogenic mechanism proceeds along the following steps: GroEL from a bacterium in the digestive or genitourinary tracts, or in the skin, invades the organism and is recognized as foreign by the immune system. Antibodies are produced which crossreact

with the human Hsp60 thus bringing this human molecule into a pathogenic autoimmune pathway, which may lead to disease. [82, 83]. This is illustrated by the numerous pathologies where anti-Hsp60 autoimmunity drives pathogenesis causing diseases whose characteristics depend on the organ/tissue most affected; for instance vessels and heart [1, 48, 89, 110]; joints [127]; salivary glands [36]; kidney [110]; skin [107]; nervous system [24]; pancreas [13]; thyroid, liver, and adrenal glands [85].

3 Hsp60 in Carcinogenesis

3.1 Chaperonopathy by Mistake

A chaperonopathy occurs when a molecular chaperone plays an etiologic-pathogenic role in the onset and/or progression of a disease [23, 83]. The chaperone may be apparently normal (although the chaperone may have undergone aberrant post-translational modifications that cannot be detected with the current methodologies) or structurally altered (mutations or post-translational modifications). These structural alterations may affect certain domains of the pathogenic chaperone, damaging one or more functional modules. Chaperonopathies may show changes in the quantity of a chaperone. These quantitative chaperonopathies are characterized by an increase or decrease of the pathogenic chaperone in certain cells and tissues [23, 82, 83]. If the increase or decrease of the chaperone is a direct contributor to the mechanism of disease as a pathogenic factor, the chaperonopathy is referred to as primary chaperonopathy. If the increase or decrease of the chaperone is the consequence of the disease, the chaperonopathy is referred to as secondary chaperonopathy. In both situations, the affected chaperone may serve as biomarker to monitor disease status, and response to treatment.

Another classification characterizes chaperonopathies based on their biologic and molecular mechanisms into by defect, by excess and by mistake. This classification helps in refining diagnosis with physiopathological implications, which is essential for making proper decisions on treatment [23, 83]. Chaperonopathies can also be grouped into genetic or acquired [81]. Genetic chaperonopathies involve a mutation in the gene of the pathogenic chaperone and are typically inherited. Acquired chaperonopathies are characterized by alterations of the chaperone protein molecule that occur after transcription, for example aberrant post-translational modifications, but the chaperone gene is normal [23, 82, 83]. The chaperoning system formed by chaperones, co-chaperones, chaperone co-factors, and chaperone receptors and interactors is widespread in the body and has canonical and non-canonical functions [82]. The former pertain to maintenance of protein homeostasis, while the non-canonical functions pertain to other cellular mechanisms and processes not related to protein quality control.

Chaperonopathy by mistake defines the situation in which an apparently normal chaperone, as far as current methodologies can determine, works against its host, in favor of the pathogen (e.g., a tumor cell) and participates in the pathogenesis of disease. An example mentioned earlier is Hsp60 compromised in inflammatory/

autoimmune processes either because it induces pro-inflammatory cytokines or because it acts as autoantigen. The latter is based on molecular mimicry between bacterial GroEL and human Hsp60, namely they share epitopes and antibodies made against the bacterial molecule crossreact with the human ortholog. However, Hsp60 chaperonopathies by mistake are not restricted to autoimmune disease but also include chronic inflammatory disorders in which the chaperonin stimulates the production of pro-inflammatory cytokines and various types of cancer [23, 82].

3.2 Hsp60 in Cancer

Quantitative patterns of Hsp60 levels in tumoral tissue characterize the neoplasms of various tissues and organs, including the gastrointestinal tract [79, 102], as illustrated in Fig. 1, breasts [33], pancreas [139], lungs [21, 22, 27, 88], kidneys [117], urinary bladder [105], oral cavity [28], uterine cervix [20], and prostate [25], Table 1. In this Table are displayed examples of cases in which Hsp60 levels are altered in tumoral as compared with normal tissue/cells. In the tumors, the levels of Hsp60 are increased (the most frequent pattern) or decreased. Concomitantly, changes occur in the distribution of the chaperonin: it accumulates in the cytosol and in the plasma-cell membrane, and it exits the cell in microvesicles (e.g., exosomes) or via Golgi.

The specific role of Hsp60 in carcinogenesis is poorly understood and under debate. Some data suggest a pro-tumorigenic role of Hsp60 through interference with apoptosis leading to cancer-cell immortality [17, 27, 72, 88]. Hsp60 levels are high at pre-neoplastic stages and remain elevated until the last stage of carcinoma [15, 102]. Therefore, Hsp60 emerges as a potential biomarker for cancer diagnosis and/or assessing prognosis. On the other hand, some findings suggest an anti-tumorigenic role for Hsp60 via inhibition of ROS overproduction and epithelial-mesenchyme transition (EMT) and favoring apoptosis [117]. Hsp60 and CD1a cells were found decreased in malignant tissue, which would mean downregulation of the

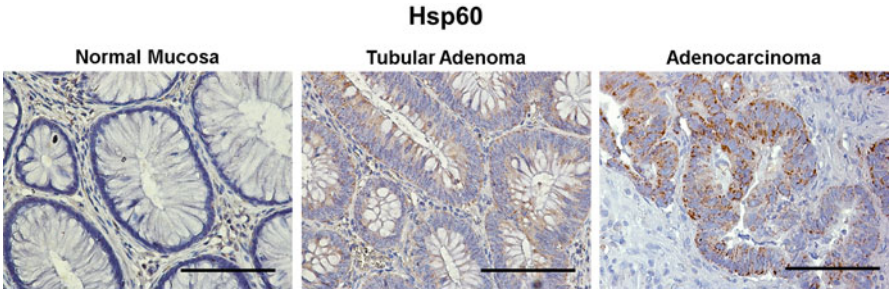

Fig. 1 HSP60 immunohistochemical pattern in large bowel tumors. Immunohistochemical demonstration of Hsp60 in human large bowel normal mucosa, tubular adenoma with moderate grade of dysplasia, and adenocarcinoma with moderate grade of differentiation. The Hsp60 (brownish coloration) levels are low in normal mucosa, high in tubular adenoma, a pre-neoplastic lesion, and even more elevated in adenocarcinoma. Magnification 400×. Scale bar 100 μm

Table 1 List of organs where HSP60 levels are altered in tumors

Tissue/Organ	Tumor	Ref.
Stomach	Gastric carcinoma; gastric maltoma	[65, 79]
Large bowel	Tubular adenoma	[102]
Breasts	Adenocarcinoma; breast ductal carcinoma	[12, 33, 39]
Pancreas	Pancreatic ductal adenocarcinoma	[139]
Lungs	Mucoepidermoid pulmonary carcinoma; bronchial carcinoma	[21, 22, 27, 87, 88, 134]
Kidney	Clear renal cell carcinoma	[117]
Ovaries	Epithelial ovarian cancer carcinogenesis; serous ovarian cancer	[53, 74]
N/A	Squamous cell carcinoma; kerathoacanthoma	[15]
Adrenal glands	Adrenocortical carcinoma; adrenal Cushing's tumor	[99, 135]
Urinary bladder	Carcinoma; transitional cell carcinoma; carcinosarcoma	[22, 64, 71, 105]
Esophagus	Oesophageal carcinoma	[42, 111]
Liver	Hepatocellular carcinoma	[76]
Prostate	Prostate carcinoma	[25, 29, 47, 61]
Brain	Astroglyoma; glioblastoma; meningioma	[5, 52, 93, 101]
Bone marrow, blood, lymph nodes	Acute myeloid leukemia; Hodgkin's lymphoma	[31, 54, 106, 119]

Adapted from Refs. [23, 83] with permission. Abbreviations: Ref. reference; N/A Not Available

immune system and inhibition of the antitumor immune response, all in favor of malignancy [15]. The role and impact of Hsp60 in neoplastic tissue may ultimately depend on the organ/tissue and the type of tumor, and on its interaction with specific factors that may differ in different tissues and organs.

4 Hsp60 in Cancer Management

4.1 Exosomes: Definition and Biogenesis

Intercellular communication is a crucial mechanism through which cells organize and coordinate their activities for the development of tissues and organs and the maintenance of physiological integrity and synchronization throughout the body. This is typically achieved by means of direct cell-to-cell communication and by the secretion of soluble molecular factors that interact with distant target cells via surface cell receptors [100]. Another means of intercellular communication has been characterized over the last few years, which consists of extracellular vesicles (EVs) that are released by all kinds of cells and travel in the body to various destinations

[100]. EVs interact with their target cells via receptor binding and internalization of their cargo (e.g., lipids, proteins, and nucleic acids) inside the recipient cell. EVs are heterogeneous and are classified based on their size and composition into exosomes (20–100 nm), microparticles and ectosomes (0.1–1 μm), apoptotic bodies (0.5–4 μm), protasomes (50–500 nm), and tolerosomes (~40 nm) [41]. EVs are generated by the internalization of vesicles inside multivesicular bodies (MVBs), forming intraluminal vesicles (ILVs), and consequent fusion of MVBs with the plasma membrane accompanied by the release of ILVs in the extracellular environment [59]. EVs may also be released through blebbing (budding out) and fission of the plasma membrane [26, 59].

Exosomes (EX) are continuously secreted from cells in both physiological and pathophysiological conditions and since they are mediators of intercellular communication they are the most studied among the EVs. Exosomes are spherical membranous, cup-shaped vesicles that range from 20–100 nm in size and are exocytosed from the plasma-cell membrane [41]. Exosomes contain proteins; lipids; and nucleic acids, including DNA, coding and non-coding RNA such as tRNA, mRNA, miRNA, and RNA. Exosome cargo is enclosed in a membranous vesicle, which protects its integrity. For example, RNA cargo is shielded from degradation by endogenous or exogenous RNases [47].

Exosomes are of endosomal origin and form ILVs inside MVBs as a result of inward budding of the maturing endosomes. MVBs containing accumulated ILVs will then follow one of these three pathways: (1) merging with lysosomes leading to their protein content degradation; (2) serving as temporary storage compartments; or (3) moving along microtubules and fusion with plasma membrane followed by exocytosis of ILVs as exosomes [131]. The molecular composition of the exosomes reflects the function of the parent cells [23, 59].

4.2 Exosomes in Disease: Cancer

Exosomes were first discovered in erythrocytes [62]. Since then, they have been described in other cell types, including B, T, and dendritic cells; reticulocytes; mastocytes; enterocytes; and platelets [58]. Exosomes are also released by erythroleukemia cells and other tumor cells [34, 35, 63], and have been implicated in various pathological processes [131] involving activation of the immune system [6] and malignant transformation [77, 86, 91]. Tumor-derived EXs can transfer biologically active molecules and thereby alter the molecular content of the recipient cell and facilitate malignant growth and metastasis [75]. Tumor cells are, as a rule, capable of releasing more EXs than normal cells, and the cargo of the tumor-derived EXs is different from that of the normal cell EXs since it contains elements that promote tumor progression and development [26].

Exosomes are abundant in blood and urine [16, 50], constituting a reservoir of biomarkers with disease specificity [37, 70]. Therefore, EXs are attractive candidates to examine with noninvasive diagnostic procedures in tumors as suggested by the

following features: (1) their cargo reflects the contents of the tumor cell in which they originated; (2) they are found ubiquitously in extracellular fluids, including blood; (3) their cargo is well preserved and resistant to: low PH, high temperatures, thawing, freezing, and RNAse degradation; and (4) their contents directly correlates with tumor staging and treatment outcome [26].

4.3 Exosomal Hsp60 in Tumorigenesis

Hsp60 and other Hsp can be secreted via the exosomal pathway by cancer cells [8, 18, 19, 78, 92, 128]. Notably, the level of Hsp60 is often increased in tumors and appears to be implicated in their initiation and/or progression. Hsp60 secretion includes association with the lipid-raft-exosomal pathway: it is found on the plasma-membrane of the tumor cells in the lipid raft region from where it translocates to exosomal vesicles [92]. Blood exosomal Hsp60 levels were evaluated in colorectal cancer patients before and after surgery [19]. Hsp60 was identified on the membrane of EXs and its levels were increased in patients before surgery but returned to normal levels following tumor ablation [19]. This was in agreement with bioinformatics and proteomic analyses of colorectal cancers indicating that the *hsp60* gene is a good diagnostic and prognostic indicator [38, 84]. Based on these and other similar observations discussed above, it can be hypothesized that exosomal Hsp60 plays a role in carcinogenesis, including metastasization.

5 Hsp60 in Cancer Therapy

5.1 Hsp and the Immune System

The non-canonical functions of chaperones encompass their interactions with the immune system. Hsp from bacterial [46, 103, 109], mammalian [2, 7, 96, 108, 112, 125], and human sources [3, 4, 11, 40, 66, 95, 124, 126]; and bacterial recombinants [14, 67, 68, 97, 98, 104, 129, 137] are capable of activating the innate immune system. Hsp can stimulate the production of proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-12), and NO and C-C chemokines [7, 94, 96, 112, 122, 129]. They also up-regulate the major histocompatibility complex (MHC) class I and II molecules, and costimulatory molecules (CD80, CD86 and CD40) through a series of signal transduction pathways that ultimately activate NF-kB and MAPKs [7, 94, 96, 112, 122, 129]. Hsp can induce proliferation, migration, and cytolytic activity of NK cells, activation of antigen-dependent T-cells paired with the production of IFN-y and T cell adhesion to fibronectin via TLR2 [122].

Cytosolic Hsp70, Hsp90, and gp96, a member of the Hsp90 family, also named GRP94 or TRA1 present in the endoplasmic reticulum, bind antigenic peptides within the cell [14, 45, 73]. Hsps have peptide-binding domains that bind exposed

hydrophobic residues of substrate proteins. The antigenic peptides are chaperoned towards MHC class I molecules at the cell surface for presentation to lymphocytes, a process termed cross-presentation [56, 73, 114]. Ultimately, the immune response leads to activation of cytotoxic T cells that provide immune protection. It has also been reported that the Hsp-peptide complex stimulates a greater immunogenic response compared with the action of antigenic-peptide on its own [10, 73]. This has inspired the idea of using Hsps as a form of vaccine adjuvants to prevent some diseases, including cancer [122].

One of the first interventions in cancer, using tumor-derived gp96-peptide complexes as a vaccine for mouse sarcoma, resulted in an anti-tumor response and induced tumor rejection and metastasis suppression [113]. Since then, autologous tumor-derived Hsp recombinants have been more thoroughly studied as means of vaccination against cancer [115, 116, 123]. Clinical trials are showing the efficiency of recombinant tumor-derived Hsp on tumors in melanoma, and colorectal and kidney cancers [9, 60, 90]. A phase III clinical trial assessed the overall survival rate in patients with stage IV melanoma based on two different treatments: (1) physician's choice of treatments being chemotherapy or surgical resection; or (2) autologous tumor-derived gp96 peptide complex vaccine [118]. The results showed no significant differences between the two treatments showing the potent effect of Hsp immune-based vaccine therapy. Other than being non-invasive, the promising clinical potential of Hsp-peptide complex as immunogen in vaccination against cancer is based on the fact that there is no need of identifying tumor-specific antigens.

5.2 Hsp60-Associated Vaccines in Tumors

Some evidence points at the therapeutic potential of Hsp60 as a novel means of vaccination in neoplasms [30, 43, 55, 57, 80, 140]. The microenvironment inside tumor cells is different from that of the normal cells; for instance, the oxygen levels are reduced in tumor cells promoting aggressiveness and resistance to therapy [120, 130, 133]. Reported observations indicate that Hsp60 has potential as antigen to induce immunity against certain types of tumors. For example, plasma collected from prostate cancer patients revealed elevated levels of Hsp60, which was considered a tumor-associated antigen [80]. Those results paralleled the levels of Hsp60 obtained from prostate cancer cells cultured in vitro under low oxygen tension, suggesting that Hsp60 is an antigen that in prostate cancer stimulates a humoral response [80]. Hsp70, on the other hand, was not altered in cancer cells compared with normal cells making Hsp60 a better candidate than Hsp70 for vaccine-based therapy [80].

Bortezomib, a known antitumor drug, was tested in murine ovarian cancer and was shown to stimulate tumor-cell immunogenicity through the action of CD8+ T cells [30]. Hsp60 and Hsp90 were both upregulated during the immune response; however, Hsp60 but not Hsp90 was the key determinant of the CD8+ T-mediated anticancer response, allowing phagocytosis of tumor cells via dendritic cells

(DC) [30]. These data support the notion that Hsp60 plays a role as immunogen in the anticancer immune response.

Human papillomavirus (HPV) is associated with cervical cancer and E6 and E7 are oncoproteins responsible for the development of malignancy in HPV [136]. We have already mentioned the advantages of using tumor-derived Hsp-peptide complexes in vaccination against cancer (Sect. 4.1). Similarly, Hsp60-E6-E7 was used as a DNA chimeric vaccine in mice suffering from HPV-mediated cervical cancer [55]. Hsp60-E6-E7 generated an antitumor response mediated by direct and cross-priming effects. Moreover, Hsp60-E6-E7 induced a more potent immunotherapeutic response in comparison with using Hsp60-E6 or Hsp60-E7 separately [55]. This could be an indication that the combination of tumor-derived Hsp60 with more than one tumor-specific antigen could have a greater effect on the immune response and on the overall antitumor effect.

Human U251MG glioma cells were retrovirally transduced with the human gene for the membrane form of macrophage colony-stimulating factor (mM-CSF) [57]. Tumor cells started dying via paraptosis and immunohistochemical staining revealed the abundant presence of Hsp60, Hsp70, and gp69 accompanying the immune reaction [57]. In support of these data is the fact that stressed apoptotic tumor cells are more immunogenic than their non-stressed counterparts [44], perhaps the consequence of great amounts of Hsp60 being on the surface of stressed apoptotic leukemia cells, as demonstrated by confocal microscopy [43].

Anti-Hsp60 IgG antibodies levels in patients with superficial bladder cancer were reported to be elevated [140]. Anti-Hsp60 IgG antibodies slightly increased in few patients after intravesical treatment with bacillus Calmette-Guérin (BCG) as a consequence of the humoral response against Hsp60 [140]. This could implicate a role for Hsp60 as an immunogen during the humoral response against bladder cancer. These data are backed by proteomic analysis highlighting the presence of Hsp60 tumor-associated antigens in sera from patients with breast cancer [39], colorectal cancer [51], and osteosarcoma [121].

5.3 Exosomal Hsp60 for Tumor Therapy

We have already discussed the potential of exosomes as intercellular mediators in cancer (Sects. 4.2 and 4.3, above), suggesting that exosome-based therapies may be developed in which EXs are employed to deliver DNA, RNA, and proteins, including antigens to alter the genome/functions of the recipient cell that may result in an antitumor response. It has been reported that tumor-derived exosomes are capable of stimulating antitumor immunity [132]. It was also investigated whether exosomes derived from heat shocked tumors would have a greater effect on tumor suppression [32]. Immunized mice with heat shocked exosomes (exosomes derived from heat shocked mouse A20 B lymphoma cells exposed to 42 °C in a water bath for 1 h) showed a greater antitumor immune response on lymphoma cells compared with their respective controls injected with tumor-derived exosomes that had not been

heat shocked. Heat shocked exosomes contained more Hsp60 and Hsp90 and had increased levels of molecules involved in immunogenicity such as MHC class I, MHC class II, IL-1β, and RANTES and increased levels of costimulatory molecules such as CD40, and CD86 than non-heat shocked counterparts. The antitumor immune response was mediated by cytotoxic T lymphocytes (CTL). Data indicate that heat shocked exosomes stimulated CD8+ T cells to mediate the antitumor effect and allowed the maturation of dendritic cells more efficiently than non-heat shocked exosomes [32].

The same data were replicated in heat-treated malignant ascitic cells of gastric cancer patients [138]. Heat shocked tumor-derived exosomes had increased levels of Hsp60 and Hsp70 and provided a more potent antitumor CTL-mediated immune response compared with non-heat shocked exosomes [138]. These data suggest that the combination of tumor-derived Hsp60-peptide complex in heat shocked tumor-derived exosomes might be a novel potent form of exosomal immunotherapeutic vaccination against tumors.

6 Conclusions

Hsp60 is a mitochondrial chaperone that has been implicated in the pathogenesis of a plethora of diseases, including carcinogenesis.

In tumorigenesis, both the quantitative and the distribution patterns of Hsp60 are altered suggesting its participation in the onset and progression of malignancy rendering it a promising candidate for diagnosis and prognosis of neoplasms. Data regarding Hsp60 pro or anti-tumorigenic role are inconclusive, most of them being in favor of its pro-tumorigenic properties through interference with apoptosis leading to immortalized cells.

Circulating extracellular Hsp60, secreted through the lipid-raft exosomal pathway, is highly increased in malignancy emphasizing further the chaperonin implication in carcinogenesis and metastasis. Measuring blood exosomal Hsp60 may be a potential biomarker for assessing malignancy.

Hsp60 was suggested as a novel means of vaccination against cancer considering its immunomodulatory properties. The use of Hsp60 as an immunogen combined with tumor-derived peptide (antigen) to activate a CTL immune reaction is at the basis of the aforementioned tumor therapy. Tumor derived Hsp60-antigen complexes showed a greater antitumor effect compared with the use of tumor-derived antigen on its own as a mean of vaccination.

Another novel method for cancer therapy through vaccination with Hsp60 emerges through the form of heat shocked tumor-derived exosomes in which Hsp60 is expressed on the exosomal surface. Heat shocked tumor-derived exosomes successfully activated a CTL immune response and allowed the maturation of dendritic cells more efficiently than non-heat shocked exosomes, and thus amplified the antitumor effect.

Hsp60 seems to be a functional player in the pathophysiology of neoplasms actively promoting tumor onset and progression followed by malignancy formation. Hsp60 tumor-based therapies provide results and a notable anti-tumorigenic effect through the interference with the immune system, specifically by activating a CD8+ T-mediated response.

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Combined Thermotherapy and Heat Shock Protein Modulation for Tumor Treatment

Abdullah Hoter, Aseel Othman Alsantely, Edreese Alsharaeh, George Kulik, and Islam M. Saadeldin

Abstract

Introduction Thermal therapy (hyperthermia) holds a promising treatment for tumor-affected patients particularly those with surgery intolerance. Recent advances and clinical trials for therapeutic purposes of heat shock proteins (Hsp) inhibitors and the astonishing progress in the field of nanotechnology pave the way for novel strategies for combined and effective treatment and targeting of the tumor cells. In here, we highlight the history of hyperthermia, as a therapeutic tool for tumors, and provide the state-of-the-art regarding the promising synergism between hyperthermia, HSP modulation and the targeted nanoparticles for tumor cell targeted therapy.

Methods A literature based collection of articles in the available search engines (PubMed and Google Scholar).

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Results We show the possible combination of thermal therapy together with Hsp inhibitors for treating cancers.

Conclusions The use of Hsp inhibitors potentiates the cytotoxic and/or anti-proliferative effects of the hyperthermia.

Keywords HSP · HSP inhibitors · Hyperthermia · Nanoparticles · Exosomes · Tumor treatment

Abbreviations

CRC	colorectal cancer
ECM	extracellular matrix
HIF	hypoxia inducible factors
HSP	heat shock protein/s
MRI	magnetic resonance imaging
MSC	mesenchymal stem cells
siRNA	small interfering RNA
SPIONs	superparamagnetic iron oxide nanoparticles

1 Introduction

Thermotherapy (thermal therapy or hyperthermia) is a type of tumor treatment that were used through 5000 years of practice by physicians, surgeons, clergy, or lay people in which body tissue is exposed to high temperatures (up to 113 °F or 45 °C) [1]. Hyperthermia is a promising treatment for a wide ranges of patients particularly those with surgery intolerance [2]. Cumulative evidence showed that hyperthermia can damage and kill tumor cells with minimal injury to the adjacent normal tissues [3]. Hyperthermia is usually a regional treatment for specific tumor lesions; however, it may be used in combination with other treatments such as chemotherapy or radiation to enhance the treatment strategy [1, 4] as summarized in Fig. 1. (1) Local tumor hyperthermia potentiates the immune system response, including tumor cell attack, tumor cell surface modulation, release heat shock proteins and exosomes which possess a direct effect on immune cells and changes the tumor microenvironments [4, 5], (2) Hyperthermia makes some tumor cells more sensitive to radiation and chemotherapy and potentiate the effects of radio- and chemotherapy [6, 7].

There are several methods of hyperthermia that are currently under study, including local (skin, esophagus, rectum, and brain tumors), regional (reproductive tract, urinary tract, respiratory system, arms, legs, abdominal organs, and tumors), and whole-body (metastatic cancers) hyperthermia [8]. Reaching but not exceeding the

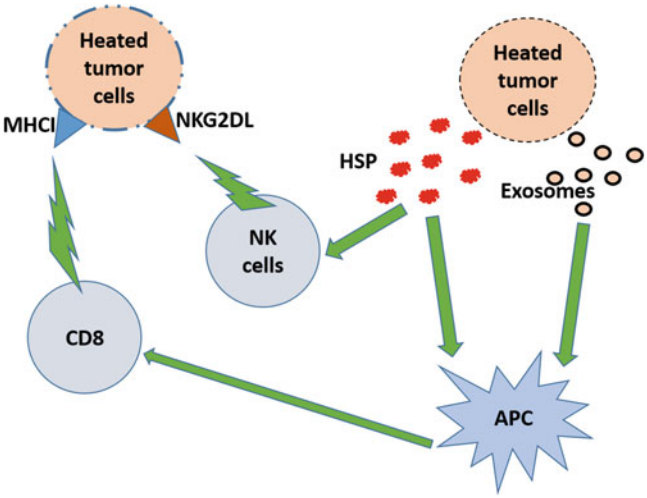

Fig. 1 Strategies of supporting immune system through hyperthermia. Tumor heated cells express MHC1 and NKG2DL that activate CD8+ T cells and natural killer (NK) cells, respectively, to attach the heated cells. In addition, tumor heated cells release heat shock proteins (HSP) that directly activate both NK cells and indirectly through activation of antigen presenting cells (APC) and in turn CD8+ cells. Furthermore, tumor heated cells release exosomes, which contain chemokines and activate APC cells too [4, 5]

desired temperature, of the tumor and surrounding tissue should accompany the thermal therapy. This can be achieved through CT (computed tomography) – aided insertion of needles with tiny thermometers into the treatment area to monitor the temperature [8].

Thermotolerance is a phenomenon in which cells become resistant to elevated temperatures. Thermotolerance might develop rapidly after the first heat treatment or during the thermal treatment at ~43.0 °C. Studies disclosed that thermotolerance developed in tumors and normal tissues as well [9, 10] and it is well correlated with enhanced synthesis of heat shock proteins [9, 11–13].

The kinetics of thermotolerance can be affected by various factors [10, 14]. For instance, thermotolerance is found physiologically in certain species as a form of estivation [15, 16]. It might be varied among certain cells of the same species [17–19]. Cells showed variability in thermotolerance because of the way of cell culture; cells grown in 3D compared with 2D culture showed reduced incidence of apoptosis and necrosis and a higher level of Hsp70 expression in response to heat shock [20].

Due to the essential role of HSP in thermotolerance, we and others propose the phenomenon “anastasis” to illustrate the survival response of thermotolerant cells [19, 21–25]. Anastasis is a term coined to outline the process of cell recovery, plasticity, resilience, or cellular resurrection from the brink of cell death [24] and might be a reason for cancer cells thermotolerance [26].

1.1 Heat Shock Proteins in Cancer Cells

1.1.1 Intracellular HSP

Overexpression of HSP is one of the key features in cancer cells which enables them to survive and develop. Several HSP including Hsp90, Hsp70, Hsp60 and sHSP perform multiple coordinated functions in tumor cells at the cellular and extracellular levels. In general, the significance of HSP in cancer comes from their implication in cancer metastasis, aggressiveness and therapeutic resistance besides their diagnostic and prognostic values [27] (Fig. 2). In this section, we briefly shed the light on the diverse oncogenic roles of major HSP such as Hsp90, Hsp70, Hsp60 and Hsp27 known in the cancer field. Hsp90, for instance, has been found to chaperone central elements along the cellular proliferation cascades which involve Erk, Src and Akt pathways [27]. In addition, it interacts with mutant oncogens and stabilizes them, thus permitting unrestricted proliferation [28, 29]. Likewise, Hsp70 plays analogous important role since silencing of Hsp70 resulted in impaired proliferation in murine mammary tumor cells [30]. Interestingly, HSP have been demonstrated to bind and stabilize the mutant p53 that is known to be mutated in more than half of cancers [31–33]. Elevated expression of Hsp90 and Hsp70 has been described in tumor cells containing mutant p53 [32, 33]. The small heat shock protein, Hsp27 (also known as HSPB1) has been shown to seriously impact the p53 mediated senescence and apoptosis [34]. These effects are of particular importance because the tumor suppression function of the wild type p53 is mostly lost upon its mutation and the mutant HSP-stabilized p53 is likely to possess oncogenic gain of function properties [35].

Another crucial aspect is the anti-apoptotic capabilities of HSP in cancer cells. These anti-apoptotic roles have been reported in many types of cancer including prostate [36], ovary [37], lung [38], liver [39], and others [27]. Hsp60, for instance, has been demonstrated to regulate apoptosis in tumor cells and its targeting by siRNA resulted in disruption of the mitochondrial function and initiation of caspase-dependent apoptosis [40]. Hsp27 and Hsp70 resist cell death *via* interacting with and inhibiting variant protein intermediates within the apoptotic pathway

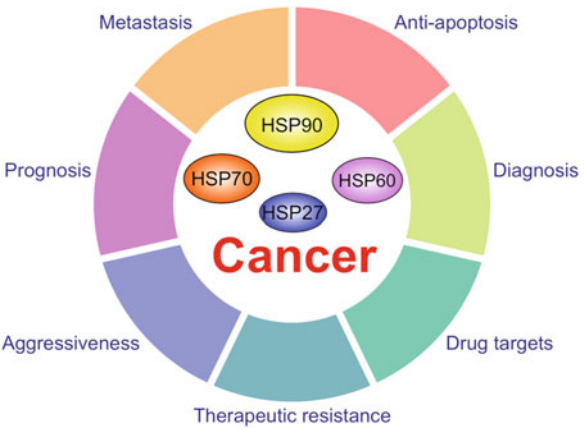

Fig. 2 The multiple aspects of HSP significance in oncology field. Hsp27, 60, 70, and 90 play a pivotal role in different aspects of cancer from the diagnosis till the therapeutics

[41, 42]. Hsp27 interferes with the mitochondrial release of cytochrome C and SMAC Diablo besides hampering caspases 3 and 9 activities [43–45]. It also hinders the extracellular apoptotic signals *via* inhibiting Fas, TNFα and TRAIL receptors' pathways [46]. On the other hand, Hsp70 blocks the c-Jun kinase of the programmed cell death and hampers the release of cytochrome C from mitochondria [41, 44]. Interestingly, Hsp90 has been found to inhibit cell senescence by chaperoning telomerase enzyme which is essential to recover eroded telomeres, thus prolonging cancer cell survival [47]. It is not surprising therefore, that co-targeting of more than one chaperone such as Hsp90 and Hsp70 has been beneficial in terms of better therapeutic responsiveness and enhanced sensitivity to anti-cancer drugs [48, 49]. Taken together, it seems that abundant expression of various HSP allow them to act coordinately and synergistically in order to afford an optimum conditions for cancer cell immortality [50].

It is well known that growing cancer cells develop mechanisms to support angiogenesis and satisfy their high demands for nutrients and oxygen. In this respect, Hsp90 has been demonstrated to activate and stabilize hypoxia inducible factors (HIF) which serves as a sensor of low oxygen content [51]. Stabilizing HIF1α is pivotal for stimulating the expression of vascular endothelial growth factor (VEGF) and subsequently creating the tumor capillary network and potentiating angiogenesis [52, 53].

Cancer metastasis is a complex process that characterizes malignant tumors and requires efficient HSP machinery. Overexpressed Hsp90 has been reported to chaperone focal adhesion kinase, integrin linked kinase and the receptor tyrosine kinases ErbB2 and MET [54]. Additionally, co-chaperones of Hsp90 contribute to tumor metastasis as seen in p23 which regulates metastasis in prostate cancer [55]. Similar to Hsp90, Hsp70 is likely to support MET expression and autophosphorylation in breast cancers [30, 56]. Moreover, Hsp27 has been reported to augment metastasis *via* supporting epithelial-mesenchymal transition (EMT) [57–59].

1.1.2 Extracellular HSP in Cancer

Despite their initial underestimation by the scientific community, the biological functions of extracellular HSP are nowadays growing dramatically. In fact, recent reports suggest that extracellular HSP are widely implicated in inflammatory and immunogenic roles [60, 61]. These observations were based on several molecular studies investigating variant HSP members both *in vitro* and *in vivo*. For instance, the secretory form of Hsp70, HSPA1A has been demonstrated to stimulate mast cells for production of tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) *via* the toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2) pathways [62–65]. HSPA1A has also been reported to induce the secretion of IL-12 from naive dendritic cells [66]. Tumor cell lines including hepatocellular carcinoma (HepG2) and murine leukemia monocytes have been described to secrete exosomes rich in HSP from different families such as Hsp60, Hsp70 and Hsp90, which enhanced the immunogenic activities of natural killer cells, macrophages and mononuclear cells [67–70]. Moreover, upon release from monocytic cell line U937, Hsp70 has been

found to stimulate the expression of matrix metalloprotease 9 (MMP-9) and augment cell motility [71]. Furthermore, extracellular Hsp70 has been demonstrated to interact with human immunoreceptors Siglec-5 and Siglec-14 trigger both anti-inflammatory and pro-inflammatory responses [72]. In colon cancer cell lines, released Hsp90β has been observed to reduce cellular adhesion and stimulate migration [73].

Clinically, several lines of evidence associate the extracellular or secretory HSP with cancer stage and progression. Hsp70 expression levels have been reported to be significantly higher in patients with liver cancer compared with control healthy group [74]. In comparison to healthy individuals, elevated Hsp70 serum levels have been detected in patients with squamous cell carcinoma [75]. High serum Hsp27 levels have been observed in many types of cancer such as epithelial ovarian cancer and were linked to tumor metastasis and progression [76, 77]. In patients with non-small cell lung cancer, measured serum levels of Hsp27 can differentiate between early and advanced stages of disease [77]. Serum Hsp90 were found significantly high in patients with cutaneous malignant melanoma compared with control subjects [78].

Collectively, it is apparent that a multitude of HSP play diverse crucial roles in the development and progression of cancer. These HSP-multifaceted functions including unlimited growth, tumor suppression prevention, increased cell survival and enhanced angiogenesis and metastasis, can therefore define the traits of cancer [50]. In accordance with these conclusions, overexpression of HSP in cancer patients has mostly been associated with poor prognosis and monitoring clinical outcome. Hence, targeting of HSP has increasingly been investigated to treat variant types of cancer.

1.2 Heat Shock Protein Modulation as a Target for Cancer Therapy

Due to their pivotal roles in cancer development and metastasis, targeting HSP has been actively researched by many investigators in an attempt to treat diverse human cancers. In the following section, we summarize different targeting approaches for crucial HSP such as Hsp90, Hsp70, Hsp60 and Hsp27, especially those approaches concerning small molecule inhibitors.

1.2.1 Targeting Hsp90

The Hsp90 family members are the most intensely investigated HSP in relation to cancer therapeutics [27, 79]. Since Hsp90 consists mainly of N-terminal domain, middle domain and C-terminal domain, variant Hsp90 inhibitors have been interestingly found to selectively target a specific structural domain within the Hsp90 molecule (Table 1 and Fig. 3). For instance, natural inhibitors derived from *Streptomyces hygroscopicus* such

Table 1 Common Hsp90 inhibitors used in preclinical and/or clinical cancer studies

Class	Inhibitor	Targeting site	References
Geldanamycin derivatives	Geldanamycin (GM)	N-terminal ATP binding domain of Hsp90	[27, 80]
	Tanespimycin (17-AAG, KOS-953)		
	Alvespimycin (17-DMAG)		
	Rataspimycin (IPI-504)		
Radicicol derivatives (These compounds share common resorcinol core)	Radiciccol (RD)	N-terminal ATP binding domain of Hsp90	[27, 80]
	Luminespib (NVP-AUY922)		
	Ganetespib (STA-9090)		
	KW-2478		
	Onalespib (AT13387)		
Purine derivatives	PU3	N-terminal ATP binding domain of Hsp90	[27, 80]
	PIIB021/ CNF2024		
	PU-H71		
	MPC3100		
	CUDC-305		
Others	SNX-5422 (PF-04929113)	N-terminal ATP binding domain of Hsp90	[81]
	NVP-HSP990	N-terminal ATP binding domain of Hsp90	[82]
Sulfoxythiocarbamate alkynes	STCAs	The middle domain	[83]
Hsp90 inhibitors with alternative mode of action	Novobiocin and KU174	C-terminal binding domain of Hsp90 and/or disruption of Hsp90-cochaperone interaction	[80]
	Cisplatin		
	Epigallocatechin-3-gallate		
	Taxol		
	Withaferin A		
	Celastrol		
	Gedunin		
	7-azapteridine core		
New compounds	Newly identified compounds (Compound 7 and 10)	C-terminal binding domain of Hsp90	[84]

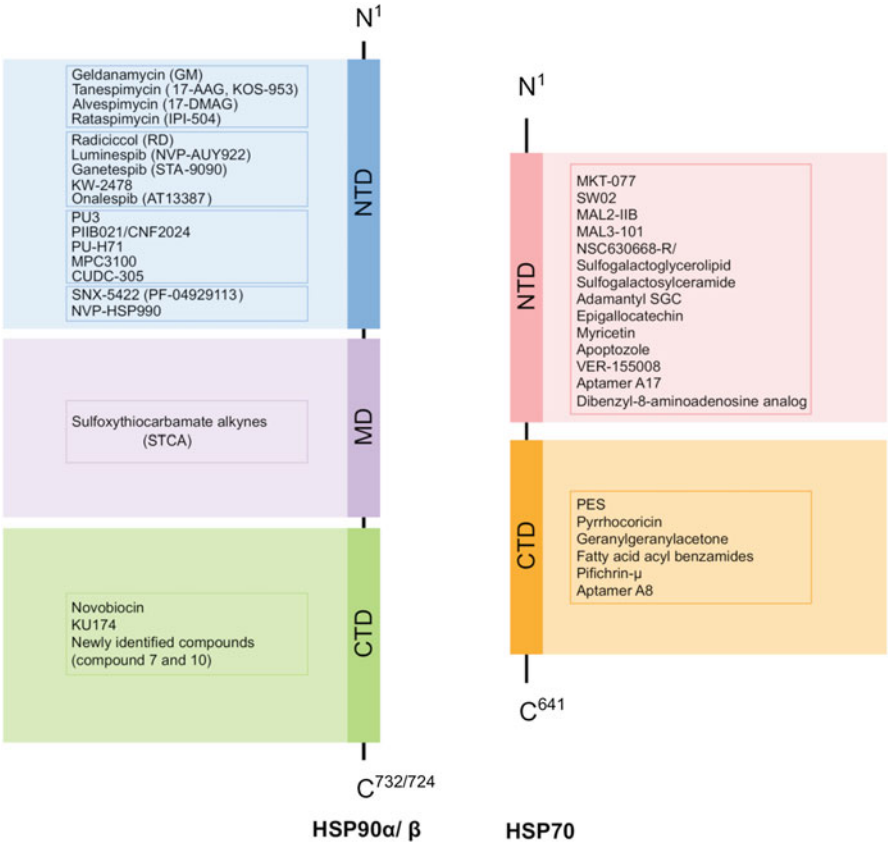

Fig. 3 Schematic representation showing variant inhibitor molecules of Hsp90 (left panel) and Hsp70 (right panel). Inhibitors of HSP are grouped according to their targeting sites (*NTD* N-terminal domain, *MD* middle domain and *CTD* C-terminal domain)

as geldanamycin (GM) perform their anti-proliferative activity through association with the ATP-binding site located in the N-terminal domain of Hsp90, thus blocking its function [85, 86]. Similarly, radicicol (RD) that was primarily obtained from *Monosporium bonorden* inhibits Hsp90 *via* occupying its ATP binding pocket, subsequently hindering its ATPase activity [87]. Unfortunately due to their hepatotoxic side effects, structural instability or poor bioavailability geldanamycin and radicicol were not used in the clinic although their *in vitro* promising effects [87, 88]. Therefore, different GM analogues like 17-AAG (tanespimycin or 17-allylamino-17-demethoxygeldanamycin) and 17-DMAG (alvespimycin or 17-dimethylaminoethylamino-17 demethoxygeldanamycin) have been developed in attempt to overcome these limitations [89, 90]. Other Hsp90-inhibiting compounds, such as sulfoxythiocarbamate alkynes (STCAs), have been recently reported to target the Hsp90 middle domain *via* attacking cysteine residues and forming thiocarbamate adducts. Interestingly, the resulting conformational changes from the thiocarbamylation

process alters the chaperoning activity of Hsp90 and hinders its binding to client proteins without interference of its ATPase activity [83].

Coumarin antibiotics, such as novobiocin and its derivatives, have been described to inhibit another ATP binding site located in the C-terminal domain of Hsp90 [91]. Binding of novobiocin to the C-terminal ATP binding site disrupts the interaction of Hsp90 with many of its client proteins such as Raf-1, v-src, mutant p53 and HER2 [91]. Recent technologies, such as plasmon resonance (SPR), have been utilized to explore various Hsp90 C- terminal inhibitors among many commercially available compounds. Interestingly, these efforts enabled Terracciano and his colleagues to report newly identified compounds targeting the Hsp90 C- terminal domain and able to induce potent anti-cancer activities [84].

In addition to previous strategies, certain compounds such as celastrol and gedunin have been demonstrated to interfere with Hsp90 binding to its co-chaperones including Cdc37 and p23 [92–94]. Furthermore, other approaches aimed to inhibit the Hsp90 interaction with its client proteins [95].

1.2.2 Targeting Hsp70

Similar to Hsp90, various molecules have been identified to inhibit Hsp70 and currently represent powerful tools in cancer therapeutics. The majority of these compounds, summarized in Table 2 and Fig. 3, are known to target either the nucleotide binding domain (N-terminal domain) or the substrate binding domain (C-terminal domain) of Hsp70. Generally, Hsp70 inhibitors are categorized into three main groups; small molecule inhibitors, protein aptamers and antibody treatment [27].

Small molecule inhibitors such as MKT-077, an analogue of cationic rhodacyanine dye, was found to target the N-terminal ATPase domain of Hsp70 and has been tested in cancer clinical trials [99]. Other small molecule inhibitors include 2-phenylethynesulfonamide (PES) or pifithrin-μ that associates with the C-terminal domain of Hsp70 and prevents its interaction with HSP40 and other protein clients such as APAF-1 and p53 [100]. Impairment of Hsp70 function leads to misfolded protein aggregation, destabilized lysosomal membrane and apoptosis. Conversely, the natural immunosuppressive compound, 15-deoxyspergualin (15-DSG), binds to the N-terminal domain of Hsp70 and blocks its ATPase activity [101]. Second generation inhibitors such as MAL3-101 and its derivatives act on the N-terminal ATP binding domain of Hsp70 and exhibit anti-proliferative activities on cancer cell lines [102]. Notably, co-treatment of cancer cells with MAL3-101 and 17-AAG or MAL3-101 with PS-341 (bortezomib) in mouse model of melanoma showed enhanced therapeutic responsiveness [103, 104]. Interestingly, VER-155008, a compound that is derived from adenosine targeting the Hsp70 ATPase domain, was able to stimulate both caspase dependent and non-caspase dependent apoptosis in breast and colon cancer cells [105]. In addition, combination therapies including Hsp90 inhibitors such as NVP-AUY922 and VER-155008 gave better anti-cancer effects in myeloma cells [106].

Table 2 Common anti-cancer Hsp70 inhibitors and their targeting sites [96]

Class	Inhibitor	Targeting site	References
	MKT-077	N-terminal ATP binding domain of Hsp70	[97, 98]
Dihydropyrimidines	SW02	N-terminal ATP binding domain of Hsp70	[97, 98]
	MAL2-IIB		
	MAL3-101		
	NSC630668-R/I		
Sulfoglycolipids	Sulfogalactoglycerolipid	N-terminal ATP binding domain of Hsp70	[97, 98]
	Sulfogalactosylceramide		
	Adamantyl SGC		
Flavonoids	Epigallocatechin	N-terminal ATP binding domain of Hsp70	[97, 98]
	Myricetin		
	Apoptozole	N-terminal ATP binding domain of Hsp70	[97, 98]
Adenosine derivatives	VER-155008	N-terminal ATP binding domain of Hsp70	[98]
Protein Aptamer	Aptamer A17	N-terminal ATP binding domain of Hsp70	[96–98]
	Dibenzyl-8-aminoadenosine analog	N-terminal ATP binding domain of Hsp70	
	cmHsp70.1mAb	Interact with Hsp70 epitope	
Small molecule inhibitor	2-phenylethynesulfonamide (PES pifithrin-μ)	C-terminal/peptide binding domain	
	Pyrrhocoricin	C-terminal/peptide binding domain	
	Geranylgeranylacetone	C-terminal/peptide binding domain	
	Fatty acid acyl benzamides	C-terminal/peptide binding domain	
	Pifichrin-μ	C-terminal/peptide binding domain	
Protein Aptamer	Aptamer A8	C-terminal/peptide binding domain	

Protein aptamers are considered among the alternative approach targeting Hsp70. A17 was demonstrated to target the Hsp70 N-terminal ATPase domain. Moreover, combined cisplatin/A17 therapy potentiated apoptosis in cancer cell lines and efficiently inhibited tumor growth in mice models of melanoma [107]. Other targeting approaches of Hsp70 include immune based monoclonal antibodies such as cmHsp70.1, which recognizes specific membrane bound Hsp70 motif [108]. These advanced approaches have been used in clinical trials with promising anticancer results [108].

1.2.3 Targeting Hsp60 in Cancer

Relative to other HSP, few compounds have been known to target Hsp60 [109]. Meng and his colleagues have classified Hsp60 inhibitors according to their origin into two main groups; derivatives natural products and synthetic compounds (listed in Table 3) [109]. Based on their mode of action, Hsp60 inhibitors have been arranged into type I inhibitors, which target the ATP binding site and interfere with the Hsp60 chaperoning activities, and type II inhibitors, which comprise compounds acting through covalent association with cysteine residues within the Hsp60 molecule. However, much of the exact mechanism of action of these inhibitors are still unclear [109]. Table 3 gives an overview about the potential modulators of Hsp60 that can be used in future cancer treatments.

1.2.4 Targeting Hsp27 in Cancer

Hsp27 is one of the major inducible sHSP known to contribute to tumor development and malignancy. Upregulation of Hsp27 has been reported in myriad cancer types where it has been linked to poor prognosis and treatment resistance [121]. In the previous section, we briefly referred to its anti-apoptotic mechanisms as well as cancer promoting roles. Here, we present a summarized overview on the potential

Table 3 Overview of common Hsp60 inhibitors [109]

Class	Inhibitor	Effect	References
Natural products	Mizoribine	binds to the Hsp60 ATPase domain leading to This direct binding inhibition of the chaperone activity of the Hsp60-Hsp10 complex	[110, 111]
	epolactaene	Unknown mechanism of action	[109, 112]
	ETB (tert-butyl ester of epolactaene)	Interacts with Cys442 of Hsp60 leading to potential proximity to potential allosteric modulation of the ATP binding pocket	[112]
	Myrtucommulone A (MC)	Interacts directly with Hsp60 leading to aggregation and misfolding of cancer related proteins	[113]
	Stephacidin B	Performs anticancer activities	[114, 115]
	Avrainvillamide	Anticancer activities	[116]
Synthetic compounds	*O*-carboranylphenoxyacetanilide	Binds to Hsp60 and suppresses hypoxia-induced HIF activation	[117]
	Gold (III) porphyrin complexes such as A prototype gold (III) complex [Au(TPP)Cl] (10)	Though poorly understood mechanisms, it inhibits Hsp60 and performs significant anticancer activities	[118–120]

approaches and inhibitors targeting Hsp27 in cancer therapeutic arena (summarized in Table 4).

It has been known that the plant bioflavonoid quercetin exhibits anti-cancer properties [122] *via* inhibition of heat shock response [123, 130]. Diverse anti-cancer activities of quercetin have been described in prostate, gastric, breast and oral cancers [131, 132]. Interestingly, quercetin has been demonstrated to down regulate casein kinase 2 (CK2) with consequent proteasomal degradation of Hsp27. Therefore, quercetin has been suggested to regulate Hsp27 in cancer cells [133, 134]. Another small molecule inhibitor, brivudin (RP101) has been revealed to inhibit Hsp27 through association of π-stacking with Phe29 and Phe33 of Hsp27 leading to apoptosis [124, 125]. RP101 has been used in clinical trials of pancreatic cancers where it increased the survival rates of diseased individuals [124]. In addition, in fibrosarcoma cells, combined RP101/gemcitabine treatment resulted in 30–50% reduction of invasiveness compared to gemcitabine alone [124].

An eminent strategy to target Hsp27 is the use of antisense oligonucleotide (ASO) which target Hsp27 mRNA. For instance, OGX-427 has been used in combination therapies treating prostate cancer where it remarkably reduced the tumor volume compared to monotherapies [127]. OGX-427 has been also used in phas I and phase II clinical studies of metastatic bladder and castrate-resistant prostate cancers, respectively [135]. Furthermore, treatment with OGX-427 resulted in enhanced sensitivity to radiation therapies in radiation-resistant lung as well as head and neck cancers besides reduction of tumor angiogenesis [136].

Difficulties in the application of antisense technology in vivo gave rise to new approaches that employs specific peptides to suppress the anti-apoptotic activity of Hsp27 [121]. Protein aptamers are designed in the form of short sequences of aminoacids associated with a scaffold protein. These aptamers aimed to modulate the activity of different cellular proteins, including oncogenes, transcription factors, signaling molecules, cell cycle regulators, and others [129]. The two aptamers PA11 and PA50 have been designed to specifically bind to Hsp27, disrupting its dimerization and oligomerization leading into impairment of cancer cell proteostasis. Although

Table 4 Common Hsp27 inhibitors and their mode of action

Class	Inhibitor	Mode of action	References
Small molecules	Quercetin	Reduces CK2 expression and increases Hsp27 degradation	[121–123]
	RP101 (Brivudine)	Inhibits Hsp27 through association of π-stacking with Phe29 and Phe33 of Hsp27 leading to apoptosis	[124, 125]
	J2 (Cross linker)	Forms a covalent bond between the cysteine of thiol groups within Hsp27	[126]
Antisense Drug	OGX-427	Antisense oligonucleotide (ASO) that targets Hsp27 mRNA	[127, 128]
Peptide Aptamers	PA11	Interferes with Hsp27 oligomerization leading to dysregulated cellular proteostasis	[129]
	PA50	Hampers Hsp27 dimerization resulting in disruption of Hsp27 mediated signaling	[129]

the application of these advanced strategies looks promising in the oncology field, certain limitations remain existing in terms of the size of the investigated protein, the presence of protein complexes, RNase containing environment [121, 129].

1.3 Targeted Cancer Thermotherapy

1.3.1 Nanoparticles in Cancer Therapy

Anticancer therapy insufficient tumor targeting, and increased side effects have directed the interest in nanomedicine for cancer therapy [137]. Nanomedicine was defined by the US National Institute of Health as 'Nanomedicine refers to highly specific medical intervention at the molecular scale for curing diseases or repairing damaged tissues, such as bone, muscle, or nerve' [137]. The nanocarriers used are (10–200) nm in size that facilitate drug uptake, fast diffusion and having a large surface area to the volume ratio [138]. Consequently, those nanocarriers with their targeting ability will be able to accumulate in the tumor site and stay longer, which increases the efficiency of the drug [138]. At the same time, they will decrease side effects and toxicity of the drug since less of the healthy tissue is exposed to it [138]. Also, nanocarriers have the potential to deliver insoluble and unstable drugs, which they can protect from degradation [138].

There are different types of nanocarriers that are named based on their composition which are: Solid Lipid, Liposomes, Micelles, Dendrimers, Polymeric, Vial, Magnetic, Carbon, and Gold carriers [137]. Those carriers are also classified into three major types of nanoparticles: one dimension, two dimensions (Carbon nanotubes), or three dimensions (Dendrimers) nanoparticles [139]. For the best results, cancer cells and biocompatibility need to be identified to select the suitable nanocarrier type that can recognize the tumor site and release the desired drug [137]. Nanocarriers can be developed to not only deliver drug but also for cancer imaging as in the case of paramagnetic nanoparticles.

1.3.2 SPIONs

Magnetic Iron oxide nanoparticles have many applications compared to other nanoparticles used in diagnosis, treatment, and treatment monitoring. Superparamagnetic iron oxide nanoparticles (SPIONs) have a smaller size compared to iron oxide nanoparticles (IONP). Those particles have a size between (20–150) nm and have a more complicated synthesis than large IONP [140–142]. They have two structural compositions either they have a magnetic particle core (Magnetite Fe_3O_4, or Maghemite γ-Fe_2O_3), that differ in their physical properties, coated with a biocompatible polymer [143, 144]. Or they can be composed of a porous biocompatible polymer where they get precipitated inside the pores [144]. SPIONs have an important role in biomedical applications [141]. For example, they are being developed for an advanced

magnetofection, which is a transfection method, and magnetic resonance imaging (MRI) [141].

SPIONs have great superparamagnetic behavior, chemical stability, high saturation magnetization, and appropriate biocompatibility for therapy [141]. Therefore, when SPIONs are used for drug delivery they will have a long blood retention time, biodegradability and low toxicity, which increase the efficiency and decrease side effects of the drug in patients [140]. Also, they have been involved in magnetic hyperthermia-based cancer therapy for their ability to enhance competency, which generates localized heat under a fluctuating magnetic field [141]. Thus, SPIONs have more advantages than other nanoparticles to be used in drug targeting or magnetic hyperthermia for colorectal cancer therapy [141].

1.3.3 Synthesis Approaches

Two approaches are used in nanomaterial synthesis: a bottom-up approach, or top-down approach. In the bottom-up approach, nanoparticles used as the building blocks for complex nanostructures and have a better chance of producing structures with less defect [143]. While the top-down approach uses larger initial structures to attain nanostructures. Additionally, SPIONs synthesis methods are subdivided into 3 general types: physical, chemical, or biological [145]. Ninety percent of the methods used in their synthesis are chemical, while the 7% physical and 3% biological methods [146]. Because chemical approaches in synthesis have more direct procedures and fast product collection. Thus, those methods are the route that will be used for mass production of therapeutic nanoparticles in the future.

1.3.4 Chemical Synthesis: Co-precipitation

Co-precipitation is the most used chemical method especially in biomedical applications [143]. This method requires the usage of Fe (II) salt in aqueous, to a base solution in the presence of oxidant [147]. Like using iron chloride ($FeCL_3$) with ferrous sulfate ($FeSO_4$). Further, Ammonia (NH_3) is usually added as a precipitating agent. The advantages of this method are that it is simple, cheap, and convenient [143]. Thus, it enables rapid large-scale production [147]. Yet, the nanoparticles product morphology form aggregations. The particles are of a large size with poor crystallization and high oxidation capability. Affecting factors of the products when using this technique are the concentration of cations, the presence of counter ions, and the pH of the solution [148]. Additionally, using anionic surfactants as dispersing agents or coating agents like proteins or starches can stabilize the product particles [148].

1.3.5 SPIONs Enhancement: Surface Functionalization

SPIONs are not stable in the aqueous environment, so they would aggregate and precipitate [138]. Therefore, a coating is required to add stability to the nanoparticles in liquid [138]. The coating can be achieved in two main approaches either during the synthesis process, or post-synthesis coatings [140]. Also, depending on the type of application those nanoparticles will be used for, the coating type will differ to provide the most stable interactions. The coating of SPIONs is similar to the coatings used for enhancement of IONPs. For example, in an application for drug delivery, the IONPs need to be coated with different moieties, which can eliminate their aggregation in blood [140]. Polyethylene glycol PEG is one of the most used in coatings for IONPs, that can be implemented in SPIONs as well [137]. Because PEG has a high solubility, biocompatibility, stability, prolonged blood circulation time, and allows bioconjugation for modifications with various functional groups [140]. However, SPIONs PEG-coated has limited binding sites available for they have a small size which limits their conjugation surface [140]. Another example, Dextran coating is used in applications for MRI imaging using IONPs, which also can be carried out on SPIONs [140]. Because it stabilizes the magnetic nanocrystals by overcoming their weak ligand-particle interactions and their easy detachment; Since they provide a cross-link using hydrogen bonds in between the iron oxide and dextran-based, which are reversible [140]. The cross-linking changes the IONPs size and stabilize the product which helps in providing a sufficient signal for MRI imaging. Hence, MRI imaging using IONPs depends on the morphology of the IO crystals [140].

1.3.6 Targeting

Advantages of SPIONs makes them good candidates for drug delivery and targeting. Pharmacokinetic profile for SPIONs is important to evaluate their biotransformation in the body in ADME parameters (absorption, distribution, metabolism, and excretion) [141]. The profiling will give information on how those nanoparticles can be used in drug delivery and targeting. The targeting of drug-containing nanoparticles can be achieved by three major approaches which are either passive targeting, active targeting, or triggered drug targeting [138]. In passive targeting, depends on the utilization of permeability enhancement, which works indirectly in specifying the tumor site [137]. While, in active targeting, it depends on the targeting of overexpressed receptors on the cancer cell surface, therefore, it targets directly to the tumor site [137]. Triggered drug targeting in the case for SPIONs, where they are targeted to the tumors by using an external magnetization on the tumor site [140]. Since the drug-coated SPION magnetic ability will enable them to move toward tumor location [149]. Multiple new researches are being conducted to investigate and implement SPIONs magnetic targeting as a new advantageous therapy which would increase the specificity of the drugs and decrease the side effects of drugs on patients. Recently, a study has used SPIONs magnetic properties

in response to an external magnetic field for targeting to a specific site [150]. They have synthesized citric acid-capped SPIONs linked to the anticancer drug, doxorubicin, by noncovalent interactions [150]. They have observed an associated drug release and a significant cellular uptake after the magnetic targeting, with low cytotoxicity [150]. Further, releasing the drug at the specific location is dependent on the effect of internal or external stimuli like the concentration of the particles and the external magnetization effect, for the case of SPIONs [149]; Where the drug can be released by dissolution, diffusion, or vehicle rupture [140].

1.3.7 Magnetic Field-Induced Hyperthermia

We have stated that SPIONs have been recently involved in inducing thermal therapy that is generated by localized heat under a fluctuating magnetic field [141]. Hence, this feature makes SPIONs to be considered as therapeutic agents without the addition of functional moieties [151]. The increase in temperatures >42 °C changes many of the functional and structural proteins [151]. This procedure causes cellular necrosis. The application of an external magnetic field with a specific alternative frequency and current, depending on the SPIONs shape and size, will cause an increase in the kinetic energy of those nanoparticles [151]. Hence, they ultimately will heat up and increase the temperature of the surrounding region [151]. Moreover, based on multiple studies, it has been observed that temperatures were retained in normal tissue, whereas elevated temperatures and loss of the extracellular matrix (ECM) were observed in tumor tissue. Additionally, the loss of the ECM increased drug diffusion into tumor cells. Also, the magnetic hyperthermia effect can be used for triggering drug-release. This can be achieved by coating the drug with a thermal sensitive label [151]. Additionally, new cancer studies have found that cancer tissue is penetrated by mesenchymal stem cells (MSC) [141]. Thus, current research is investigating the possibility to combine MSC with SPIONs by endocytosis [141]. Therefore, the applied magnetics hyperthermia will be more specific since the MSC are placed in between tumor cells [141]. In summary, SPIONs have several therapeutic applications that can be developed for personalized combination therapies for CRC patients.

1.4 Combined HSP Targeting with Hyperthermia

The approach for HSP targeting in combination with hyperthermia was elegantly described in the work of Ito et al. [152, 153]. Firstly, they developed in situ vaccination for tumor treatment. Tumor cells will be a target for immune system *via* release of Hsp70-tumor antigen complexes at the tumor site, and the recruitment of immune effector cells, including APC, to the tumor subsequently occurred as a consequence of the inflammation. Hyperthermia was selectively induced in the tumor cells by the mean of magnetite cationic liposomes (MCL). Based on their

results, they proposed that intracellular hyperthermia by means of MCL is an in situ vaccination therapy for cancer [152]. Moreover, they confirmed the results by Hsp70 gene therapy; human Hsp70 gene was introduced in the cells by cationic liposomes and hyperthermia was induced by exposing the MCLs to an alternating magnetic field for 30 min. The temperature at the tumor (melanoma) reached 43 °C and was maintained by controlling the magnetic field intensity. The combined treatment strongly arrested tumor growth over a 30-day period, and complete regression of tumors was observed in 30% of the treated mice [152]. Lately, Ito et al. targeted Hsp90 through geldanamycin concomitantly with thermosensitive ferromagnetic particles-mediated hyperthermia. Results showed HSP inhibitor exerted an antitumor effect by increasing the cells' susceptibility to hyperthermia in both *in vitro* and *in vivo* models [153]. Similarly, Vriend et al. [154] showed that treatment with a single, short course, with a relatively low dose of Hsp90 inhibitor (Ganetespib) potentiated the cytotoxic as well as radio- and chemosensitizing effects of hyperthermia and reduced the thermotolerance in cervix cancer cell lines. Moreover, it has been demonstrated that Hsp70 inhibition in combination with magnetic fluid hyperthermia generated a synergistic effect and could be a promising target to enhance magnetic fluid hyperthermia therapeutic outcomes in ovarian cancer [155]. In addition, the co-inhibition of Hsp70/Hsp90 with quercetin plus 17-DMAG significantly increased apoptosis in hyperthermia-treated cancer cell HNE1 both *in vitro* and *in vivo* as well as synergistically sensitized nasopharyngeal carcinoma cells to hyperthermia [48].

2 Conclusions

A number of challenges must be overcome before hyperthermia can be considered a standard treatment for cancer [3, 7]. Many clinical trials are being conducted to evaluate the effectiveness of hyperthermia in combination with other therapies for the treatment of different cancers. There are numerous challenges in creating the most effective SPION that is ideal for its intended application. Those obstacles include finding the suitable particle morphology, coating, and determining the best concentration with the lowest toxicity for the most effective therapy [156]. Moreover, the ability to target the nanoparticles to the tumor location with low specificity is difficult since the SPIONs depend on probe-based delivery for targeting or applying an external magnetic field. Likewise, problems in drug delivery includes: (1) the probability that modifications to conjugate the drug to the nanoparticle might change its properties, (2) drug distribution in the body, (3) releasing the drug to enzymatic digestive organelles, like endosomes and lysosomes, that causes drug digestion and decrease its effect, and (4) the probability of losing the magnetization of SPIONs when it undergoes a large number of coatings and a large number of chemical reaction [140]. Moreover, one of the recent challenges is found in the ability of the SPIONs to cross the blood-brain barrier (BBB) and target glioblastoma cells at the same time [157]. Also, oral administration of SPIONs, which is more preferred by

patients, was found to have a lower therapeutic efficiency compared to direct injection in the bloodstream [157]. Thus, challenges in drug administration need to be considered in new drug development [157]. In addition, research with HSP inhibitors together with SPIONs targeted different HSP such as Hsp90, Hsp70, and others would prevent resistance as well as potentiate the cytotoxic and/or antiproliferative effects of the hyperthermia.

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Small Molecule Inhibitors Targeting Heat Shock Response Pathways: Lessons from Clinical and Preclinical Studies in Cancer Therapeutics

Daniel Zhang, Dorothy Wang, and Bin Zhang

Abstract

Introduction Heat shock response (HSR) pathway is a highly conserved cellular process. HSF1 is a master transcriptional regulator responsible for the expression of several important heat shock proteins (HSP), which can effectively protect critical client proteins from misfolding and degradation, thus maintaining intracellular integrity under stressed conditions. Recent studies have demonstrated the direct connections between HSR players and tumor cell survival, validating HSR players as novel molecular targets in anticancer treatment. Small molecule screening has produced some promising HSR inhibitors for anticancer treatment. In this article, we aim to summarize the main findings from HSR inhibitors on recent clinical and preclinical studies.

Methods The authors reviewed all the relevant papers of HSR inhibitors with an emphasis on human and animal studies.

Results More than 18 unique chemical identities have been discovered with confirmed inhibition of HSR pathway. Among them, two natural products and their derivatives are currently in various phases of clinical studies. Detailed works are required to define the exact mechanisms of actions (MOA) for these compounds.

Conclusion Many hurdles in clinical application still need to be effectively addressed, such as undesirable drug toxicity and off-target effects; narrow therapeutic window; poor PK/PD profiles, etc. Recent reports on synergistic drug combination, advanced prodrug design, smart nanoparticle packaging, and RNA aptamer selection offer promising solutions to overcome these challenges. Future advancements in this fast-growing area can potentially lead to the next-generation cancer therapeutics.

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Abbreviations

HCS	high content screening
HSE	heat shock element
HSF1	heat shock factor 1
HSP	heat shock protein
HSR	heat shock response
HTS	high throughput screening
MOA	mechanism of action
NCT	ClinicalTrials.gov identifier number
PD	pharmacodynamics
PK	pharmacokinetics
SAR	structure-activity relationship
target ID	target identification

1 Introduction

Cancer is a malignant disease characterized by uncontrolled cell growth. Genetic and epigenetic alterations can activate oncogenes whose activities are necessary for tumor initiation and maintenance, a phenomenon called "oncogene addiction." [75]. As a result of this oncogenic transformation, cancer cells are known to exhibit "stress phenotype", such as high levels of DNA damage, aneuploidy, and reactive oxygen species [21]. These cancer cells constantly express mutated oncogenes with misfolded protein structures. Comparing to their normal counterparts, the transformed malignant cells demand a much higher expression level of molecular chaperone proteins, including HSP70, HSP90 and HSP27, etc., to preserve protein homeostasis for tumor cell survival [6]. The tumor dependency of molecular chaperone machinery creates an attractive model of cancer therapeutics by selective targeting key HSR players [17].

Heat shock factor 1 (HSF1) is the master transcription factor responsible for controlling the heat shock response [15]. HSF1 is normally maintained in an inactive monomeric state through binding to a complex containing heat shock protein (HSP) 70, HSP90 and HSP40 (Fig. 1). When cellular stress occurs by the multiple known causes of protein misfolding (such as cancer cell transformation, DNA damage, etc.), HSF1 dissociates from the chaperone complex, translocates into the nucleus, and forms active phosphorylated trimers. This activated HSF1 trimer thus binds to the heat shock element (HSE) in the Promoter regions of many heat shock proteins. As a result, activation of HSF1 leads to the mRNA induction and translation of HSP70 and other chaperone family proteins. In addition, HSF1 has a large number of target genes encoding proteins with versatile cytoprotective functions [2]. This highly conserved protective machinery is now believed to be adopted by the majority of

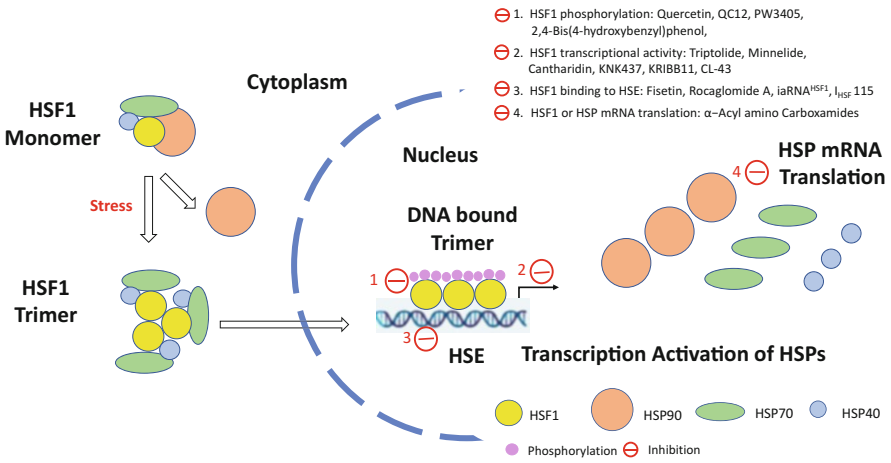


Fig. 1 Illustration of HSF1 trimerization and activation inside the cell with four types of inhibitory mechanisms of small molecules. (1) Inhibition of HSF1 phosphorylation or HSF1 dephosphorization; (2) Interference of HSF1 trimer binding to HSE; (3) Blocking HSF1 mediated transcription activation; (4) hampering mRNA translation of HSF1 and HSP. For a total of 18 compounds in this review (including two prodrugs), 14 compounds show some evidence in one type of the inhibitory mechanisms of HSF1 (listed above), although more detailed studies are essential. The MOAs for the remaining four compounds need to be determined

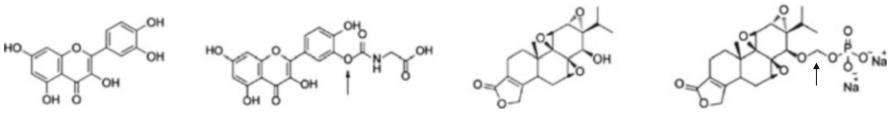
cancer cells, through which the so-called "protein folding pressure" can be mostly relieved to facilitate the survival of malignant cells [33].

Since HSF1 essentially controls the expression of all cellular HSP in tumor cells, it has been proposed to be a novel anticancer target with "non-oncogene addiction" features [68]. Dai and colleagues demonstrated that genetic HSF1 deficiency in transgenic mice inhibits tumorigenesis in a mouse skin cancer model. Similar results were also observed in genetic models containing oncogenic mutations of the RAS oncogene or inactivating mutations in the tumor suppressor p53 [14]. Other target validation results from different groups also confirmed that tumor cells have a much greater dependence on HSF1 function compared to normal cells, suggesting that cancer cells are becoming "HSF1 addicted" [43]. Thus, small molecule intervention pf tumor HSR pathway, as monotherapy or combined with other drugs, has been suggested to be a novel avenue for cancer therapeutics [15].

In recent years, efforts have been made to screen and identify small molecule HSR inhibitors [16]. Through various compound screening platforms, such as reporter based high throughput screening, image-based phenotypic screening and chemical SAR approach, more than 18 unique chemical identities or structure derivatives have been discovered with confirmed inhibition of HSR pathway (Fig. 1; [65]). According to Clinincaltrial.gov, two of the natural products, Quercetin [59] and triptolide [49] and their respective prodrugs (QC12 and Minnelide), are in various stages of clinical trial studies (Table 1 and Fig. 2). Seven of these compounds were reported to have positive antiproliferative effects in the mouse xenograft models carrying different tumor cell lines, including breast, skin, colon, hepatoma, and pancreatic cancer lines (Table 2 and Fig. 3). The remaining seven compounds

Table 1 Compounds and their prodrugs targeting HSF1 pathways with human clinical data in cancer treatment

	Chemical property	Mechanism of action (-) inhibition	Animal preclinical study	Human clinical study
Quercetin	Natural product	(-)HSF1 phosphorylation	Oncology Report 2016	Phase 1, Clinical Cancer Research 1996 (Ferry et al.)
			PLOS One 2017	Phase 2 (ID#: NCT03476330)
			Scientific Report 2016	
			Chinese J Cancer Res 2016	
			Apoptosis 2017	
			Cancinogenesis 2000	
			Cancer Prevention Research 2009	
			Molecular Cancer Therapeutics 2008	
QC12	Analog prodrug	A prodrug of quercetin		Phase 1, Ann Oncology 2001 (Mulholland et al.)
Triptolide	Natural product	(-) HSF1 transcriptional activity	Cancer Research 2007	Phase 1, Eur J Cancer 2009 (Kitzen et al.)
			Ann Thorac Surg 2015	
			Acta Pharmacologica Sinica 2015	
			BMC Cancer 2016	
			Molecular Cancer Therapeutics 2003	
			PLOS One 2012	
			Oncology Report 2014	
			Oncotarget 2016	
			Front Oncol 2019	
Minnelide	Analog prodrug	A prodrug of triptolide	Pancreatology, 2016	Phase 1 (NCT01927965), completed in 2016
			J translational Medicine 2019	Phase 1 (NCT03347994), active
			J Clinical Oncology 2016	Phase 1/1b (NCT03129139), active
			PLOS 2017	Phase 2 (MCT03117920), active.
			J Med Chem 2015	
			J. Am. Coll. Surg 2017	



Quercetin QC12 Triptolide Minnelide

Fig. 2 Chemical structures of four inhibitors of HSR pathway with available human clinical trial data. Both quercetin and triptolide are of natural origin. QC12 and Minnelide are prodrugs for quercetin and triptolide, with arrows pointing to the respective hydrolysis sites *in vivo*

Table 2 Compounds targeting HSF1 pathways with animal model data in cancer treatment

	Chemical property	Mechanism of action (−) inhibition	Animal preclinical study	Animal xenograft models
PW3405	Synthetic compound	(−) HSF1 phosphorylation	Manuscript in preparation	Mouse xenograft (pancreatic cancer)
Cantharidin	Natural product	(−) HSF1 transcriptional activity	Cell Physiol Biochem 2017	Mouse xenograft (breast cancer)
			Oncogenesis 2018	Mouse xenograft (pancreatic cancer)
			Environ Toxicol 2016	Mouse xenograft (skin cancer)
			Molecules 2017	Mouse xenograft (hepato-cellular carcinoma)
KNK437	Synthetic compound	(−) HSF1 transcriptional activity	Clinical Cancer Research 2001	Murine squamous cell cancer
			Oncogene 2019	Mouse xenograft colorectal cancer
KRIBB11	Synthetic compound	(−) HSF1 transcriptional activity	JBC 2011	Mouse xenograft (colon cancer)
			Clinical Cancer research 2018a	Mouse xenograft (myeloma)
		Direct binding to HSF1	Clinical Cancer research 2018b	Moust xenograft (myeloma)
			Oncotarget 2017	Mouse orthotopic xenograft (breast cancer)
Fisetin	Natural product	(−) HSF1 binding to HSE	Onco Rep 2017	Mouse orthotopic xenograft (hepatoma cancer)
			Cell Death Dis-ease 2019	Mouse xenograft (pancreatic cancer)
			Carcinogenesis 2015	Mouse xenograft (colon cancer)
			Int J Biol Macromol. 2019	Mouse xenograft (breast cancer)
Rocaglamide A	Natural product	(−) HSF1 binding to HSE	Science 2013	Mouse xenograft (leukemia)
			Am J Transl Res 2016	Mouse xenograft (pancreatic cancer)
			Cancer Letters 2017	Mouse xenograft (myeloma and leukemia)
CCT251236	Synthetic compound	TBD, pirin inhibition	J Med Chem 2017	Mouse xenograft (ovarian cancer)
			Clin Cancer Res 2018	Mouse xenograft (myeloma)

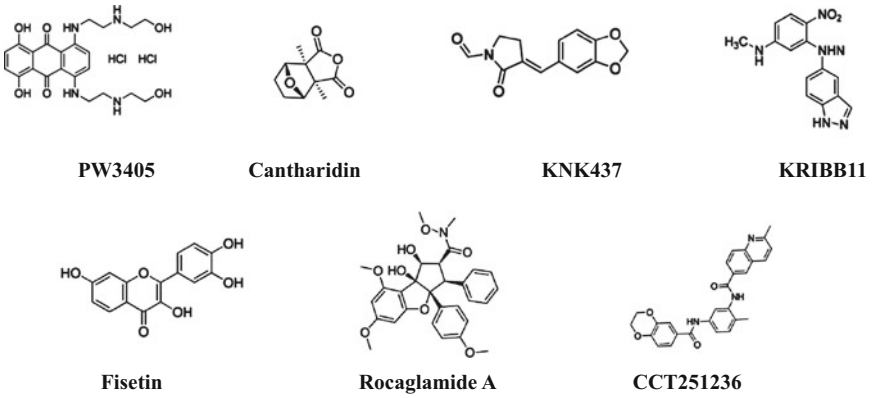


Fig. 3 Chemical structures of seven inhibitors the HSR pathway with data available in various animal xenograft models. Cantharidin, fisetin, rocaglamide A are natural compound products while PW3405, KNK437, KRIBB11, CCT251236 are synthetic compounds

Table 3 Compounds targeting HSF1 pathways with cancer cell line data available

	Chemical property	Mechanism of action (−) inhibition	Cell line study	References
2,4-Bis (4-hydroxybenzyl) phenol	Natural product	Dephosphorylation of HSF1	NCI-H460 human lung cancer line	J Nat Prod 2014
α−acyl amino Carboxamides	Synthetic compound	(−) HSF1 mRNA translation	Multiple Myeloma INA-6 line	J Med Chem 2017
Cardenolide CL-43	Natural product	(−) HSF1 transcriptional activity	HCT-116 human colon cancer line	Oncotarget 2018
$I_{HSF}115$	Synthetic compound	(−) HSP mRNA translation	A panel of 33 cancer lines	Nucleic Acid Res 2017
$iaRNA^{HSF1}$	RNA aptamer	(−) HSF1 binding to HSE	A panel of 5 cancer lines	PLOS One 2014
NZ-28	Synthetic compound	TBD	Multiple Myeloma IS line	Cancer Res 2006
			Prostate carcinoma PC-3 line	
Stresgenin B	Natural product	TBD	A panel of 6 cancer lines	J Antibiot (Tokyo) 1999
4,6-Disubstituted pyrimidine	Synthetic compound	TBD, CDK9 inhibition	Osteosarcoma U2OS line	Medchemcomm 2016

and one RNA aptamer are in the early stage of preclinical studies, with only cell line data available (Table 3 and Fig. 4). More detailed works are required to define the exact mechanisms of actions (MOA) for these compounds. As shown in Fig. 1, the inhibitory mechanisms can be primarily categorized into four types: (1) Inhibition of HSF1 phosphorylation or HSF1 dephosphorization; (2) Interference of HSF1 trimer

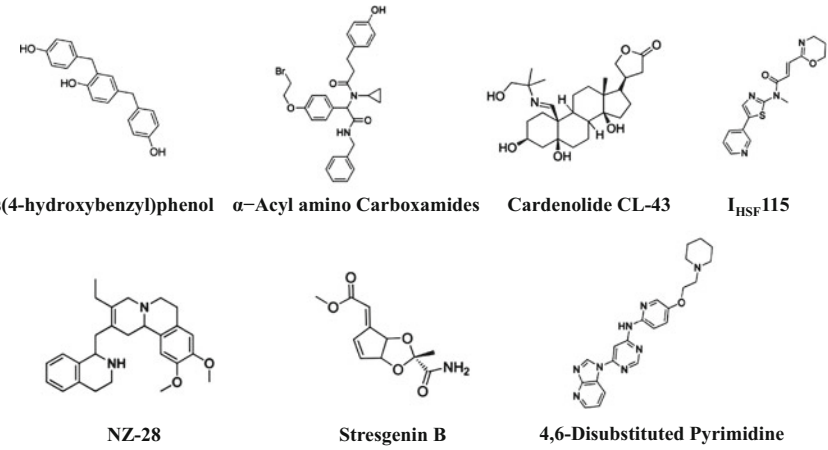


Fig. 4 Chemical structures of seven inhibitors of HSR pathway with data available only in various cancer cell lines. These inhibitors include three natural products (2,4-Bis(4-hydroxybenzyl) phenol, cardenolide CL-43, stresgenin B), four synthetic compounds (α−acyl amino carboxamides, $I_{HSF}115$, NZ-28, 4,6-disubstituted pyrimidine) and one RNA aptamer ($iaRNA^{HSF1}$)

binding to HSE; (3) Blocking HSF1 mediated transcription activation; (4) hampering mRNA translation of HSF1 and HSP. The undefined MOAs and diverse chemotypes make the clinical study of these drug candidates less predictable. In this chapter, the authors reviewed the recent progress and lessons learned from human and animal model studies. Several excellent reviews are also available on the current status of MOAs, chemical structures and screening of HSR inhibitors [16, 28, 65].

2 HSR Inhibitors Currently in Human Clinical Studies (Table 1 and Fig. 2)

2.1 Quercetin

Quercetin is one of the bioactive flavonoids found ubiquitously in fruits, vegetables and beverages [59]. Previous publications established its role as a natural antiproliferative agent against various cancer cell lines from diverse lineages and mouse xenograft models [44]. Using a gel shift assay, Nagai et al. reported that quercetin down-regulates HSF1 by the decrease of HSF1-HSE binding, rather than inhibition of HSF1 trimerization [47]. Yang and colleges used quercetin-loaded liposomes to treat rats with R3230 breast adenocarcinoma. An increase of the tumor destruction/endpoint survival *in vivo* was observed as compared to the control

treatment group [83]. Importantly, this study confirmed that HSF1 is required for quercetin-induced cancer cell death and quercetin can directly down-regulate HSF1. Moreover, quercetin was also tested in other xenograft mouse models, including hepatoma [92], ovarian cancer [39, 40], breast cancer [25, 34, 64], colon cancer [25, 54, 81]. In addition to its inhibitory role in the heat shock response pathway, these animal studies showed that quercetin can also perform its anti-cancer effects through modulating cyclins, pro-apoptotic, autophagy, PI3K/Akt and mitogen-activated protein kinase (MAPK) molecular pathways, etc.

Quercetin has been proposed as a sensitizer and protects non-cancer cells from the side effects of conventional cancer therapies [59]. The safety and potential usefulness of quercetin for cancer treatment have been documented in both animal experiments and a phase I clinical trial. The first clinical trial phase I study was done in the UK [20]. The authors investigated the pharmacokinetics of quercetin with short i.v. infusion and found 1,400 mg/m^2 as the safe bolus dose. The maximum tolerated dose was determined as 1,700 mg/m^2 three weekly. The anti-tumor effects were recorded with confirmed inhibition of lymphocyte tyrosine kinase activity. However, dimethyl sulfoxide was used as a solubilized vehicle and was unsuitable for further clinical development of quercetin.

According to clinicaltrial.gov, several clinical trials of quercetin are currently registered for the treatment of COPD, Hepatitis C and Type II diabetes, etc. Some of these trials are designed to study Quercetin as a dietary supplement for cancer prevention. For monotherapy of Quercetin, a phase II clinical trial is currently active to investigate its effects on chemoprevention for squamous cell carcinoma in patients with Fanconi anemia. Quercetin was designed to be orally administered for a maximum total daily dose of 4000 mg/day for 24 months (ClinicalTrials.gov Identifier: NCT03476330). The clinical data is expected to be available after 2023.

2.2 *QC12 (Analog Prodrug of Quercetin)*

In order to overcome the solubility issue of quercetin, a synthetic chemistry effort was undertaken to produce water-soluble derivatives of quercetin [46]. The most promising candidates of these synthetic compounds, called QC12, was selected. QC12 can be hydrolyzed *in vivo* and releases quercetin and glycine. Unfortunately, QC12 or quercetin was not detected in plasma following oral administration of QC12, confirming it was not orally bioavailable. As a result, QC12 entered the clinical phase I study with i.v. administration. The authors can detect peak QC12 concentration at >100 μM in plasma. Although quercetin was found in all patients following i.v. infusion. The relative bioavailability of quercetin is estimated to be only 20–25% released from QC12. This unsatisfactory Phase I results prevented QC12 for further clinical application [61].

2.3 Triptolide

Triptolide has been reported as one of the most potent inhibitors of heat shock response pathway [10, 49]. Westerheide et al. identified triptolide as an inhibitor of the HSF1 pathway through small molecule screening [76]. Although triptolide does not inhibit the earlier steps in the HSF1 multistep activation process, including trimer formation, hyperphosphorylation, or translocation and binding HSP70 promoter, the inhibitory effects of triptolide on HSP70 expression was reported to be at the level of transcription by interfering with the proper activity of the C-terminal transactivation domain of HSF1. Significantly reduced tumor cell viability was reported after triptolide was incubated with pancreatic cell lines, including PANC-1 and MiaPaCa-2. Triptolide also induces pancreatic cancer apoptosis via inhibition of heat shock protein 70 at mRNA level [52]

A number of mouse xenograft models were adopted to evaluate the anti-cancer effects of triptolide. Some representative examples including breast cancer [35, 36, 79], Prostate cancer [26], lung cancer [57, 58], pancreatic cancer [52], gastric cancer and melanoma [78, 82], etc. Comparing to all known HSR pathway inhibitors, triptolide exhibits a broader spectrum of anticancer activity against the common types of human cancer.

Using a semi-synthetic derivative of triptolide F60008, a phase I and PK/PD study were performed in patients with advanced solid tumors [31]. Twenty patients were enrolled, but hematological side effects were reported such as mild grade anemia. Other mild grade toxicities included constipation, fatigue, vomiting, diarrhea and nausea. Importantly, two lethal events were documented with increased caspase-3 activity and overt apoptosis in neutrophils and monocytes. PK data showed high variability between tested patients. The narrow therapeutic window and undesired water solubility largely limited the clinical application of triptolide [73, 74]. However, the prodrug of triptolide, namely Minnelide, has become an excellent candidate in the clinical study (next). In addition, a recent report on the smart drug delivery system can be an alternative solution to address toxicity issues of triptolide [78].

2.4 Minnelide (Analog Prodrug of Triptolide)

Most human clinical studies of triptolide were performed with its analog prodrug Minnelide. Minnelide is a phosphonooxymethyl prodrug with three times more water solubility than its parent molecule triptolide. Minnelide can be converted into triptolide by phosphatase *in vivo* [51]. Chugh et al. reported that Minnelide is as effective as triptolide in inhibition of pancreatic tumor cells both *in vitro* and *in vivo* [12]. In two mouse xenograft models of human colon adenocarcinoma and ovarian cancer, Minnelide was effective in reducing or eliminating tumors with a well-tolerated safety profile [51]. Banerjee and colleagues reported that Minnelide

induces cell death in a number of pancreatic cancer cell lines and reduces tumor volume in multiple xenograft mouse models, including pancreatic cancer and hepatoma [5]. Similar results were also reported on several pancreatic and melanoma xenograft models [18, 63]. Minnelide also exhibits inhibitory effects of HSP70 on the human gastric tumor xenograft mouse model, both as a single agent and in combination with chemotherapy agent CPT-11 [3]. Using an *in vivo* imaging system, Giri and colleagues reported Minnelide significantly decreased leukemic burden in multiple xenograft models of acute myeloid leukemia at doses easily achievable in patients [23]

The first open-label, phase I, safety clinical trial of Minnelide was completed in patients with advanced gastrointestinal tumors in 2016 (ID: NCT01927965). The primary objective of this study was to determine the maximum tolerated dose and the dose-limiting toxicities of Minnelide. Although no phase I data is currently available, a continued Minnelide phase II study is expected to begin for treating gastrointestinal malignancy [63]. According to Clinicaltrial.gov, there are three active clinical trials of Minnelide in human cancer treatment. A phase 1, open label, pilot study was initiated in 2018 on the pharmacokinetic and pharmacodynamic property of Minnelide with adult patients of AML (ID: NCT03347994). Minnelide capsule was also tested alone or in combination with protein-bound paclitaxel in patients with advanced solid tumor (various cancer types). This open label, phase 1 trial started in 2017 and is expected to be finished in 2021 (ID: NCT03129139). Promising response data from early stage trials has led to a phase II, international open-label trial of Minnelide in patients with refractory pancreatic cancer (ID: NCT03117920, [55]).

Interestingly, all current inhibitors of heat shock response in the clinical trial study are of natural origin or prodrug of natural compounds. Expectations are particularly high for positive results of Minnelide as monotherapy or combined treatment with other cancer drugs. Meantime, more data on animal PK/PD, toxicity, SAR analysis are becoming available for synthetic HSR inhibitors. Human clinical studies of this category of synthetic compounds are likely to be increased in the near future.

3 HSR Inhibitors Currently in Preclinical Animal Studies (Table 2 and Fig. 3)

3.1 PW3405

PW3405 was discovered as a potent heat shock response pathway inhibitor via a large-scale, unbiased, high content image-based screening in our group [89]. This synthetic compound demonstrated a nanomolar potency against HSF1 granulation after heat stress. Our study showed that the decrease of heat shock response is achieved through inhibition of HSF1 phosphorylation at the Ser326 activating site.

Thus, a potential intracellular kinase inhibitory mechanism was proposed [90]. The results from *in vivo* pancreatic cancer PC-3 xenograft models showed encouraging results with reduced tumor volume at a well-tolerated dose schedule (manuscript in preparation). Currently, PW3405 alone, or in combination with chemotherapy agents, are being investigated with several relevant mouse xenograft models. The results will be reported in due course.

3.2 Cantharidin

Cantharidin is a type of terpenoid secreted by the blister beetle Mylabris phalerata. A cell-based screening led to the discovery of this natural compound with potent activity against HSF1 [29]. Li and colleagues reported that cantharidin inhibits the growth of triple-negative breast cancer cells *in vitro* [37]. After the treatment of cantharidin, the tumor growth in MDA-MB-231 and MDA-MB-468 xenografts mice was reduced through inducing apoptosis of tumor cells. Moreover, the combination of cantharidin and antiangiogenic therapeutics presents additive antitumor effects against pancreatic cancer xenografts *in vivo* [80], although an unfavorable proangiogenic side effect was recorded. Anticancer effects of cantharidin were also reported in mouse skin cancer xenografts [38]. Using liposomal encapsulated cantharidin, increased anticancer effects were reported in a HepG2-bearing hepatocellular carcinoma xenograft model [91]. Although cantharidin has been a traditional Chinese remedy, there is no human clinical trial data recorded in clinicaltrial.gov.

3.3 KNK437

KNK437 was first reported as an inhibitor of HSF1 and HSP induction in human colon carcinoma cell [85]. The compound inhibits HSP expression at the mRNA level while it does not increase thermos-sensitivity in nontolerant cells. Since then, several animal tumor studies have been reported. In a mouse transplantable tumor model, Koishi et al. reported that KBK437 can inhibit thermotolerance via the inhibition of HSP72, thus improving the efficacy of clinical fractioned hyperthermia [32]. Another recent study showed KNK437 can inhibit colorectal cancer *in vivo*, with a significant reduction of HSP40 family member A1 expression [84]. Similar to quercetin, KNK437 may not be sufficiently potent for clinical use as high concentrations of compound are required to demonstrate inhibitory activity. The use of such high concentrations of low potency compounds increases the likelihood of off-target effects [53].

3.4 KRIBB11

Yoon and colleagues reported a synthetic compound named KRIBB11 that can directly bind to HSF1 in a western blot analysis [86]. It was proposed that the association of HSF1 and KRIBB11 can further inhibit the transcription process of heat shock proteins. In the same study, KRIBB11 can inhibit the growth of colon cancer cells in BALB/c nude mouse xenograft regression model. These results, particularly the evidence of direct HSF1 binding, promoted *in vivo* animal study of this compound. KRIBB11 was intraperitoneally administrated in a myeloma xenograft model, a significant decrease in tumor volume was observed [22]. At the molecular level, a significant reduction of HSP27 protein expression was documented in the KRIBB11 treated tumor group comparing to the control group. Using an orthotopic xenograft mouse model, KRIBB11 and AKT inhibitor MK-2206 in combination can result in the synergistic killing of breast cancer cells and inhibit tumor growth [7]. Recently, Parekh reported that KRIBB11 exhibits primary myeloma cell killing and cytotoxicity in stromal coculture, thus eliminating tumor cell protection inside the bone microenvironment in myeloma [50]. Although KRIBB11 exhibits modest *in vivo* efficacy in xenograft models as a single agent, its efficacy of HSF1 inhibition may be further improved by rational combination therapy.

3.5 Fisetin

Kim and colleagues reported their results of fisetin, a dietary flavonoid, on its inhibitory activity of HSF1 [30]. The downregulation of HSP70, HSP27 and BAG3 by fisetin significantly reduces the cellular levels of Bcl-2, Bcl-xL and Mcl-1 proteins, followed by apoptotic tumor cell death. Further analysis indicated that fisetin inhibits HSF1 by blocking the binding of HSf1 to HSP70 promoter. Treatment of colon cancer xenograft mice model with fisetin caused inhibition of HCT-116 cell growth *in vivo*. Another example is the results from a mouse liver cancer model. The orthotopically implanted tumors were inhibited by fisetin with a prolonged survival rate [39, 40]. Recently, a mouse xenograft model with luciferase expression in human pancreatic PANC-1 tumor cells was reported [27]. Bioluminescence can be emitted after the injection of luciferin intraperitoneally in a living mouse. Using this noninvasive method, tumor volume was recorded kinetically with significant size reduction in the fisetin treated group as compared to the control group. In an effort to improve the solubility and therapeutic index of fisetin, poly (lactic acid) nanoparticles loaded with fisetin were also developed. The data showed that drug-loaded nanoparticles were superior to that of free drug solution when tested against HCT116 colon cancer cells *in vitro* and antitumor test in a xenograft 4 T1 breast cancer model *in vivo* [19].

3.6 Racoglamide A

Santagata et al. adopted a well-designed reporter-based assay to screen for inhibitors of HSF1 activation. With a diversified library of 300,000 compounds from the NIH Molecular Libraries Probe Center Network, racoglamide A was identified as an inhibitor of HSF1 activation with nanomolar potency against multiple cancer cell lines [62]. Importantly, racoglamide A can significantly suppress tumor growth in an M0–91 acute myeloid leukemia xenograft mouse model with no evidence of systemic toxicity. In another study, the combination of racoglamide A and a human circularly permuted TRAIL (CPT) exhibits an efficient treatment towards mice xenografted with the CPT-resistant human acute T-cell leukemia cell line Molt-4 [77]. Furthermore, racoglamide A was reported to reduce the tumor size in a patient-derived pancreatic cancer xenograft mouse model without noticeable toxicity *in vivo* [73, 74]. Since racoglamide A is a natural Chinese herb compound with a relatively safe profile, this inhibitor or its derivatives may possibly advance to human study pending more efficacy data in animal models.

3.7 CCT251236

Using an image-based phenotypic screen, Cheeseman et al. reported the discovery of a new chemical probe, bisamide (CCT251236) as a potent inhibitor of the HSF1 stress pathway [9]. Efforts have been made to make analogs to improve solubility and bioavailability of this bisamide compound series with satisfactory mouse pharmacokinetics data. Importantly, CCT251236 displays efficacy in a human ovarian carcinoma xenograft model. In addition. CCT251236 demonstrates relatively low toxicity and was well tolerated in a mouse multidose tolerability study. The Pirin protein was proposed to have a possible role in the bisamide phenotype and the cellular effects of modulating the HSF1 pathway [9, 11]. In addition, significant anti-myeloma efficacy was observed in a myeloma xenograft mouse model after the treatment of CCT251236 through oral administration [50]. Although this group of HSR inhibitors has shown promising results in different xenograft mouse models, more animal model data, including PK/PD, MOA and biomarker studies, are essential for their advancements to the human clinical trial. Recent progress on nanoparticles and prodrugs can accelerate this translational process.

4 HSR Inhibitors in Early Preclinical Studies (Cancer Cell Lines, Table 3 and Fig. 4)

Seven compounds and one RNA aptamer were reported with antiproliferative effects in various cancer cell lines (Table 3), although publicly available data are limited for many compounds in this group. 2,4-Bis(4-hydroxybenzyl) phenol was discovered to

induce the dephosphorylation of HSF1 at Ser326 [87], a similar HSF1 inhibitory mechanism as PW3405 [89]. This compound can induce growth arrest and apoptosis of NCI-H460 human lung cancer cells. Using a smart synthetic library, Bach et al. found another HSR inhibitor, namely alpha-acyl aminocarboxamides, which can induce apoptosis in multiple myeloma cells [4]. Cardenolide CL-43, a natural compound, was identified through a heat-shock element-luciferase reporter system [48]. CL-43 can effectively inhibit the levels of all major HSP in the HCT-116 colon cancer line with no cytotoxicity observed in human fibroblasts.

An *in-silico* screening of lead-like library along with a cell-based assay led to the discovery of compound $I_{HSF}115$ [70]. This compound can bind to an isolated HSF1 DNA binding-domain fragment *in vitro* and inhibit its transcriptional activity. $I_{HSF}115$ exhibits a broad anticancer capacity in a panel of 33 cancer lines, with high sensitivity observed in multiple myeloma lines. In another study, Zaarur et al. identified NZ28 after screening of 20,000 compounds from several diversity compound libraries [88]. This compound potently inhibits the induction of HSP by heat shock, proteasome, and Hsp90 inhibitors in a variety of cell lines. An NZ28 analog, called emunin, strongly sensitizes myeloma cells to proteasome and HSP90 inhibitors as well as prostate carcinoma cells to proteasome inhibitors. Importantly, both NZ28 and emunin cause potent inhibition of HPS72 induction after heat shock in all tested cell lines.

Another phenotypic screen of 200,000 small molecules identified 4,6-disubstituted pyrimidines as a potent inhibitor of the HSF1 stress pathway [60]. Efforts on SAR and analog analysis led to the improvement of the HSF1 pathway inhibition to 14 nM potency with a U2OS human osteosarcoma tumor cell line. Interestingly, biochemical data showed high binding affinity (sub-micromolar to the single-digit micromolar range) of selected 4,6-pyrimidines to CDK9, suggesting possible roles of CDK9 in the inhibitory function of HSR. Unfortunately, this chemical series showed high clearance in mouse pharmacokinetic experiments, which were unsuitable for progression into the animal model study. Lastly, Stresgenin B was isolated as an inhibitor of the HSR pathway from a culture broth of Streptomyces sp. AS-9. This natural product showed inhibition of heat-induced reporter gene expression, including HSP70, HSP 90 and HSP110. Significant cytotoxicity against a panel of 6 cancer lines was documented with single to double-digit micromolar potency [1]

In summary, inhibitors in this category are either in their early stage of the preclinical study or have chemical liability (such as PK issues) that prevent them from entry into animal and human testing. It is also possible that the relevant animal data have not become publicly available at this point.

5 Lessons Learned from Preclinical and Clinical Studies

5.1 Improvement of Therapeutic Window by Prodrug Design and Nanoparticle Packaging

Many inhibitors in the HSR pathway show unfavorable chemical properties (low solubility and bioavailability) with a narrow therapeutic window. Treatments with

increased dosages usually lead to undesirable cytotoxicity. Recent advancements of prodrug design research make it possible to intentionally design current HSR inhibitors as prodrugs for improved therapeutic effects [56]. FDA has already approved at least 30 prodrugs, providing a promising direction for the prodrug development of HSF1 pathway inhibitors. On the other hand, some of the inhibitors are of natural origin with significant challenges in the medicinal chemistry approach and prodrug design. Nanoparticles or liposome encapsulation can be introduced for the packaging of these natural compounds with improved efficacy [72]. With the advance of cancer biomarker research and imaging technology, this type of nanomedicines can be better targeted to cancerous tissues with precision.

5.2 *Combined Application with Chemotherapy Drugs or Drugs against a Different Cancer Target*

Shevtsov et al. recently published an excellent review on combination therapy of current anticancer drugs with molecular chaperone inhibitors [66]. An optimal drug combination has been proposed to simultaneously target cytoprotective mechanisms (i.e., heat shock response pathway) and malignant proliferation drivers (oncogenes, signal transduction players, cell cycle players, etc.) Such a combination strategy can allow these drugs to act synergistically while reducing doses of individual drugs and related unfavorable side effects [42]. Several HSR inhibitors have been applied to combinational therapy with synergistic anticancer effects. For example, Xiong et al. reported that triptolide can significantly enhance the antiproliferative effects of doxorubicin in human breast cancer line MCF-7 and MDA-MB-468 [79]. In combination with curcumin, triptolide also significantly reduces tumor cell proliferation in the ovarian cancer SKOV-3 line [41]. KBIBB11 and CL-43 also exhibit similar synergistic effects when combined with AKT inhibitor [7] and conventional chemotherapy agents [48]. Thus, screening for the best combination of these drugs may be the quickest way to create a novel cancer therapy with HSR pathway inhibitors.

5.3 *Target Validation and Use of RNA Aptamer*

Due to the complex nature of HSR pathways, the underlying molecular mechanisms for the majority of HSR pathway inhibitors are not clearly identified. Only two inhibitors, namely KRIBBII and $I_{HSF}115$, demonstrate direct biochemical binding evidence to HSF1 *in vitro*. More detailed target identification studies are essential for the success of future drug development. Alternatively, recent publications on the RNA aptamer approach can open a different avenue to address this critical issue [61]. The complex tertiary folded structure of RNA aptamer makes it possible to achieve superior binding affinity and selectivity for a presumed cellular target. Currently, one aptamer drug has already been approved by the FDA [69], so more advanced projects can be initiated in this promising area [45].

5.4 *HSF1 as a Potential Biomarker for Clinical Trial Design and Prognosis Prediction*

It has been reported that elevated levels of HSF1 are generally associated with poor prognosis [8]. After analysis of over 3000 cancer patient samples, Wan and colleagues reported HSF1 overexpression as an unfavorable prognostic biomarker for a number of solid tumors, including breast cancer, hepatocellular carcinoma, non-small-cell lung cancer and pancreatic cancer, but not in osteosarcoma [71]. The recent advances in high-resolution imaging at single cell level, in combination with new molecular probes of cell types and metabolic states, will allow sensitive detection of HSF1/HSP expression of tumor samples in real time [13]. Thus, HSF1 pathway biomarkers can enable the development of tools for early cancer diagnosis, drug response prediction and therapeutic monitoring. Future clinical trial designs will particularly benefit from the better translation of basic science insights at the single cell level towards precision cancer treatment [24].

6 Conclusions

Significant progress have been made in the drug discovery and development of small molecule inhibitors against HSR pathways. Along with RNA aptamers, prodrugs, nanoparticles and combined therapy approaches, novel HSF1pathways-based cancer therapeutics will be created with tremendous clinical values.

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Ethical Approval for Studies Involving in Humans This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving in Animals This article does not contain any studies with animals performed by any of the author.

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Multifaceted Roles of Heat Shock Factor 1 (HSF 1) in Cancer

Meng Xu and Chengkai Dai

Abstract

Introduction Heat shock proteins (HSPs) are molecular chaperones, which facilitate protein folding, trafficking, and proteasomal degradation. As the master regulator of the evolutionarily conserved heat-shock, or proteotoxic stress, response, heat shock factor 1 (HSF1) governs the stress-inducible *HSP* expression transcriptionally, thereby guarding proteomic stability and promoting cell survival of proteotoxic stress. This cytoprotective mechanism, however, is hijacked by cancer cells to alleviate intrinsic, chronic proteotoxic stress and support their malignant growth.

Methods A narrative review of publications investigating the role of HSF1 in cancer was conducted.

Results The literature review revealed that distinct from its inducible and transient activation by environmental stressors, HSF1 becomes constitutively active via diverse mechanisms within cancerous cells. Moreover, the pro-oncogenic roles of HSF1 appear to be multifaceted, beyond safeguarding the proteome, including enhancing survival, reprogramming metabolism, evoking genomic instability, and even promoting immune evasion. Importantly, unlike primary cells, cancerous cells highly depend on HSF1 for their growth and survival, a phenomenon referred to as "Non-oncogene Addiction".

Conclusions In contrast to its widely acclaimed cytoprotective role under stressful conditions, HSF1 acts as a potent pro-oncogenic factor. Besides being a valuable prognostic marker, emerging evidence supports HSF1 as a promising target for anticancer therapies.

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Abbreviations

APC complex	anaphase-promoting complex
AR	androgen receptor
CAFs	cancer-associated fibroblasts
CaMKII	calcium/calmodulin-dependent protein kinase II
EMT	epithelial-mesenchymal transition
HCC	hepatocellular carcinoma
HSF	heat shock factor
HSP	heat shock proteins
HSR	heat shock response
MSR	metabolic stress response
PLK1	Polo-like kinase1
PSR	proteotoxic stress response
RNAi	RNA interference

1 Introduction

Organisms inevitably encounter a wide array of environmental insults. In turn, environmental insults provoke diverse biological stresses, including genotoxic, oxidative, metabolic, and proteotoxic, to the cells. To cope with these stresses, specific cellular responses are promptly initiated to maintain the cellular homeostasis and, ultimately, the fitness of organisms. Among these diverse responses is the heat-shock, or proteotoxic stress, response (HSR/PSR), one of the most evolutionarily conserved cytoprotective mechanisms [56]. Following exposure to heat stress, cells increase the induction of a group of heat shock proteins (HSPs) to prevent protein misfolding and aggregation. Based on their molecular weighs, HSP are classified as HSP100, HSP90, HSP70, HSP60, HSP40, and some small HSPs [45]. In the past 30 years, works by numerous groups have elucidated the essential function of HSP as molecular chaperones, including facilitating protein folding, trafficking, complex assembly, ubiquitination, and proteasomal degradation. In summary, HSPs are vital to cellular proteome homeostasis or proteostasis [11].

A small group of transcriptional factors, named heat shock factors (HSFs), control the induction of *HSPs* in response to proteotoxic stress. Among them, HSF1 is the best characterized. Importantly, genetic ablation of HSF1 in mice and primary cells abolishes the induction of *HSP* by heat stress, pinpointing HSF1 as the master regulator of the HSR/PSR[12]. In sharp contrast to its broadly acclaimed role in maintaining proteostasis, accumulating evidence over the past 10 years has uncovered that HSF1 acts as a key enabler of malignancy [14, 47]. It has been demonstrated that HSF1 functions to support cancer cell proliferation, survival, invasion, and metastasis. Moreover, HSF1 is frequently overexpressed in a broad range of

human cancers and its transcriptional activation is positively correlated with poor patient survival [47]. In this chapter, we summarize the mechanisms leading to constitutive, autonomous activation of HSF1 within cancer cells. Furthermore, we emphasize the multifaceted roles of HSF1 in promoting tumorigenesis. Lastly, the potentials of HSF1 as a novel biomarker and therapeutic target for human cancer are discussed.

2 HSF1 Becomes Constitutively Activated Within Cancer Cells

It has been reported that HSP can suppress HSF1 activity, suggesting an autoregulatory mechanism controlling the intensity and duration of the HSR/PSR. In particular, the HSP90-containing complex has been proposed to repress HSF1 activation in unstressed cells. This canonical model suggests that HSP90 physically sequesters HSF1 monomers to prevent its trimerization, nuclear translocation and DNA binding [90]. However, emerging findings in budding yeast show that HSP70, rather than HSP90, dynamically interact with and repress HSF1 [88]. Moreover, a recent study also indicates that HSP90 only interacts with HSF1 trimers transiently during the recovery phase of the HSR/PSR [33]. Thus, it still remains unclear how HSF1 is repressed in mammalian cells under non-stress conditions.

In contrast to its inducible and transient activation by acute heat shock, recent studies reveal the constitutive activation of HSF1 in cancer cells. This abnormal activation in cancer cells reflects their intrinsic, chronic stressed state. Mechanistically, several events likely account for the chronic proteotoxic stress in cancer cells, including aneuploidy and numerous genetic mutations, the burden of excessive protein synthesis driven by hyperactive mTORC1 signaling, and protein damage ascribable to acidic and hypoxic tumor microenvironments.

2.1 Activation of HSF1 by the Oncogenic RAS Signaling Pathway

Posttranslational modifications, particularly phosphorylation, have been shown to play an important role in HSF1 transcriptional activation during heat shock. Phosphorylation at Ser326 was identified as a key modification leading to HSF1 activation during heat shock [26]. Unsurprisingly, cancer cells exploit various signaling pathways, mainly via phosphorylation at multiple sites, to render HSF1 constitutively active. The RAS/MAPK signaling pathway, also known as RAS-RAF-MEK-ERK signaling cascade, controls cell cycle entry and proliferation in the cells. Of note, it is estimated that activating mutations of the RAS signaling cascade frequently occur in approximately 30% of all human cancers, highlighting its prominent role in oncogenesis [9]. Following hyperactivation, owing to either activating

mutations, frequent activation of upstream receptor tyrosine kinases (RTKs), or inactivation of the tumor suppressor NF1, RAS signaling mobilizes numerous downstream effectors that govern a wide array of cellular processes to drive malignancy [13, 55].

Recent findings unveiled that heat shock activates RAS/MAPK signaling and that MEK inhibition blocks the Ser326 phosphorylation on HSF1 and its mediated HSR/PSR [67]. Unexpectedly, it is MEK, rather than ERK, that activates HSF1 via Ser326 phosphorylation. Thus, HSF1 is a second substrate for MEK, in parallel with ERK. Naturally, oncogenic RAS signaling drives the constitutive activation of a large number of human cancers. Of interest, ERK suppresses HSF1 activation indirectly via a negative feedback mechanism. Mechanistically, ERK phosphorylates MEK at Thr292/386 to repress the MEK-mediated Ser326 phosphorylation on HSF1 [67]. In addition to MEK, mTOR and p38 MAPK were also reported to phosphorylate HSF1 at Ser326 *in vitro* [7, 17]. Nonetheless, it still remains unclear whether mTOR or p38 MAPK directly interacts with HSF1 *in vivo*.

2.2 *Activation of HSF1 by Other Oncogenic Signaling Pathways*

Moreover, HSF1 can be phosphorylated by other kinases responding to various oncogenic or acute stress stimuli. For instance, HSF1 transcriptional activity is induced following Ser230 phosphorylation by calcium/calmodulin-dependent protein kinase II (CaMKII) [30]. Emerging evidence has established a critical role of CaMKII in modulation cancer cell proliferation, invasion, and metastasis [74]. Thus, CaMKII may transduce diverse stimuli to the downstream effector HSF1 to promote tumor progression. Also, protein kinase A (PKA)-mediated phosphorylation at Ser320 activates HSF1 for protein quality control [87]. Given that its activation by a second messenger cAMP has been implicated in tumor initiation and progression, PKA signaling can promote carcinogenesis partially via activating HSF1 [52]. Furthermore, HSF1 phosphorylation on threonine 142 by casein kinase CK2 is required for its transcriptional activity and DNA binding [62]. Since the proliferation of tumor cells is associated with activation of CK2, it is plausible that HSF1 may contribute to the oncogenic potential of CK2 [69]. Moreover, Polo-like kinase1 (PLK1) phosphorylates HSF1 at both of Ser419 and Ser216, contributing to the pro-oncogenic function of HSF1 in both transcriptional-dependent and -independent manner [34, 36].

2.3 *Inactivation of HSF1 by Metabolic Stress Signaling*

AMP-activated kinase (AMPK), a highly conserved master regulator of metabolism, plays a pivotal role in preserving cellular energy homeostasis by conserving ATP

consumption but enhancing ATP production simultaneously [24]. Quickly responding to elevated cellular AMP/ATP or ADP/ATP ratio, AMPK becomes activated following Thr172 phosphorylation by upstream kinases. The central upstream kinase responsible for Thr172 phosphorylation in response to energy stress is the liver-kinase-B1 (LKB1), a tumor suppressor causally implicated in human Peutz-Jeghers syndrome [27]. In turn, activated AMPK mediates the metabolic stress response (MSR) via phosphorylating numerous downstream effectors to regulate diverse biological processes, including lipogenesis, gluconeogenesis, autophagy, glycolysis, fatty acid oxidation, and protein synthesis [12]. Thereby, AMPK preserves energy homeostasis.

Of interest, metabolic stress can regulate the HSR/PSR via AMPK. After activation by metabolic stress, AMPK directly interacts with and phosphorylates HSF1 at Ser121 [16]. As an inhibitory modification, Ser121 phosphorylation impedes HSF1 nuclear translocation. Accordingly, both glucose deprivation and anti-diabetic drug metformin, through activation of AMPK, suppress the HSF1-mediated HSR/PSR. Importantly, either glucose deprivation or metformin treatment can retard tumor growth, due to the proteomic instability induced by HSF1 inactivation in human cancer cells [16]. Thus, through the suppression effect of AMPK on HSF1, there is a crosstalk between the metabolic and proteotoxic stress responses. Consistently, germline mutations in the *LKB1* gene cause Peutz-Jeghers syndrome, a cancer-predisposition disorder. Hypoactivation of AMPK, due to *LKB1* mutations, leads to HSF1 activation to promote tumorigenesis.

2.4 *Other Signaling Pathways Suppress HSF1 Through Phosphorylation*

It is reported that phosphorylation of HSF1 at Ser303 is mediated by glycogen synthase kinase 3 (GSK3). This negative modification promotes HSF1 binding to 14-3-3ε, resulting in sequestration of HSF1 in the cytoplasm and transcriptional repression [71]. Interestingly, Ser303 phosphorylation is also necessary for subsequent sumoylation of HSF1 at Lys298, causing inhibition of its transactivation [29]. Thus, inactivated GSK3 signaling in some human cancers could lead to HSF1 activation.

In addition to GSK3 signaling, JNK signaling could also inhibit HSF1. JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase, phosphorylates numerous downstream effectors to regulate differentiation, growth, and apoptosis, following activation by diverse extracellular and intracellular cues [3]. One of its substrates is HSF1. JNK can phosphorylate HSF1 at Ser363, an inhibitory modification [15]. In support of its inhibition on HSF1, JNK deficiency stimulates HSF1 activation in tumor cells [13]. Intriguingly, HSF1 physically prevents JNK activation, revealing a mutual suppression between HSF1 and JNK [63]. As the key upstream kinases activating JNK, the putative tumor suppressor *MKK4* and *MKK7*

are often mutated in some human cancers, supporting a tumor-suppressive role of JNK. It is conceivable that inactivated JNK signaling in some human cancers could cause HSF1 activation to facilitate malignancy.

3 HSF1 Enables Oncogenesis Through Multifaceted Mechanisms

Congruent with elevated expression and constitutive activation of HSF1 in many types of human cancer, HSF1 enables oncogenesis. The concrete evidence establishing HSF1 as a powerful modifier of carcinogenesis came from *in vivo* studies using genetically engineered mouse models. In mice, *Hsf1* deficiency suppresses the chemical-induced skin carcinogenesis as well as the tumorigenesis driven by a 'hot-spot' *Trp53* mutation [14]. An independent group also reported that the genetic deletion of *Hsf1* in mice impaired the lymphomagenesis due to *Trp53* deficiency [49]. Since then, accumulating evidence has revealed that HSF1 contributes to HCC development by supporting hepatic steatosis and metabolic syndrome [32], facilitates breast cancer initiation and progression by regulating the expression of hypoxia-inducible factor 1 via the RNA-binding protein HuR [23], promotes mammary tumorigenesis and metastasis in Her2/Neu transgenic mice [80], and stimulates the NF1-associated carcinogenesis by augmenting RAS/MAPK signaling [13].

Moreover, the pro-oncogenic effect of HSF1 has also been demonstrated using mouse xenograft models. In those studies, shRNA-mediated *HSF1* knockdown blocks the transformation of human mammary epithelial cells induced by HER2/NEU [48], impairs the growth of HCC cells *in vitro* and *in vivo* [19], and suppresses the growth, invasion, and metastasis of human melanoma cells [21, 51]. Of note, in stark contrast to cancer cells' addiction, HSF1 is dispensable for the growth and survival of primary cells [14, 18]. Consistently, primary cells and mice deficient for HSF1 remain viable under non-stress conditions [14, 81]. Nonetheless, the central question of how HSF1 enables oncogenesis remains.

3.1 Guarding Proteomic Stability in Cancer

The best-known transcriptional targets of HSF1 are HSPs, such as HSP90, HSP70 and HSP27. As molecular chaperones, those HSPs play an essential role in maintaining the conformation and stability of a large number of cellular proteins. Among these client proteins of HSPs are many prominent oncoproteins, including, but not limited to, BRAF, AKT, c-MET, Androgen Receptor (AR), ERBB2/HER2 [11, 25, 79], as well as mutant driver oncoproteins generated *de novo* in cancer, such as mutant TP53, BCR-ABL, and EML4-ALK [11, 42, 77]. One good example is

mutant TP53, whose stability is highly reliant on HSP90 in cancer cells. Compared to the wide-type TP53 proteins, which are tightly regulated by MDM2 and CHIP (carboxy-terminus of HSP70-interacting protein) in normal cells, the oncogenic gain-of-function mutant TP53 proteins are markedly stabilized in the cancer cell. Mechanically, mutant TP53 complexes with HSP90 to block the E3 ligase activities of endogenous MDM2 and CHIP, thereby preventing its ubiquitination for proteasomal degradation [42]. Besides HSP90, HSP70 and HSP40 are also involved in stabilization and degradation of mutant TP53 [83].

As a consequence of destabilizing numerous HSP' client proteins, HSF1 deficiency evokes increased global protein ubiquitination and aggregation in malignant cells [67]. Importantly, a small fraction of these detergent-insoluble protein aggregates can further form amyloids, enriched for β-sheet structures and causally associated with several neurodegenerative disorders in humans. Thus, it seems that both neural cells and malignant cells are susceptible to amyloidogenesis [10]. Congruent with the crucial role of MEK in activating HSF1, pharmacological blockade of MEK similarly provoke protein aggregation and amyloidogenesis in cancerous cells. Indeed, both HSF1 and proteasome operate in concert to contain amyloidogenesis in malignant cells, although the levels of amyloids are still elevated compared to non-transformed cells. Nonetheless, combinatorial inhibition of HSF1 and proteasome markedly enhances amyloidogenesis, leading to toxicity in cancer cells, but not in primary cells. This unique vulnerability of cancerous cells to proteomic perturbations may have therapeutic implications. As a proof of concept, combined inhibition of MEK and proteasome markedly suppresses the growth and survival of cancer cells both *in vitro* and *in vivo* [67]. In aggregate, HSF1 critically guards proteomic stability in cancer cells and inhibits tumor-suppressive amyloidogenesis, thereby empowering robust tumorigenesis.

3.2 Preventing Cell Senescence and Promoting Survival

Multiple studies have reported that HSF1 suppresses cellular senescence triggered either by the HER2/NEU oncogene in breast tumor cells [48] or by DNA damage [35]. Heavily reliant on its activity, HSF1 acts the anti-senescence effect either through p53-p21 dependent pathway [48, 53] or p53-independent pathway [35]. Additionally, it was reported that HSF1 inhibition also induces apoptosis in multiple tumor models [18, 38, 44, 72]. Mechanistically, beyond activating the expression of HSP70 and BAG3 (Bcl-2 associated athanogene domain 3) to stabilize the anti-apoptotic BCL2 protein [31], HSF1 also down-regulates some pro-apoptotic factors, including SMAC, XAF1, and BAX [44, 70, 72]. Thus, through antagonizing cellular senescence and apoptosis, HSF1 promotes oncogenesis.

3.3 *Contributing to Genomic Instability and Alleviating Genotoxic Stress*

It was reported that expression of a dominant-negative HSF1 mutant inhibits aneuploidy in prostate carcinoma cells [73]. Subsequently, another study revealed that HSF1 blocks mitotic exit via direct interaction with CDC20 to promote aneuploidy. In details, Polo Like Kinase 1 (PLK1) phosphorylates HSF1 at Ser216, which induces its physical interaction with CDC20, thereby sequestering CDC20 away from the anaphase-promoting complex (APC complex) and preventing its degradation [41]. Of note, this aneuploidy-promoting effect is independent of the transcriptional activity of HSF1 and depends on defective TP53 functions [34].

Although genomic instability is causally related to tumorigenesis, cancer cells need to contain the genotoxic stress associated with genomic instability for survival and proliferation. It was reported that HSF1 promotes cell survival of genotoxic stress by inducing cell cycle arrest at the G2/M phase for DNA repair [43]. This HSF1-governed checkpoint activation depends on TP53, as HSF1 could direct TP53 recruitment to target gene promoters for transcriptional response to DNA damage [46]. In addition, a recent study reported that HSF1 regulates genome integrity in cooperation with PARP1 in response to DNA damage [22]. By forming a ternary complex with PARP1 and PARP13, HSF1 promotes PARP1 activation and its redistribution to DNA lesions and DNA damage-inducible gene loci. Thereby, HSF1 supports the growth of *BRCA1*-deficient mammary tumors partly via facilitating DNA repair to alleviate genotoxic stress [22]. Thus, HSF1 could facilitate tumorigenesis by both inducing genomic instability and alleviating genotoxic stress.

4 Enhancing Tumor Cell Invasion and Metastasis

Moreover, HSF1 can promote cell migration and epithelial-mesenchymal transition (EMT), which is a critical step for tumor cell invasion and metastasis. Several studies reveal that HSF1 promotes metastatic progression in distinct tumor model, including lung cancer [80], HCC [19], melanoma [39, 68], osteosarcoma [89], PDAC [5] and ovarian carcinoma [54]. In $Hsf1^{+/+}$ mouse mammary epithelial cells expressing the *HER2/NEU* oncogene, transforming growth factor beta (TGFβ) stimulates EMT, indicated by reduced expression of the epithelial marker E-cadherin and increased expression of mesenchymal markers, including SLUG and Vimentin [80]. By contrast, this TGFβ-induced EMT is impeded in $Hsf1^{-/-}$ cells. Moreover, *HSF1* knockdown impairs the transcription of several EMT-inducing genes, including *SLUG, SNAIL, TWIST1* and *ZEB1*, suppressing the EMT and migration induced by TGFβ in ovarian cancer cells [39]. Therefore, by regulating the EMT process, HSF1 promotes cancer invasion and metastasis.

4.1 Augmenting Critical Oncogenic Signaling Pathways

Key oncogenic signaling pathways drive cancer initiation and progression. One of them is the RAS-RAF-MEK-ERK cascade. As a direct downstream substrate of MEK, HSF1 is activated by RAS signaling and further promotes the HSP90-mediated KSR1 stabilization [13, 67]. Given that KSR1 is a key scaffold protein required for the assembly of RAF-MEK-ERK complexes, this feedforward loop can augment the oncogenic effects mediated by RAS signaling [13, 37]. Like KSR1, many oncoproteins are the clients of HSP90. Thus, this mechanism of action of HSF1 is expected to augment a number of diverse oncogenic pathways.

In addition to this HSP-mediated oncoprotein stabilization, a transcription-independent action of HSF1 has been uncovered recently. Under proteotoxic stress, JNK becomes activated and phosphorylates both RAPTOR and mTOR, leading to dissociation of mTORC1 and suppression of protein translation [63]. Through physical sequestration of JNK apart from mTORC1, HSF1 maintains the integrity and activity of mTORC1 [63]. To support rapid proliferation and growth, tumor cells highly depend on the mTORC1-mediated translation machinery. Accordingly, mTORC1 signaling is frequently hyperactivated in a broad range of human cancer. Thus, by relieving the suppression imposed by JNK and augmenting mTORC1 activity, HSF1 supports malignant growth.

4.2 Reprogramming Cancer Metabolism

Tumor cells can reprogram metabolic pathways to facilitate malignant transformation. It was reported that *Hsf1* deficiency reduces glucose uptake, revealing the role of HSF1 in glucose metabolism [14]. Another study in HCC indicates that genetic ablation of *HSF1* results in loss of *LDHA* expression and activity, a pivotal enzyme mediating glycolysis and the Warburg effect [8]. Conversely, in non-transformed cells, HSF1 increases cellular uptake of glucose [14], which is an essential fuel for rampant cancer cell growth [28]. Mechanistically, HSF1 suppresses the expression of thioredoxin-interacting protein (TXNIP), a repressor of glucose uptake through regulation of GLUT1 [78].

Apart from enhancing glucose uptake, it was demonstrated that HSF1 stimulates lipogenesis through suppression of AMPK signaling [32, 64]. Intriguingly, as a substrate of AMPK, HSF1 reciprocally suppresses AMPK by physically evoking its conformational switching, leading to impaired AMP binding to the γ subunits, enhanced PP2A-mediated Thr172 de-phosphorylation, impeded LKB1-mediated Thr172 phosphorylation, as well as retarded ATP binding to the catalytic α subunits [64]. As lipids are crucial for the membrane synthesis required for rapid proliferation in cancer cells, this lipogenic effect of HSF1, mediated via AMPK suppression, promotes the growth of melanomas *in vivo*. It is worth noting that this AMPK repression is also a transcription-independent pro-oncogenic mechanism of HSF1 [64]. Thus, at least by favoring glycolysis and lipogenesis, HSF1 supports malignant growth.

4.3 Modulating Tumor Immune Evasion

A role of HSF1 in modulating immune responses was firstly uncovered in the *Hsf1−/−* mice challenged with endotoxin, which demonstrated an exaggerated TNFα production [81]. Subsequent studies showed that HSF1 suppresses the transcription of TNFα to regulate immune responses [61, 66, 82]. TNFα has been shown to induce anti-tumor immunity [4]. Intriguingly, a recent study reveals that phosphorylated HSF1 at Thr120 by PIM2 binds to the *PD-L1* promoter and induces its expression [84]. The PD-1/PD-L1 interaction constitutes a key mechanism for cancer cells to evade anti-tumor immune responses [6]. Thus, this study suggests that HSF1 could facilitate tumor cells to evade immune surveillance partly via upregulating PD-L1.

4.4 Promoting Oncogenic Growth Non-cell-autonomously

The evidence mentioned above all pinpoints that HSF1 facilitates tumorigenesis in a cell-autonomous manner. However, a new study indicates that HSF1 also enables malignant progression in a non-cell-autonomous manner [60]. HSF1 is frequently activated in cancer-associated fibroblasts (CAFs), where it drives a transcriptional program to induce the expression of TGFβ and SDF1 to support the malignant growth of adjacent cancer cells. Importantly, depletion of HSF1 in stromal fibroblasts delays the growth of xenografted MCF-7 breast cancer cells *in vivo* [60]. Moreover, it was reported that intracellular HSP70 proteins could be released via exosomes from human peripheral blood mononuclear cells [40]. Extracellular HSPs either induce pro-inflammatory cytokine in human monocytes [1], or suppress protein aggregation in recipient cells to maintain organismal proteostasis via exosome-mediated transmission [65]. Thereby, secreted HSPs can also promote tumor growth non-cell-autonomously. Collectively, via multifaceted mechanisms, HSF1 potently enables oncogenesis in both cell-autonomous and non-cell-autonomous manners.

5 HSF1 Is a Potential Therapeutic Target and Prognostic Marker

In light of its potent role in promoting tumorigenesis, it is reasonable to target HSF1 for cancer therapy. To date, a number of small-molecule compounds have been shown to inhibit the transcriptional activity of HSF1, including quercetin [50], triptolide [75], 2,4-Bis(4-hydroxybenzyl) phenol [85], rocaglates [59], and KRIBB11 [86]. As most compounds were identified using HSF1 reporter systems,

unsurprisingly, many of them lack the target specificity and inhibit HSF1 indirectly. Furthermore, given the emerging evidence indicating the transcription-independent actions of HSF1 [34, 63, 64], new screening strategies should be considered to discover effectively HSF1-targeting molecules. Alternative approaches to target HSF1 include RNAi-based gene silencing and RNA aptamer technology. Both have been demonstrated to be effective in impairing the malignant phenotypes of human cancer cell lines [57, 76].

In accordance with the crucial role of HSF1 in supporting oncogenesis, an increasing body of evidence reveals that high expression of HSF1 positively correlate with poor patient survival in a broad spectrum of human cancers, including breast cancer [58], lung cancer [47], HCC [19], melanoma [44], prostate cancer [2], colon cancer [47], multiple myeloma [20], and pancreatic cancer [18]. Interestingly, it was reported that the HSF1-regulated transcriptional program in malignant cells is distinct from the canonical HSR/PSR [47]. And, the "HSF1-cancer signature", encompassing a collection of 456 HSF1-bound genes, displays a remarkable correlation with shortened survival in patients inflicted with breast, lung, or colon cancer [47], demonstrating excellent prognostic value. Likely, increased HSF1 expression and activation of HSF1 reflect the heightened intrinsic proteotoxic stress closely associated with malignant progression.

6 Conclusions

Whereas genomic instability is widely recognized as a hallmark of cancer, little is known about the role of proteomic stability in cancer. Now, accumulating evidence suggests that proteomic stability enables malignancy, sharply contrasting with its broadly acclaimed roles in antagonizing neurodegeneration and aging. Distinct from its canonical stress-inducible, transient activation by environmental stressors, HSF1 remains constitutively active within cancer cells to provide extra chaperoning capacity necessary to contain chronic proteotoxic stress. Unlike primary cells, where their proteome is stable and well protected, the fragile and compromised proteomic stability in cancer cells renders them highly addicted to HSF1 and vulnerable to proteomic perturbations. In aggregate, HSF1 is a promising target for anti-cancer therapies.

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Exploring the Role of Heat Shock Proteins in the Development of Gastric Cancer

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Abstract

Introduction Worldwide, gastric cancer (GC) is one of the most deadly carcinomas ranking fifth in the order of occurrence and third in mortality. The effective management of GC patients remains poor even with the noticeable advancements in surgical, radio- and chemo-therapeutic approaches. One of the major problems is the failure in early diagnosis of the disease and non-availability of efficient prognostic biomarkers. Identification of key players contributing towards initiation and progression of the disease helps in formulating strategies for the effective treatment of gastric cancer. Recent studies have implicated the dysregulation of heat shock proteins (Hsp) in the occurrence of human malignant tumors, suggesting their potential to be explored as biomarkers in the diagnosis and prognosis of gastric cancer. In here, we aim to summarize information available on implication of dysregulation of Hsp in gastric tumorigenesis and current Hsp based approaches being exploited for the treatment of gastric cancer.

Methods Authors reviewed the findings of all relevant reports available showing association of Hsp in the initiation, progression, metastasis, and treatment of gastric cancer.

Results Review of dysregulation of major Hsp including HSP27, HSP40, HSP60, HSP70, and HSP90 and role of heat shock factors (HSFs) in gastric tumorigenesis suggests their importance in the molecular pathogenesis of gastric cancer. Hsp based biomarkers are being explored for their diagnostic and prognostic value. The Hsp based approaches including the use of Hsp inhibitors and Hsp based vaccines provide new directions to GC treatment. The information on HSp70 polymorphism and its association with *H. pylori* and EBV positive gastric cancer is also being presented.

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Conclusions The differential expression of various Hsp has been implicated as a key event in the initiation and progression of tumor growth and metastasis. Therefore, Hsp based approaches are being explored for diagnosis, prognosis and treatment of cancer. Although Hsp inhibitors and vaccines are finding their application in cancer management, still substantial focussed efforts are required to develop a widely accepted treatment regime involving Hsp. Nevertheless, a few of these Hsp inhibitors have been promoted to the next clinical phases with encouraging outcomes. Increased efficiency has been accomplished in the combined therapy as compared to the independent monotherapy since inhibitors in combination overcome the resistance resulting from the single inhibitor approach.

Keywords Cancer therapy · Differential expression · Gastric cancer · HSP · HSP inhibitors · HSP polymorphism

Abbreviations

EBV	Epstein-Barr virus
GC	gastric cancer
H. pylori	*Helicobacter pylori*
HSF	heat shock factor
Hsp	heat shock protein
HSP	heat shock protein family
SNP	single nucleotide polymorphism

1 Introduction

Worldwide, gastric cancer (GC) is one of the most deadly carcinomas ranking fifth in the order of occurrence and third in mortality. The effective management of GC patients remains poor even with the marked advancement in surgical, radio- and chemo-therapeutic approaches. One of the major problems is the failure in early diagnosis of the disease and non-availability of efficient prognostic biomarkers. Therefore, comprehensive understanding of the molecular pathogenesis of GC and identification of key players contributing towards initiation and progression of the disease is required to help in devising strategies for the effective treatment of gastric cancer. The leads derived from investigations also help in deciding targets for the development of biomarkers and personalized drugs. Recent studies have implicated the dysregulation of heat shock proteins in the occurrence of human malignant tumors, suggesting their potential to be explored as biomarkers in the diagnosis and prognosis of different cancers including gastric cancer. Furthermore, Hsp have been reported to contribute towards acquired resistance of cancer cells to radio- and chemotherapy, two important regimes followed in cancer therapy.

2 Heat Shock Proteins

Cell Survival, proliferation and apoptosis ensures the regulation of a normal tissue development and maintenance necessary for the well-being of an organism. Activation of oncogenes, loss of activity of tumor suppressor genes, and aberrant mismatch repair machinery promote reprogramming of key metabolic pathways. Along with normal cells, cancer cells also adapt to withstand deprivation of oxygen and nutrients to ensure their uninterrupted proliferation. A competition arises between cancer and normal cells for the utilization of the limited resources and in accordance to Darwinian Theory, nature selects the one better fit for the extreme environment. The stressed environment of a cell causes activation or deactivation of certain proteins to combat the struggle for its survival. Heat shock proteins are one such family of proteins, which modify their role under normal and stresses conditions. Hsp are also known as stress proteins and constitute a large family of evolutionary conserved molecular chaperones produced by cells in response to various environmental and pathophysiological stimuli to protect cells from degradation, oxidative stress and thermal stress. Hsp also play a significant role in cancer development and progression as they regulate cellular proliferation and differentiation (Fig. 1). These proteins primarily work as chaperones by affecting protein folding and maintaining the structure and functions of their downstream proteins [62]. Table 1 gives a brief account of heat shock proteins implicated in gastric cancer.

3 Mode of Action

A substantial amount of efforts have been put in the recent times to understand the mode of action of different HSP, particularly in relation to the development of cancer. In the following text, we attempt to briefly highlight the mode of action of some of the important Hsp playing a role in cancer development.

4 HSP27

The phosphorylation regulated activation of Hsp27 in response of various stresses has been reported to have a crucial role in the formation of tumor and subsequent events leading to metastasis [96]. In normal cells, the unphosphorylated Hsp27 can form multimers whereas the phosphorylated Hsp27 forms large oligomers leading to the loss of chaperonage function of the protein [80]. The conformational changes in the unphosphorylated versus phosphorylated protein occurs through interaction with the specific proteins including b-catenin, procaspase-3 and histone deacetylase 6 protein. The activated p38 MAPK phosphorylates Hsp27 at Ser15, Ser78, Ser82, and Thr143 sites [30]. It has been observed that inhibition of Hsp27 phosphorylation

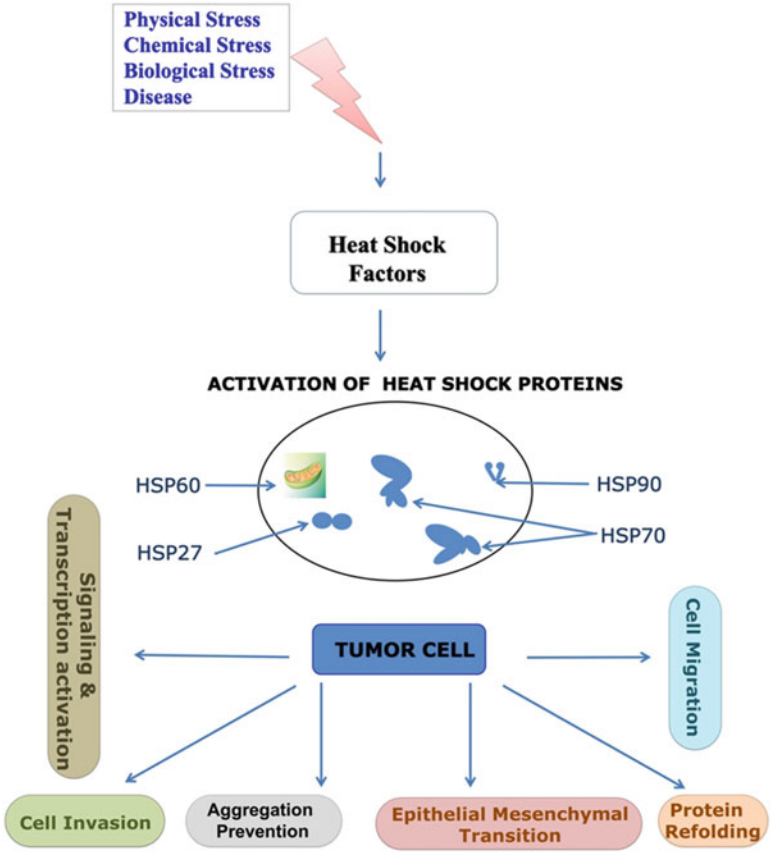

Fig. 1 Overview of the implication of heat shock proteins (Hsp) in gastric cancer. Heat shock factors (HSF) perceive various stress signals and in turn activate different heat shock proteins. Differential expression of these Hsp mediate various cellular events associated with the initiation, progression and metastasis of cancer

slows down the proliferation, migration and invasion of cancer cells to the adjacent tissues, thereby controlling metastasis [26]. The Hsp27 also inhibits apoptosis by inhibiting transcription of *p53* gene facilitated by *p21* gene [71].

5 HSP40

Overexpression of Tid1, a member of the HSP40/DNAJ family of proteins, acts as a tumor suppressor by regulating p53-mediated apoptosis [11]. Tid1 interacts with p53 via DnaJ domain causing resistance to stress through inhibition of mitochondrial localization of p53 [93]. There are two isoforms of Tid1, large and small, known for their opposite roles. Large Tid1 promotes whereas small Tid1 isoform suppresses

Table 1 Summary of important Hsp implicated in gastric cancer

S. No.	Family	Hsp	Encoding Gene	No. of Amino Acids	Localization	References
1.	Large HSP	Hsp110	*HSP110*	858	Cytosol/ nucleus	[97]
		GRP170	*HYOU1*	999	ER	[97]
2.	Small HSP	Hsp10	*HSPE1*	102	Mitochondria	[33]
		Hsp27	*HSPB1*	205	Cytosol/ nucleus	[33]
3.	HSP40 (DNAJ)	DNAJA1	*DNAJA1*	397	Cytosol	[76]
		DNAJA3/ Tid1	*DNAJA3*	480 (I)	Cytosol (I)	[106]
			(isoforms I and II)	453 (II)	Mitochondria (II)	
		DNAJA4	*DNAJA4*	426	Cytosol, nucleus	[106]
		DNAJB1	*DNAJB1*	340	Cytosol	[106]
		DNAJB4	*DNAJB4*	337		[106]
		DNAJB6	*DNAJB6*	335		[106]
		DNAJB8	*DNAJB8*	232		[106]
		DNAJB9	*DNAJB9*	223	ER, nucleus	[106]
		DNAJC6	*DNAJC6*	913		[106]
		DNAJC12	*DNAJC12*	107		[106]
		DNAJC25	*DNAJC25*	360		[106]
4.	HSP60	Hsp60	*HSPD1*	573	Cytosol, mitochondria	[19]
5.	HSP70	Hsp70	*HSPA1A*	641	Cytosol	[38]
		Hsp70–2	*HSPA1B*	641	Cell surface	[38]
		HSC70	*HSPA8*	646	Cytosol	[38]
		GRP75	*HSPA9*	679	Mitochondria	[38]
		GRP78	*HSPA5*	654	ER	[38]
6.	HSP90	Hsp90A	*HSPC1*	732	Cytosol	[87]
		Hsp90B	*HSPC3*	724	Cytosol	[87]
		GRP94	*HSPC4*	803	Cytosol, ER	[87]
		TRAP1	*HSPC5*	704	Mitochondria	[87]

apoptosis [89]. Knockdown of Tid1 negatively regulated the cancer cell migration through depletion of interleukin-8 production indicating its role in the suppression of angiogenesis [49]. Erdj3, a DNAJ homolog, abundantly found in the endoplasmic reticulum binds to KSHV Kaposi sarcoma-associated herpesvirus K1 (KSHVK1) protein leading to the inhibition of cell death [102].

Similarly, another member of the DNAJ family, HLJ1, suppresses tumor growth while inversely affecting tumor invasion. Transcription factor YY1 and activator protein 1 activate HLJ1. Curcumin increases the expression of HLJ1 via JNK/JunD pathway leading to the inhibition of tumor invasion and metastasis [94]. Contrary to

the action of these members of HSP40 family, DNAJB1, DNAJB8 and DNAJC6 have been suggested to stimulate tumor progression and migration of cancer cells in different type of cancers [17, 69, 107].

6 HSP60

Hsp60 consists of three main domains namely, apical, intermediate and equatorial domains and plays an important role in the regulation of immune system by acting as an antigen for B and T lymphocytes [61]. Hsp60 binds directly to cyclophilin D (CypD) in the mitochondria of cancer cells. CypD is a permeability transition pore component in a multi-chaperone complex, which also contains Hsp90 and Tumor Necrosis Factor Receptor-Associated Protein-1. It has been observed that silencing of gene for Hsp60 induces CypD-dependent mitochondrial permeability transition, caspase-dependent apoptosis and tumor growth suppression, proposing Hsp60 to be a novel regulator of a cytoprotective chaperone network inhibiting CypD-dependent tumor cell death [23].

7 HSP70

Some of the widely investigated members of this family of HSP implicated in cancer development include Hsp72, Hsp70B, HSC70, HspA8, and GRP78. The Hsp72 and Hsp70B are stress inducible whereas HSC70, HspA8 and GRP78 are constitutively expressed proteins. Hsp70 with anti-apoptotic properties acts as a facilitator for tumorigenesis through the inhibition of intrinsic and extrinsic pathways. Hsp70 binds to BAX and block its mitochondrial translocation in the intrinsic pathway. Also, it inhibits oligomerization and the association of Apaf-1 with procaspase-9 to form apoptosome [83]. Hsp70 also binds to death receptor DR4/5 and blocks the Apo-2 L/TRAIL-induced formation of the death inducing signaling complex (DISC) [31]. Cancer cell survival is promoted by the inhibition of lysosomal membrane permeabilization through lysosome stabilization involving Hsp70 binding to endolysosomal bis-phosphate [70]. Also, Hsp70 is reported to regulate tumor progression and metastasis involving transcription factors Hif1α and NF-Kβ [16, 20].

8 HSP90

Members of HSP90 family are evolutionary conserved and constitutively expressed during folding, stabilization, activation, maturation, function, and proteolytic degradation of several associated proteins viz. ERBB2, EGFR, CDK4, BRAF, CRAF, HER2, AKT, MEK, p53, and HIF-1α [105]. The Hsp90 binds to other associated proteins through N-terminal ATPase domain. The inhibition of Hsp90 expression

eventually leads to the inhibition of its associated proteins resulting into the blockage of multiple signaling pathways triggering growth, apoptosis, evasion, angiogenesis, invasiveness and metastasis [12, 110].

9 Differential Expression of Hsp in Gastric Cancer

Elevated expression of Hsp is widely accepted as a signature event in multiple cancers. The transcriptional level expression of Hsp is regulated by the action of upstream transcriptional regulators and heat shock factors (HSFs). HSF1 helps in the promotion of tumor invasion and metastasis as hyperactivation of heat shock factor 1 leads to overexpression of Hsp. There are a number of HSFs including HSF1, HSF2, HSF3, HSF4 and HSFY bearing a conserved N-terminal DNA binding domain and C-terminal transactivation domain [29, 85]. Among all the heat shock factors, HSF1 is considered as the master regulator of Hsp expression [1, 103]. Various members of HSP family show dysregulated expression that can play a remarkable role as biomarkers in the diagnosis of cancer.

10 HSP70

Seventy kD HSP70, a representative family of HSP, comprises of major cognate heat shock protein (HSC) isoforms, which are constitutively expressed and stress-inducible. HSP70 family is comprised of 13 members including HspA1, HspA2, HspA5, Hsp70B, HspA8, and HspA9 [60, 106]. HSP70 interacts with mutated or altered proteins including products of several oncogenes and tumor suppressor genes [53]. HSP70 is essential for the maturation, regulation and transformation of proteins involved in cell growth. HSP70 can be induced in response to different environmental stresses including heat, heavy metals, chemicals, toxins, hypoxia, oxidants, and various viral and bacterial infections. One of the primary functions of HSP is the protection of body cells against such stresses [5, 81].

HSP70 is crucial for tumorigenesis and its overexpression has been correlated with the increased cell proliferation and malignancy. Overexpression of Hsp70 has been reported in various malignant tumors that closely relates its role in tumor formation, tumor immunity, and resistance to apoptosis [39, 47, 84]. In gastric cancer also, overexpression of Hsp70 might play an important role in promoting cell growth, resistance to treatment, prognosis and inhibiting apoptosis [63]. Thus, it is widely conceived that specific inhibition of Hsp70 expression may regulate the proliferation and survival of human gastric cancer cells. Several studies have suggested an association between Hsp70 expression and prognosis of different types of cancer. However, the expression of Hsp70 is not associated with advance tumor-related characteristics or with the prognosis of gastric cancer. Inhibition of the

expression of Hsp70 has been observed to reduce the growth of GC cells [8, 53, 57, 63].

Hsp72, a member of HSP70 family, is overexpressed in gastric cancer cells in contrast to that in adjacent normal tissues. Expression of this Hsp is mediated by the binding of HSF 1 with specific sites located in the promoter region of the *hsp70B* gene, such that incorporation of the hsp70B promoter into genes exploited in suicide gene therapy. Hsp72 has shown the potential to be used as a diagnostic or prognostic marker [100].

11 HSP40

A eukaryotic homologue of bacterial DnaJ protein, HSP40, is a large protein of DNAJ family that contains a J-domain, which helps in establishing an interaction with HSP70 by stimulation of ATPase activity resulting in chaperonage of HSP70 i.e. protein folding, unfolding, translocation, translation, degradation [36, 45, 88]. Mostly all members of HSP40 family are found to be overexpressed in various cancers including gastric cancer [42, 46, 72]. Overexpression of Hsp40 has been observed in GC tissues through immunohistochemistry and further confirmed by using immunoblotting. However, there was no relation of Hsp40 expression on the survival of GC patients. In addition, reduced expression of Hsp40 was correlated with the undifferentiated type of GC tissues [42].

12 HSP60

Hsp60 is located in the mitochondria and plays key role in the transport and folding of mitochondrial proteins. Similar to Hsp40, Hsp60 also interacts with Hsp70. Hsp60 overexpression has been reported in different cancers such as lung cancer, cervical cancer and gastric cancer [8, 24, 41]. It has been reported that Hsp60 provides drug resistance in cancer therapy and therefore, its inhibition lead to the reversal of drug resistance [52]. High expression of Hsp60 in GC tissues and its association with male gender was also observed. However, Hsp60 expression could not be used to predict prognosis in GC subjects [24].

13 Hsp27

Hsp27, a member of the family of small HSP, performs important roles in tumor initiation, progression, invasion, metastasis and suppression of cell death leading to resistance to cancer therapy [9, 25]. Overexpression of Hsp27 and its association with the tumor size, metastasis and staging has been reported in GC tissues by using

immunohistochemistry (IHC) techniques. Hsp27 expression has been associated with the aggressive tumor behavior leading to metastasis [24]. Phosphorylation of Hsp27 resulting from the activation of p38 mitogen activated protein kinase (MAPK) causes conformational changes resulting in the smaller size of the oligomers, and dissociation of the complex and ultimately loss of chaperone activity whereas unphosphorylated Hsp27 normally present in the cytosol can form multimers required for normal chaperone function [58, 59]. Association of Hsp27 expression with lymph node metastasis has been detected in gastric cancer.

14 HSP90

Members of this family of HSP display anti-apoptotic properties and play significant role in folding, stabilization and degradation of several associated proteins involved in oncogenesis [7, 105]. Enhanced expression of Hsp90 has been observed in GC tissues along with its association with lymph node metastasis and poorly differentiated histological type GC. Higher expression of Hsp90 was associated with favorable prognosis and increased overall survival rates [24] suggesting it to be an independent prognostic factor in GC. Loss of Hsp90 expression may lead to the development of more aggressive phenotypes and ultimately causing poor prognosis or resistance to cancer therapy.

15 Diagnostic and Prognostic Applications of HSP in Gastric Cancer

Early diagnosis of gastric cancer is problematic and unreliable as majority of the available biomarkers lack sensitivity and specificity for the screening of the disease. Hence, there is an urgent need of developing more efficient and reliable biomarkers as a pre-requisite for the meaningful management of gastric cancer. Out of 81 GC cases analysed, Isomoto et al. [42] reported 55 and 18 cases showing overexpression of Hsp70 and Hsp40, respectively raising a hope of using expression levels of these two HSP as biomarkers in GC. However, there was no effect on patients' survival rate and also no association with clinicopathological parameters questioning their use as prognostic markers.

A study on GC patients suffering from gastric adenocarcinoma found overexpression of HSP27 in 50% of the cases using IHC technique. Overall, 30% mildly stained, 61% moderately stained and 9% highly stained gastric cancer tissues were observed. A significant association of expression of Hsp27 with tumor size and tumor staging has been suggested [13]. Another study, a meta-analysis on 624 gastric cancer patients, revealed significance association of its expression with GC incidence indicating a crucial role of Hsp27 in tumorigenesis.

A study on the expression of Hsp60 was observed in 223 GC patients by using immunohistochemistry and overexpression of this protein was correlated with poor overall survival, high invasiveness and shorter recurrence-free survival, making it a potential target for prognosis. Overexpression of Hsp60 was observed in 55% of the 66 GC patients. The mild, moderate and high intensity of staining was reported in 32%, 48% and 20% of the cases, respectively. Half of the GC patients were observed with high expression of Hsp90. The intensity of IHC staining was mild in 45%, moderate in 47% and intense in 8% gastric cancer cases. There was no clinical association of Hsp90 expression with gastric cancer. The diagnostic role of Hsp90 has not been observed in cancers including GC [14]. There is an evidence in support of prognostic value of Hsp90 in GC indicating overall survival rates with respect to overexpression. Hsp90 has also been assessed as an independent prognostic factor for GC patients. However, Hsp27 and Hsp60 could not qualify as a prognostic factor to assess the prediction of the disease recurrence.

The study on correlation between expression of Hsp27, Hsp60 and Hsp90 suggested that expression of Hsp27 and Hsp60 had maximum correlation followed by correlation between Hsp27 and Hsp90 and between Hsp60 and Hsp90 [24]. Transcriptome analysis revealed that Hsp90 has a diagnostic and prognostic potential as a biomarker for cancer [10].

16 HSP70 in *H. pylori* and EBV Positive Gastric Cancer

Helicobacter pylori, a spiral, Gram-negative microaerophilic bacterial pathogen, is categorized by WHO as a class I carcinogen in gastric cancer. The bacterium is reported to infect the stomach of nearly 50% of the world population [104]. *H. pylori* affects the process of apoptosis that takes place in stomach, predominantly in the upper part of the gastric glands comprising 2–3% of all the epithelial cells [90]. The main genetic characteristic of *H. pylori* infection pertaining to the upper gastrointestinal disorders is the presence or absence of CagA and VacA strains that affect the apoptosis [34, 48]. The effect of *H. pylori* on Hsp70 expression in gastric cancer cell line MKN7 has been observed indicating a time dependent inhibition of Hsp70 expression by CagA and VacA positive strains that may be associated with apoptosis. Further, a complete lack of Hsp70 expression was recorded when exogenous CagA was added to CagA and VacA positive cells implying that the presence of CagA to the cells infected with *H. pylori* positive for CagA and VacA accelerated the inhibitory effect of bacteria and its cytotoxins on HSP70 expression [91]. Inhibition of Hsp70 expression due to *H. pylori* infection causes the breakage of cell defence system provided by Hsp70.

Epstein-Barr virus is a ubiquitous human herpes virus whose primary infection causes mononucleosis, Burkett's lymphoma, nasopharyngeal carcinoma, autoimmune diseases, and gastric cancer. Deregulation of Hsp70 has been reported in EBV positive gastric cancer [82]. Infection with EBV in both latent and lytic phases can cause the disease. 2-phenylethynesulfonamide (PES), a small molecular

inhibitor of Hsp70, has been reported to suppress replication and carcinogenicity of Epstein–Barr virus via inhibiting the molecular chaperone function of Hsp70 [98]. Epstein–Barr Virus Nuclear Antigen 1 (EBNA1) is essential for the maintenance of the EBV DNA episome, replication and transcription. Over-expression of Hsp70 enhanced the expression of EBNA1 indicating its potential in therapeutic applications [98].

17 Genetic Polymorphism of HSP70 in Gastric Cancer

HSP70 shows immunogenicity that activates both arms of the immune system, innate and adaptive immune responses. Human HSP70 family comprises of three genes namely, *HSPA1A*, *HSPA1B* and *HSP1L* that code for protein Hsp70-1, Hsp70-2 and Hsp70-hom, respectively. These genes are located in the major histocompatibility complex, 92 kb telomeric region of the complement C2 locus on Chromosome 6. *HSP1A* and *HSP1B* genes encode for a heat inducible protein whereas *HSP1L* encodes for a non-heat inducible protein. A number of single nucleotide polymorphisms (SNPs) have been detected in these genes, which could affect the expression of the host gene leading to cancer susceptibility. The most commonly found SNPs are at positions +190 of *HSP70-1* gene (rs1043618), +1267 of *HSP70-2* gene (rs1061581), and +2437 of *HSP1L* gene (rs2227956).

The *HSP70-2* A>G polymorphism in the coding region at +1267 position is a synonymous mutation which could affect the secondary structure. Also, a gender specific inclination of this SNP has been observed in the Japanese population. However, no such observation has been reported in other populations, possibly due to the difference in the genetic background. On the contrary, the *HSP70-hom* T>C polymorphism is a non-synonymous mutation at position 493 that may affect the chaperone activity of HSP70. An increased risk of cancer in individuals with the AG/GG genotype in *HSP70-2* was also observed [73]. Another study reported the GA genotype of HSP70-2 and TT genotype of *HSP70-hom* associated with the increased risk of gastric cancer. The observed proportion of GA, GG and AA genotypes in *HSP70-2* in gastric cancer remained 67%, 18% and 15%, respectively indicating that the heterozygous genotype is more important as compared to homozygous genotypes for this particular +1267 position in *HSP70-2* [21]. In corroboration, another study, a meta-analysis on Caucasian population, reported that the G allele polymorphism may interfere with the secondary structure and stability of mRNA causing predisposition and gastric tumorigenesis [50]. Likewise, the observed proportion of TT, CT and CC genotypes in *HSP70-hom* at −2437 in gastric cancer were 87%, 13% and 0%, respectively suggesting that the C carrier genotypes reduce the risk of development of gastric cancer [83]. This observation is contrary to a study on breast cancer wherein allele C in *HSP70-hom* was found to be predominant. The discrepancies between these studies imply that polymorphism vary in various cancers.

At the protein level, the *HSP70-hom* +2437 polymorphic site leads to a substitution of methionine to threonine amino acid at position 493 [65]. This amino acid substitution could alter the polarity of this protein as methionine is hydrophobic whereas threonine is hydrophilic [66]. The said alteration may affect the efficiency of the *HSP70-hom* protein by lowering the strength of the hydrophobic interactions between the chaperone and the target protein.

The C/G genotype in *HSP70-1* at position +190 has also been associated with non-atrophic gastritis and gastric cancer with an observed increase in the odd ratios from non-atrophic gastritis to gastric cancer whereas C/C genotype showed no association with gastric cancer. Allele C at +190 position of *HSP70-1* has been found significantly associated with non-atrophic gastritis and gastric cancer when compared to normal tissues. However, this SNP at +190 is located in the 5′ untranslated region of the *HSP70-1* gene and therefore could not directly alter the amino acid sequence, however, this region has controls over cellular localization, stability and translational mechanisms. Therefore, such an alteration may affect the expression and function of the gene. Therefore, SNPs in *HSP70–1* have been considered as possible risk factors for pre-cancerous lesions and gastric cancer [77].

The polymorphism at position +1267 in *HSP70-2* and at position +2437 in *HSP70-hom* revealed a lack of allele or genotype association with gastric cancer. The lack of combination of the three genotypes G/G, A/G and T/T in *HSP70-1*, *HSP70-2* and *HSP70-hom* in gastric cancer was observed whereas it was present in one-seventh of the non-atrophic gastritis group and one-fourth of the normal individuals group under study. This absence of combination suggests that the combination may have a defense role towards gastric tumorigenesis. The association between HSP70 polymorphism and gastric cancer is not just because of the loss of chaperonage activity. It has been reported that linkage disequilibrium of HSP with other genes located in the HLA region on p arm of chromosome 6 also contributes to the occurrence of GC [21].

18 Inhibitors of HSP

Hsp70 is an important target for cancer therapy as Hsp70/Hsp90 ATPase is regulated by its inhibition. Transcription of *HSP70* gene is regulated by heat shock factor 1, a transcription factor that gets activated in response to stress [54]. Inhibition of Hsp70 expression can be achieved by blocking HSF1. Activity of HSF1 can be inhibited or blocked by inhibitors namely diterpenetriperoxide, triptolide, benzopyrene and flavonoid quercetin. A list of inhibitors of different HSP and their source is given in Table 2.

Cisplatin is a drug frequently used in the chemotherapy of various cancers including gastric cancer as it induces cell death via apoptosis pathway. Overexpression of Hsp70 inhibits anti-apoptotic action of cisplatin and thereby impeding the cancer therapy. ADD70 (AIF-derived decoy for Hsp70) is an Hsp70 inhibitor which sensitizes tumor cells to cisplatin [54].

Sp1 (Specificity Protein 1) is a transcription factor responsible for the transcription of several pro-survival genes including *HSP70* and *HSF1* [2]. High expression levels of Sp1 and Hsp70 have displayed reduced survival rate and poor prognosis in gastric cancer patients [99]. The inhibitor triptolide/minnelide reduces the expression of O-GlcNAc transferase (OGT), which further inhibits the activity of Sp1. Similar to triptolide, mithramycin, another inhibitor for Sp1, has been observed to reduce the viability of gastric cancer cell lines in a dose and time dependent manner resulting into the downregulation of *HSP70* and *HSF1* suggesting thereby that *HSP70* and *HSF1* are downstream genes for Sp1.

HSP40 family members are known to systematize the effect of chemotherapy drugs. To target Hsp40, a derivative of phenoxy-N- arylacetamides (a small molecule inhibitor) has been used efficiently as an inhibitor of Hsp40 [6]. Another heat shock protein 40 inhibitor, KNK437 (HSP inhibitor I), a benzylidene lactum compound has been found to inhibit HSP expression explored *in vitro* in colon cancer cells [109]. EGF receptor inhibited by BMS-690514 has been reported to downregulate the expression of Hsp40 and promote apoptotic effects produced in erlotinib resistant non-small cell lung cancer [18].

19 HSP70 Based Vaccines

Various HSP possess immunological properties that lead to the enhancement of immune system of specific antigens. Hsp antigens are being used in clinical practice for the development of cancer vaccines. Both innate and adaptive immune systems can be stimulated by Hsp that make them a good candidate for vaccine development.

One of the large HSP, Hsp110 primarily induced by heat shock, recognises and binds to its target protein antigens inducing dendritic cell-mediated cross presentation, aiding as a catalyst to increase the potential of antigen targeted vaccines in different cancers [111]. Increase in the anti-tumor efficacy of cytotoxic T lymphocyte epitope E7 has been observed when inoculated with Hsp110 in cervical cancer [78]. Hsp110 based vaccination produced promising consequences in mouse models of intestinal adenoma [108]. Similarly, another large HSP, GRP170 gets primarily induced by glucose deprivation and shows the same properties as Hsp110 for vaccine development. GRP170 has displayed candidate cancer antigen for the development of cancer vaccines [101].

Hsp70 is one of the most crucial and important elements of HSP based vaccine development due to its immunogenic properties. Clinical trials of phase I level (NCT00027144 and NCT00030303) investigated the toxicity and efficiency of Hsp70 in chronic myelogenous leukaemia [92]. Patients suffering from cervical neoplasia have been tested with vaccines containing Hsp70 [92]. In response to mediated adaptive cell system, endocytosis of Hsp70 stimulates the major histocompatibility pathway consisting antigen presenting cells and cytotoxic T-lymphocytes. Tumor derived Hsp70 can be used as a cancer vaccine [95]. In innate system, Hsp70 induces the release of inflammatory cytokines leading to the increase of expression

Table 2 Data on some Hsp inhibitors

S. No.	Hsp	Inhibitor	Target Site	Source	Clinical Evaluation Status	References
1.	Hsp27	Quercetin	HSF1	Bioflavinoid	No	[40]
		RP101/ Bromovinyldeoxyuridine/ Brivudine	HSF1	Antiviral nucleoside	NCT00550004	[37]
		TDP/ 1,3,5-trihydroxy-13,13-dimethyl-2H-pyran [7,6-b] xanthone	–	*Garcinia oblongifolia*	No	[22]
		OGX-427	–	–	NCT00959868: Phase I trial	[51]
					NCT01120470: Phase II trial	
2.	Hsp40	KNK437	–	Benzylidene lactam	No	[109]
		BMS-690514	–	–	No	[18]
3.	Hsp70	Pifithrin-μ/ 2-phenylethynesulfonaamide (PES)	Substrate binding domain	–	No	[32]
		15-deoxyspergualin	Nucleotide binding domain	Natural immunosuppresssant	No	[68]
		VER-155008	ATPase domain	Adenosine		[64]
		cmHsp70.1	TKD motif	–	NCT02118415: Phase II trial	[79]
		Epigallocatechin	Nucleotide binding domain	Flavonoids	No	[27]
4.	Hsp90	Geldanamycin	ATP-binding site	*Streptomyces hygroscopicus*	No	[4]
		Radicicol	ATP-binding site	*Monosporium bonorden*	No	[86]
		17-AAG/ Tanespimycin/ 17-allylamino-17-demethoxygeldanamycin		Geldanamycin	No	[55]
		17-DMAG		Geldanamycin	NCT00088868	[55]
		IPI-504/ Retaspimycin hydrochloride		Geldanamycin	Phase II trial	[35]
		WK88–1		Geldanamycin	–	[43]

		Onalespib/ AT13387		Radicicol	NCT01294202, NCT01685268, NCT00878423, NCT01246102	[28]
		Ganetespib/ STA-9090		Resorcinol	–	[74]
		Debio0932		Purine	NCT01168752: Phase I	[44]
		PU-H71		Purine	–	[44]
		Luminespib/ NVP-AUY922/ VER-2296		Radicicol	–	[75]

of co-stimulatory molecules [3, 56]. Hsp70 expressed on the tumor cell surface activates the lytic machinery of NK cells against them [67]. A comparison between Hsp70 vaccines directly derived from the tumor cells and vaccines derived from the fusion of dendritic and tumor cells suggested that the latter performs more efficiently by reversing the immuno-tolerance of cancer cells [15].

20 Conclusions

A substantial number of studies have established the role of Hsp in the occurrence of different cancers. In general, the differential expression of various Hsp has been suggested to be a key event in the initiation and progression of tumor growth, and finally inducing metastasis. Researchers and medical practitioners are exploring the application of Hsp based approaches for diagnosis, prognosis and treatment of cancer. Although Hsp inhibitors and vaccines are finding their application in cancer management, still substantial focussed efforts are required to develop a widely accepted treatment regime involving Hsp. Nevertheless, a few of these Hsp inhibitors have been promoted to the next clinical phases with encouraging outcomes. The Hsp inhibitors are being evaluated independently and in combination with other inhibitors also. Increased efficiency has been accomplished in the combined therapy as compared to the independent monotherapy as inhibitors in combination overcome the resistance resulting from the single inhibitor approach.

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Heat Shock Proteins in Atrial Fibrillation

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Abstract

Introduction Heat shock proteins (HSP) are a group of chaperones, which have been shown to protect cells and organs, especially the heart, against harmful and cytotoxic conditions. More recent attention has focused on the role of HSP in irreversible remodeling of atrial fibrillation (AF) which is the most common clinical tachyarrhythmia and a significant contributor to morbidity and mortality. This chapter sets out to investigate the link between HSP and atrial remodeling processes in AF. Understanding the association between HSP and complex pathophysiological processes of AF-associated remodeling could lead to the development of specific therapeutic interventions.

Methods PubMed was searched for studies using both the search terms "Heat Shock Proteins" and "Atrial Fibrillation" and relevant abbreviations. The database was searched up to Oct 10, 2019.

Results The chapter attempts to show that HSP have cytoprotective role on atrial cardiomyocytes during AF involving electrical remodeling, structural remodeling, inflammatory processes and oxidative stress. Furthermore, potential implications of HSP in the management of AF are also briefly discussed. HSP may serve as the predictors of AF type and recurrence in the future while and anti-HSP antibodies may serve as novel therapeutic modalities in postoperative AF.

Conclusions HSP play an important role in different stages of AF occurrence and development, and HSP have predictive value for the occurrence of AF. The treatment of HSP will provide new ideas for the treatment of AF.

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Abbreviations

AF	atrial fibrillation
APD	action potential duration
Ca^{2+}	calcium
CABG	coronary artery bypass surgery
DAMPs	danger associated molecular pattern
ERP	effective refractory period
H_2O_2	hydrogen peroxide
HSP	heat shock proteins
Hsp70	heat shock protein 70
HSPB	small HSP
IL-10	interleukin-1
LAD	left atrial diameter
PICP	carboxyterminal propeptide of type I procollagen
PIIINP	amino-terminal propeptide of type III procollagen
ROS	reactive oxygen species
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
TGF-β	transforming growth factor beta
TLR2	toll-like receptors 2
TNF	tumor necrosis factor

1 Introduction

Heat shock proteins (HSP) are a group of chaperone proteins, mainly including Hsp70, Hsp60, Hsp90 and HSPB (small HSP), which are endogenous protective proteins and biomarkers of cell stress response. Indeed, they can preserve cellular integrity by maintaining proteins in their correctly folded state under various stresses [3]. Recently, it has reported that HSP have a protective effect in some cardiovascular disorders such as atherosclerosis [50].

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice and is associated with increased morbidity and mortality. AF is a modern epidemic, since recent data indicates that nearly 35 million people (20.9 million males and 12.6 million females) suffer from AF worldwide while the incidence of AF continues to increase mainly due to aging of the population and improved survival of patients with other cardiovascular diseases [7]. AF is a complicated and heterogeneous arrhythmia occurring in diverse clinical settings. Besides local triggers, atrial electrophysiological and structural abnormalities that constitute atrial remodeling seem to play an important role in AF development and persistence. Accumulating evidence suggests that HSP are implicated in AF development and perpetuation.

We aim to provide a concise overview of the current evidence regarding the role of HSP in atrial remodeling processes as well as in the development and maintenance of AF. The potential clinical implications of HSP in the management of AF are also discussed.

2 HSP and Electrical Remodeling in AF

Data from several studies have been demonstrated that HSP are involved in restoring atrial action potential duration (APD) and effective refractory period (ERP), parameters affected by cellular calcium (Ca^{2+}) overload provoked by atrial tachyarrhythmias. Overexpression of HSPB6, HSPB7, HSPB8 can also reduce the formation of F-actin stress fibers showing a protective role against tachypacing-induced Ca^{2+} transients reduction [22]. One of the mechanisms of L-type Ca^{2+} current downregulation is associated with the imbalance in kinase and phosphatase activities [6]. It has been indicated that HSPBs bind to L-type Ca^{2+} channel directly to protect it from the increased phosphatase activity and impaired kinase regulation [12]. Besides, Brundel et al. [5] demonstrated that phosphorylated HSPB1(Hsp27), pre-induced by nontoxic heat shock or HSP-inducers GGA in tachypaced HL-1 cardiomyocytes, could prevent the shortening of the APD by restoring L-type Ca^{2+} current inactivated by the overload of cellular Ca^{2+} during the early stage of AF [43]. It is well known that during AF the mRNA transcription of cell membrane L-type calcium channels and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) are down-regulated, resulting in atrial electrical remodeling, contractile dysfunction and perpetuation of AF. Hsp70 binds to the N-lysine site of the SERCA and maintains its activity, thereby inhibiting cellular calcium overload [45].

Interestingly, some HSP may show cytoprotective properties in electrical remodeling by interacting with some ion channels directly. Hsp70 and Hsp90 in the cytoplasm participate in the process of HEGR protein maturation. HERG is a human gene which encodes the α subunit of cardiac rapid potassium currents IKr [41]. Eventually, they can increase IKr density modulating APD shortening [10]. Other HSP that have been found to interact directly with ion channels include HSPB5, which interacts with Na^+ channels [19], and Hsp70 which interacts with voltage-gated calcium channels [29], reinforcing the potential role for HSP in preventing atrial electrical remodeling. Collectively, HSP can maintain calcium homeostasis in cardiomyocytes and protect various ion channels function against rapid and irregular activation, while reducing the likelihood of induction and maintenance of AF.

3 HSP and Structural Remodeling in AF

Structural remodeling is a major factor in the occurrence and maintenance of AF associated with fibrosis, apoptosis and cell damage, connexin dysregulation, disruption of microtubule network, and myolysis. The function shared by Hsp70 and

Hsp27 reversed AF-related structural remodeling by preventing myolysis and F-actin stress fiber formation in cardiomyocytes [5]. Zhang D et al. used the tachypaced Drosophila as an experimental AF model, and increased HSP expression by heat shock, HSP-inducers GGA and BGP-15. It was shown that some HSPBs, particularly HSPB1, protected the heart against contractile dysfunction and structural changes [52]. A more detailed study elucidated the HSPB1 effects that are mediated via restoring contractile protein and microtubule (α-tubulin) levels [15]. Moreover, it has been indicated that induction of Hsp72, a member of the Hsp70 family, attenuated angiotensin II-induced α-SMA expression and fibrosis formation in rat left atrial fibroblasts [47]. As mentioned above, Ca^{2+} overload in AF can activate calpain. The activated calpain degraded contractile proteins resulting in myolysis [21]. However, calpain activity can be attenuated in HSPB1 overexpressing Drosophila [52].

The transforming growth factor beta (TGF-β) signaling pathway is strongly associated with the increase of atrial fibrosis in both experimental models and humans with AF [39, 46]. It has been demonstrated that Hsp70 modulates the phosphorylation of Smad2, thus inhibiting TGF-β-induced epithelial-mesenchymal transition [31]. On the contrary, circulating Hsp70 enhanced fibronectin and collagen I synthesis of human vascular smooth muscle by TGF-β1 upregulation [11]. However, the protective or unfavorable role of extracellular Hsp70 in atrial remodeling requires further investigations. Collectively, HSP can restore atrial contractile function via stabilization of cardiomyocyte structure and regulation of fibrosis formation, thus prevent AF mediated cardiac damage.

4 HSP and Inflammatory Reactions in AF

Inflammatory pathways are involved in atrial electrical and structural remodeling promoting AF initiation and perpetuation [13, 27]. An increasing body of evidence indicates that HSP are implicated in inflammatory pathways. An immediate release of Hsp70 into the circulation and a modulation of Toll-like receptors (TLR)2 and TLR4 on monocytes after coronary artery bypass surgery (CABG) has been observed [9]. Remarkably, an increase of the expression of TLR2 [48] and TLR4 [20], a class of important receptors in inflammatory and immune reactions, was found in atrial tissue of patients with AF. TLR in immune cell surface can recognize danger associated molecular pattern (DAMPs), and subsequently induce the expression of proinflammatory genes [14, 44], which ultimately promote atrial remodeling. In addition, transcription factor NF-κB and AP-1 play an important role in the expression of proinflammatory genes. Extracellularly, Hsp70 stimulates pro-inflammatory cytokine production in the performance of activating NF-κB via TLR2 [35]. In this context, Hsp27 increased NF-κB activation through TLR2 and TLR4 in mouse hearts after global ischemia [18]. In the same line, extracellular Hsp60 as a ligand of TLR4, induced myocytes apoptosis through the TLR4–MYD88–p38/NF-κB pathway [23], which may increase AF burden. In fact, Hsp27

seems to play an anti-inflammatory role by upregulating the content of interleukin-1 (IL-10) and by downregulating expression of tumor necrosis factor (TNF) [16] which are associated with AF [30]. Furthermore, the anti-inflammatory effect of Hsp27 is mediated by inhibition of TLR4 expression and NF-κB activation [8].

5 HSP and Oxidative Stress in AF

Experimental and clinical data indicate that oxidative stress is implicated in thepathophysiology of atrial remodeling. The mechanistic links between atrial remodeling and oxidative stress are complex with several underlying diseases and conditions may affect these pathways [26]. In atrial myocardium of patients with chronic AF, Hsp60 and Hsp10 expression were found to be more than 2.3-fold and 2.4-fold increased, respectively [42]. Interestingly, Hsp10 and Hsp60, acting synergistically or independently, maintain mitochondrial integrity and capacity for ATP generation [32].

HSP modulate mitochondrial function as well as reactive oxygen species (ROS) during oxidative stress. It has been shown that increased toxic ROS in tissues activate TLR2 and TLR4, and subsequently up-regulate the expression of IL-1, IL-6 and TNF-α [51], thus promoting AF. Under conditions of oxidative stress, the ROS induced by NADPH oxidase, such as hydrogen peroxide (H_2O_2) and other free radicals [25], affect biomolecules and ion channels contributing to atrial remodeling [24, 38]. Overexpression of HSPB1(Hsp27) typically leads to a significant decrease in the production of ROS [2]. Also, Hsp70 markedly inhibited H_2O_2-induced apoptosis provoked by oxidative stress in mouse myogenic cells [17]. Undoubtedly, further studies are needed in order to elucidate the role of HSP in oxidative stress related to atrial remodeling.

6 Clinical Application of HSP

Hsp70 is involved in multiple aspects of the AF-related remodeling process. Patients with persistent AF appear to have higher anti-Hsp70 antibody levels at baseline compared to controls with paroxysmal AF [28]. However, no significant differences were observed in the serum Hsp70 and anti-Hsp70 antibodies levels between AF patients and controls [28]. Higher HSP70 levels in the bloodstream may also be associated with AF progression, AF following bypass surgery, and AF recurrence after catheter ablation [28]. In the same line, a prospective study demonstrated that preoperative and postoperative circulating Hsp70 levels were associated with postoperative AF [37]. Moreover, when Hsp70 levels increase $\geq$0.025 ng/ml ($p = 0.038$) or anti-Hsp70 levels increase to $\geq$2.5 μg/ml ($p = 0.033$), AF recurrence rates are significantly higher after catheter ablation [28]. Hence, the levels of circulating Hsp70 and anti-Hsp70 antibodies could be used as predictors of AF type and predictors of recurrence. There are also relative data regarding other HSP.

Specifically, serum Hsp27 levels were correlated with left atrial diameter (LAD), left atrial voltage, fractionated intervals, and predicted AF recurrence after catheter ablation (Y. F. [16]). Anti-Hsp65 antibodies levels were also independently associated with postoperative AF (OR, 1.41; $P = 0.04$) [34]. Furthermore, circulating anti-Hsp65 antibodies can recognize stressed cell surface Hsp60. Therefore, the variation of anti-HSP antibodies may be related to postoperative AF, even though the precise mechanism is unclear.

The prognostic role of myocardial HSP have also been investigated. Wei L et al. [49] indicated that expression of Hsp47 in AF myocardial tissue was positively associated with the levels of serum carboxyterminal propeptide of type I procollagen (PICP), amino-terminal propeptide of type III procollagen (PIIINP) ($r = 0.83$, $P < 0.01$; $r = 0.842$, $P < 0.01$) as well as with the left atrial diameter ($r = 0.780$, $P < 0.01$). Additionally, the concentration of serum PICP, PIIINP were significantly increased in patients with AF ($P < 0.05$). Interestingly, in atrial tissues of ischemic hearts, elevated Hsp70 induced by GGA can prevent ischemia-induced atrial conduction abnormalities and inhibit ischemia-related AF [40]. Thus, the HSP may have therapeutic value in AF. A clinical study recruited 80 patients undergoing CABG and showed that intracellular Hsp70 had a negative independent association with postoperative AF rates (OR = 0.90; 95% CI 0.84 to 0.99, $p = 0.02$) [33]. These data suggest that intracellular HSP may have a prognostic role in patients with AF.

Recent studies have focused on the role of HSP in AF associated with various heart diseases. In rheumatic heart disease patients, Hsp27 was an independent predictor of AF (OR = 0.81, 95% CI 0.34–0.90, $p = 0.03$). It has been suggested that Hsp70 can protect AF patients from stroke by preventing thrombosis without increasing bleeding risk when aspirin was concomitantly administered [1]. However, the relationship between Hsp70 and AF has not been confirmed in all cardiomyopathy models, a fact that could be attributed to the inconsistent disease status of each cardiomyopathy model [36]. Bianca C. et al. had shown that long-term overexpression of Hsp70 was unable to relieve abnormalities in the structure and function of heart in a mouse model which develops heart failure and AF [4]. Therefore, no firm conclusions can be made regarding the role of HSP in AF associated with other heart diseases.

Collectively, the presented evidence in this section suggests that HSP appear to be considered as a novel therapeutic target in AF. Moreover, the levels of specific HSP and anti-HSP antibodies could be used as predictors of AF progression and AF recurrence.

7 Conclusions

In conclusion, various HSP members have cytoprotective role on atrial cardiomyocytes and thereby prevent AF development and progression. Detailed investigations of atrial remodeling in animal models and clinical studies suggest that HSP improve calcium homeostasis and restore L-type calcium current preventing APD and AERP shortening. HSP can also modulate inflammation. Of

note, HSP act as a DAMP to activate TLR2 and TLR4 in immune cells, which subsequently activate proinflammatory pathways and promote AF. Intracellular HSP render the myocytes resistant to fibrosis and myolysis during stress. This favorable effect is mediated by stabilizing mitochondrial function and attenuating oxidative stress. Indeed, induction of HSP may represent a promising therapeutic intervention in order to modulate cardiac function and maintain normal sinus rhythm after bypass surgery or after myocardial ischemic attack. Finally, HSP and anti-HSP antibodies may serve as novel predictors of AF progression and recurrence (Fig. 1).

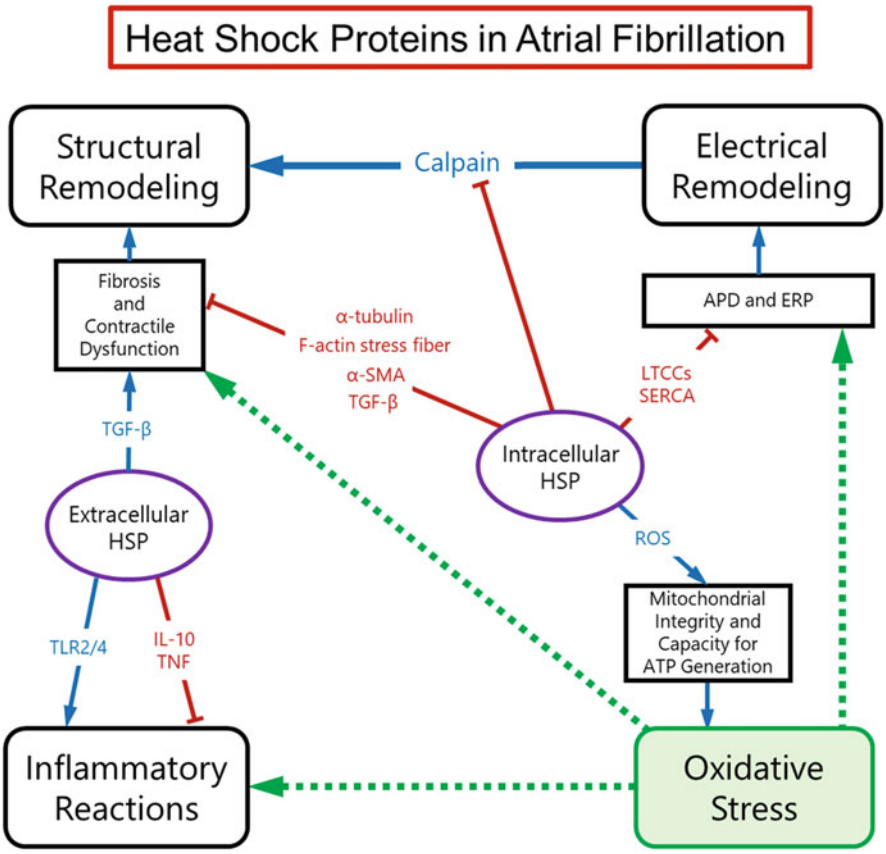

Fig. 1 Heat shock proteins in atrial fibrillation. HSP members show cytoprotective role on atrial cardiomyocyte via various mechanisms, thereby prevent AF development and progression. HSP improve calcium homeostasis and restore L-type calcium current preventing APD and AERP shortening**.** Intracellular HSP can restore atrial contractile function via stabilization of cardiomycyte structure and regulation of fibrosis formation. HSP act as a DAMP to activate TLR2 and TLR4 in immune cells, which subsequently activate proinflammatory pathways and promote AF. And HSP modulate mitochondrial function as well as attenuate oxidative stress during oxidative stress
APD action potential duration, *ERP* effective refractory period, *IL-10* interleukin-10, *LTCCs* L-type calcium channels, *ROS* reactive oxygen species, *SERCA* sarcoplasmic reticulum Ca^{2+}-ATPase, *TGF-β* transforming growth factor beta, *TRL2/4* Toll-like receptors 2/4, *TNF* tumor necrosis factor

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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Threading Microarrays into Novel Applications

Anusha Kishore and Carsten Zeilinger

Abstract

Introduction HSP90 is known as a stabilizer of the proteome and is required for many newly synthesized proteins and introducing damaged proteins back into the refolding chaperone cycle. Due to its key position and interaction with several hundreds of proteins in a cell, it is a target for noxious cells and a responsive sensitive biomarker for cellular stress. Cellular stress is when unfolded proteins are formed by, e.g. mutational events, changes in the osmolality, or redox status. One challenge is to monitor and discriminate the cellular answers to relevant and reliable signals. The aim of this narrative review is to provide an overview of currently available microarray applications using HSP90 as the target.

Methods Pubmed search was performed for available studies on microarray-oriented techniques.

Results Different strategies have been used to measure the presence of HSP90, e.g., immunologically, by turnover, or binding activities. Protein microarrays have a broad application range. As a highly miniaturized assay system they are used to study protein-ligand or protein-protein interaction and used to measure the presence of a target as a diagnostic tool. Q-Dots have interesting electrical and optical properties. The exploitation of its optoelectronic properties for sensing plays a momentous role in the biomolecular diagnostic assay for HSP90.

Conclusions Target-oriented trawling of natural product compound libraries can help to identify novel compounds that inactivate the HSP90 function or increase the affinity for the natural ligand or client or help to restore the activity. HSP90 is a

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biomarker used in future as a stress marker for disease treatments, observing endurance status of athlete or life style management.

Keywords Cancer · Protein Microarray (PMA) · Protein-ligand interaction · Protein-protein interaction (PPI) · Quantom dots (Qdots) · Target-oriented screening

Abbreviations

17-AAG	17-N-allylamino-17-demethoxygeldanamycin
Aarsd1	muscle specific cochaperone (alanyl-tRNA synthetase domain-containing 1)
AIF	apopotosis inducing factor
Apaf1	apoptotic protease activating factor 1
CdSe	cadmium selenide
CgA	chromogranin A
CTD	c-terminal domain
Cy5	Cyanin 5
IgA	Immunoglobulin A
MD	middle domain
NTD	N-terminal domain
PMA	protein microarray
PPI	protein-protein interaction
PU-H71	6-amino-8-[(6-iodo-1,3-benzodioxol-5-yl)thio]-*N*-(1-methylethyl)-9*H*-purine-9-propanamine
Q-dots	quantum dots
SPR	surface plasma resonance
ZnS	zinc sulfide

1 Introduction

Elevated HSP90 levels can be detected as an early response of stress and is cancer therapies' target due to its increased susceptibility for high affine compounds which often result from natural products and their derivatives or new such as geldanamycin (**1**), reblastatin derivatives (**2**), radicicol (**3**) newly developed (**4–6**) or isolates (**7, 8**, Fig. 1) [13, 16, 27, 29, 40, 53, 61, 63, 66, 69, 75, 76, 78, 79]. Meanwhile, a respectable portfolio of new inhibitors exists acting on Hsp90 with different affinities and oral disposable [69, 70]. In addition, HSP90 is also acting as a fate selector in the evolutionary process in a way that only proteins identified by HSP90 mediated activity by HSP90 [56, 70] [15, 23, 34, 54]. It has the meaning that the structure of the client-binding site selects and determines the fate of the client proteins, whereas proteins, which do not fit become removed. This is also a relevant point where the

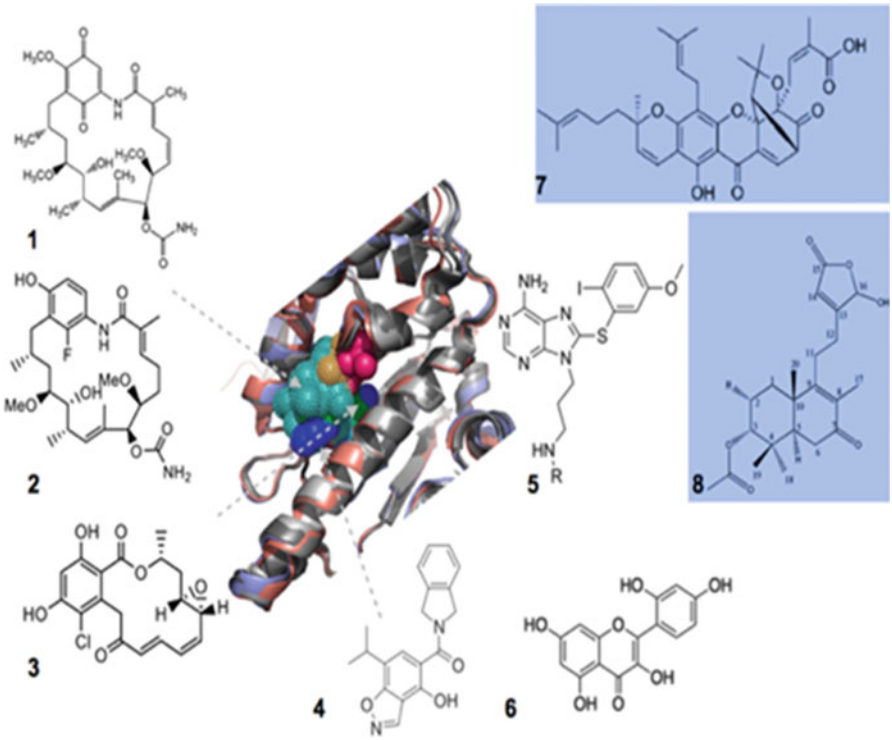

Fig. 1 HSP90 pocket volume occupancy by ATP/ADP and inhibitors. Different HSP90 crystal structures (PDB codes: 3TOZ; 2iws; 1yet; 1amw; 5uc4) of the N-terminal domain aligned with ligand or inhibitors. To emphasize filled volumes, small molecules (ATP, magenta; ADP orange; geldanamycin (**1**), aquamarine; radicicol (**3**), green and benzoisoxazole KUNB31 [27]; (**4**), blue) are visualized as spheres. Other interesting novel candidates like reblastatin derivative (**2**), purine derivative, PU-H71, [33], (**5**), N-acyl-N-alkyl sulfonamide, morin (**6**) or natural products gambogic acid (**7; [**79]) and labdane diterpernoid (**8; [**61]) are not shown in the crystal

chaperone system can fail when uncorrected and unfolded proteins blinding the cellular proteome. Therefor techniques are required, which help efficiently analyze the HSP90 levels as a function of the cellular stress response. On the other hand, techniques required for testing the effect of compounds that are needed to identify high affine compounds or tools for identifying producers of compounds that bind or inactivate the HSP90 function. Furthermore, it is important to avoid off-target effects to sustain an appropriate effect towards pathogenic cells [42].

Heat shock proteins (HSP) are a big class of proteins that exist in prokaryotic and eukaryotic organisms. They have been conserved throughout evolution. Due to their significant part in protein homeostasis, they are instituting in all major cellular compartments and required for the adaptive response to stress conditions [8]. HSP of Mammals have been classified into six groups based on their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSP including HSP27 [24]. Larger HSP have ATP-dependent function while smaller ones work in an

ATP independent way. The HSP90 interacts with hundreds of client proteins, which are important in the development of the steroid receptor complex and other [11, 48]. The HSP70 family is essential for protein synthesis, translocation, and folding and is the mediator for diverse cellular functions [55]. HSP60 family is significant in protein stability [28].

HSP have a molecular chaperone and anti-apoptotic activity. As molecular chaperon HSP catalyze the appropriate folding of misfolded proteins and evade their aggregation [22]. As anti-apoptotic protein HSP interfere with cell death at different stages [20]. At post mitochondrial level HSP27 binds to cytochrome c while HSP90 or HSP70 binds to the apoptotic protease activating factor 1(Apaf-1), thus inhibiting caspases activation and apoptosis. HSP70 association with apoptosis-inducing factor (AIF) blocks the caspase-independent cell death [45]. HSP gives stability or proteasomal degradation of proteins under stress (protein triage process). Thus, HSP contribute to cell survival.

These protective functions of HSP make cancer cells dependent on them as cancer cells must extensively rewire their metabolic and signal transduction pathways. Expression or activity of HSP90, HSP70, and HSP27 is abnormally high in many cancer cells, and other death stimuli increase it further. HSP70-I confers tumorigenicity in cancer cells, make them resistant to anti-cancer drugs like Cisplatin or Imatinib, and it renders cancer cells resistant to the immune response from cytotoxic cells like monocytes. Antiapoptotic activities of HSP90 helps cancer cells in survival [21, 31, 73, 74].

1.1 HSP90 as a Stress Marker

Since HSP90 has an important target role in anti-cancer treatment, HSP90 levels is a relevant biomarker. The early detection of biomarkers is crucial in many diseases, such as different cases of cancer, cardiovascular disorders, pathological conditions, and also monitoring cellular processes (Fig. 2). Another challenge is the identification of biomarkers in sports physiology to monitor training success and analyze

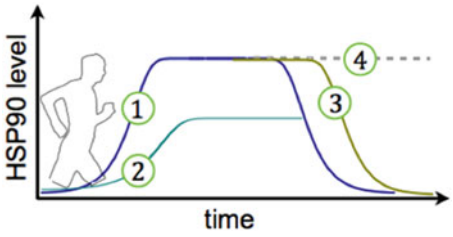

Fig. 2 HSP90 a biomarker for stress. The HSP90 level as a function different stress levels with (1) under high stress or endurance, with (2) with lower stress and regeneration and reduced HSP90 levels or (3) delayed HSP90 reduction with different regeneration time and (4) remaining elevated HSP90 levels as a diseased level

nutrition and metabolic health, hydration status, muscle status, endurance performance, injury status, risk, and inflammation [4]. Recently it was shown that a novel muscle-specific cochaperone Aarsd1 is required for muscle differentiation and may help in the muscle regeneration process [12]. There are wide-spreading application techniques meanwhile available to identify biochemical, hormonal, and immunological biomarkers [26, 32, 65]. Most of the relevant biomarkers are measured as cellular stress responses resulting from and measured as elevated values from oxidative stress, malondialdehyde, isoprostane, enzymatic anti-oxidants, cortisol, blood urates, copeptin, alpha-amylase, secretory IgA, chromogranin A (CgA), lysozyme, miRNAs and heat shock proteins (HSP). In addition, biomarkers can result from different tissues or physiological background [14].

The detection of HSP as a biomarker in tissue and bodily fluids such as blood and urine requires techniques for specific diagnosis. Newer results indicate that HSP90α biomarker for liver cancer in the early stage using ELISA technique for detection [4]. This can help to evaluate limits for endurance exercises as well as for personalized records. The main problems are the detection limits, sensitivity, and artificial quenching by foreign compounds, e.g., albumin, etc. [43, 47, 67]. To circumvent limitations and to provide the highest reliable sensitivities, biomarkers need to be identified by different techniques. The physical presence of HSP is monitored by immune blots, proximity assays, ELISA, mass spectrometry, SPR or protein microarrays, and also Raman-based detection systems [51]. ELISA and related systems use the affinity of antibodies or aptamers as detection sensors while the signal is enhanced and detected electrically or optically. Due to the high miniaturization grade, protein microarrays are a powerful tool in monitoring the presence of HSP biomarkers, using a known fixated probe (antibody, aptamer, ligand) against multiple unknown samples. These technologies are under continuous optimization and, in some cases, can detect picogram of biomarkers. Mobile technologies detect physiological and simple-analyte biomarkers such as DNA, mlRNA, protein, peptides, or small molecules; most of them have been endorsed by the US FDA and explored for the improvement and cost-reduction of healthcare-services. These technologies comprise around 664 non-invasive molecular biomarkers and the 592 potential minimally invasive blood molecular biomarkers available for monitoring, diagnostics or theragnostic purpose [51]. Technologies like micro-fluidics or micro-electrical sensors for protein biomarker detection can be developed for personalized applications as well for point of care (POC) diagnostic of disease markers, which, in future, could provide reliable and low-cost sensors for monitoring the physiological status [2] (Fig. 2).

1.2 HSP90-Oriented Screening of Compound Libraries by the Use of Protein Microarrays (PMA)

The curiosity for HSP inhibitors has lately found an extra motivation, because heat shock proteins can also serve as a target in the treatment of various pathogenic

diseases, such as Malaria and Leishmania, as well as a neurological disorder [7, 30, 41, 44, 50, 57, 64]. Natural HSP inhibitors bind to the ATP binding pocket of the protein and prevent the ATPase activity. As an evolutionary adaptation process different natural producers used HSP90 as a target for their product portfolio to inhibit HSP90 chaperone activity. Natural products like radicicol (**3**) isolated originally from *Monosporium bonorden* [10] or other from the mold *Chaetomium chiversii*, an endophyte of the Mormon tea *(Ephedra fasciculata*) and geldanamycin (**1**), a bacterial ansamycin from *Streptomyces hygroscopicus* bind with high affinity into the ATP binding site (Fig. 1) [68, 72]. Geldanamycin has high hepatotoxicity; thus, it has been suspended from the phase I trial. Its analogue like 17-AAG, is the first-in-class inhibitor and has entered phase II trials.

Meanwhile, more anti Hsp90 inhibitors developed, e.g. reblastatin derivatives (**2**) and other (**4–7**) (Fig. 1). In comparison to the natural ligand ATP/ADP the inhibitors occupy different places in the pocket. Interestingly, ATP/ADP (Fig. 1, magenta, orange) is more orientated to the inner right side of the pocket whereas geldanamycin to the upper left (Fig. 1, aquamarine), and radicicol (Fig. 1, lime) to the lower left and intermediate lower left (Fig. 1, blue) a novel compound with 50-fold higher selectivity for Hsp90ß developed recently by the Blagg group [27]. The binding and replacement activities of the natural ligand and their derivatives often in the nM range and more complex assay such folding or cellular assay gave activities in the lower µM range.

Protein microarrays are growing as beneficial tools for biopharmaceutical research. These micro spot arrays have boundless potential to fast-track not only elementary biological discovery but also drug development. Protein microarrays systems can detect with very low material consumption presence of a target or used as functional assay system for detection of protein/ligand and protein/protein interaction [17, 35, 71] (Fig. 3). Microarrays allow highly parallel detection and quantification in a rapid, low-cost and low sample volume format [1].

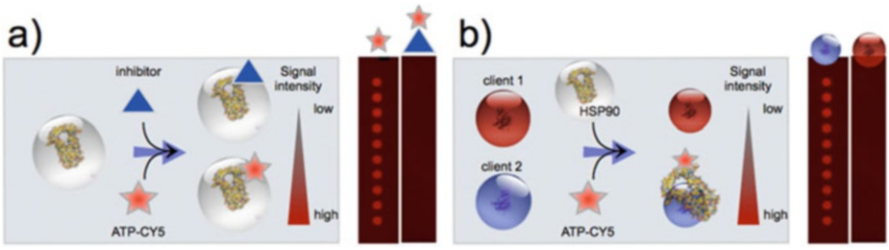

Fig. 3 HSP90 microarrays in protein-ligand or protein-protein applications. (**a**) Protein microarray application with spotted HSP90 on microarray binding labeled ATP-CY5 or not in the presence of an anti HSP90 inhibitor such as radicicol. The fluorescence intensity is visualized by bound ATP-CY5 whereas displaced in the presence of anti HSP90 inhibitor. A typical binding experiment with and without inhibitor in presence of ATP-CY5 shows ten spots in a row of HSP90 (right scheme). (**b**) Microarray-based PPI activity of HSP90α on putative client proteins. Binding on client was monitored by ATP-CY5 binding on HSP90 whereas clients do not bind ATP (pers. comm., ms in prep.)

To measure protein interactions, such as protein/ligand (small molecules), protein/protein, or other interactions with a specific method on a miniaturized chip, they must first be immobilized on the microarray [9, 18, 19]. For this purpose, the proteins must be brought onto the chip with a spotter (printer), via contact or contactless method. There are different types of microarrays. These include functional, reverse phase, and analytical chips [9, 39, 51]. For example, analytical microarrays are used to evaluate binding affinities and protein expression levels of complex protein solutions. Meanwhile it is possible to generate microarray based libraries of target proteins by cell free expression from linearized cDNA templates [59]. For this purpose, often provided with an antibody or aptamers on carrier glass. The incubation of the protein solution is carried out on the chip. Reverse-phase protein chips are closely related to the analytical ones. A complex solution, e.g. cell lysates become analyzed. In this type carrier glass slides are supported by a layer of nitrocellulose [1]. After immobilization on the nitrocellulose arrays, the lysate is incubated with binder (bait) molecule, e.g. antibodies or aptamers, which have a high affinity for the analyte protein to capture molecules. The binder can then be provided with detectable substances (fluorescent, chemiluminescent or colorimetrically detectable). It is important that a reference protein is tested as standard on the chip, so that protein quantification can be performed. This type of microarray is particularly useful for the detection of diseases associated with altered proteins due to an abnormal pathway of synthesis. In this case, the presence of a biomarker candidate molecule is monitored [77]. In functional protein chips, these consist of immobilized, in the best-case, highly pure proteins or protein domains. This microarray is used to test the biological activity of the peptides. Various interactions, such as protein against RNA, DNA or substances can thus be analyzed.

Various materials are available for the surface treatment of glass slides, which ensure the immobilization of the printed proteins. It is essential for later activity measurements to maintain the desired active conformation. In addition, a sufficient binding capacity should be present, so that the signal in the subsequent detection is sufficiently strong and can be distinguished from the background noise well. To ensure that the activity of the proteins does not decrease, it is necessary to store them in a moist, cool environment until they are immobilized. By adding glycerol in the storage buffer, the protein is stabilized in the wet state. To uniformly align the proteins on the chip, it is possible to use metal ions with high affinity to specific structural motifs. For example, histidine hexamer tags can be used on the proteins that have an affinity for cobalt or nickel ions. However, it is possible that the tags attached to the proteins by N- or C-terminal decrease their activity. As a further coating, there are aldehyde or epoxy surfaces which covalently interact with the amines of the proteins. With this surface activation, the proteins are bound in random orientation.

The advantage of this immobilization is that the proteins show a strong adhesion to the carrier through the covalent bond, whereby harsher conditions for further processing of the chip can be used. However, the covalent bond may be accompanied by a disadvantage at the same time. With such a strong interaction, the protein can be “pulled apart”, thereby losing activity. Likewise, the random orientation of

the proteins on the carrier can be achieved by a gel coating, via poly-L-lysines or nitrocellulose membranes. The advantage of these surfaces is that it is immobilized only via adsorptive interactions, which means a significantly milder procedure for the proteins. The disadvantage, however, is that the adsorbed proteins can be relatively easily re-washed by subsequent purification steps. Almost all surfaces described, except with the affinity binding, lead to non-specific binding of the proteins. The purer the protein, the more meaningful is the result obtained from the experiment. The non-specific adsorption of molecules requires that after adsorption of the proteins to be analyzed, the remaining free adsorption sites must be blocked to keep the background signal as low as possible. This is often achieved with a solution of bovine serum albumin or a milk protein solution.

1.3 Novel Applications by Protein Microarrays (PMA)

1.3.1 Quantum Dots as a Sensor for Biomarker

Quantum dots in microarrays, offer very exclusive features that allow detection at pg/mL concentration. Quantum dots offer notable photostability and brightness. They do not display photobleaching common to organic fluorophores. The high emission amplitude for Q-Dots results in a better signal to noise ratio of the final image. Quantum dots are tiny semiconductor crystals. They have broad excitation range and size tune-able narrow emission wavelength. This is due to quantum confinement effect. It has a core-shell structure. The core is made up of CdSe that absorbs and emits radiation while the shell is made up of ZnS. The shell layer is transparent, i.e. it a has high bandgap; it has structure like core to confine excitement till core. Shell increases quantum yield. Conjugated Quantum dots like Streptavidin conjugated are used in detection system. Five to ten molecules of Streptavidin are covalently attached to per nanocrystals through an additional layer of polymer on them [46] The high sensitivity allows to monitor binding and the presence of a target molecule, but can be used in the displacement mode a differential signal and therefore selective quantification by using inhibitors and ligands (Fig. 4).

Using quantum dots in detection system ensures high degree of improvement in target detection, time of detection, sensitivity and reduction in assay cost. It also increases multiplexing capabilities. They offer large excitation coefficient, high photostability, low photobleaching, large stroke shift and inertness to pH change [3, 5, 6, 36].

Studying Mutational Effects by PMA

HSP90 is highly conserved during a billion years of evolution [62]. Sustaining the functional architecture of the proteins may be the main reason for non-tolerable exchanges [38]. Additionally, more than 10% of most proteomes are clients of HSP90 [60]. In vitro experiments reveal the drastic influence of mutations in the

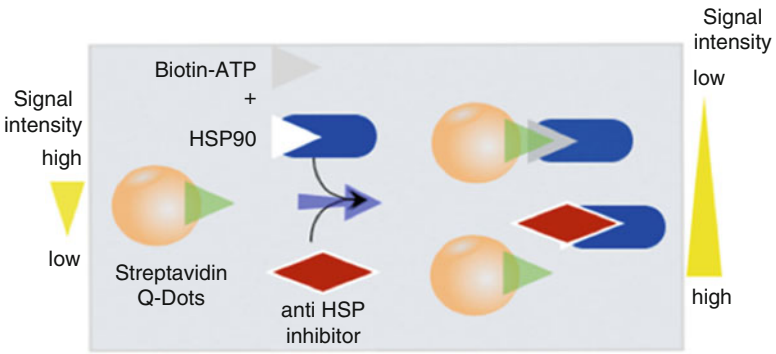

Fig. 4 Microarray-based detection of HSP90 by spotted Q-dots. Spotted Q-dots with coupled streptavidin can bind to Biotin-labeled ATP. HSP90 binds to ATP and induce signal quenching whereas in the presence of an anti HSP90 inhibitor no signal quench occurs (pers. comm., ms in prep.)

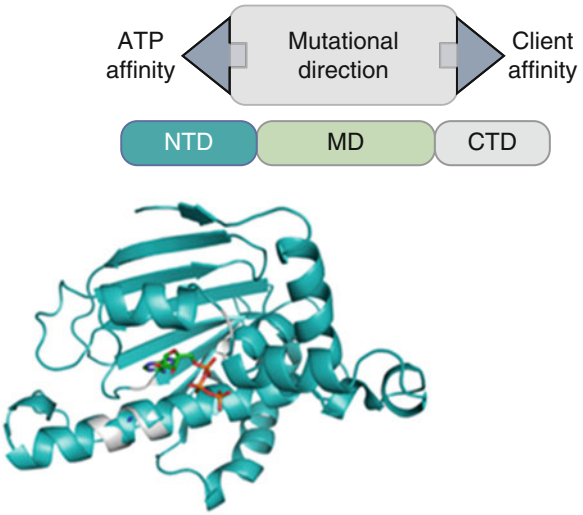

Fig. 5 Scheme of mutational consequences for the HSP90 proteome. Amio acid exchanges elicited by mutations can influence the affinity for the natural ligand ATP transition to ADP or release of ADP in different and also influence the affinity towards cochaperones and clients which happens always in the evolutionary processing

HSP90 protein with consequences for affinities on the ligand-to- or protein-to-protein interaction (Fig. 5) [25, 52]. However, analysis of the NTD between humans and pathogens revealed that some variations exist, and ligand and compound assays have shown that differences in the binding affinities exist [58]. Therefore it was concluded that these differences are sufficient to be a selective target. Some drastic effects by amino acid exchange can be monitored by analysis of the hydropathy as a function of the sequence. As shown in Fig. 6, the exchange of isoleucine to lysine has a drastic influence on the hydropathy. Other exchanges phenylalanine or lysine to arginine reduces hydropathy little. Analysis of the ATP binding revealed that the exchanges had different influences on the ATP-binding activity. The exchange from isoleucine to lysine, e.g., enhanced the ATP binding whereas other exchanges caused loss of ATP-binding (Fig. 6). (pers. comm. manuscript in prep).

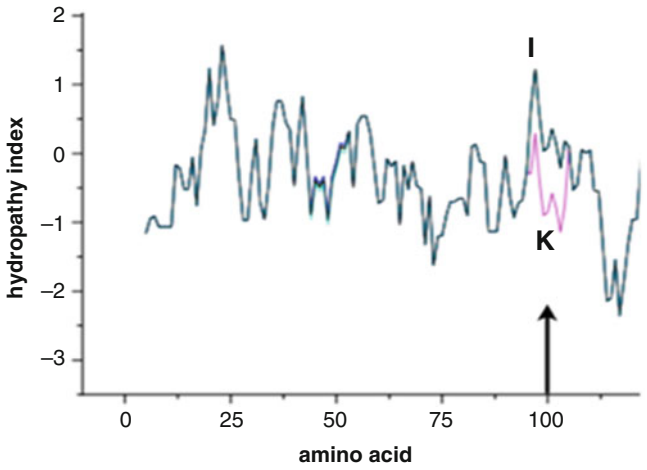

Fig. 6 Effect of the amino exchange on the hydropathy of HSP90. The graph represents the hydropathy index scanned at a window size of 9 as a function of the first 100 N-terminal amino acids of HtpG, whereas a single exchange from I (isolysine) to K (lysine) influence the hydropathy passage drastically

Variations in the HSP sequence have been identified earlier as a hub for natural producers of anti-HSP compounds such as Geldanamycin by S. hygroscopicus [37, 49]. It can be shown that several homologues from Streptomyces strains exist, which may be producers of anti HSP90 compounds. It can be suggested that some other organisms that have aberrant amino acid sequences in the conserved ATP pocket could be producers of secondary metabolites with an ATP-displacing effect. To improve whether there are already sequenced HSP90 homologs that have the mutated positions, putative amino acid substitutions suggested were transferred to a bacterial wild-type sequence and stored in a FASTA file and thread the information repetitive into the searching algorithm (Fig. 7). With the sequence containing all the mutations, a protein-BLAST search was carried out for organisms naturally carrying these amino acids at the mutation positions. To increase the likelihood of finding hits based on more distant organisms, the homologous or identical groups were excluded. In order to further increase the diversity of the specified organisms, this group was also excluded. The best 100 sequences found were downloaded and searched for the mentioned substitutions via a sequence comparison. As indicated in Fig. 5 in the NTD structural model relevant positions emphasized which have in the region some variations. The surrounding amino acids, however, seem to be more variable and have different motives within the 100 organisms considered. However besides the strongly conserved regions, some exchanges in the random site and also in the mainly conserved regions exist, indicating that producers of anti HSP90 compounds can exist. However, related consequences may exist for the client binding site the middle domain. Sequence alignments reveal that the middle domain is more variable than the NTD and may indicate that the difference is responsible for the proteome plasticity.

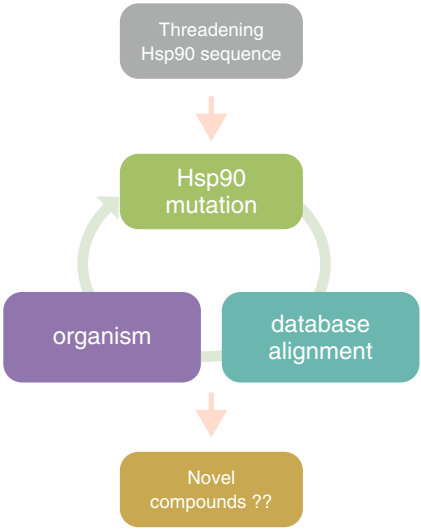

Fig. 7 Strategy for using mutations in the HSP90 structure for identification of producers of novel compounds (pers. comm., ms in prep.). The available Hsp90/HtpG proteome database (BLAST) will be scanned with positions of known antiHsp90 producers and using later raw extracts of identified organism for testing in Hsp microarrays

2 Conclusions

The HSP90 proteome with cochaperones and hundreds of client proteins always threaded through an evolutionary adaptation process. Fixed positions in protein architecture such as NTD allow not massive changes without drastic consequences. Therefore is this position during the evolution a target for natural products and compounds, a target for pathogens, a target for drug therapies, and a target for the development of biomarker assays.

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Ethical Approval for Studies Involving Human This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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Regulation of Kaposi's Sarcoma-Associated Herpesvirus Biology by Host Molecular Chaperones

Elisa Kirigin, Duncan Kyle Ruck, Zoe Jackson, James Murphy, Euan McDonnell, Michael Obinna Okpara, Adrian Whitehouse, and Adrienne Lesley Edkins

Abstract

Introduction Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with development of the human diseases Kaposi's sarcoma, Primary Effusion Lymphoma and Multicentric Castleman's Disease. KSHV establishes a chronic latent infection in hosts, with periods of viral lytic replication, where both latent and lytic virus cycles contribute to malignancy, most often in the immunodeficient host.

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Methods Here we review the published literature from Pubmed, Scopus and related databases with keywords relating to molecular chaperones, heat shock proteins and KSHV biology.

Results Lytic KSHV replication and latent infection is regulated by cell stress and functionality of KSHV proteins relies on folding and conformational stabilization which is mediated by host chaperone systems, including Hsp90, Hsp70 and the endoplasmic reticulum stress response, as well as chaperone-mediated host signaling pathways.

Conclusion Dependence on the host cell chaperone systems means that inhibitors have been developed which have anti-viral effects at non-toxic concentrations and hence represent a possible avenue for effective therapeutic intervention in the future.

Keywords Cancer · Heat shock protein (Hsp) · Human herpesvirus 8 (HHV8) · Kaposi's Sarcoma-associated herpesvirus (KSHV) · Molecular chaperones · Oncovirus

Abbreviations

17-AAG	17-allylamino-17-desmethoxygeldanamycin
17-DMAG	17-(2-dimethylaminoethy1-amino)-17-demethoxygeldanamycin
CEACAMI	carcinoembryonic antigen-related cell adhesion molecule 1
CHIP	C-terminus of Hsp70 interacting protein/STUB1
DNMT1	DNMT-associated protein1
DUB	Deubiquitinating enzyme
EBNA	EBV-encoded nuclear antigen
EBV	Epstein-Barr virus
GA	Geldanamycin
HAART	highly active antiretroviral therapy
HDAC1	histone deacetylase 1
HPV	human papillomavirus
HSP	heat shock protein
HSV	Herpes simplex virus
HYOU1	hypoxia up-regulated 1 protein
IFN	interferon
IRF	interferon regulatory factor
Iκκ	inhibitor of KappaB kinase
KS	Kaposi's sarcoma
KSHV	Kaposi sarcoma-associated herpesvirus
LANA	latency associated nuclear antigen
MCD	multicentric Castleman's Disease
NAP1L1	nucleosome assembly protein 1-like 1
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells

NPM	nucleophosmin
PEL	primary effusion lymphoma
RRE	RTA-responsive elements
RSK/ERK	p90 ribosomal S6 kinase and extracellular signal-regulated protein kinase
RTA	replication and transcription activator
RTC	replication and transcription compartments
STAT3	signal transducer and activator of transcription 3
STIP1	stress inducible phosphoprotein 1/Hop
SUMO	small ubiquitin modifier
TIVE cells	telomerase-immortalized human umbilical vein endothelial cells
UPR	unfolded protein response
vFLIP	viral FLICE-like inhibitory protein
vIRF	viral interferon regulatory factor

1 Introduction

Infection is a major cause of cancer worldwide. Human tumour viruses account for ~15% of new cancers globally (~1.5 million cases/year). Kaposi's sarcoma-associated herpesvirus (KSHV) is a DNA virus classified as a Group 1 carcinogen, required for the development of all forms of Kaposi's sarcoma (KS) [21], a highly vascular tumour of endothelial lymphatic origin. KSHV infection is also associated with two lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's disease (MCD). KS is epidemic in sub-Saharan Africa due to high rates of HIV infection and is considered an AIDS defining cancer. Notably, in South Africa, KS is in the top 10 cancers identified in men, women and children, and is the third most prevalent cancer in African men (2013). Despite some reductions in KS incidence due to effective Antiretroviral Therapy (ART) and well-controlled HIV infection [106], many individuals with well-managed HIV infection still develop progressive KS. In addition, in areas of sub-Saharan Africa with sporadic or limited ART access, KS remains a major cause of morbidity and mortality, including in children. Despite this, there are no KSHV-specific antivirals or vaccines, and current treatments rely on immune modulation and systemic administration of cytotoxic agents. As such, KS-associated immune reconstitution inflammatory syndrome (KS-IRIS) is a major contributor to KS-related deaths in Africa where its incidence is higher than in developed countries primarily due to more advanced KS disease and lower chemotherapy availability [89]. Consequently, there is an immediate need for research into understanding the biology of KSHV and for the development of specific, efficacious KSHV-targeted therapeutics.

Like other herpesviruses, KSHV persists as a latent infection with episodes of lytic replication which give rise to infectious virus. The lytic and latent phases are associated with expression of distinct proteomes and both have been linked to tumour development and which must be expressed and assembled inside the host

cell. The determinants of folding are encoded in the amino acid sequence, but the action of host molecular chaperones is necessary as a catalyst to ensure proper folding on a biologically relevant timescale. Molecular chaperones are a class of cellular proteins which function to promote the successful transition of a linear peptide sequence into a functional three-dimensional protein structure in a process known as folding. In addition to *de novo* folding, chaperones also prevent aggregation and promote refolding of proteins whose structure has been perturbed by cell stress. KSHV, like other viruses, has several essential proteins which are dependent on host molecular chaperone systems for stability and conformational regulation. The roles of HSP90, HSP70 and ER family chaperones in regulating the expression, folding and stability of important KSHV proteins have been studied and suggest that inhibition of chaperone systems may be a promising therapeutic avenue for the development of new clinical strategies for KSHV-related malignancies.

1.1 KSHV Lifecycle and Biology

KSHV is a large, enveloped, double-stranded DNA herpesvirus of the genus rhadinovirus [151, 152]. It encodes more than 90 open reading frames (ORFs). As with all herpesviruses, KSHV exists in either a latent state, where viral gene expression and genome replication is minimal, or undergoes lytic replication, where viral gene expression is pervasive and replication is productive, resulting in the formation of mature, infectious virions [6]. The KSHV life cycle is outlined in (Fig. 1)**.** KSHV is capable of infecting a broad range of human cells, including B lymphocytes, monocytes, epithelial cells, endothelial cells and keratinocytes, via interactions between its envelope glycoproteins and various cell surface proteins, namely heparin sulfate, integrins and Ephrin A2 [26]. Entry is mediated via the activation of native cellular signaling pathways and is followed by un-coating and translocation of the KSHV genome to the nucleus, where it is circularised [26, 117]. This circular genome is termed an episome and is maintained as a highly ordered chromatin structure that undergoes minimal gene expression during the latent state of infection [117]. During latency, almost none of the viral genes are expressed, the exceptions being LANA (Latency-associated nuclear antigen), vCyclin, vFLIP (viral FLICE-like inhibitory protein), kaposins, vIRF3 and a repertoire of virally-encoded miRNAs [143] (Fig. 2). LANA is crucial for episome maintenance, being necessary and sufficient for latent replication of viral DNA and tethering the episome to mitotic chromosomes [143]. As KSHV does not express any proteins directly for DNA replication during latency, association of LANA with host proteins is crucial to mediate replication during this stage [117]. This enables the episome to be evenly segregated into daughter cells upon mitotic division of the parental host cell [143].

Latent infection enables KSHV to establish a life-long infection in the host, primarily in a reservoir of latently-infected B-cells and, to a lesser extent, monocytes [46]. In a population of such cells, a small percentage will spontaneously undergo

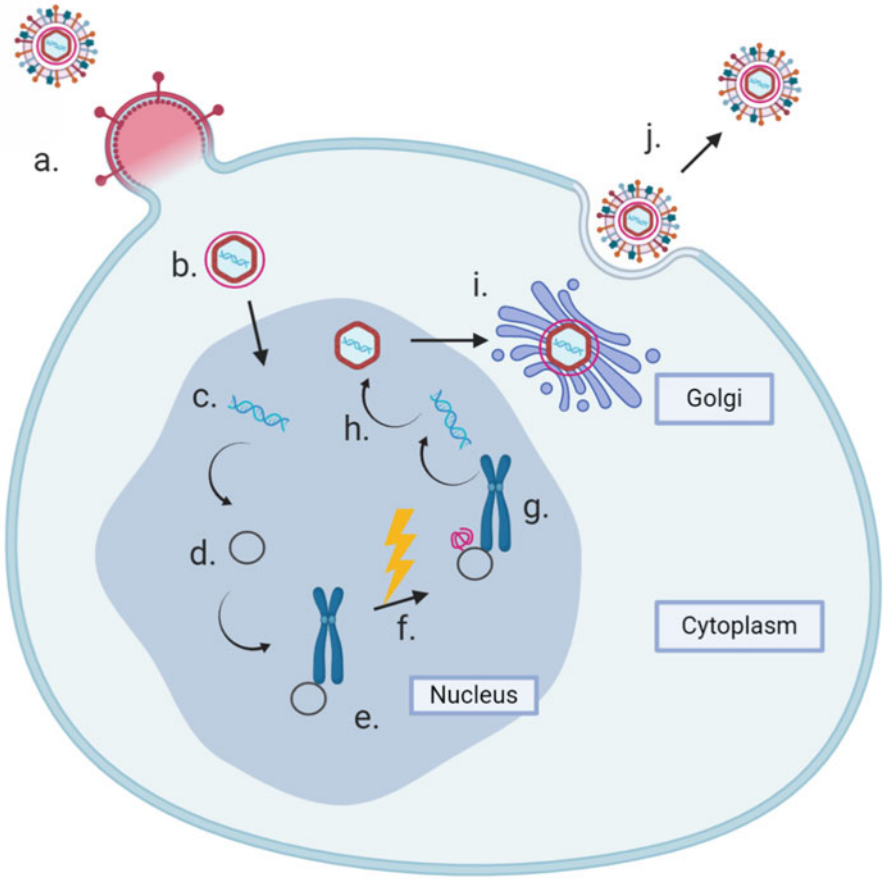

Fig. 1 KSHV life cycle. (**a**) The virus enters a naïve B lymphocyte and the viral capsid is uncoated. (**b–c**) The viral genome is translocated to the nucleus. (**d–e**) The viral episome is formed in the nucleus where it associates with the host genome. (**f–g**) Upon a stress factor (e.g. immunocompromised patients) viral reactivation occurs resulting in the transcription of new viral genome. (**h**) The new genomes are packaged into the viral capsid. (**i**) The viral capsid is trafficked to the Golgi where it acquires the tegument layer studded with viral proteins. (**j**) Finally, the virus fuses with the cell membrane where the viral capsid is enveloped with the glycoprotein studded envelope lipid bilayer. The newly released virus can then go on to infect new cells

lytic reactivation, resulting in the production of mature virions which go on to infect further B-cells and monocytes, alongside spreading to endothelial and epithelial tissues [53]. Reactivation of KSHV to its lytic life cycle is a highly complex process, requiring combinations of cellular and viral factors, and is known to be induced by multiple compounds, conditions and cellular stresses [118]. Curiously, lytic reactivation of the latently KSHV-infected primary effusion lymphoma (PEL) cell line appears to mirror the maturation of plasmablasts to plasma cells, through

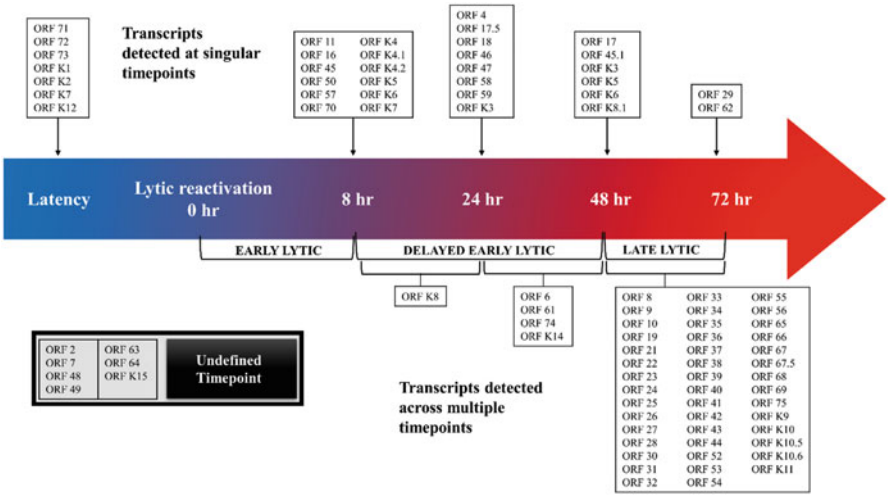

Fig. 2 Transcription timeline of Kaposi's Sarcoma-associated herpesvirus genes from latency through lytic reactivation. Timepoints annotated occur post lytic activation. Expression timeline generated from published datasets [10]

activation of XBP-1 s via endoplasmic reticulum (ER) stress brought on by the unfolded protein response (UPR) [154]. This furthers the idea that KSHV has co-opted certain cellular stress responses in order to promote its own replication, and which has been observed in multiple other viruses like hepatitis C virus and other herpesviruses, namely herpes simplex virus γ134.5 and human cytomegalovirus [154].

The lytic cycle proceeds as a sequence of gene cascades, which can be classified into 3, temporally sequential stages; immediate early (IE), early (E) and late (L) [118] (Fig. 2). Replication and trans-activator (RTA) is the major IE gene and is the initial trigger, being both necessary and sufficient, for reactivation [114]. It mediates reactivation by binding to RTA-responsive elements (RREs) in the promoters of IE (including its own), E and L viral genes (and host genes), complexing with various viral and host proteins and inducing transcription from these loci both directly and indirectly [154]. KSHV replication and transcription activator protein (RTA) also activates expression from its parent gene and as such is an auto-activator, as well as being a trans-activator [114]. Additional IE genes expressed during this time (up to ~10 h post-induction) tend to reflect RTA in that they are activators of transcription or modulators of cellular behaviour, including ORF45, kaposins, ORF48, ORF29b and ORF70 [156, 164] (Fig. 2).

Early genes are expressed roughly 10–24 h post-induction and tend to encode functions that mediate viral gene expression and DNA replication [114, 118]. They include ORF6, ORF57, ORF74, ORF37, ORF21, ORF65, PAN RNA, vIL-6, vIRF1 and further kaposins [10] (Fig. 2). Of these, ORF57 is particularly important in driving reactivation as it facilitates the stabilization and export of intronless viral

mRNAs and thus is a crucial mediator viral gene expression [77, 78, 124, 125]. Unlike latent replication, lytic DNA replication is performed by KSHV-encoded proteins and proceeds from a different origin of replication (Ori-Lyt) via a rolling-circle mechanism, resulting in amplification of the viral DNA [115]. As with latent replication, lytic replication and viral gene transcription occurs entirely in the nucleus, and during this stage, KSHV induces the formation of replication and transcription compartments (RTCs), which aggregate and expand as the virus enters the late phase of the lytic cycle [14]. Interestingly, RTCs have been shown to concentrate Hsp70 proteins, whose ATPase activity is required for viral transcription, DNA replication and ORF57 function [14].

Beyond 24 h post-induction, KSHV expresses L-genes, which primarily encode structural proteins, including those of the capsid, tegument and envelope glycoproteins, alongside several associated factors [10]. Assembly and egress proceed with the assembled capsid budding through the double-membrane of the nuclear envelope, acquiring tegument proteins on the way to Golgi body [86]. Here it is trafficked to the cell periphery, where its envelope fuses with the plasma membrane, releasing mature virus into the extracellular milieu for further subsequent infections (Fig. 1).

1.2 KSHV Structure

The linear KSHV genomic DNA is packaged into a t = 16 icosahedral nucleocapsid in the nucleus and wrapped in a proteinaceous tegument layer at the Golgi apparatus and then a lipid bilayer envelope applied at the cell membrane [40]. The protein envelope layer is studded with numerous viral encoded glycoproteins, gB (ORF8), gH (ORF22), gL (ORF47), gM (ORF39), gN (ORF53), ORF68, and K8.1 (Fig. 3). The glycoproteins are important in the cellular tropism of the virus, for example K8.1A determines B cell tropism, independent of the viral heparin sulphate binding activity which is mediated by gB [47, 144]. The tegument layer appears to be divided into an outer and inner layer, with viral proteins found in these layers (e.g. ORF 45, ORF52, ORF11, ORF21, ORF33, ORF63, ORF64, and ORF75) thought to play roles in the primary infection of cells, facilitation of genome entry and likely early viral replication events.

The nucleocapsid is composed of viral proteins ORF26, ORF25 (major capsid protein), ORF62, ORF65 (small capsid protein), ORF17 and ORF17.5 [159]. It is thought that some of the proteins found in the capsid (ORF19 and ORF32) are also associated with the tegument layers, with these proteins playing a role in the transport of viral proteins to the nucleus via the microtubule and dynein networks. ORF32 and ORF19 are also important in the assembly of the proteins within the tegument layer, and anchorage of the capsid on penton-proximal triplexes [40]. These capsid-tegument associated proteins are well documented in alpha- and betaherpesviruses where they have been shown to be important in the propagation of the virus [40].

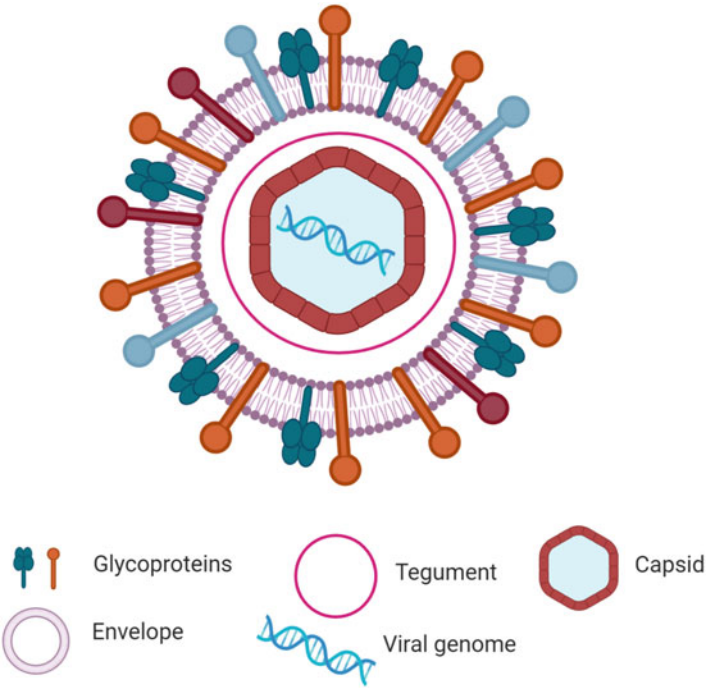

Fig. 3 KSHV virion structure. A schematic representation of the virion structure of KSHV. The genome is surrounded by capsid and tegument layers before being enveloped in a lipid bilayer studded with viral glycoproteins

In addition to the viral proteins found in the virion, a number of host cell proteins are also packaged, particularly of note here are members of the heat shock family of proteins, Hsp90 and Hsc70 [18]. Hsp90 and Hsc70 have been shown to play vital roles in the replication cycles of KSHV and other viruses and will be further discussed in this chapter. The main components of the virion are shown in (Fig. 3). The most recent KSHV virion structure resolved by cryo-EM was reported in 2018 at a resolution of 4.2 Å [39]. The capsid comprises of 46 unique conformers of the major capsid protein (MCP/ORF25), the smallest capsid protein (SCP/ORF65) and triplex proteins (Tri1/ORF62 and Tri2/ORF26).

1.3 KSHV Associated Malignancies

KSHV is the etiological agent for the development of the endothelial malignancy Kaposi's Sarcoma (KS) and two lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's disease (MCD) [25, 28, 128]. All three diseases arise mainly in the context of immune deficiency due to HIV infection

or iatrogenesis, in addition to the endemic and classic forms of the disease. There are no standard treatment guidelines for KSHV-associated tumours, and current options include targeting cancers through surgical excision, chemotherapy and radiotherapy. Currently, the most effective treatment of AIDS-associated KS is highly active antiretroviral therapy (HAART), which works mostly through re-establishment of the patient's immune system to limit tumour progression [142]. Likewise, iatrogenic associated KSHV malignancies are treated by the removal of immunosuppressants to restore the patient's immune system, although this in turn can lead to graft rejection [133]. Immunotherapies have also demonstrated clinical efficacy for the treatment of MCD such as Rituximab (anti-CD20), Tocilizumab (anti-human IL-6 receptor) and Siltuximab (anti-IL6 chimeric monoclonal antibody) [127, 141, 153].

Substantial advances in the understanding of the KSHV life cycle and related diseases have been made since its discovery, although currently there are still no vaccines or effective direct therapeutic options available for the prevention or management of its associated pathologies. However, progress has been made in the identification of potential therapeutic targets and in subsequent development of treatments acting on both the lytic and latent life cycle of KSHV. Promising novel treatments include lytic induction therapy involving the efficient reactivation of all latently infected tumour cells into the lytic cycle while concurrently exposing the cells to lytic replication inhibitors [123]. Lytic replication inhibitors have been developed against a variety of viral and cellular targets. The only clinically trialed KSHV lytic inhibitors target the viral DNA-polymerase, for example acyclovir, valganciclovir and foscarnet sodium [20, 58, 140]. However, a number of inhibitors which target cellular proteins integral to the KSHV lytic life cycle have been developed which include inhibitors against Hsp70, UAP56 and mTOR [14, 111, 124, 125].

1.4 KSHV, Protein Folding and Molecular Chaperones

Molecular chaperones are a large group of proteins involved in the regulation of protein homeostasis in cells. Central to this function, is the ability of chaperones to control protein synthesis, folding, assembly, translocation and degradation [50]. The fundamental information controlling the three-dimensional structure of a protein is encoded in the primary amino acid sequence, but the linear chain needs to fold such that the amino acids assume the correct orientation in space to be functional [7]. Folding on a biologically relevant timescale in a crowded macromolecular environment in the cell requires molecular chaperones as catalysts. The largest group of molecular chaperones are members of the heat shock protein (Hsp) family and include a majority of isoforms which function as ATP-dependent machines within large complexes to support folding, prevent aggregation and regulate degradation. Chaperone reliance is increased in the presence of cellular stress which has dramatic deleterious effects on protein structure. The need for chaperone-regulated folding and degradation is also altered in human disease, including age-related

degeneration and cancer. Chaperone systems support malignancy by providing an environment that can withstand the associated environmental stresses (e.g. hypoxia, nutrient deprivation) and which can support increased demand for protein folding associated with high growth rates whilst simultaneously stabilizing oncogenic driver mutant proteins [50].

As cellular proteins rely on chaperone systems, so do viral proteins, particularly since the latter are often multifunctional and more structurally complex than cellular homologues. Viruses also tend to rapidly produce large quantities of particular proteins simultaneously and this leaves proteins vulnerable to aggregation and misfolding, making chaperones a necessity to stabilize and correctly fold viral proteins to their functional conformations [56]. Viral proteins are often exquisitely sensitive to perturbation of host chaperone systems, even if they are highly similar to human homologues. This is best exemplified by the comparison of the vSrc oncoprotein from *Rous sarcoma* virus with the human equivalent cSrc. The proteins display 98% sequence identity and yet vSrc is a stringent Hsp90 client relying on the chaperone for stability and protection from degradation, while cSrc is only slightly affected by Hsp90 inhibition [22, 96, 158]. Many viral proteins will interact with host chaperones, and viruses may specifically encode for viral proteins capable of sequestering and regulating chaperone systems. For example, the T antigen of SV40 encodes the J domain most commonly found in Hsp40 cochaperones which recruits host Hsp70 isoforms for virion assembly, viral replication and transformation [24, 82, 129]. Host chaperone systems provide the correct environment to support both the folding of viral proteins, as well as maintaining host biological processes required viral related processes.

The link between cellular stress and viral reactivation from latency [6, 13, 41, 55, 160, 161] further suggests that KSHV is closely associated with the molecular chaperone systems in the host cell. Many of the stresses that induce lytic replication, including hypoxia, oxidative and ER stress, have also been reported to increase expression of Hsp chaperones. It is therefore not surprising that several reports have identified roles for molecular chaperones in both the latent and lytic phases of the KSHV viral life cycle, predominantly focusing on the major molecular chaperones from the Hsp70 and Hsp90 families.

1.5 KSHV and Hsp90

Heat shock protein 90 (HSP90) isoforms are one of the best studied molecular chaperone families in eukaryotic cells. Hsp90 primarily binds to partially folded or unstable folding intermediates to retain these labile proteins until required for activity. Hsp90 interacts with over 300 proteins (for a comprehensive and regularly updated list please see the Picard lab website at https://www.picard.ch/downloads). Known anecdotally as ‘the cancer chaperone’, Hsp90 interacts with a significant number of client proteins important in cancer development, including a wide range of kinases, ligases and other signaling intermediates, many of which are mutated in

tumours [87]. HSP90 isoforms include the inducible Hsp90α, constitutively expressed Hsp90β [104], the endoplasmic reticulum (ER) Grp94/Gp96 [100] and the mitochondrial TRAP1 [5]. Cytosolic Hsp90β and Hsp90α are also secreted to the extracellular environment, where they function as chaperones, signaling molecules and immune regulators [23, 43, 51, 61, 64, 71, 135, 139]. Hsp90β is essential in the mouse [146], while Hsp90α is not [63].

The dependence of viruses on HSP90 appears to be universal, with Hsp90 fulfilling multiple roles, including acting as a receptor for viruses, directly binding to viral proteins [3, 48, 74, 163], assisting in the nuclear localisation of viral proteins [107] and acting as a prominent co-factor for viral replication [29, 56, 75, 83, 92, 119]. In particular, the largest group of Hsp90 viral client proteins are polymerases [37, 83, 107] and many of these, such as telomerase and DNA polymerase share features with their cellular counterparts, allowing Hsp90 to facilitate common mechanistic steps involving these viral proteins as alternatives to the human homologues [56]. In addition, Hsp90 and the associated chaperone Hsp70 have been identified as components of the KSHV virion purified from lytically infected B cells [18, 165].

Hsp90 has been considered a druggable target in cancer for many years [138]. The first line Hsp90 inhibitors focused on the natural ansamysin geldanamycin (GA) (and its derivatives 17-N-allylamino-17-demethoxygeldanamycin/17-AAG and 17-Dimethylaminoethylamino-17-demethoxygeldanamycin/17-DMAG) which bind the N-terminal Hsp90 ATPase domain and compete with ATP for binding [65]. The geldanamycin-derivatives have low solubility and stability and consequently recent studies have produced a range of pharmacokinetically improved and chemically distinct N-terminal inhibitors, including purine scaffold–based PU-H71 [76] and BIIB021 [95], resourcinol derivatives AUY922 [80] and KW-2478 [108], and fully synthetic NVP-BEP800 [101, 102]. The structural similarity of the Hsp90 isoforms, means that these compounds will target both cytosolic and organelle Hsp90 and result in degradation of labile client proteins which are often mutated cancer drivers. Despite promising *in vitro* and *in vivo* studies, results from clinical trials have been disappointing due to unacceptable side effects [110]. However, inhibition of Hsp90 in viral dependent cancers may still be a feasible option. Due to the heavy reliance on chaperone systems, viral replication may be sensitive to non-toxic concentrations of Hsp90 inhibitors [56], effectively reducing the minimum concentration required for activity and hence showing potential to ameliorate at least some negative side effects. Hsp90 inhibition (using 17-DMAG, AUY922, and PU-H71 in the nanomolar range) or shRNA-mediated Hsp90 depletion was effective at inducing apoptosis in a number of KSHV infected PEL cell lines [35, 109, 151, 152]. Consistent with this observation, BIIB021 and PU-H71 were preferentially cytotoxic towards PEL cells compared with other lymphoma types, and blocked the expression of cellular protein regulators of the cell cycle and apoptosis [62, 109]. Hsp90 inhibitors GA, 17-AAG and radicicol were capable of suppressing KSHV viral particle production at low concentrations [72]. Additionally, Hsp90 inhibitors downregulated levels of entry receptor Ephrin A2 (EphA2) and EphB2 proteins, which are receptors that facilitate KSHV infection and upregulate latent persistence [35]. Sangivamycin, a protein kinase C inhibitor,

decreased PEL cell viability compared to KSHV-uninfected B lymphoma cell lines, in part by suppressing Akt phosphorylation [147]. Akt activation has proved to be essential for PEL cell survival, as shown by Akt inhibitors, which suppress PEL cell proliferation compared to KSHV-uninfected cells [150]. Akt is an Hsp90 client protein [17], which explains the synergistic cytotoxic effect with co-treatment with the Hsp90 inhibitor GA and sangivamycin [147].

The efficacy of Hsp90 inhibition in KSHV infected cells is thought to be due to destabilization of key proteins which rely on Hsp90 for function. Hsp90 interacts with and stabilises both viral and human proteins required for the lytic and latent phases of the life cycle as well as infection, and Hsp90 inhibitors can inhibit proliferation of infected cells and induce degradation of viral proteins. Hsp90 interactions have been identified for a number of KSHV proteins by immunoprecipitation, including LANA, K1 and vFLIP [35, 151, 152]. In addition, isolation of complexes using the Hsp90 inhibitor PU-H71 conjugated to a resin identified a number of proteins, including but not limited to DNA polymerase (ORF K9), primase (ORF56), vIL-6 (ORF K2), vCyclin (ORF72), major and minor triplex capsid proteins (ORF25 and ORF62), and the envelope glycoprotein (ORF22), which putatively represent Hsp90 associated complexes and/or client proteins [109]. However, only a few of these interactions have been validated or studied in any mechanistic detail.

LANA, K1 and vFLIP have been partially characterised as Hsp90 clients. Immunoprecipitation studies have found latently associated nuclear antigen (LANA) isoforms to interact with a multitude of cellular proteins, including nucleotide-binding proteins, cytoskeletal components, chaperones and transcription factors [121]. A number of Hsp and chaperones, including Hsp90β (HSPC3), three Hsp70 isoforms (endoplasmic reticulum HSPA5/BiP, mitochondrial HSPA9 and cytosolic HSPA8/Hsc70) and two chaperonins (CCT1 and CCT3) were identified in LANA containing complexes isolated from stably transfected BJAB cells [35]. The Hsp96 (endoplasmic reticulum Hsp90) precursor and Hsp70 were also identified in LANA immunoprecipitates from a BC-3 cell line nuclear extract and Hsp90 and LANA co-localised in the nucleus of TIVE cells [34, 35]. The interaction with Hsp90 required the N-terminal domain of LANA, and both the interaction and expression levels of LANA were reduced upon Hsp90 inhibition, with LANA's nuclear distribution pattern being altered. Hsp90 inhibition induced ubiquitination and proteasomal degradation of LANA, inducing apoptosis and resulting in reduced tumour progression in a KS xenograft model [35]. These data strongly suggest that LANA is an Hsp90 client protein and is reliant on chaperone systems for stability and function.

Together with Hsp90β, the endoplasmic reticulum (ER) associated Hsp40 (DNAJB11) binds the N-terminal domain of the KSHV transmembrane glycoprotein K1 [151, 152]. K1 shares structural similarities with the human B-cell receptor (BCR) and is able to transform murine fibroblasts [131]. Hsp90 ATPase activity and chaperoning function is needed for optimal K1 expression, and Hsp90 or Hsp40 depletion by siRNA diminished K1 expression preventing it from promoting cell survival. The Hsp90α isoform binds to K1 without Hsp40 [151, 152], but the

involvement of Hsp40 in Hsp90β binding suggests a functional role for Hsp70 isoforms in stabilization of K1.

The interaction of Hsp90 with vFLIP (ORF71) was predicted based on pathway analysis of Hsp90 complexes isolated with resin-bound PU-H71 [109]. vFLIP is a viral homologue of the human protein, cFLIP, and both proteins are involved in blocking apoptosis via induction of NF-kB signaling [66]. vFLIP was detected by Western blot in the PU-H71 complexes, while the interaction of the cFLIP isoform, which is highly active at inducing NF- κβ signaling with Hsp90, was determined by immunoprecipitation. Inhibition of Hsp90 function with PU-H71 induced degradation of vFLIP and IKKγ in PEL and TIVE cells, in addition to reducing both Akt phosphorylation and NF- κβ transcriptional activity [109]. The Hsp90 inhibitor BIIB021 also blocked the interaction of vFLIP with the Iκκ complex subunits to block NF- κβ activity [62]. Interestingly, PU-H71 did not reduce LANA, vCyclin or vIL6 levels in treated PEL cells in this study. Furthermore, while PU-H71 inhibited NF-κβ signaling and significantly reduced tumour burden in a PEL mouse model, it did not trigger substantial spontaneous reactivation of the virus in the JSC-1 cell line model [109].

In addition to direct interaction with viral proteins, Hsp90 also regulates a number of key host signaling pathways that impinge on viral and tumour biology. A large majority of the host proteins identified in PU-H71 complexes from KSHV infected cells were related to apoptosis or autophagy, which suggests a causal role for Hsp90 in networks regulating survival of PEL. This is supported by the fact that PU-H71 synergised with an inhibitor of the Bcl2 family of proteins to induce apoptosis in PEL [109]. In particular, Hsp90, alone or in combination with co-chaperones, appears to play an important role in the NF-kB pathway. TRADD, TRAF2, Iκκα, Iκκβ, Iκκγ, p65/RELA, RELB, NF-κβ p50 and NF-κβ p52 were all identified in PU-H71 isolated complexes [109]. In lymphoma cell lines, including those with KSHV infection, Hsp90 helps activate the NF-κβ pathway by enhancing the association of NF-κβ with Iκβ kinase (IKK) [35], in a manner that involves the Hsp90 co-chaperones, Cdc37 and FKBP51 [73]. The Hsp90 inhibitors reduced Iκκ co-localization with Hsp90 [36] and blocked phosphorylation of Ser32 and Ser36 of inhibitor of κβ (Iκβ)α in KSHV-infected PEL cells. This stabilization ultimately induced the inhibition of the constitutive NF-κβ transcriptional activity upon which PEL proliferation is dependent, leading to caspase-induced apoptosis in KSHV-infected PEL cells [32, 72]. Notably, the fact that KSHV causes NF-κβ pathway activation and Hsp90 plays a regulatory role in this activation may suggest an underlying interaction between the KSHV proteins responsible for NF-κβ activation and Hsp90. An example is vFLIP, which associates with complexes containing Hsp90 and Iκκ. Additionally, targeting extracellular Hsp90 (exHsp90) reduces both TNF-α and KSHV induced NF-kB activation [44]. exHsp90 also induces MAPK activation and hence exHsp90 inhibition reduces KSHV-initiated MAPK activation to inhibit MEK and ERK phosphorylation which ultimately reduces KSHV transcription and virion release [119].

The early lytic product of ORF74, viral G-protein-coupled-receptor (vGPCR), is homologous to the human interleukin-8 receptors CXCR1 and CXCR2. vGPCR

activates G proteins to stimulate cell signaling pathways such as phosphatidylinositol 3-kinase (PI3K) and phosphoinoside-dependent kinase (PDK, an Hsp90 client) [99]. Like vGPCR, the latent and lytic expression of ORF K1 contributes to KSHV persistence by maintaining the replication and transcription activator protein RTA (ORF50). Additionally, K1 and vGPCR proteins are both associated with Hsp90 signaling pathways such as PI3K, Akt and mTOR pathways in both B cells and endothelial cells. Activation of K1 and vGPCR cause signaling cascades that promote cellular survival [151, 152]. The inhibition of Hsp90, and therefore its role in these pathways, is suggested to contribute to suppression of KSHV replication [72].

Soluble or cell surface extracellular Hsp90 (exHsp90) is a cofactor for activating intracellular signal transduction pathways and KSHV genes to support KSHV persistence [119]. exHsp90-CD91 (LRP1) interactions, for example, regulate signaling from KSHV's EphA2 receptor and also increase cell invasiveness in other tumour types [61]. Through interactions with CD91 and other cellular receptors, exHsp90 is significant in the immune response to virus-infected cells and their associated tumours [44]. This exHsp90-CD91 interaction is particularly prominent in the presentation of viral antigens by dendritic cells during the immune response to KSHV [15, 130]. Increased Hsp90 secretion, or exHsp90 interactions with protein clients at the cell surface may enhance specific immune responses to KSHV-infected tumour cells [44].

Because Hsp90 associates with more than half of the human kinome [134], it is likely that KSHV kinases, having a homology to human homologues, represent putative Hsp90 clients. Hsp90 interacts with the KSHV ORF36, which encodes a serine protein kinase (vPK) that is equivalent to human S6 kinase B1 (S6KB1) and is key in the development of malignancy and cell proliferation [19]. As confirmed by the protein-protein interaction split *Renilla* luciferase complementation assay (SRLCA), the Hsp90 interaction was shown to occur in the 215–257 aa region of ORF36 and the 110–180 aa region of Hsp90, which lies in the N-terminal domain [148]. vPK expression in HeLa cells was reduced upon Hsp90 inhibitor 17-DMAG treatment [132]. Together with Hsp90, the Hsp90 kinase specific cochaperone Cdc37 is an essential component of the Iκκ complex (the major kinase complex of the NF-κβ pathway) where it may be involved in latency regulation [32]. The latently expressed kaposin B contributes to the secretory phenotype in infected cells by binding and activating the stress-responsive kinase MK2, [38] potentially providing a role of Hsp90 in the kinase regulation at the latent phase. CDK6 is the major kinase partner of viral cyclin (vCyclin) in PEL cells [70]. vCyclin, expressed from the latent ORF72 gene, is the viral homologue of cyclin D and maintains latency by forming a kinase complex with CDK6 and modulating the cell cycle and cell proliferation [59, 90]. Its mRNA transcripts are also reduced with Hsp90 inhibition [119]. KSHV vCyclin and cellular CDK6 kinase phosphorylate nucleophosmin (NPM) on threonine 199 (Thr199) in *de novo* and naturally KSHV-infected cells, as well as in primary KS tumours. This phosphorylation promotes interaction between NPM and LANA, and also identifies a role for Hsp90, many of whose clients comprise of kinases including CDKs [122].

1.6 Hsp70 and KSHV

Hsp70 isoforms are ATP-dependent chaperones which function in *de novo* and stress related protein folding, as well as a range of other cellular processes. There are 13 Hsp70 isoforms in the human genome expressed in all subcellular locations of the cell [68]. Hsp70 isoforms exhibit low affinity for client proteins when in an ATP bound form. The low basal ATPase activity of Hsp70 is stimulated by the conserved HPD motif in the J domain of partner Hsp40 cochaperones [155]. Hsp70 isoforms have a prominent role in viral protein folding where they associate with 15–20% of newly synthesized proteins during their biogenesis [137]. Generally, Hsp70 homologues are active in plant and animal viruses in viral replication, assembly, disassembly, and cell-to-cell movement [4]. In KSHV, Hsp70 isoforms are important for the assembly and activation of preinitiation complexes at the origin of DNA replication, as has been observed in several prokaryotic species, eukaryotes such as yeast, and the HPV and HSV viruses [93, 136]. Virally associated Hsp70 isoforms include the constitutively expressed heat shock cognate protein 70 (Hsc70/HSPA8) [121] the stress-inducible Hsp70 (Hsp72/HSPA1A), the endoplasmic reticulum isoform BiP (Grp78/HSPA5) and the mitochondrial Grp75 (HSPA9) [14]. These are required for KSHV lytic replication to stabilize essential viral proteins, activate viral promoters, and form viral RTCs [14].

Immunofluorescence analyses show that Hsp70 isoforms are redistributed from the cytoplasm to the nucleus on the periphery of KSHV RTCs, where they form nuclear foci during early lytic replication. Co-localization of the Hsp70 isoforms with viral DNA supports a role in viral DNA replication, capsid assembly and cell viability. Hsp70 isoform recruitment to RTCs could enable sequestering of misfolded or modified proteins away from RTCs, or remodelling or degradation of proteins to delay cellular pathways, such as apoptosis [14].

After Hsp90, Hsp70 is considered the second druggable chaperone of oncogenesis and consequently a number of new Hsp70 inhibitors have been developed. Inhibition of Hsp70 isoforms focuses on direct targeting of the Hsp70 ATPase (such as VER155008) [101, 102] or inhibition of protein complexes in the Hsp70 ATPase cycle (such as JG98, which targets the Hsp70-BAG interaction; and MAL3-101 based compounds targeting the interactions between Hsp40 and Hsp70 [2, 91]. In KSHV infected cells treated at non cytotoxic concentrations of the Hsp70 inhibitor VER155008, the levels of the early lytic protein ORF57, late minor capsid proteins and the RTA protein were reduced. Similar to the effects of Hsc70 depletion, which decreases RNA levels of many lytic and latent viral transcripts, VER155008 inhibited KSHV transcription, translation and lytic replication [14]. The simultaneous inhibition of both Hsp90 and Hsp70 is a suggested antiviral strategy, as chaperone inhibition would be detrimental both to cell survival and virus specific functions that are dependent on chaperone isoforms. Combining Hsp70 and Hsp90 inhibitors could eradicate latent harboured KSHV [14] and potentially overcome the resistance-associated upregulation of Hsp70 and other stress pathways observed with N-terminal Hsp90 inhibition.

HSPA5 is an endoplasmic reticulum (ER) Hsp70 molecular chaperone protein that binds to newly synthesized proteins during their translocation into the ER and serves as a core regulator of ER homeostasis [149]. During the late stages of the KSHV lytic cycle, surplus viral proteins are synthesized through the ER and consequently modulation of ER homeostasis is critical for progression of the KSHV lytic cycle. HSPA5 is critical during KSHV lytic replication and its downregulation can affect the completion of the KSHV lytic cycle because of the essential role in ER homeostasis [11, 126]. The KSHV tegument protein ORF45 and glycoprotein ORF47 upregulate the ER HSPA5 isoform upon viral reactivation, while HSPA5 knockdown impaired the viral lytic cycle [27]. ORF47/45-A and ORF47/45-B are two novel spliced gene products of KSHV ORF47-ORF46-ORF45 gene locus, which are localized in the ER, and both upregulate HSPA5 expression and induce phosphorylation of p90 ribosomal S6 kinase and extracellular signal-regulated protein kinase (RSK/ERK) during KSHV lytic replication. A 38-aa region in ORF47/45 is responsible for the upregulation of HSPA5 by promoting a specific unfolded protein response (UPR) signaling pathway, while a 103-aa region is responsible for the phosphorylation of RSK/ERK [27]. Therefore, the activation of HSPA5 is an important molecular event required for KSHV lytic replication.

The majority of data available to date suggest a role for Hsp70 isoforms in lytic KSHV replication. However, Hsp70 activity may also be relevant to latent KSHV infection, through its binding to the latently expressed kaposin ORFK12C which amongst the genes ORF59 and PAN, is one of the most upregulated genes responsive to RTA induction [60]. Tandem mass spectrometry (MS/MS) has been widely used to identify viral protein interactors. The MS/MS analysis of critical latent protein LANA has revealed its interaction with Hsp70 amongst other chaperones [34]. In the related murine gammaherpesvirus 68 (MHV68), Hsc70 interacts with the LANA homologue and recruits it to infected cell nuclei. Inhibition or knockdown of Hsc70 impairs MHV68 lytic replication and viral protein expression [121]. Furthermore, KSHV LANA and the EBV LANA-analogue EBNA interact with selected host Hsp70 chaperones and Hsp40 cochaperones [35, 162].

1.7 Endoplasmic Reticulum Chaperones and KSHV

In addition to the regulation of ER Hsp70, KSHV regulates and interacts with other ER chaperones. The early KSHV gene K4.2 encodes an ER resident protein that interacts with and inhibits the plasma cell-induced-ER resident protein (pERP1) [156]. As well as a lytic enhancer and binder of KSHV RTA [30, 33], K4.2 enhances glycoprotein expression, and interferes with immunoglobulin assembly to weaken the adaptive immune response. pERP1 is highly expressed in MCD and KS lesion cells which strongly suggests a role in the KSHV life cycle and malignancy, potentially classifying it as a tumour marker and target for future antitumour treatments [86].

The KSHV viral homologue vIL-6 functions actively in the ER in the autocrine promotion of proliferation and survival of latently infected PEL cells [30, 33]. KSHV latent vIL-6, like its human homologue, can bind the gp130 receptor [53] but unlike cellular IL-6, vIL-6 can signal without the involvement of the gp80α subunit of the IL-6 receptor [31] to activate the JAK/STAT, MAPK and PI3K/Akt cellular signaling pathways [98, 112]. Binding of vIL-6 to gp130 produces angiogenic factors that enhance vascular remodelling and permeability and activates the aforementioned pathways in many cell types that are unresponsive to cellular IL-6, contributing to PEL, MCD and KS phenotypes [98].

KSHV's viral glycoprotein H (gH), encoded by the lytically expressed ORF22 with its chaperone gL (ORF47) controls the attachment and entry of KSHV into target cells. HeLa cells transfected with the gL expression plasmid showed gL colocalisation with the ER's luminal chaperone calreticulin [69], however, the introduction of the viral gH relocalised gL away from the ER, demonstrating a host-like interplay of viral chaperones, highlighting ORF22's role in inhibiting the colocalization of calreticulin and ORF47. Interactions of ORF K1 with calreticulin have been suggested previously [88], where K1 retains B cell antigen receptor (BCR) complexes in the ER to prevent host immune response.

KSHV viral interleukin-6 (vIL-6) (encoded by ORF K2) is a KSHV latent and lytic protein which induces tumorigenesis and inflammation by modulating host cell signaling pathways. All KSHV-associated diseases have appreciable levels of vIL-6; and vIL-6 can interact with other proteins in the ER of the host cells [9]. The retention and stability of vIL-6 in the ER is key to most of its cellular functions. The ER membrane protein calnexin maintains the folding and cellular distribution of vIL-6 [30, 33] while the gp130 signal transducer promotes vIL-6 ER exit, which is consistent with a recent study identifying more than 40 unique calnexin peptides and 9 unique gp130 peptides interacting with vIL-6 [57]. Calnexin depletion corresponds to a decrease in intracellular vIL-6 levels but did not lead to an overall reduction in vIL-6 secretion. Transfection of a gp130 expression vector into calnexin-depleted cells rescued vIL-6 expression. The interaction of IL-6 with calnexin was specific to the viral isoform, since the human homologue (hIL-6) did not bind calnexin [30, 33].

Affinity purification followed by mass spectrometry analysis of vIL-6 to identify its interacting partners revealed hypoxia-upregulated protein 1 (HYOU1) as a novel interacting partner of vIL-6. HYOU1, also known as 150-kDa Oxygen-regulated protein 1 (ORP150), is an ER-localized molecular chaperone whose expression is upregulated during hypoxia and ER stress in cells [113]. The life cycle of KSHV is greatly influenced by hypoxia, suggesting HYOU1 could be relevant to KSHV biology and pathogenesis. Interestingly, the expression of endogenous KSHV vIL-6 protein during latent and lytic cycles is modulated by the expression of HYOU1. Knockdown of HYOU1 in BCBL1 PEL, latent TREx BCBL1, lytic TREx BCBL1-RTA and HEK293 cell lines reduced expression of intracellular vIL-6 protein but LANA, RTA and K8.1 levels were unchanged [57]. vIL-6 induced migration of endothelial cells and vIL-6-mediated survival of serum-deprived endothelial cells was dependent on the expression of HYOU1 [57]. The expression of HYOU1 positively influenced the binding of vIL-6 to gp130 thereby promoting

vIL-6-mediated Signal Transducer and Activator of Transcription 3 (STAT3) signaling [30, 33]. STAT3 signaling is known to play several pro-carcinogenic roles during KSHV infection, including promoting cell proliferation and angiogenesis in a KSHV-associated cancer mouse model [97] and has been identified as an Hsp90 client [116]. In PEL cell lines, STAT3 activation contributed to the differentiation, migration, proliferation, angiogenesis and survival of the tumour cells. These established functions of STAT3 in KSHV-associated diseases are promoted by STAT3-mediated transcriptional activation of survivin, DNMT1 and CEACAMI [8, 84, 157].

1.8 KSHV and Nucleophosmin

During latency, LANA effectively prevents the transcription of ORF50, a KSHV lytic reactivation gene which codes for replication and transcription activator (RTA) protein and a promoter of the progression of KSHV latency to lytic replication [94]. vCyclin is another viral gene expressed during KSHV latency and shares the same promoter element with LANA during transcription [45]. vCyclin forms an effective kinase complex with hCDK6 (a known Hsp90 client protein) and this vCyclin-CDK6 complex phosphorylates several cellular protein substrates such as pRb, p27KIP1, p21CIP1, ORC-1, CDC6, caldesmon, and Bcl-2 [79, 105, 122, 145]. Sarek et al. [122] showed that vCyclin-CDK6 also phosphorylates a nucleocytoplasmic chaperone and phosphoprotein called nucleophosmin (NPM) at Thr199 in KSHV-infected cells. This phosphorylation of NPM by vCyclin-CDK6 promotes the binding of NPM with LANA, recruiting histone deacetylase 1 (HDAC1) for an interaction with LANA which inhibits KSHV lytic gene expression by promoting the de-acetylation of LANA [122]. Also in the nucleus, the nucleosome assembly protein, NAP1L1 is a histone chaperone and nucleocytoplasmic shuttling protein that transports histones to the nucleus and interacts with LANA N- terminal domain [67].

1.9 Putative Candidate KSHV Chaperone Clients Inferred from Proteomics Datasets

Currently, mechanistic detail on these interactions and the biological consequences thereof is restricted to selected host chaperones (namely Hsp70 and Hsp90 isoforms) and to key viral proteins. Herpesviruses use mimicry that forces the host cell to produce viral proteins that are structurally and functionally similar to host counterparts and which can disrupt homologue functionality and contribute to KSHV-related diseases [120]. Some notable KSHV viral mimics such as vPK, vIL-6, vCyclin, vGPCR and K1 have already been associated with human chaperones,

but it is likely that other KSHV host homologues may similarly interact. Based on this, and the understanding of chaperone function in cells, we predict that important classes of KSHV proteins including ubiquitin ligases, kinase and signaling mimics are good candidates for evaluation as chaperone clients.

A KSHV based proteomics interactome suggests that a large number of KSHV proteins may interact with host chaperone complexes (Figs. 4 and 5) [42]. Notwithstanding the need for independent experimental validation, our analysis of these supplementary data predict that as many as 88 KSHV proteins can be isolated in complex with at least one chaperone isoform, albeit with differing confidence. The interactome analysis identified a number of the published interactions between KSHV and host proteins, including LANA, K1 and vFLIP, in addition to novel interactions. The identification of interacting Hsp from different classes in complex with similar KSHV proteins suggests functional Hsp chaperone partnerships, and complexes which are consistent with known chaperone interactions. For example, of the 13 human Hsp70 isoforms, 10 were identified with one or more KSHV proteins. In addition, a number of Hsp40 cochaperone isoforms were identified as interacting with similar KSHV proteins to Hsp70. The Hsp40 isoforms identified were predominantly those with promiscuous or broad client binding specificity (including DNAJA1-4, DNAJB2, DNAJB4, DNAJB11 and DNAJB12) and which are involved in Hsp70 mediated *de novo* and stress related protein folding pathways in all compartments of the cell [81]. Consistent with the importance of the ER and UPR

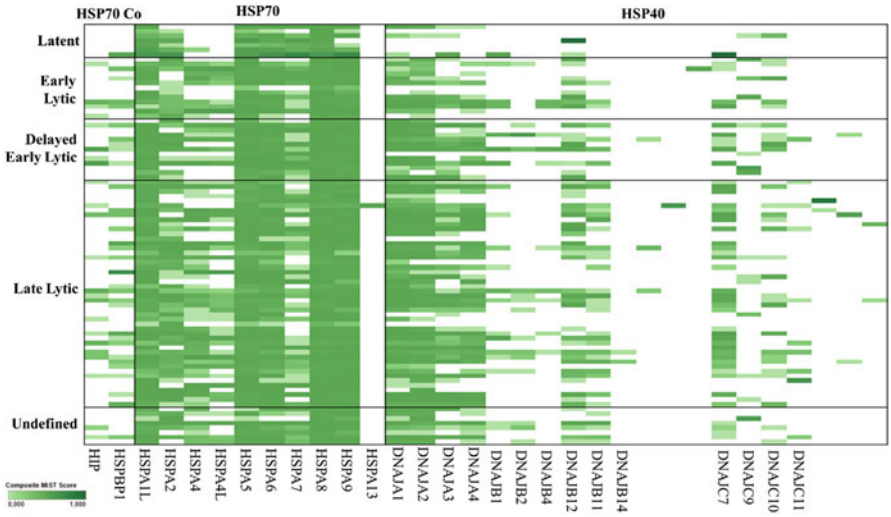

Fig. 4 Heatmap illustrating interaction intensity between KSHV proteins and human Hsp70 and cochaperones predicted from proteomics analyses. This figure shows the overview of putative interactions between Hsp40, Hsp70 and other Hsp70 co-chaperones (Hsp70 co) with KSHV proteins, where major classes and isoforms are indicated. The composite MiST score indicates the interaction intensity. Data extracted from published supplementary proteomics dataset [42]. The full-size image (zoomable to reveal names of individual proteins on both axes) is available online as supplementary data

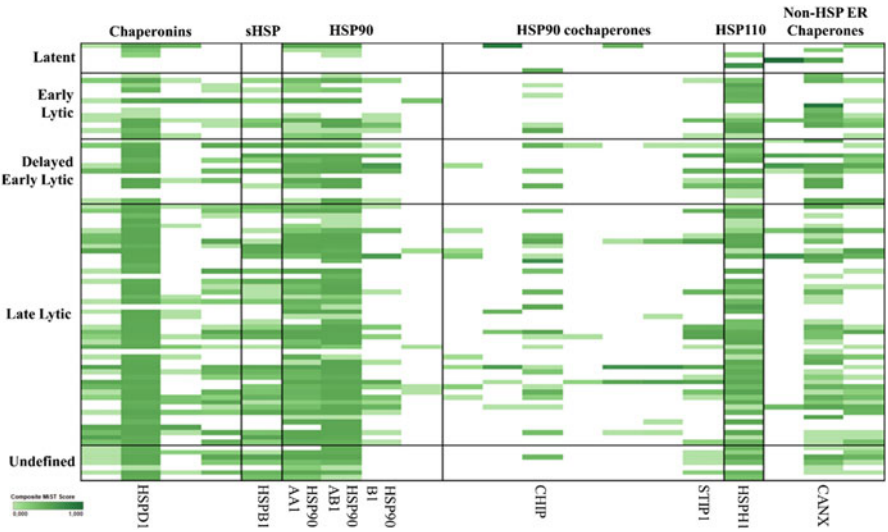

Fig. 5 Heatmap illustrating putative complexes between KSHV proteins and Hsp90, Hsp110, chaperonins and associated cochaperones predicted from proteomics analyses. This figure shows the overview of putative interactions between chaperones with KSHV proteins, where major classes and isoforms are indicated. Data extracted from published supplementary proteomics dataset [42]. The composite MiST score indicates the interaction intensity. The full-size image (zoomable to reveal names of individual proteins on both axes) is available online as supplementary data

in KSHV biology, a number of ER chaperones were identified in putative complexes with KSHV proteins, including DNAJC1, DNAJC10, DNAJB11 and DNAJB12, calreticulin, calnexin and Hsp47. Of the Hsp90 isoforms identified, most KSHV proteins interacted with the essential Hsp90β isoform (HSP90AB1) followed by the stress inducible Hsp90α isoform (HSP90AA1) and then the ER Hsp90 isoform Gp96 (HSP90B1). It is interesting to note the potential for isoform specific interactions suggested by the interactome, since Hsp90β and Hsp90α are known to have some unique clients and isoform selective inhibition of Hsp90 is currently being pursued as an improved anti-cancer strategy [104]. Additionally, Hsp90 cochaperones STIP1 and CHIP interact with a number of the Hsp90 interacting proteins, consistent with the role of these chaperones in balancing protein folding and degradation pathways respectively [12, 49]. The possible interaction with between KSHV OR22 and the small Hsp, Hsp27 (HSPB1), is strengthened by the fact that Hsp27 plays a role in virus induced oxidative stress in the gammaherpesviruses herpes simplex virus 1 (HSV1) which showed enhanced replication in the presence of Hsp27 (Fig. 5) [103].

A third of human E3 ligases have Hsp90 associations [134], and Hsp70/Hsp90 complexes play important roles in protein degradation through interactions with E3 ligase and cochaperone CHIP that directs chaperone bound proteins to proteasomal degradation [49]. KSHV encodes ubiquitin ligases and deubiquitinating enzymes (DUB) for exploitation of the ubiquitin proteasome system to facilitate viral gene

expression and replication by removing ubiquitin chains from proteins and interfering with cellular pathways. Particularly, KSHV is able to modulate the host cell small ubiquitin-like modifier (SUMO) system to overcome the host antiviral immunity that limits KSHV lytic reactivation [52]. Consistent with this, ORFs K3 and K5U are identified in putative complexes with both Hsp90 and CHIP (Fig. 5). Additionally, cellular client E3 ligases may indirectly link interacting KSHV proteins to Hsp90 and Hsp70 chaperone systems, or E3 ligases encoded by KSHV may remodel chaperone complexes associated with protein turnover [49]. Another large family of Hsp90 client proteins are kinases, which depend on Hsp90 and/or the kinase specific chaperone/cochaperone Cdc37 for functionality [1, 134]. It is likely that KSHV kinases may require Hsp90.

KSHV viral interferon regulatory factors (vIRFs) 1–4 are encoded by the respective late lytic ORF K9, ORF K11, ORF K10.5 and ORF10 genes. vIRFs are homologous to cellular IRFs [54] and function within the RTA to activate lytic promoters. The vIRFs disrupt the antiviral interferon (IFN) response by inhibiting transcription of inflammatory signals. vIRFs usually bind cellular IRFs and inhibit their ability to activate transcription, dysregulating the IFN antiviral response and preventing cycle arrest to increase the oncogenic potential of KSHV-infected cells [85]. vIRF3 is constitutively expressed in the nuclei of KSHV-infected hematopoietic cells (PEL and MCD) and not in KS lesions. vIRF3 is one of the few viral genes expressed in nearly all latently infected PEL cells, where it is crucial for their survival; its knockdown by RNA interference reduces proliferation and induces apoptosis in cultured PEL cells [16]. hIRF1, hIRF2 and hIRF3 are known Hsp90 client proteins, suggesting that vIRFs may also require Hsp90.

Viral polymerases are the most common Hsp90 client proteins, and an interaction between the KSHV DNA polymerase (ORF9) and Hsp90 and Hsp90 cochaperones STIP1 and CHIP is predicted by the proteomics data. Given the central role of Hsp90 in viral polymerase function, and the targeting of DNA polymerase by anti-KSHV agents, it would be interesting to confirm this interaction and determine if Hsp90 inhibitors synergise with the lytic inhibitors like acyclovir, valganciclovir and foscarnet sodium currently being trialed for KSHV therapy.

2 Conclusions

A number of important KSHV proteins rely on host chaperone systems for folding and stability, and inhibition of chaperones *in vitro* and in mouse models provides proof-of-concept that targeting this cellular system may be effective as a treatment for KSHV-related malignancies, either alone or in combination with other inhibitors. Chaperones like Hsp90 and Hsp70 function by interaction and stabilization of viral proteins, as well as involvement in host processes that impinge on virus replication and oncogenesis. There is evidence for regulation of both lytic and latent phases of KSHV infection. There however remains a need for a more comprehensive and integrated analysis to understand mechanistic details of chaperone requirements and

complexes during different phases of KSHV infection, and during malignancy. In particular, analysis of the role of cochaperones in KSHV biology may be important, since numerous cochaperones fine-tune chaperone systems in ways that may be more amenable to selective intervention. In addition to being potential therapeutic targets, viral proteins also represent interesting and important models to understand principles of protein folding and requirements for chaperone involvement, particularly in the case where viral and human homologues exist and have differing stability and chaperone requirements. Therefore, focusing on comparative analyses of folding of viral and host homologues may provide useful insight into principles governing chaperone client selection and overall protein stability.

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Role of Heat Shock Factors in Diseases and Immunity

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Abstract

Introduction Studying the role of HSFs during external stimuli induced conditions, diseased conditions and host pathogen interactions is considered to be much needed one due to their rapid involvement and regulation. Therefore, the roles of HSF in diseases were summarized in this chapter.

Methods Journals and data bases (Pubmed, Scopus and Google Scholar) were surveyed with the keywords "HSF", "Diseases" and "Immunity". Pertinent works were chosen for discussion.

Results Protein homeostasis is one of the important molecular mechanisms required to maintain the cellular systems in a balanced manner. Various factors such as oxidative stress, increased temperatures, heavy metals and pathogenic invasion affect the protein homeostasis in which the heat shock response plays a crucial role to sort out the condition. Many reports on heat shock related responses have revealed the involvement of two major classes of chaperone molecules such as Hsp and HSF. HSF are transcriptional regulators of heat shock related genes/proteins during both normal and cellular stresses thereby they plays a significant role in cellular and protein homeostasis.

Conclusion In this chapter, we have discussed about the involvement of HSFs in protein and cellular homeostasis in connection to their functions, organizations, and role in diseases. Altogether, we suggest that HSF is considered to be one of the most crucial phenomena in various biological processes and molecular functions including immunity.

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Abbreviations

ALS	amyotrophic lateral sclerosis
AMPK	5′-AMP-activated protein kinase
CDK4	cyclin-dependent kinase 4
DNA	Deoxyribonucleic Acid
HIF1α	hypoxia-inducible factor 1α
HSE	heat shock element (s)
HSF	heat shock factor (s)
HSP	heat shock family
Hsp	heat shock protein (s)
HSR	heat shock response (s)
mRNA	messenger RNA
MS	mass spectrometry
PTM	post translational modification (s)
ROS	reactive oxygen species
SBMA	spinal and bulbar muscular atrophy
SOD 1	superoxide dismutase 1
SUMO	small ubiquitin-like modifier
TauT	taurine transporter
UPS	ubiquitin mediated proteolysis
α-syn	α-synuclein

1 Introduction

All the eukaryotic organisms respond to environmental factors such as oxidative stress, temperature, infection, etc., by eliciting variety of immune and HSR. During temperature fluctuation, a typical response from most of the organisms takes place, and to rectify the condition, Hsp are generally functioning as mediators. Even though, the involvement of heat shock response plays a major role in combating the environmental stimuli, the activation/inactivation relies on the HSF, transcriptional regulators of Hsp [17]. These chaperone molecules appear to have multifaceted functions in controlling the cellular integrity and homeostasis [34, 41]. For example, it is known that HSF from human beings and in particular the two most-characterized HSF family members – HSF1 and HSF2 exhibit an unanticipated complexity in their structure, domains, DNA-binding, PTMs, interacting partners and regulation which deciphers that they have various roles against wide range of stresses. Although, the functions of Hsp and their activating factors, namely, HSF have remained obscure since the discovery of the heat shock phenomena due to the

complexity of regulatory mechanisms [8]. Thus, studying the regulation of HSF will provide a basic idea of molecular and regulatory mechanism during any external stimulus associated to heat stress. In this milieu, this chapter summarizes the basic functions, characteristics, involvement of HSF in infections and diseases.

1.1 Heat Shock Factors – Functions, Characteristics, Organization and Role in Diseases

1.1.1 Heat Shock Response and Importance of HSF

Typically, heat shock responses and their regulations are under the control of a conserved regulatory switch/mechanism referred to as the HSF. Indeed, the conservation of Hsp and HSF between many eukaryotic organisms suggests that they had an ancient relationship that was crucial for survival under certain circumstances throughout the evolution [51]. Basically, synthesis of Hsp is regulated by interaction of the HSF transcription factors with HSE present in the promoter regions of the associated heat shock gene [25, 49]. The first defined evidence for a specific HSF that can bind to HSEs and regulate the Hsp expression was obtained through DNA–protein interaction studies in *Drosophila melanogaster* nuclei [38]. More studies demonstrated that, in contrast to a single HSF in invertebrate organisms, multiple forms of HSF are expressed in vertebrates and plants [1]. Consequently, the complexity of heat shock response is directly/indirectly driven by the HSF which could make them to play a vital role in wide range of cellular mechanisms ranging from oxidative stress to pathophysiological activities (Fig. 1). The exposure of organisms against diverse array of stresses including an exposure to heavy metals, infection with viruses and bacteria, pharmacologically active small molecules, oxidants and amino acid analogues appear to trigger the heat shock response in connection with the involvement of HSF, Hsp and HSE. Common to these sorts of stresses, the effects on protein folding, protein biosynthesis, translocation and assembly into the native protein or protein complex are tightly regulated by HSF [4]. Accordingly, through the elevated synthesis/regulation of required molecular chaperones, rapid and precise heat shock response will get activated against the specific environmental and physiological stress signals by repairing the protein damage (such as unfolding) to reestablish the protein and cellular homeostasis [29].

1.1.2 Organization and Regulation of HSF

Generally, HSF binds to a DNA sequence specific motif HSE, which is characterized by an arrangement of the motif tandem repeats “AGAAN”. Many copies of the HSEs are found in the promoters of heat shock genes in most of the eukaryotic organisms. The human genome encodes six HSF isoforms namely HSF1, HSF2, HSF4, HSF5, HSFX and HSFY [17]. HSF1 and HSF2 are the well studied isoforms in the HSF

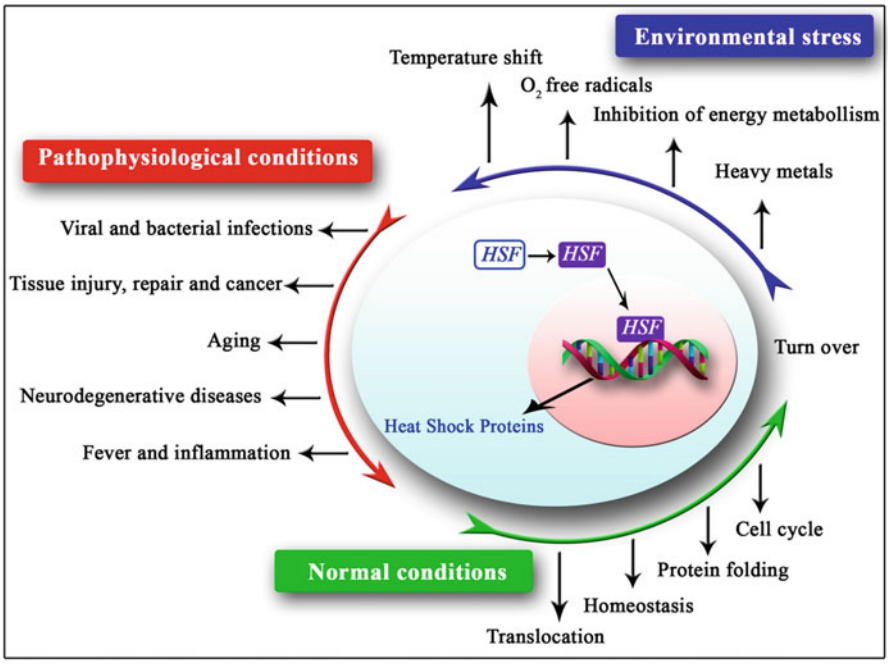

Fig. 1 Overview of HSF. The image illustrates the role and involvement of HSF on (i) how cells triggers the heat-shock response to regulate the transcription of genes that encode molecular chaperones and heat-shock proteins; (ii) cytoprotection against disease, infections and (iii) translocation, protein folding, and protection against the misfolded proteins

family because of their recognized roles in the stress responsive gene expression and association with immunity and disease [1]. However, the exact regulatory roles of other HSF isoforms have not been explored yet.

The activation cycle of HSF at particular stimuli is a multistep and highly coordinated process incorporating sequence specific HSEs that tune each and every step of the cycle. The most studied HSF1 and its mode of activation is illustrated in Fig. 2. During normal growth conditions, HSF1 exists mostly in an inactive form, possibly as a monomer [2]. In response to external stimuli, HSF1 converts into a DNA-binding-competent form, which is found to make an active homotrimer (trimerization of HSF composed of three identical units). This sort of activation results in accumulation of active HSF in the nucleus due to strong bipartite nuclear localization signals thereby they undergo hyper-phosphorylation events. Nevertheless, it is likely to be that HSF1 is activated through different signaling mechanisms depending on the type of stimuli, as it can differentiate between temperature mediated stress and other type of stresses at normal conditions [32]. Although, the natural expression of heat shock genes during differentiation and development of cells are well studied in many animal species, but the underlying

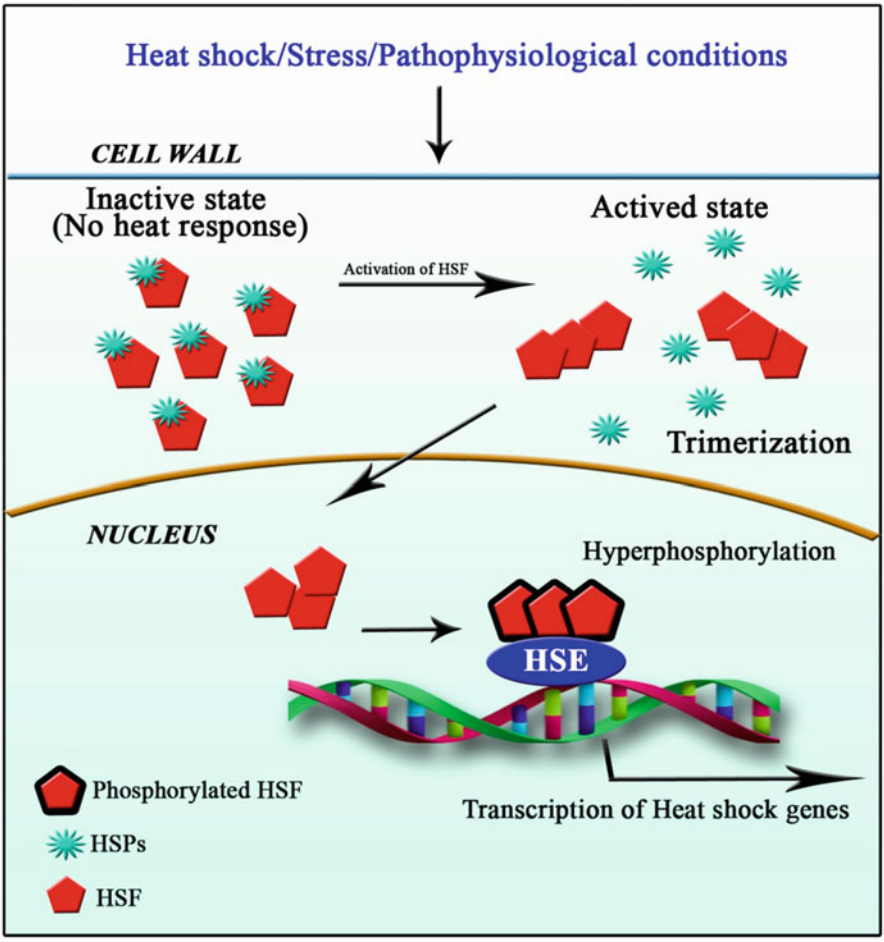

Fig. 2 Activation of HSF. The image represents the activation of heat shock response in connection with Hsp and HSF. During stressed conditions, inactivate HSF (monomers) turns to active form by forming HSF trimers. Subsequently, the active forms of HSF undergo hyperphosphorylation to elicit the transcription of other heat shock responsive genes to control the condition

mechanisms responsible for this regulation during development and differentiation are poorly understood and yet to be studied in detail.

1.1.3 PTMs and HSF

It is well known that PTMs are the regulatory switches of a protein. Almost, all the heat shock responses require the PTM associated changes to either activate or inactivate the players involved in the cascade. A MS study by Xu et al., revealed

that many PTMs play a crucial role in the regulation and expression of HSF [9, 52]. Phosphorylation, acetylation, SUMOylation, ubiquitination, etc., sites were present in the HSF which were explored using Tandem MS studies. In fact, these PTMs are thought to influence the regulation at every step of the HSF activation cycle due to the identification of PTM sites in large number. Phosphorylation of HSF1 in human is strongly linked to normal metabolism and any defect in the phosphorylation levels modulates the existing conditions. Specifically, AMPK, which plays a critical role in the cellular energy metabolism, directly inactivates the HSF1 under the unique metabolic conditions of cancer and during metabolic stress [14, 22]. Even though, novel PTM sites of HSF have been identified and explored with the use of high throughput proteomic technologies, the functions and characteristics of many of these PTMs are still unclear and yet to be studied at *in vivo* conditions. Thus, exploring the HSF and their responsible PTMs attract researchers worldwide to unveil the mystery during normal and external stimuli induced conditions.

1.1.4 Importance of Small Molecules in HSF Activation

Several endogenously and exogenously occurring small molecules have reportedly activate HSF especially HSF1 [50]. In many instances, those small molecules were found to be the inducers of HSF1 transcriptional activity. Certainly, the chemical properties of these small molecules have provided further insights and activation of the heat shock responses. Especially, sulfhydryl reactivity is a typical feature of several HSF1 activators, affects the function and its interacting regulatory proteins such as SIRT1, Hsp90, or other upstream regulatory kinases [15]. In addition, HSF1-mediated transcriptional activation is induced by endogenous electrophilic nitrated lipids as well as a,b-unsaturated aldehydes [E.g. acrolein, 4-hydroxy-2-nonenal, 15-deoxy-D- prostaglandin J2 (15d-PGJ2) and 10-nitro-octadecenoic acid (nitro-oleic acid)]. Electrophilic and ROS can also stimulate the activation of HSF1, which are formed during pathophysiological and normal physiological processes [33]. Recently, the study of small molecules mediated regulation of HSF is emerging as a trend among the researchers worldwide which strongly validates the involvement of compounds in addition to other factors such as PTMs.

1.1.5 Relationship Between HSF, Diseases and Immunity

For the past few decades, the importance of heat shock response in diseases and immunity has been studied very critically. Indeed, HSF have vital roles in brain development and function in regulating genetic programs of neuronal migration and the formation and maintenance of neuronal synapses and the resistance against proteotoxic stress [21]. Additionally, the modulation of HSF activity with age-related diseases such as neurodegeneration has been widely documented for the past decades. It is known that neurodegenerative diseases are closely associated

with differential expression of the protein control machinery (misfolding of crucial proteins). Though, the differentially regulated HSF do not cause neurodegenerative diseases directly, rather it appears to cause protein aggregation and misfolding by decreasing the chaperone expression, thereby it causes poor protein quality control, neuronal cell death and dysfunction and disease progression [37]. Accordingly, various studies conducted in cell lines, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus* models of neurodegenerative diseases clearly demonstrated that enhanced protein-folding capacity with elevated level of HSF expressions, as well as chaperone molecules and other targets have significant therapeutic potentials [37].

HSF and Cancer

Elevated levels of molecular chaperone expression have been experimentally observed in many cancers with increased metastatic potential and resistance to drug therapy. The expression of both HSF1 and HSF2 has been identified to be modulated during cancer with considerable evidences indicating the distinct roles in tumorigenesis [12]. In most of the cases, HSF1 is considered to be the master regulator of heat shock response in eukaryotes, which is highly conserved protective mechanism among the other HSF. HSF1 function also enhances the survival under many pathophysiological conditions including bacterial infections and cancer. But, how it directly involves in cancer and malignancy remains mostly unknown. It is proposed that, key cancer-related proteins such as AKT, p53, BCR–ABL1 fusion, RAF1, CDK4, ERBB2, Cyclin D, HIF1α and hormone receptors are greatly dependent on the molecular chaperones for their stability and activity. And also, it is not surprising that the alterations in HSF1 and other HSF have an impact on oncoprotein function and abundance [12]. For example, a study reports that inhibiting or eliminating the HSF1 protects mice from tumors induced by hotspot mutation in the tumor suppressor p53 protein or mutations in the oncogene, RAS. In cell culture also, HSF1 supports malignant transformation by coordinating a large network of core cellular and molecular functions such as survival, proliferation, glucose metabolism and protein synthesis. Certainly, these effects of HSF1 on oncogenic conversion are not limited to mice systems alone; diverse origins of human cancer lines also show greater dependence on HSF1 function to maintain the protein homeostasis, survival and proliferation than their non-transformed equivalents. At the same time, as it enhances the organismal survival under most conditions, HSF1 has the opposite effects also in supporting the fatal occurrence of cancer cells [13].

HSF and Neurodegenerative Diseases

By providing a rapid response against thermal stress, the heat shock response coordinated by HSF has protective effects during numerous pathophysiological conditions including neurodegenerative diseases [2]. The neurodegenerative

diseases/disorders such as Amyotrophic lateral sclerosis, Huntington disease, Parkinson's disease, Alzheimer's disease, etc., have been reported with the involvement of molecular chaperones in their disease progression. A short description about each disease is given in this section.

1. Huntington Disease and HSF

Abnormal development of polyglutamine (polyQ)-encoding regions cause 14 neurodegenerative diseases among which the most common are Huntington disease, spinocerebellar ataxia and SBMA. Generally, abnormal level of polyQ causes the protein misfolding and aggregation, leading to death and cellular dysfunction [19]. In this aspect, reports suggest that Hsp and HSF1 may serve as excellent drug targets for Huntington disease therapeutics [19]. An antibiotic called geldanamycin (antitumor agent) is known to regulate HSP90 by binding to the ADP/ATP-binding domain of the protein. Basically, HSP90 binds to HSF1 and keeps it in a native state (inactive state). The basic mode of action of geldanamycin is that it can bind to HSP90, causing it to release the bound HSF1. Subsequently, HSF1 activates itself by homotrimerization mechanism, and translocates into nucleus and stimulates the synthesis of HSP70. As a result, these HSP70s then reduce the toxicity in cell. Another compound named celastrol identified from a plant (used in Chinese herbal medicine) is generally used for the treatment of bacterial infection mediated chills, fever and rheumatoid arthritis. Supplement of celastrol activates HSF1, which then triggers the heat shock response by increasing the levels of Hsp such as HSP40, HSP70, etc. Collectively, these activities help to reduce Huntingtin toxicity inside the cell [20]. But, researchers are looking further to characterize celastrol by showing how it interacts with HSF1 to disclose the underlying mechanisms.

2. Parkinson's Disease and HSF

Parkinson's disease is one of the most frequently occurring neurodegenerative diseases characterized by a progressive inhibition of dopaminergic neurons primarily due to the formation of Lewy bodies (abnormal aggregates of protein) inside the substantia nigra of brain and aggregation of α-syn protein. Earlier study suggested that overexpression of HSP70 in Parkinson's disease model of *D. melanogaster* showed that it prevented the dopaminergic neuronal loss and α-syn toxicity [3]. It was validated in human cell lines also by expressing the constitutively active HSF1, which resulted in the decreased α-syn inclusions and increased HSP70 protein levels [28]. It is reported that PTMs such as phosphorylation and neddylation play major roles in the activation/inactivation of HSF1 in connection with Parkinson's disease. As of now, it is unclear whether crosstalks between PTMs exhibit increased/decreased expression or activity of the HSF in Parkinson's disease. Also, the details about the involvement of other HSF in Parkinson's disease are not explored in detail.

3. Alzheimer's Disease and HSF

Experimental evidences suggests that the amyloid-β (Aβ), a toxic neuropeptide released after the proteolysis of β-amyloid precursor protein that aggregates and misfolds, contributing to the neuronal loss in cerebellum and hippocampus of brain

is one of the significant indications of Alzheimer's disease [16, 43, 46]. Some reports revealed that, pharmacological activation of HSF1 by inhibiting the interacting HSP90 in an Alzheimer disease mouse model mitigated the memory loss induced by Aβ aggregation [11]. In the Purkinje cells of cerebella, patients with Alzheimer disease showed a reduced Aβ aggregation appeared to contain depleted HSF1 and the molecular chaperones. On the other hand, overexpression of the HSF1 molecules in mice models rescued Purkinje cell numbers from neuronal dysfunction and lowered Aβ levels [31]. Thus, the differential expression of HSF also determines the disease progression of Alzheimer's disease and these HSF could be considered as potential drug targets to treat the disease. In this milieu, HSF1 was the most studied one and other HSF and their roles during Alzheimer's diseases are not yet explored in detail.

4. ALS and HSF

ALS is a progressive neurodegenerative disease that generally affects the motor neurons of brain and spinal cord, thereby ultimately leads to complete paralysis. As a clinical diagnosis, brain motor neurons affected by ALS accumulate the biomarker protein aggregates such as SOD1. Based on the current understanding, HSF1 is hypothesized to protect the brain motor neurons by activating TauT expression and related molecular chaperones. Meanwhile, increased expression of the TauT protein enhances taurine accumulation in brain motor neurons, in turn acts as an antioxidant by decreasing the motor neuron loss triggered by the SOD1 dysfunction [23]. Thus, the increased susceptibility of ALS brain motor neurons result in protein aggregation and oxidative stress appear to be closely associated with reduced HSF1 activity [10]. In this context, the hyperactivation of HSF1 could aid in treating the ALS disease.

HSF and Bacterial Infections

It is reported that HSF have a crucial role during bacterial infection also. Since it has chaperone roles in the cellular systems, they are closely attached with the protein organization (such as folding, etc.,) and control machinery. The conserved immune regulatory pathways such as MAPK, Insulin signalling pathway, etc., activates in coordination with the heat shock response as a primary immunity to fight against the invading pathogenic bacteria in a model host, *C. elegans.* It is well known that *C. elegans* has been used to study the host pathogen interactions for the past few decades against various infections [5–7, 18, 24, 30, 35, 36, 44, 48]. Earlier report suggests that a conserved heat shock pathway that requires HSF-1 is important for innate immunity against bacterial pathogens in *C. elegans* [47]. It was hypothesized that Hsp activated in an HSF-1 dependent manner could be the possible effectors responsible for innate immunity in *C. elegans* [39]. Another report also suggested that HSF1 plays a vital role during *Staphylococcus aureus* infection in *C. elegans* [40]. These reports collectively suggested that, in addition to the role of HSF in

aging, cancer, neurodegenerative diseases, etc., they also involved in host innate immune system during pathogenic infection.

1.1.6 HSF and Its Turnover Mechanism

HSF transcription factor is an acidic, strong activator similar to that of many other positive regulatory transcriptional factors resembles many other heat shock regulatory genes. Two complementary and independent approaches on the two widely studied acidic transcriptional activators have revealed their highly stable DNA binding properties during the activation. Alternative models also been proposed about the HSF activation by showing the recycling as a key component, chaperone mediated disassembly or UPS pathway-mediated protein turnover. More precisely, the well studied endogenous HSF1 protein has a relative half-life in the range of 13–20 h [45, 53]. Unlike the long half-life of the HSF1 protein, the half-life of corresponding mRNA transcript is only about 100 min [26]. The reason for HSF1 having such long half-life is to readily accessible during the exposure to any cytotoxic stress conditions, so that it can able to contribute with its cytoprotective effects immediately. The histone acetyltransferase E1A protein (EP300) enhances the stability of HSF1 through acetylation on its lysine residues (lys208 and lys298, etc.,), hence preventing it from proteasomal degradation mediated by ubiquitination/polyubiquitination mechanism [42]. On the other hand, phosphorylation of HSF1 at serine 216 aminoacid residue provides signals for proteolysis degradation by the E3 ubiquitin ligase SCF/b-TrCP during mitosis [27]. Characteristically, HSF1 is the well studied transcriptional factor among the other HSF in which the turnover mechanism has been elucidated more precisely. But, rest of the HSF family members and their turnover mechanisms have not been explored yet.

2 Conclusions

Over the past few decades, understanding of the HSF and their structure, organization, regulation and function has rapidly increased by providing new molecular and mechanistic insights into their roles in protein misfolding, regulation, metabolism, neurodegenerative diseases and cancer. However, numerous unanswered questions remain pertaining to the activation and regulation of the HSF and heat shock response. Thus, it will be more important to gain a systematic understanding of the complete network of HSF isoforms, their structures and how they interact within the HSP and with other proteins to regulate the gene expression. Although HSF and their regulations are closely connected to PTMs, we still have a meagre understanding of their precise and context-dependent roles in regulation during normal/disease conditions. Though, it should be significantly noted that as more PTM sites were identified on HSF, the ultimate function of these PTMs still requires more comprehensive studies which could be possible by applying the systems biology approach

(including pull down assays, MS, mutant based studies, etc.,). Moreover, the basic mechanisms by which HSF are regulated in the broad circumstances of metabolism, cancer signalling pathways or protein misfolding in neurodegeneration are yet to be elucidated critically using suitable model organism(s). At this juncture, a widespread understanding of HSF and their family of transcription factors could offer a basis for antagonists or selective small molecule agonists, which may provide new therapeutic interventions against different diseases.

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Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Humans This article does not contain any studies with animals performed by any of the authors.

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Heat Shock Proteins and Pain

Xin Chen, Amanda Smith, Christopher Plummer, and Wei Lei

Abstract

Introduction Pain, especially chronic pain, is a health issue affecting about one-third of the population all over the world. The sensitization of pain is highly associated with inflammatory responses to tissue damage and other harmful signals. The heat shock proteins (HSP) have been demonstrated to play central rolesin the inflammatory responses and pain via regulating the protein folding, maturation, activation, and degradation of proteins in varioussignal pathways. The objective of this chapter was to summarize the role of HSP in pain and pain drug-induced signal pathways and antinociception.

Methods The authors reviewed papers of different HSP with an emphasis on pain and pain management.

Results We highlight some basic concepts of pain and inflammatory responses, discuss the impact of inflammatory mediators, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), on the sensitization of pain. We also summarize the role of HSP in different types of pain in animal models and human diseases and discuss the impact of HSP on pain drug-induced signalsand behavior.

Conclusions HSP are highly involved in pain and pain treatment and could be targeted for improving therapeutic outcomes, even though the exact mechanisms remain unclear.

Keywords Cytokine · Heat shock protein · Inflammation · Kinase · Opioid · Pain

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Abbreviations

17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
Bdnf	brain-derived neurotrophic factor
BiP	binding immunoglobulin protein
CCI	chronic constriction injury
CGRP	calcitonin-gene-related peptide
cHsp60	*C. trachomatis* Hsp60
CLR	c-type lectin receptor
CNS	central nervous system
COX	cyclooxygenase enzymes
CP	chronic bacterial prostatitis
CP/CPPS	chronic bacterial prostatitis and chronic prostatitis/chronic pelvic pain syndrome
DAMP	danger-associated molecular pattern
DRG	dorsal root ganglion
ELISA	enzyme-linked immunosorbent assay
EP1	Prostaglandin E2 receptor 1
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde-3-phophate-dehyrogenase
Grp	glucose-regulated protein
HDAC6	histone deacetylase 6
Hsc	heat shock cognate
Hsp	heat shock protein
IL-1β	interleukin (IL)-1 beta
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
JAK-STAT	Janus kinase-signal transducer and activator of transcription
MAPK	mitogen-activated protein kinase
MD-2	myeloid differentiation factor 2
MEF2	myocyte enhancer factor 2
MEK2	mitogen-activated protein kinase kinase 2
MOR	mu opioid receptor
NF-κB	nuclear factor kappa-B
NLR	NOD-like receptor
NOS	nitric oxide synthase
PAMP	pathogen-associated molecular pattern molecule
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PID	pelvic inflammatory disease
PKC	protein kinase C
PNS	peripheral nervous system
PRR	pattern-recognition receptor

RLR	retinoic acid-inducible gene-1-like receptor
ROS	reactive oxygen species
sHsp	small heat shock proteins
SOCS3	suppressor of cytokine signaling 3
SOD	superoxide dismutase
SP	substance P
SRF	serum-response factor
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TPA	12-o-tetradecanoylphorbol 13- acetate
TRP	transient receptor potential
TRPM3	transient receptor potential melastatin-3
VR1	Vanilloind receptor subtype 1
ZDF	Zucker diabetic fatty

1 Introduction

The heat shock proteins (HSP) are ubiquitous and occur in all living organisms and primarily serve as molecular chaperones [1–3]. They bind to and promote the folding and maturation of the newly synthesized polypeptide chains. The HSP have been classified into six families based on their molecular weight, including small Hsp, Hsp40, Hsp60, Hsp70, Hsp90, and large Hsp [4, 5]. The HSP continuously express under normal conditions and are crucial for maintaining normal physiological functions of cells [5, 6]. The expression of HSPis also induced by environmental, pathological, or physiological stress [7]. As such, HSP are highly involved in numerous diseases, such as cancer, aging, heart failure, and inflammatory diseases [8–12]. The HSP have also been reported to modulate the function of a variety of immune cells to the expression of inflammatory mediators by innate immune cells [7, 13, 14]. Pain is a symptom of inflammation and inflammation-related diseases [15, 16]. Through the inflammation, the activated immune cells release prostaglandins, cytokines, chemokines, proteases, neuropeptides, and growth factors. In consequence, the inflammatory mediators bind to the nociceptors and enhance the sensitization of pain [17, 18]. The interactions between nociceptors and innate immune cells have been reported in various types of pain, including inflammatory pain, neuropathic pain, and cancer pain [16, 19, 20]. HSP are shown to be involved in pain signaling through a variety of mechanisms even though the exact mechanism remains incompletely understood. In this chapter, we will discuss the contribution of inflammatory responses on pain generation and summarize the impact of HSP on modulating pain signaling and pain treatment.

1.1 Inflammation and Pain

Pain, especially chronic pain, is a serious health concern, affecting over one-third of the world's population [21]. The chronic pain is affecting not only the quality of life but also economic matters [22, 23]. As described by the International Association for Study of Pain (IASP), pain is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [24]. As a natural physiological response to tissue damage and dangers signals, inflammation plays pivotal role on sensitizing peripheral pain [16], via mediating the generation of pro-inflammatory cytokines, chemokines, prostaglandins, neuropeptides, and growth factors [25–31].

1.1.1 Pain Signaling

Pain serves a physiological role to identify potentially harmful conditions and prevent tissue damage. The perception of pain is a complex signaling pathway caused by the interaction between peripheral nociceptors and primary efferent neurons, and the higher-order processing center in the brain [15, 32]. The transmission noxious stimuli from receptors to the central nervous system (CNS) occurs in three major stages: transduction, transmission, and modulation. During transduction, stimuli that activate pain receptors are translated into electrical signals that can be relayed through effector neuron signaling. Transmission occurs through the ascending pathway. Stimuli are carried from the primary efferent neuron to the dorsal horn of the spinal cord, and eventually to the thalamus in the brain [33, 34]. Here, sensory information such as the severity and location of the pain is processed. Finally, modulation involves the activation of afferent neurons pathways [16].

Pain can be classified on the basis of several different characteristics. Categories can be developed based on location, duration of onset or the body system affected. While these categorizations can be helpful in clinical assessments, division by mechanism of actions creates a more accurate description of different pain models and their underlying mechanisms [34, 35]. By this method, pain can be divided into three major classes: nociceptive, neuropathic and inflammatory. Nociceptive pain, at a physiological level, plays a key role in recognition of the presence of noxious stimuli and injury. Nociceptive pain occurs when receptors are activated, and the signaling pathway is initiated. Major nociceptive pain can further be classified into radicular, somatic, and visceral pain, dependent on the location and characteristics of the pain presentation [36, 37]. Neuropathic pain is associated with damage to the peripheral nerve and the presence of allodynia [18]. Allodynia occurs when a typically innocuous stimulus is able to activate nociceptors and cause pain. Because it does not serve a physiological purpose, neuropathic pain can be described as pathological. It is commonly caused by inflammatory or metabolic diseases, although it can result from any condition affecting the peripheral nervous system (PNS) or CNS that can cause nerve damage [38]. Inflammatory pain occurs as a

result of immune system activation. While inflammation targets infection and initiates tissue repair, it can also affect pain responses such as hyperalgesia, allodynia, and sympathetic maintained pain. Inflammatory pain can be classified into chronic and acute pain. Acute inflammatory pain is typically short-lived and intense. It is often a result of noxious stimuli and is mediated by A-delta fibers [20]. In comparison, chronic inflammatory pain persists beyond the expected duration of recovery from injury. It is mediated by C fibers. Inflammatory pain can also be activated by mediators and cytokines produced by the immune response [39]. This association depicts the relationship between inflammatory processes and pain perception.

1.1.2 Inflammation and Pain

Inflammation is a component of the immune response, and aids in the elimination of foreign substances and initiating tissue repair [17]. Both immune and non-immune cells are able to recognize noxious stimuli through pattern-recognition receptors (PRR), including toll-like receptors (TLR), c-type lectin receptors (CLR), NOD-like receptors (NLR), and retinoic acid-inducible gene-1-like receptors (RLR) [40]. These receptors recognize the danger-associated molecular patterns (DAMP), such as pathogen-associated molecular pattern molecules (PAMP) [41]. The immune cells (including the residential and infiltrated immune cells) in the nervous system can also recognize the neuropeptides, such as substance P (SP), calcitonin-gene-related peptide (CGRP), and glutamate released by the activated neurons [15]. Upon stimulation, PRRs activate the intracellular signaling, including nuclear factor kappa-B (NF-kB), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) via different pathways. Consequently, activation of these signaling pathways promotes the productions of inflammatory mediators, such as bradykinins, prostaglandins, cytokines IL-6, IL-1β, and TNF-α, and other mediators [42]. The receptors expressed at the nociceptor's peripheral terminal recognize and respond to those inflammatory molecules resulting in excitation of nerve fiber and sensitazition of pain [43].

Kinins, such as bradykinin, kallidin, and their metabolites, can activate B1 and B2 receptors causing different types of pain in human and animal models [44–48]. Bradykinin binds to B2 receptors leading to the sensitization of pain[47, 49]. Oliveira and colleagues demonstrated the block the activity of B1 and B2 receptor using pharmacological inhibitors or genomic editing reduced the fibromyalgia-like and paclitaxel-induced pain models [44, 45]. The activation of B1 and B2 receptors also contributes to tibial fracture pain via cyclooxygenase enzymes (COX)-1/COX-2 signaling pathway [50].

An injury to tissue is shortly followed by a release of arachidonic acid which could be converted into prostaglandins, such as prostaglandin E2 (PGE2), by COX-1/COX-2. PGE2 is a potent inflammatory mediator that is responsible for recruiting neutrophils, macrophages, and mast cells to the site of inflammation. PGE2 can also directly activate nociceptive signaling by binding to prostaglandin

E (PE) receptors promoting central and peripheral sensitization [51]. Antagonists to certain isoforms of PE receptors have been studied clinically to treat hyperalgesia. A tricyclic prostaglandin E2 receptor 1 (EP1) antagonist, SC51322, GW-848687X, has been shown to be effective at managing symptoms of hyperalgesia in animal models [52].

Cytokines IL-6, IL-1β, and TNF-α are also playing important roles in the sensitization of pain. The concentrations of pro-inflammatory cytokines are elevated in different types of pain [53–58]. In a study utilizing IL-6 knockout mice, it was determined that IL-6 likely had a role in the development of neuropathic pain following a nerve injury, and the administration of anti-IL-6 antibodies had shown effectiveness at reducing allodynia [59]. IL-1β is involved in peripheral nerve damage through binding to IL-1β receptors and consequently enhancing pain transmission. It can also induce the production of other pro-inflammation mediators, such as IL-6 and IL-17 maintaining both the immune response and the pain. TNF-α is an important component of the pain pathways associated with a variety of diseases, such as inflammatory bowel disease, rheumatoid arthritis, and neuropathic pain [60–62]. Because of its role in the pain pathway, TNF-α has been used as a target for developing drugs for the treatment of those diseases.

1.2 Heat Shock Proteins and Pain

The expression of heat shock genes usually undergoes a rapid, vigorous and transient acceleration as a result of the living organisms' response at a cellular level to unfavorable conditions such as heat shock, and other stressful situations of many different origins [63]. As a result, heat shock proteins (HSP), or stress proteins, which are the commonly referred products of these genes, have been found to be upregulated in those situations including inflammation and pain [64]. HSP are highly conserved proteins in both eukaryotic and prokaryotic organisms that perform chaperone functions by stabilizing new proteins to ensure correct folding or by helping to refold proteins that have been damaged by the cell stress [65]. Family members of HSP, with their molecular sizes ranging from 10 to more than 100 kDa, are categorized according to their molecular sizes and include small Hsp, Hsp60, Hsp70, Hsp90, and large Hspsubclasses [66, 67]. High molecular weight HSP are ATP-dependent chaperones, whereas small Hsp act in an ATP-independent fashion [68]. Among those subclasses, HSP in the first five subclasses have been found to be closely related to pain and pain treatment. As molecular chaperones, HSP interact with and promote the maturation of their client proteins [7]. More and more research has revealed that HSP also regulates the activation of proteinsinvolved in signaling transduction associated with multiple physiological functions. HSP have been proven to modulate the production of inflammatory cytokines, chemokines, and other mediators via regulating theintracellular signaling pathways, such as MAPKs and NF-κB [69–74]. As discussed above, inflammatory mediators play critical roles in pain sensitization. HSP can also be released to extracellular environment and

activate receptor-mediated signal transduction [75–78]. Here we will discuss how HSP are involved in pain process.

1.2.1 Small Heat Shock Proteins(sHsp)

Small heat shock proteins (sHsp) mainly refers to Hsp10 and Hsp30 in the family of HSP because their molecular weights are comparably smaller than other family members. Structurelly, sHsps are very specail in HSP family since they don't have an ATPase domain. Based on the sequence homology [79], ten sHsps have been identified in the human genome.

Different members in the sHsp family have been discovered to play different roles in a variety of biological process inside human body. Hsp10, as a subclass of 10 kDa, highly conserved, mitochondrion-resident Hsp proteins, has been confirmed to have played major roles in development differentiation and specialized tissue-specific functions [80]. Another subclass of sHsp widely distributed in various tissues [81], Hsp30 is a predominantly heat-inducible HSP that is believed to be a critical player in cell survivial under stress conditions.

As part of the HSP family, sHsp share the common molecular chaperon-like activity as other family members in preventing aggregation of proteins/peptides. Moreove, they are also actively participate in a large variety of many more cellular functions other than stress tolerance and protein folding including cell cycle, differentiation, protein degradation, cell death, maintaining cytoskeletal integrity, signal transduction and development etc. [82–88]. As a result, family members in sHsp, through their interactions with several clients, have demonstrated neuro- and cardioprotection as well as exhibited potent anti-apoptotic, anti-inflammatory activity and pro-antiogenic property [89].

The structure characteristics of sHsp, having a flexible binding pocket which leads to large binding capacity, help to establish unique mechanisms of action for the family members which are very different from the other more sophisticated chaperon proteins in the Hsp family such as Hsp70 and Hsp90. Therefore sHsp is able to bind efficiently to a large number of non-nantive proteins with variable structures and molecular weights [90]. Consequently, sHsp can work more efficiently in preventing irreversible aggregation processes. On the other hand, because of the lack of ATPase domain, sHsp cannot further process the bound proteins as the ATP-dependent chaperones can. Their general roles are believed to mostly trap the non-native proteins in a state so that the ATP-dependent chaperones will be able to refold them to their native states with the assistance from sHsp [91]. Because of their great substrate compability, sHsp play an important role in readily recover a huge variety of non-native proteins and help convert them to their native states. Without sHsp, many non-native proteins could escape from the chaperone protein refolding process and could aggregate and became desfunctionalized. Therefore, sHsp is considered as the central cellular component mostly work on the inhibition and modulation of protein aggregation by improving disaggregation process after stress [92, 93]).

Several studies have been carried out to examine the dynamic changes in the expression of sHsp in pain situations and the possibility of regulating the expression and function of sHsp in pain alleviation. In the proteomic analysis of vincristine-induced neuropathic pain rat cerebral cortex tissues, Li and his colleagues dosed the control group and the pain model group of male SD rats with intraperitoneal (*i.p.*) injection of either saline or vincristine. The proteomic expression profiling analysis indicated significant upregulation of Hsp10 in the pain model group together with the upregulation of the two other neuropathic pain associated proteins DJ-1and ATP synthase D as well as the downregulation of Cu-Zn superoxide dismutase (SOD) which is also a neuropathic pain associated protein. Their results suggested that Hsp10, together with DJ-1, ATP synthase D, and Cu-Zn SODmay be involved in the central mechanisms of vincristine-induced neuropathic pain [1].

Among all the sHsp, Hsp27 is one of the most attractive targets. Continued investigation of Hsp27 revealed that it responds to cellular stress conditions not only heat shock but also oxidative stress and chemical stress. In addition to its well-documented properties as an anti-apoptotic agent under conditions of chemical stress [94], Hsp27 can also functionalize as an antioxidant during oxidative stress, by lowering the levels of reactive oxygen species (ROS) [95, 96]. Several clinical studies on Hsp27 have been reported. In patients with chest pain, significantly higher Hsp27 antibody levels than controls were detected and Hsp27 antibody concentrations showed strong associations with age and hypertension [97]. Interestingly, in clinical research on patients with coronary heart disease, while the plasma level of Hsp27 was detected by enzyme-linked immunosorbent assay (ELISA) analysis, Zhang and colleagues found that increased Hsp27 plasma level was associated with acute myocardial ischemia but not with chest pain syndrome [98]. Further investigation from Heidari-Bakavoli et al. about the plasma level of Hsp27 in patients after acute coronary syndrome indicated that serum Hsp27 concentrations were elevated in the early hours following acute coronary syndrome but fell back to normal levels after about 12 h from the onset of chest pain [99].

Constitutively expressed by a subpopulation of adult primary sensory neurons at low levels, Hsp27 has been found to play a protective role in sensory neurons [100]. The neuropathic pain after peripheral nerve injury was found to be associated with the dramatic upregulation of the expression of Hsp27 mRNA and protein in the dorsal root ganglion (DRG), which could activate the TLR4 signaling and consequently contribute to neuropathic pain [101]. Furthermore, high levels of Hsp27 expression has been virtually detected in all injured sensory neurons [102]. Recently, exogenous Hsp27 has been approved to be able to reduce the apoptosis of sensory neuron after NGF withdrawal *in vitro* [103]. Additionally, after peripheral nerve injury and NGF withdrawal, exogenous Hsp27 also significantly increased the survival of sensory neurons. Based on these findings, Hsp27 was probably an important factor for survival of adult sensory neurons [104]. While using type 2 diabetic Zucker diabetic fatty (ZDF) rats on a long-term type 2 diabetes rat model, Brussee et al. detected the early rises of Hsp27 mRNA levels in the rats developed distal degenerative sensory neuropathy accompanied by selective long-term pain syndrome [105]. A follow-up study from the same research group found

out that the overexpression of human Hsp27 level was able to protect sensory neurons from diabetes. Taken together, overexpression of human Hsp27 in diabetic mice help to protect from a good range of neuropathic abnormalities such as slowing of sensory conduction velocity, loss of footpad thermal sensation, mechanical allodynia loss of epidermal innervation [106].

1.2.2 Heat Shock Protein 60 (Hsp60)

Mainly found in the mitochondrial, Heat shock protein 60 (Hsp60) is considered as a mitochondrial protein with its major responsiblility being the correct folding and function of mitochondrial proteins [107]. Under normal conditions, Hsp60 is constitutively expressed. Its expression can be induced by different types of stressors such as heat shock, oxidative stress and DNA damage [108]. The fact that Hsp60 has been found to be expressed endogenously in astrocytes, neurons, microglia, oligodendrocytes and ependymal cells all over the brain suggests that this chaperon family may have active participation in a lot of brain functions not only in normal conditions but also in pathological condition including inflammation and pain [109]. Inaddition to the mitochondrial, Hsp60 also has been discovered to be accumulate in the cytosol and plasma membrane, so that it can reach to the extracellular space. Through the interaction with immune cells, Hsp60 participates in a great number of inflammatory and autoimmune processes, like some affecting the nervous system such as pain [110, 111].

Variable expression levels of Hsp60 have been found to be associated with pathological conditions. With gene expression level is regulated by microRNAs in both physiological and pathological processes such as chronic pain, the Hsp60 expression level has been found to be increased by 1.85-fold after the deletion of the brain-derived neurotrophic factor (Bdnf) gene in DRG neurons, but the miR-1 associated transcription factors myocyte enhancer factor 2 (MEF2) and serum-response factor (SRF) have their expression levels remain unchanged [112]. In an aminal study, quantitative real-time polymerase chain reaction (PCR) for the mRNAs encoding Bax, Bcl-2, Hsp60, mitogen-activated protein kinase kinase 2 (MEK2), and inducible nitric oxide synthase (iNOS) was performed after the rats was having chronic constriction injury of sciatic nerve that led to allodynia, hyperalgesia and disability, The results showed a selective a down-regulation of Hsp60 mRNA and up-regulation of Bax and MEK2 mRNA in pain and disability rats. These findings might indicate cell death in specific midbrain regions [113]. While trying to elucidate the mechanisms of protein kinase C (PKC) γ in regulation neuropathic pain and detect proteins that were associated with the function of PKCγ in neuropathic pain, a chronic constriction injury-induced neuropathic pain rat model in which PKCγ knockdown in the spinal cord was successfully carried out with stable RNA interference. Proteins obtained from the spinal cords were analyzed with proteomic instruments and data revealed that Hsp60 was upregulation together with some heat shock cognate 71 kDa protein Hsc70 in the

chronic constriction injury-induced neuropathic pain rat with PKCγ knockdown [114].

Elevated Hsp60 has been found in a variety of clinical condictions. In one clinical study, patients with arthritis were scored on formal joint assessment which included swelling and tenderness, pain and limitation of motion. The Hsp expression levels in synovial membranes were analyzed via immunohistochemical approches which showed a significantly higher intensity of staining in Juvenile Chronic Arthritis patients than in controls as Graeff-Meeder et al. found out. Their data suggested that T cell regulatory mechanisms that control the development of arthritis maybe partially resulted from T lymphocyte reactivity to Hsp60 [115]. In another clinical study trying to determine whether serum antibody to chlamydia trachomatous antigens altered the risk of *C. trachomatis* pelvic inflammatory disease (PID), Incident *C. trachomatis* and *Neisseria gonorrhea cervical* infection over 33 months for 280 females were prospectively evaluated. Clinical PID with lower abdominal pain and having uterine and adnexal tenderness on pelvic examination were identified and examined. The data from this study demonstrated that 2- to 3- fold increased risk for *C. trachomatis* PID could be predicted from the detection antibody to *C. trachomatis* Hsp60 (cHsp60) [116]. In a large case-control study of patients with coronary heart disease, increased risk for coronary heart disease was found to be associated with elevated Hsp60. The risk of coronary heart disease could be increased by the combined levels of Hsp60 and anti-Hsp60 antibody and the release of Hsp60 could be induced by acute myocardial infarction [117]. The results from another clinical study on 710 patients with acute cardiac chest pain indicated that increased titers of anti-human Hsp60 could predict an adverse 1-year prognosis independent of another cardiovascular risk factor including age, hypertension, diabetes, and smoking [118].

1.2.3 Heat Shock Protein 70 (Hsp70)

As another family of molecular chaperones expressed in response to stress, Heat shock protein 70 (Hsp70), just like other family members, can bind to its protein substrates and stabilizes them against denaturation or aggregation until conditions improve [119]. What makes Hsp70 special in the family is that it has multiple responsibilities during normal growth such as the folding of newly synthesized proteins [120, 121], the subcellular transport of proteins and vesicles [122], the formation and dissociation of complexes [123], and the degradation of unwanted proteins [124]. Because of all of the above functions associated with Hsp70, it serves as protein quality control and turnover during both normal and stress conditions. It has a broad susbtrate specificity and can largely shape protein homeostasis [125]. It is not surprising that Hsp70 has been implicated in a wide range of diseases, including cancer, neurodegeneration, allograft rejection, and infection. Multiple members have been identified in this subclass and they are are usually ubiquitously expressed and highly conserved [126]. The major isoforms of Hsp70 subclass that can be found in all the cellular compartments in eukaryotes are: Hsp72 (HSPA1A)

and heat shock cognate 70 (Hsc70/HSPA8) in the cytosol and nucleus, binding immunoglobulin protein (BiP) (Grp78/HSPA5) in the endoplasmic reticulum (ER), and mtHsp70 (Grp75/mortalin/HSPA9) in mitochondria [127]. Some of the functions of Hsc70 and Hsp72, which are the two cytosolic isoforms, are believed to be redundant, but Hsc70 is constitutively expressed while the transcription of Hsp72 is found to be highly responsive to stress. In the ER and mitochondria, the Hsp70 family members are thought to exihibit very specific functions and have unique substrates. For example, mtHsp70 is involved in the import and export of proteins from the mitochondria, and BiP plays key roles in the folding and quality control of ER proteins.

Since Hsp70 is a group of critical molecular chaperones in cell survival signaling and protein homeostasis, a lot of research has been done in the relationships of Hsp70 with pain and pain treatments. In a proteomic analysis of differential proteins related to the neuropathic pain and neuroprotection in the DRG following its chronic compression in rats, Zhang et al. found out that glyceraldehyde-3-phophate-dehyrogenase (GAPDH) and Hsp70 were significantly upregulated in the DRG following chromic compression of DRG in rats compared with the normal control [128]. This result was confirmed by a follow-up study from the same group thatdetected theupregulation of GAPDH and Hsp70 were detected in DRG underlying neuropathic pain, which suggested that concurrent process of nerve injury and neuroprotection in the course of neuropathic pain have existed [129].

Some animal studies have shown that upregulated Hsp70 proteins are associated with various pain conditions. The results from an animal study in horses with colic showed that high plasma Hsp72 concentration might indicate circulatory deficits but was not associated with clinical signs of colic [130]. While Hsp72 was thought to confer neuroprotection against acute neurological injury, an experiment carried out by Vizcaychipi et al. on C57BL/6 wide type and Hsp72 overexpressing transgenic mice concluded that Hsp72 overexpression is associated with the prevention of postoperative hippocampal-dependent and -independent memory deficit induced by anesthesia and surgery [131].

1.2.4 Heat Shock Protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is one of the cell's central protein regulators. Through specific and direct interactions with its client proteins, it promotes proper protein folding and maturation which happens late in the protein maturation process [132]. As the central protein regulator, Hsp90, through various mechanisms, regulates a large number of key proteins of the cell such as the translation, interaction partners, location and activation states of hundreds of clients proteins, including receptors, channels, transcription factors signaling kinases pathological proteins, etc. [133]. Through these mechanisms, Hsp90 plays an incredibly impactful role on a wide range of physiology and signaling processes like proliferation/survival, neuronal development, cardioprotection, analgesia and inflammation.

Considering the many critical roles of Hsp90 is playing as the cell's central signal transduction regulator and its importance in inflammation and/or pain signaling, our knowledge of Hsp90 on the context of pain via the opioid receptor system is still comparatively very scarce. Recently Streicher reviewed the known roles of Hsp90 in indirectly regulating the initiation and maintenance of the pain state. This review also explored how opioid analgesic drugs initiated the Hsp90 regulation on signaling and antinociceptive responses, with a special emphasis on extracellular signal-regulated kinase (ERK) MAPK signaling [134].

Nevertheless, Hsp90 has been cleaerly indicated in the pain phenomena according to a number of studies. In a study using intrathecal lipopolysaccharide, a classic TLR4 agonist, to explore TLR4-mediated pain modulation, it is found that lipopolysaccharide, when co-administrated with a drug that enhanced Hsp90-mediated TLR4 signaling, was able to induce robust allodynia. On contrary, intrathecal co-administration of a Hsp90 inhibitor, a TLR4 inhibitor, a microglia/monocyte activation inhibitor, and interleukin-1 receptor antagonis could be prevented or reversed the induced allodynia. These resultes suggested that Hsp90 was playing an important role in TLR4-dependent pain phenomena [135]. A follow-up study from the same research group discovered that through TLR4/myeloid differentiation factor 2 (MD-2) and interleukin-1β, intrathecal morphine-3-glucuronide may cause pain enhancement, while interleukin-1 receptor antagonist, minocycline (microglial inhibitor) and (+)-and (−)-naloxone could blocked or reversed this pain enhancement. 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), an well-kown Hsp90 inhibitor, was found to be able to block the hyperalgesia as well as *in vitro* M3G-induced TLR4 signaling [136]. Results from an inflammatory pain rat model identified increased expression of Hsp90 as phosphoproteins after phosphoproteomic analysis of electroacupuncture analgesia. Intraplantar injection of complete Freund's adjuvant into the rat hind paw induced inflammatory hyperalgesia which led to the increased level of phosphoproteins of Hsp90 in the spinal cord dorsal horn. These data indicated that Hsp90 was involved in an electroacupuncture analgesia mechanism [137]. To evaluate the *in vivo* participation of Hsp90 in monoarthritic rats, Nascimento et al. found out that in ipsilateral DRG of inflamed animals there was an increase in Hsp90 mRNA levels. While administration of 17-DMAG, an Hsp90 inhibitor, in the first hours could attenuate monoarthritis-induced allodynia by reversing the Hsp90 upregulation and cleavage which resulted in the attenuation of glial activation and neuronal sensitization that eventually might correlate with the clinically observed pain alleviation. Their data indicated Hsp90 also involved in monoarthritis pathophysiology [138]. The mitochondrial Hsp90 isoform, TRAP1 variants were also found to be consistently upregulated with choronic pain with functional disorders [139].

1.3 Heat Shock Proteins and Pain Management

Heat shock proteins (HSP), especially Hsp70 and Hsp90, can modulate intracellular signal transduction which has attracted more and more attention in the drug

discovery field. Previous studies demonstrated that HSP are highly involved in the pain drug-induced signaling and antinociception via different mechanisms, such as the modulation of inflammation and MAPKs signaling pathways.

1.3.1 sHsp

Hsp27 is the major member in the subclass of sHsp that has been closely associated with pain management. It has been discovered that Hsp27 is interacting with some anesthetics, which indicates the active participation of Hsp27 in pain and pain management. When after pretreating cultured A10 cells with pentobarbital, the longest-established anticovulsant, they are then stimulated by vasopressin or 12-o-tetradecanoylphorbol 13- acetate (TPA). Hsp27 levles were found to be elevated by vasopressin but the levels of Hsp70 remain unchanged. At the clinically used pharmaceutical concentration of pentobarbital, the accumulation of Hsp27 by vasopressin or TPA in vascular smooth muscle cells could be inhibited [140]. The phosphorylation of Hsp27 induced by thrombin was discovered to be markedly suppressed by a short-acting hypnotic-sedative drug named midazolam. This inhibition of thrombin-induced phosphorylation of Hsp27 at both Ser-15 and Ser-85 in cardiac myocytes was believed to be very similar to SB203580 and PD169316 which are inhibitors of p38 MAP kinase. Taken together, it is strongly suggested that p38 MAP kinase activation in cardiac myocytes is at least responsible for the thrombin-induced Hsp27 phosphorylation, and that the inhibition of the thrombin-induced Hsp27 phosphorylation by midazolam is via suppression of p38 MAP kinase activation [141]. While antinociceptive tolerance and upregulated Hsp27 expression in the dorsal horn of the rat spinal cord would be resulted from chronic morphine infusion, morphine's antinociceptive effect in morphine-tolerant rats could be partially restored by melatonin pretreatment could partially restore which might through reversed morphine-induced Hsp27 upregulation [142].

1.3.2 Hsp70

Hsp70 has also been found to interact with other drugs or proteins in pain treatment. The possibility that pain perception and processing in the CNS results in cellular stress and may influence HSP expression was examined in a rat model of morphine dependence and withdrawal carried out by Sharma et al., Massive upregulation of Hsp72 was seen in CNS during withdrawal phase in the untreated group with morphine dependence and withdrawal symptoms but not in the cerebrolysin, a mix of potent growth factors, treated group, suggesting that cerebrolysin could attenuate Hsp73 expression in the CNS and include neuroprotection against morphine-induced hyperalgesia [143]. In the evaluation of the mechanisms underlying tongue-referred pain associated with tooth pulp inflammation, the number of Hsp70-immunoreactive neurons in trigeminal ganglion was significantly increased after complete Freund's adjuvant application to the molar tooth compared with

vehicle-applied rats, indicating that the Hsp70-TLR4 signaling in complete Freund's adjuvant treatment plays a pivotal role in tongue-referred pain associated with tooth pulp inflammation [144]. To explore the role of the nucleus tractussolitarii in the protective mechanism of pre-moxibustion on gastric mucosa, nucleus tractussolitarii were damaged in rats and pre-moxibustion treatment at the Zusanli (ST36) point followed. The increased expression level of Hsp70 was exhibited together with epidermal growth factor content, which suggested the increased Hsp70 expression level might be protective against acute gastric mucosal injury following moxibustion pretreatmentat the Zusanli point [145]. Hsp70 was also found to be upregulated together with vanilloid receptor subtype 1 (VR1) in colorectal distension-induced visceral hypersensitivity rats stimulated with moxibustion and the upregulation of Hsp70 might contribute to the visceral pain relief in visceral hypersensitivity rats [146]. While wet cupping therapy is a complementary therapy in pain management, the mechanism research of this therapy in rats revealed that the expression of Hsp70 and ss-endorphins were significantly higher in the keratinocytes of the treated group than in the control group which led to the conclusion that the benefit of wet cupping therapy in terms of pain reduction in rats could be mediated by the expression of Hsp70 and ss-endorphine [147]. In type I diabetic animals, continuous expression of interleukin (IL)-10 was demonstrated to be able to alter TLR4 expression in the DRG with increased expression of Hsp70 in conjunction with the reduction of pain [148]. Another study on the transient receptor potential melastatin-3 (TRPM3) medicated nociceptive-like responses in *Hydra vulgaris*, Malafoglia et al. found out that both heat shock and TRPM3 specific agonist (i.e., pregnenolone sulfate) induced the modulation of Hsp70 and the nitric oxide synthase (NOS), two genes activated by transient receptor potential channels (TRP)-mediated heat painful stimuli in mammals and these effects could be inhibited by a TRPM3 antagonist (i.e., mefanamic acid) [149]. In the searching for endogenous "immune brakes" to inhibit the key receptors in central pain sensitization and neuroinflammation, Fan and his co-workers used paeoniflorin to induce Hsp70/TLR4 signaling and found tout that paeoniflorin significantly induced suppressor of cytokine signaling 3 (SOCS3) expression both *in vitro* and *in vivo* and promoted the efflux of Hsp70 from the cytoplasm to the extracellular environment and could markedly attenuate incision-induced postoperative pain [10].

Related clinical studies have also demonstrated the important role of Hsp70 in pain and pain treatment on patients. Significantly increased levels of cytokines (TNF-α and IL-1β) and Hsp70 were observed in seminal plasmas from patients with chronic bacterial prostatitis (CP) compared with chronic bacterial prostatitis and chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) patients who had significantly lower Hsp70 level than that in controls [150]. A follow-up study from the same group further confirmed that Hsp70 had an important protective role in the regulation of cell functions in CP patients while CP/CPPS was probably detrimental to the function of T cells and consequently suppressed the expression of Hsp70 [151]. With balneotherapy being one of the most commonly used nonpharmacological interventions for osteoarthritis, the study on its mechanism of action in relieving pain and stiffness and

improving physical function indicated a limited increase level of Hsp70 in patients under balneotherapy, which warrant further investigation [152].

Interestingly, physical excises have been discovered to be able to interact with Hsp70 in the process of alleviating pain. In an animal study with male Sprague-Dawley rats, Chen and colleagues found out that after chronic constriction injury of the sciatic nerve, greater Hsp72 expression and lower TNF-α or IL-1β level in rats with swimming or treadmill exercise than those without exercises. This finding suggests that decreased peripheral neuropathic pain, reduced TNF-α and IL-1β expression, and increased Hsp72 expression after chronic constriction injury (CCI) of the sciatic nerve can be achieved through progressive exercise training [153]. To futher investigate the underlying mechanism of exercise on the development of diabetes-associated neuropathic pain, the same group demonstrated that diabetes-associated neuropathic pain, including thermal hyperalgesia and mechanical allodynia in rats could be markedly decreased by progressive exercise training. And in rats with streptozotocin-induced diabetes, exercise can increase the expression level of Hsp72, but not TNF-α and IL-6, in the spinal cord and peripheral nerves, which might be the underying mechanism of clinically observed pain alleviation [154].

1.3.3 Hsp90

Several studies found that Hsp90 inhibition reduced morphine dependence and withdrawal responses in *in vitro* and *in vivo* models [155, 156]. Most recently, inhibitors targeting the C-terminus of Hsp90, such as KU-32, were able to promote neuronal survival and reduce diabetic peripheral neuropathy [157–159]. When testing the effect of 1 nM of (+)-Naloxone, an opioid receptor antagonist, on morphine-induced activation of microglia EOC13.31 cells, Tsai and collegues found out that enhanced microglia activation and migration, decreased α-tubulin acetylation, induced Hsp90 fragmentation and histone deacetylase 6 (HDAC6) expression were associated with 1 μM morphine, but all these can be inhibited by pretreatment of (+)-naloxone (1 nM) [160]. Streicher and colleagues have explored the role of Hsp90 in opioid-induced mu opioid receptor (MOR) signaling and pain. They found that Hsp90 inhibition strongly blocked morphine-induced anti-nociception in post-operative and HIV neuropathic pain models by an ERK MAPK mechanism in mouse brain [161], and the alpha isoform of Hsp90 mainly contributed to those activities [162]. In contrast, Hsp90 inhibition in the spinal cord enhanced morphine anti-nociception in several pain models (unpublished data). These findings indicate that Hsp90 could be a future target for improving the therapeutic index of opioid drugs. Another animal study of male rats with morphine-induced persistent sensitization revealed that protraction of neuropathic pain could be mediated by spinal DAMPs and pharmacological attenuation of Hsp90 together with other proteins could persistently reverse morphine-prolonged allodynia [163]. In an *in vivo* evaluation of the involvement of Hsp90 in monoarthritic rats, Nascimento et al. demonstrated that Hsp90 mRNA levels increased in ipsilateral DRG of inflamed animals, and administration of an Hsp90 inhibitor, 17-DMAG, attenuated monoarthritis-induced

allodynia in the first hours through reversing the Hsp90 upregulation and cleavage which led to attenuation of glial activation and neuronal sensitization that might correlate with the observed pain alleviation. Their data indicated the role of Hsp90 in monoarthritis pathophysiology [138].

2 Conclusions

Heat shock proteins, especially Hsp70 and Hsp90, have shown pivotal roles in the regulation of pain, even though the mechanisms are not fully understood. HSP could regulate pain signals viamodulating maturation and/or function the intracellular signaling components associated with inflammatory responses. The extracellular HSP could bind to the receptors, such as TLR4, on the cell membrane and promote the sensitization of pain. Although the number of studies on the function of HSP in pain signaling and pain management is still limited, they have revealed that HSP could involve in the activities of numerous pain relieving drugs including opioids. Consequently, some of the HSP, such as Hsp27 and Hsp70, have been identified as the drug targets for managing pain signaling. The mechanisms remain unclear but mightrelate to their functional rolein inflammatory responses. A few studies demonstrated that Hsp90 could modulate the opioid-induced anti-nociception via affecting ERK MAPK signaling pathway. Overall, the HSPmay be potential targets for improving the performance of pain drugs, including increasing the anti-nociception and eliminating the adverse effects. However, more studies are needed to determine the underlying mechanisms.

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HSF1 Regulates Cellular Senescence: Role of the DHRS2-MDM2-p53 Pathway

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Abstract

Introduction Heat shock transcription factor 1 (HSF1) regulates lifespan, age-related pathologies and cancer development. These functions of HSF1 have mainly been attributed to the maintenance of proteostasis by activating the expression of heat shock protein (Hsp) genes. However, increasing attention has been attracted to non-canonical functions of HSF1 linked to cell proliferation and metabolism through the regulation of diverse non-Hsp genes. In here, I aim to describe experimental evidence that the DHRS2-MDM2-p53 pathway mediates HSF1 depletion-induced cellular senescence (HDIS) and to discuss the potential significance of this finding in tumorigenesis and aging.

Methods The author reviewed relevant papers of the DHRS2-MDM2-p53 pathway and HDIS, focusing on tumorigenesis and aging.

Results Acute depletion of HSF1 induced cellular senescence in human diploid fibroblasts. Several lines of evidence indicated that HDIS is mediated by activation of the DHRS2-MDM2-p53 pathway but not through decreased expression of Hsp or increased proteotoxic stress. Accumulating evidence in literature suggests that DHRS2 is an important regulator of tumorigenesis, underlying the above findings. In addition, there is substantial evidence that HSF1 regulates cellular senescence through multiple pathways.

Conclusions To better understand the molecular mechanisms of HDIS and its *in vivo* functions will provide new insights into roles of HSF1 in tumorigenesis and aging and effects of HSF1-targeting therapies.

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Abbreviations

CDK2	cyclin dependent kinase 2
DHRS2	dehydrogenase/reductase 2
DOX	doxycycline
HDF	human diploid fibroblast
HDIS	HSF1 depletion-induced cellular senescence
HSF1	heat shock transcription factor 1
Hsp	heat shock protein
Mieap	mitochondria-eating protein
NAMPT	nicotinamide phosphoribosyltransferase
PGC1α	peroxisome proliferator-activated receptor γ coactivator-1α
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
SA-β-gal	senescence-associated β-galactosidase
shRNA	short-hairpin RNA
SDR	short-chain NAD/NADP-dependent dehydrogenase/reductase
TERT	telomerase reverse transcriptase

1 Introduction

Heat shock transcription factor 1 (HSF1) activates the expression of heat shock protein (Hsp) genes in response to proteotoxic stress and, thereby, maintains proteostasis [1]. In an orthodox view, prominent phenotypes of HSF1-deficient organisms, such as shortened life-span, acceleration of age-related neurodegeneration, and tumor suppression, are explained by the accumulation of misfolded protein aggregates [1]. However, accumulating findings reveal that HSF1 functions in more extensive biological networks that are linked to cell growth, survival and metabolism [3, 16, 30]. In response to various signals linked to cell proliferation and metabolism (e.g. MEK and AMPK), HSF1 is regulated through post-translational mechanisms, such as covalent modifications (e.g. phosphorylation, acetylation and ubiquitination), and by proteasome-mediated degradation and nuclear localization, trimerization and DNA-binding [16, 30]. Furthermore, HSF1 remodels a transcriptional program of Hsp and diverse non-Hsp genes to regulate fundamental cellular functions depending on the situation [3, 34]. These findings have attracted attention as non-canonical HSF1 functions in the development of age-related phenotypes and tumors. Given the critical role of cellular senescence in aging and tumor suppression [17, 22, 24], an interesting hypothesis is that HSF1 regulates cellular senescence; however, more information is needed to test this.

1.1 Cellular Senescence

Cellular senescence is a complex process in which proliferating cells undergoes irreversible growth arrest in response to various forms of stress [17, 22, 24]. In 1961, Hayflick and Moorhead reported that cultured normal human diploid fibroblasts (HDFs) progressively lose proliferative capacity during repeated cell divisions, finally leading to stable growth arrest [21]. They termed this state "replicative senescence", based on the hypothesis that it reflects cellular phenotypes of aged organisms. Replicative senescence was later attributed to telomere attrition resulting from telomerase deficiency [22]. Subsequently various forms of stress, including DNA damage, replication stress and oncogene activation, were shown to induce "premature" senescence much faster than "replicative" senescence (days vs. weeks to months). Other hallmarks of senescent cells include (1) global epigenetic alteration, which is reflected by the formation of senescence-associated heterochromatin foci, (2) secretion of inflammatory cytokines and matrix-degrading proteases, which is known as "senescence-associated secretory phenotype (SASP)", (3) increased activity of lysosomal enzymes, usually detected by staining for senescence-associated β-galactosidase (SA-β-gal), and (4) activation of various stress signaling pathways, such as p53 and NF-κB pathways. It is widely accepted that cellular senescence functions as an important anticancer barrier and contributes to the efficacy of cancer therapies; however, senescence can also have a tumor-promoting activity in a context-dependent manner [13]. Remarkably, recent studies demonstrate that selective removal of senescent cells in rodents suppresses development of age-related pathology and functional decline in various organs, such as the kidney, heart and fat, and even prolongs life-span [2, 17]. These findings indicate that senescence is an important cellular basis of aging, which offers a new therapeutic avenue for age-related diseases [17, 40, 45]. Indeed, various senolytic agents, many of which induce apoptosis or suppress SASP, are currently in pre-clinical or clinical trials.

1.2 Acute Depletion of HSF1 Induces Cellular Senescence

Previous studies documented that HSF1 has a modulatory role in cellular senescence [28, 35]. For example, short hairpin RNA (shRNA)-mediated knockdown of HSF1 promotes oncogene-induced senescence in the human mammary cell line, MCF10A, and maintains genotoxic stress-induced senescence in an HDF line, TIG1. These effects of HSF1 knockdown were shown to be associated with decreased levels of Hsp and increased expression of the Cyclin Dependent Kinase 2 (CDK2) inhibitor, p21; however, the link between these two events remains to be determined (Fig. 1). We found that acute depletion of HSF1 by itself induces senescence in different primary HDF lines (MRC5, TIG1 and TIG3), and in a telomerase reverse transcriptase (TERT)-immortalized HDF line [39]. To study the molecular mechanisms

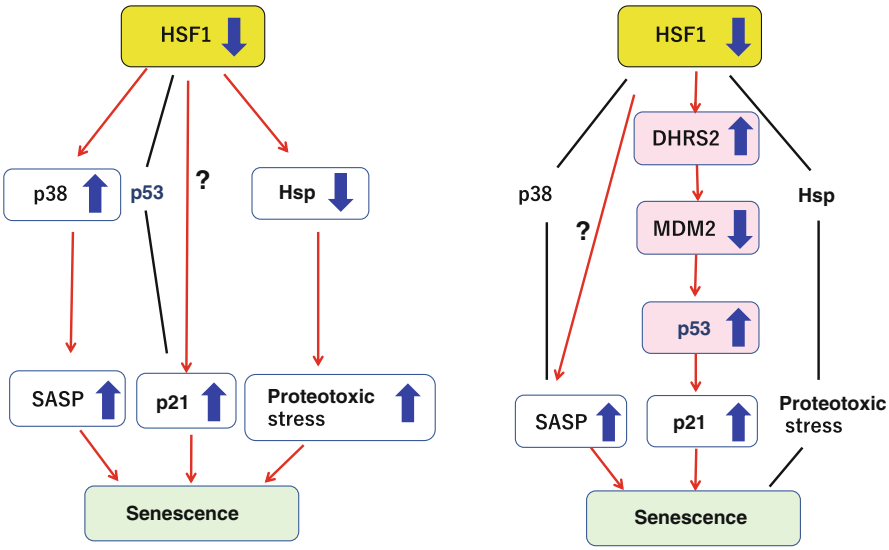

Fig. 1 Models for HDIS. A model based on our study (right) and a previous model (left) [28] are illustrated. Because Kim et al. studied the role of HSF1 in the context of DNA damage-induced cellular senescence, their model has been modified to facilitate comparison with ours. Kim et al. proposed that reduced Hsp levels and activation of the p38-SASP pathway, but not activation of the p53 pathway, is involved in HDIS. By contrast, we propose that the DHRS2-MDM2-p53 pathway, but not perturbation of Hsp-mediated proteostasis or activation of the p38 pathway, is involved in HDIS. Upwards and downwards blue arrows indicate increased and decreased levels/activities, respectively. The involved and uninvolved pathways are indicated by red arrows and black lines, respectively

underlying the HSF1 depletion-induced cellular senescence (HDIS), we employed a TERT-immortalized HDF line in which doxycycline (DOX)-inducible expression of shRNA targets the non-coding region of the *HSF1* gene. DOX treatment of this cell line reproducibly induced HSF1 depletion and subsequent senescence, as shown by irreversible growth arrest, morphological changes, increased expression of SASP, and increased numbers of SA-β-gal-positive cells and cells containing senescence-associated heterochromatin foci. We demonstrated that HDIS was dependent on activation of the p53-p21 pathway (Fig. 1). However, HDIS was not accompanied with decreased Hsp expression or increased levels of global proteotoxic stress, as measured by polyubiquitinated protein levels. In a reciprocal experiment, an inhibitor of Hsp70 family proteins increased proteotoxic stress but not senescence. The upregulation of p53 during HDIS resulted from reduced proteasomal degradation following MDM2 ubiqutin ligase (MDM2)-induced polyubiquitination. Collectively, these data indicate that the activation of p53-p21, but not reduced Hsp gene expression, mediates HDIS (Fig. 1).

HSF1 regulates cell proliferation in a manner unrelated to its transcriptional activity. For instance, HSF1 inhibits mitotic exit by directly interacting with CDC2 [29]. However, our analysis showed that wild-type HSF1 suppressed HDIS

but that HSF1 mutants defective in transcriptional activity did not. More specifically, HSF1 mutants lacking the trimerization domain or activation domain and two missense mutants defective in DNA-binding activity, all of which failed to induce expression of Hsp70 in response to proteotoxic stress, failed to suppress HDIS. Together, our findings indicate that low levels of a transcriptionally active HSF1 trimer are present under non-stressed conditions, contributing to repression of HDIS, but not basal expression of Hsp.

From the above observations, we reasoned that HSF1 depletion affects gene expression of an MDM2 regulator(s). To search for such candidates, we performed microarray gene expression analysis of DOX-treated model cells. We found that the mRNA levels of Dehydrogenase/Reductase 2 (DHRS2), a putative inhibitor of MDM2 [12], were the most significantly increased among 144 previously reported MDM2 regulators. Subsequent quantitative RT-PCR and immunoblot analyses confirmed the increases in *DHRS2* mRNA and protein levels, respectively, after HSF1 depletion in association with the HSF1 transcriptional activity. Interestingly, overexpression of wild-type and mutant HSF1 proteins affects *DHRS2* expression, raising the possibility that HSF1 directly regulates transcription of *DHRS2* [20, 23]. To examine binding of HSF1 to the promoter region of the *DHRS2* gene, we performed chromatin immunoprecipitation followed by quantitative PCR using primer sets covering potential heat shock responsive elements consisting of more than three inverted nGAAn repeats. However, we did not detect direct binding of HSF1 to the *DHRS2* promoter. These results indicate that HSF1 suppresses the expression of DHRS2 to basal levels in an indirect manner. Another possibility is that HSF1 regulates *DHRS2* gene expression by direct recognition of a non-canonical sequence in the promoter, perhaps in collaboration with other transcriptional regulators. Finally, we studied functions of DHRS2 in HDIS. Introduction of shRNAs targeting *DHRS2* partially suppressed HSF1 depletion-induced p53 stabilization and HDIS. In conclusion, we propose that the DHRS2-MDM2-p53 pathway plays an important role in HDIS (Fig. 1).

1.3 Role of HDIS in Tumorigenesis

The critical role of HSF1 in tumorigenesis was demonstrated in a seminal study by Dai et al. who showed in mouse models that HSF1 deficiency suppressed carcinogenesis induced by chemicals, or an activated RAS oncogene, or a p53 mutant [9]. In addition, HSF1 is overexpressed in various types of human tumors cells, and its elevated expression and activity correlate with aggressiveness and/or progression of tumors [26]. The influence of HSF1 activity on growth and survival of cancer cells is mediated by different pathways [10, 16]. First, cancer cells depend on the increased activity of the HSF1-Hsp pathway for their tolerance of proteome perturbations resulting from various intrinsic and extrinsic proteotoxic stresses and genetic and/or epigenetic alterations [47]. Second, HSF1 facilitates Hsp-mediated folding and/or stabilization of diverse proteins essential for cell growth and survival, including

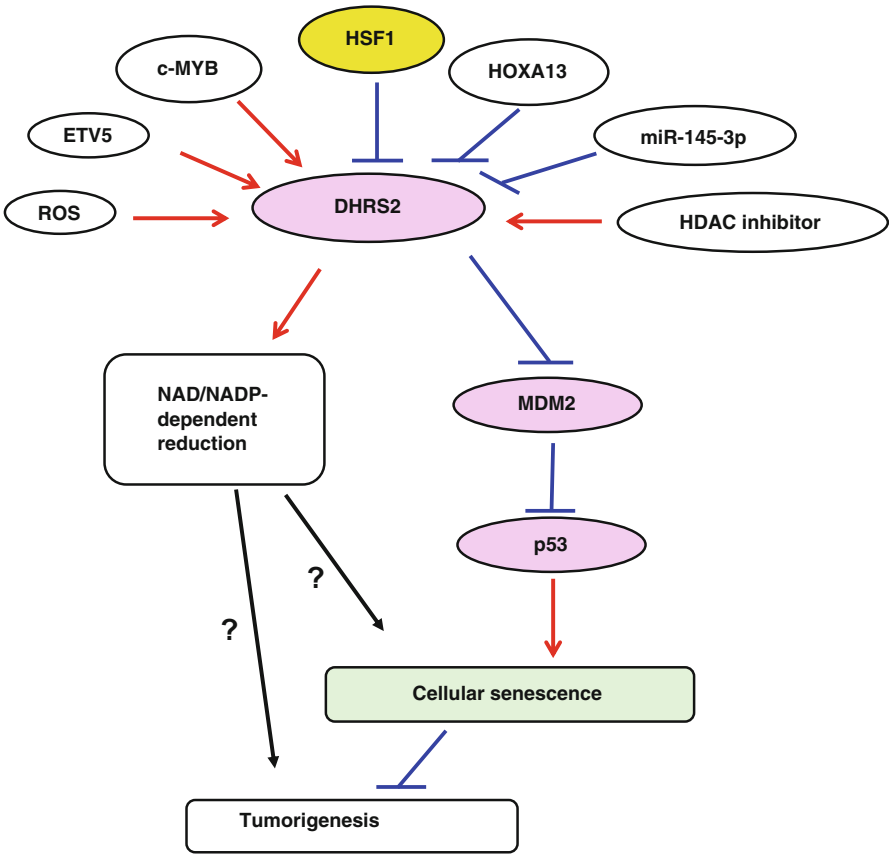

Fig. 2 Regulation and function of DHRS2 in tumorigenesis. DHRS2 functions as an NAD/NADP-dependent reductase and MDM2 inhibitor. HSF1 depletion induces expression of DHRS2. c-MYB and ETV5 bind to the promoter of the *DHRS2* gene and activates its expression. ROS and HDAC inhibitors stimulate expression of DHRS2. Conversely, HOXA13 suppresses expression of DHRS2 through binding its promoter. *miR-145-3p* targets transcripts of *DHRS2* and suppresses its expression. The DHRS2-MDM2-p53 pathway mediates cellular senescence which leads to tumor suppression. The role of DHRS2 reductase activity in tumorigenesis is unknown. See text for details

signaling proteins (e.g. AKT), DNA repair factors and mutant oncoproteins (e.g. SRC and BCR-ABL). Third, HSF1 directly regulates expression of various non-Hsp genes critical for cancer cell growth and survival [3, 34]. Accumulated data indicate that the DHRS2-MDM2-p53 pathway has tumor suppressor activity; therefore, as described below, it may play a significant role in the HSF1-mediated regulation of tumorigenesis and in the anti-tumor effects of HSF1-targeting therapies [27] (Fig. 2).

1.3.1 The DHRS2-MDM2-p53 Pathway

DHRS2 is a member of the *s*hort-chain NAD/NADP-dependent *d*ehydrogenase/*r*eductase (SDR) family [15, 43]. DHRS2 was originally identified as a 27 kD nuclear protein that was upregulated by butyrate-induced growth arrest in HepG2 human cancer cells, and was thus termed as Hep27 [14]. DHRS2/Hep27 is also localized in the cytoplasm and mitochondria [12], but the mechanism regulating its subcellular localization remains to be clarified. The SDR family of enzymes are evolutionarily conserved among eukaryotes and catalyze reactions with a wide spectrum of substrates, including retinoids, steroids, fatty acid derivatives and xenobiotics [15, 43]. Of note, a substantial body of evidence indicates that *DHRS2* is a tumor suppressor gene. *DHRS2* maps to chromosome 14q11.2, which is a region characterized by high-frequency loss of heterozygosity [5, 11]. Downregulation of DHRS2 is associated with tumor cell development and progression in esophageal squamous cell carcinoma [52]. Consistent with the study that first identified DHRS2/Hep27 [14], cell cycle arrest of tumor cells induced by treatment with histone deacetylase inhibitors is mediated by DHRS2 [32, 38]. Moreover, overexpression of DHRS2 suppresses growth and motility of cancer cells [52]. The tumor-suppressive effect of DHRS2 is at least partly mediated by the MDM2-p53 pathway [12, 52]. Paradoxically, increased expression of DHRS2 is associated with aggressiveness or drug resistance of tumors [19, 31]. Furthermore, DHRS2 expression is negatively regulated by the microRNA, *miR-145-3p*, whose overexpression exhibits tumor suppression [44]. These discrepancies might be partly explained by two different functions of DHRS2 (MDM2 inhibition and reductase enzymatic activity). The role of DHRS2 reductase activity in tumor cell biology is poorly understood and needs further study.

Some oncogenic signals stimulate expression of *DHRS2*. For instance, ETV5, a member of the Ets transcriptional factor family, binds to the promoter region of the *DHRS2* gene and induces its expression in a manner dependent on oxidative stress [36]. c-MYB also upregulates *DHRS2* expression through binding to its promoter [12]. Elevated expression of these proto-oncogenes may potentiate HDIS. In another case, HSF1 knockdown induces cellular senescence after ErbB2-induced transformation in an immortalized human mammary epithelial cell line, MCF10A [35]. This may be partly explained by synergistic activation of the DHRS2-MDM2-p53 pathway by ErbB2-induced oncogenic stress and HSF1 loss. This notion is consistent with our preliminary data, which showed that in the immortalized human epithelial cell line, RPE-1 cells, HSF1 knockdown alone produced little senescence. However, senescence was significantly induced after introduction of activated RAS oncogene in association with synergistic increases of DHRS2 and p53 protein levels (Oda, Yamashita, unpublished).

Conversely, some oncogenic molecules may promote tumorigenesis by suppressing the expression levels or activity of DHRS2. Such a case was recently documented. Homeobox A (HOXA)13, an oncogenic transcriptional factor contributes to gastric carcinogenesis by negatively regulating the DHRS2-MDM2-p53

pathway through direct inhibition of *DHRS2* transcription [19]. It would be interesting to further clarify the molecular mechanism of *DHRS2* gene expression, focusing on the role of HSF1, in tumor promotion and suppression, and cancer therapy.

1.4 Role of HDIS in Age-Related Pathologies

Does HDIS contribute to age-related phenotypes in HSF1-deficient organisms? If so, what is the role of the DHRS2-MDM2-p53 pathway? Addressing these questions will help elucidate the role of HSF1 in age-related diseases and longevity. Although overt cellular senescence is not documented in HSF1-deficient mice, mitochondrial abnormalities and increased oxidative stress, which are known to be tightly linked with cellular senescence [7], were observed [4, 51]. As depicted in Fig. 3, there are multiple pathways through which HSF1 deficiency can cause mitochondrial dysfunction and increased oxidative stress. First, HSF1 directly activates transcription of peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α), a central transcriptional regulator of mitochondrial biogenesis and remodeling [33]. Second, HSF1 also directly activates transcription of nicotinamide phosphoribosyltransferase, an enzyme functioning in the NAD^+ salvage pathway [42]. Levels of NAD^+ are critical for the enzyme activities of sirtuins, a family of NAD^+-dependent deacylases and

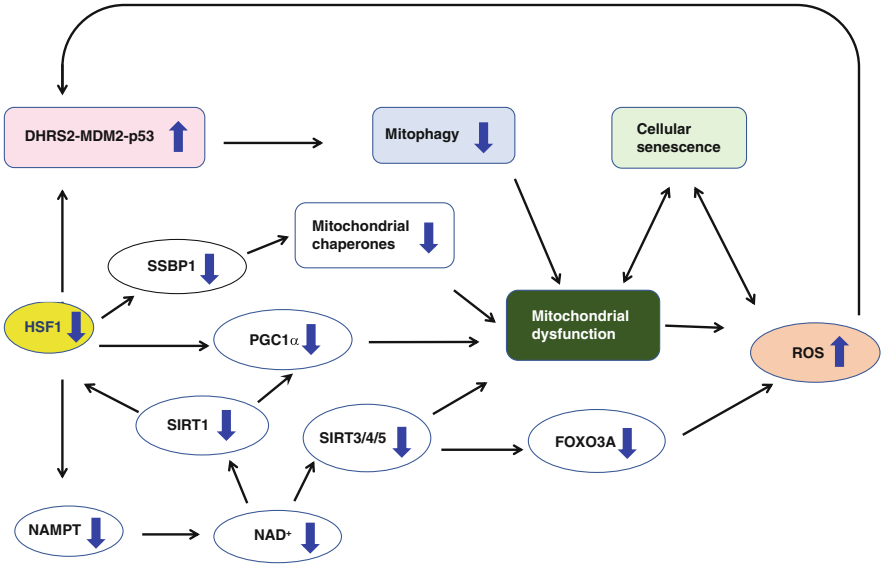

Fig. 3 Hypothetical model of molecular network linking between HSF1 deficiency and mitochondrial dysfunction: possible involvement of the DHRS2-MDM2-p53 pathway. See text for details. Upwards and downwards blue arrows indicate increased and decreased levels/activities, respectively. *NAMPT* nicotinamide phosphoribosyltransferase

ADP-ribosyl transferases. Mitochondrial sirtuins (SIRT3–5) play crucial roles in mitochondrial function and prevention of various age-related disorders [49]. In addition, SIRT1-mediated deacetylation stimulates the activity of PGC1α [48]. SIRT1 also suppresses the acetylation-induced inhibition of HSF1 [50], thus forming a positive feedback loop. Sirtuin-mediated stimulation of FOXO3A plays a critical role in expression of reactive oxygen species (ROS)-detoxifying enzymes, such as Manganese superoxide dismutase and Catalase; therefore, decreased activity of this pathway leads to increased levels of ROS [18]. A recent report by Nakai and colleagues revealed that HSF1 binds and recruits mitochondrial single-strand DNA-binding protein 1 (SSBP1), which is involved in replication of mitochondrial DNA, to the nucleus where the HSF1-SSBP1 complex promotes expression of mitochondrial and cytosolic chaperones under exposure to proteotoxic stress [46]. Thus, HSF1 deficiency might cause mitochondrial dysfunction through impaired proteostasis in mitochondria. This notion is supported by knockdown of SSBP1, which moderately reduces mitochondrial transmembrane potential even in unstressed conditions [46]. Overall, HSF1 plays various roles in the biogenesis and functional maintenance of mitochondria.

Mitophagy, autophagic removal of dysfunctional or excessive mitochondria, plays an essential role in the quality control of this organelle [41]. Thus, impaired mitophagy leads to accumulation of dysfunctional mitochondria. The mitochondrial kinase, PINK1, and the ubiquin ligase, Parkin, collaborate to mediate the major mitophagy pathway. In stressed or aged cells, p53 binds Parkin in the cytoplasm and inhibits mitophagy, resulting in accumulation of dysfunctional mitochondria [25]. Conversely, the p53-inducible protein, mitochondria-eating protein (Mieap) contributes to repairing dysfunctional mitochondria [37]. Mieap-mediated quality control of mitochondria is frequently inactivated in p53-deficient tumors. The resulting accumulation of dysfunctional mitochondria is proposed to contribute to tumorigenesis. However, the functional significance of Mieap in age-related phenotypes or cellular senescence remains unknown. Thus, activation of the DHRS2-p53 pathway during HDIS may suppress mitophagy and promote accumulation of dysfunctional mitochondria, boosting progression of cellular senescence. In addition, because ROS is known to stimulate DHRS2 expression [8], the DHRS2-p53 pathway might amplify senescence signaling in a positive feedback loop.

In summary, we hypothesize that HSF1 deficiency may affect multiple pathways leading to mitochondrial dysfunction and induce activation of the DHRS2-MDM2-p53 pathway, which cooperatively promote HDIS. It is also tempting to speculate that DHRS2 might be activated as a reductase as well as an MDM2 inhibitor in response to increased ROS. Interestingly, a recent transcriptome analysis revealed that expression of DHRS7, a member of the SDR family, is commonly elevated in eight different senescence model systems using HDFs and human vascular endothelial cells [6].

2 Conclusions

Acute depletion of HSF1 transcriptional activity induces cellular senescence in HDFs through activation of the DHRS2-MDM2-p53 pathway. However, many aspects of the molecular mechanisms of HDIS, such as mitochondrial functions, epigenetic alteration and SASP expression, remain to be studied. Other important questions include whether or not HDIS occurs *in vivo* and whether or not it regulates development of tumors and age-related phenotypes. Study of these problems in comparison with other senescence models will provide novel insights into the mechanisms and functions of HDIS.

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Disclosure of Interests The author declares he has no conflict of interest.

Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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Huntingtin Yeast Two-Hybrid Protein K (HYPK): An Intrinsically Unstructured Heat Shock Inducible Protein with Diverse Cellular and Molecular Functions

Nitai P. Bhattacharyya, Srijit Das, Kamalika Roy Choudhury, Swasti Raychaudhuri, and Jayeeta Ghose

Abstract

Introduction Huntingtin Yeast Two-Hybrid Protein K (HYPK) was identified as huntingtin interacting protein in yeast 2 hybrid (Y2H) assay in 1998. Since then, HYPK has been characterized and shown to participate in diverse cellular functions. HYPK is shown to possess chaperone like activity in *vitro* and in *vivo* and regulated by Heat Shock Factor 1 (HSF1) by binding to the promoter of *HYPK* in response to heat shock (HS). The goal of the present review is to describe and evaluate current information of HYPK.

Methods We searched published literatures and various databases to get information of HYPK.

Results Result is divided into two parts. In first part, we briefly describe regulation of genes coded for heat shock proteins (HSP) and noncoding RNAs by HSF1. Role of HSP in different biological processes/functions is also summarized. Next, we

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discuss biophysical, biological characteristics and regulation of HYPK in the light of regulation of genes coding for HSP. HYPK is an intrinsically unstructured protein with chaperone activity and regulated by HSF1 in response to HS. Like many HSP, HYPK suppresses heat shock response via an auto-regulatory loop, binds to nascent polypeptides and interacts with more than 70 proteins, including transcription factors HSF1, TP53, RELA/p65. Role of HYPK-TFs interaction in modulating target genes of TFs remains unknown. HYPK involves in cell proliferation, cell cycle regulation, apoptosis and autophagy.

Conclusions Taken together, we conclude that HYPK is a heat shock protein with chaperone activity. Based on the result, activation of HYPK could be one of the approaches to reduce aggregates and toxicity in HD.

Keywords Apoptosis · Autophagy · Cell cycle · Chaperone · Heat shock response · HYPK · Intrinsically unstructured protein · Nascent peptides

Abbreviations

HS	heat shock
HSE	heat shock element
HSF	heat shock transcription factor
HSP	heat shock proteins
HSR	heat shock response
HTT	huntingtin
HYPK	Huntingtin Yeast Two-Hybrid Protein K
IDP	intrinsically disordered protein
IUP	intrinsically unstructured protein
lncRNA	long non-coding RNA
miRNA	microRNA
TF	transcription factors
Y2H	yeast two-hybrid

1 Introduction

Exposure to cellular stresses like heat shock, oxidative agents, heavy metals, hypoxia, ischemia, acidosis and others triggers protein misfolding leading to formation of aggregates of the misfolded/damaged/unfolded proteins. Cells respond to heat shock (HS) by synthesizing set of proteins known as heat shock proteins (HSP). Even though, induction of HSP was initially identified in response to HS in fly [100], it is now known that HSP is induced in repose to diverse cellular stress signals. Heat shock proteins are mostly chaperones and can be classified as (a) Heat shock 90 kDa proteins (b) Heat shock 70 kDa proteins, (c) Chaperone DnaJ, also known

as heat shock protein 40 kDa (Hsp40) and (d) small heat shock proteins [38]. In the present review, we present experimental evidences to show that Huntingtin Yeast Two-Hybrid Protein K (HYPK) with theoretical molecular weight 14.7 kDa is a heat stress responsive protein with chaperone activity. In the first part, we briefly discuss regulation of classical HSP by the evolutionary conserved HSF1, other properties of HSP and then describe in next sections experimental evidences to show that HYPK is regulated by HSF1 in response to HS and other stress signals and participated in diverse cellular functions.

1.1 Regulation of Heat Shock Proteins by HSF1

Among different members of heat shock factor (HSF) family present in vertebrates, HSF1 is the prime transcription factor for regulation of HSP [2]. HSF1 upon activation in response to HS moves to nucleus from cytoplasm, is modified by phosphorylation, sumoylation, acetylation etc. and binds to specific DNA sequence motif, known as heat shock element (HSE) (review [128]). A functional HSE contains a series of pentameric unit arranged as inverted arrays of sequence 5′-nGAAnnTTCn-3′; at least three pentamers are required for interaction with HSF1 [3, 128]. Although HSF1 activates or represses hundreds of protein coding genes in response to HS in diverse organisms, only a small fraction of these genes harbour functional HSE. Occupancy of HSF1 to HSE at promoters of positively correlated genes is about 27% while only 7% of the promoters of negatively regulated genes are occupied by HSF1 [92]. Various transcriptional studies in cells in response to HS reveal some common features. These are in brief (a) during HS, expressions of HSP and few other chaperones are induced by binding HSF1 to the promoters, (b) many induced genes in response to HS are not direct targets of HSF1 and (c) HSF1 binds to the promoters of some genes that are not induced by thermal tress [81, 84, 92, 99, 122, 130]. HSF1 regulated gene expression is dependent on cell type [64], phase of cell cycle [129], metabolic state [17] and pathological state like cancers [84]. Deregulated genes in response to HS without having HSE could be due to global modification of chromatin landscape [23, 51, 130]. In addition, altered expression of microRNA (miRNA), a negative regulator of protein coding genes in general [8], may also contribute to the deregulation of protein coding genes without HSE in response to HS. Some of the miRNAs are also regulated by HSF1 directly by binding to the promoters and reviewed [28]. Long non coding RNA (lncRNA), functional RNA with length > 200 nucleotide without potential for protein coding, is emerging as a major regulator of transcription [40, 49]. Out of thousands of lncRNAs only few lncRNAs like NEAT1, GAS5 and TERRA are known to be regulated by HSF1 directly in response to HS [70, 75, 134]. These lncRNAs induced by HS may regulate expressions of protein coding genes directly by binding with the chromatin [40, 49] or indirectly by interacting with miRNA [123]. Role of non-coding RNA in deregulating expressions of protein coding genes in response to HS is not extensively studied. Several miRNA and few lncRNA are

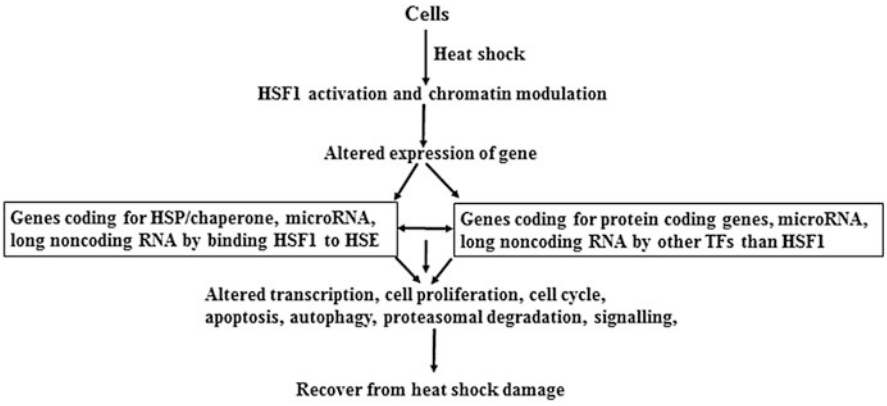

Fig. 1 Transcription response to heat shock and interconnections of genes. Double headed arrow represents various types of interactions like mRNA-microRNA, lncRNA-chromatin, lncRNA-mRNA and lncRNA-miRNA [54]

regulated in response to HS and likely to contribute to the deregulated genes in response to HS. Other transcription factors than HSF1, whose expression is altered during heat shock response (HSR) may also contribute to the deregulate protein coding genes directly. For example, serum response factor (SRF) is identified as a novel regulator of cytoskeletal genes during HSR and regulates many genes in cells treated with HS [81]. Summary of the possible mechanisms of deregulation of protein coding genes, microRNA and lncRNA and their interconnections in response to HS is shown in Fig. 1.

1.2 Functions of Heat Shock Proteins

HSP plays an essential role in proteostasis. Transiently induced HSP ensure folding of stress induced damaged/misfolded and unfolded proteins [57] or proteasomal degradation depending on the extent of damage [63, 107] and appropriate folding of proteins during protein synthesis through binding with newly synthesised proteins [42, 56]. These functions are achieved through chaperone activities of HSP and interactions with other proteins. In addition to the main functions of HSP as molecular chaperone, they might have secondary functions in antigen processing and presentation [87] and modulation of immune system [144]. HSP is also associated with other transcription factors and regulate expressions of genes. Direct or indirect cooperation of different HSP with TF like TP53 [135, 138], NFκB/RELA/p65 [15, 72, 79, 93, 141], CREB [104], MYD88 [11], RUNX2 [77], SOX11 [39], HNF4A [65], STAT1 [82], STAT3 [19, 110], HIF1A/HIF-1α [73, 85], cMYC,

EZH2 [74] and SREBP [71] and transcription modulator SMAD2 [133] in different conditions modulates transcription of many genes.

Altered expressions of genes coding for HSP or genes coding for proteins other than HSP directly or interacting with other proteins involve in different biological processes. HSP90 interacts with CDK4, CDK11p110 and Wee1 and regulates cell cycle. Levels of *HSP90* vary throughout the different phases of cell cycle. Increased ATP levels during mitosis may also alter the functions of HSP90. This result shows that HSP participates in cell cycle and reviewed [61]. HSP interact with pro-apoptotic genes like caspases, anti-apoptotic gene like BCL2, signalling proteins like JNK, NFκB and AKT and modulate apoptosis [10]. Heat shock protein HSP90AA1/HSPC1/HSP90A enhances DNA repair activity by modulating cell cycle check point kinase CHK1. Inhibition of the heat shock protein sensitizes cells to chemotherapeutic agents by destabilizing CHK1. HSPB1, a family member of small heat shock protein, has been implicated in ATM mediated DNA repair. Role of HSP90AA2/HSPC2 and HSPA1A (70 kDa family member) in repair of different types of DNA damage has been identified [34, 111].

Autophagy is a physiological process for removing misfolded proteins, aggregates of misfolded proteins and damaged organelles by macroautophagy, microautophagy or mitophagy. In chaperone-mediated autophagy, soluble proteins with a specific pentapeptide motif are recognized by heat shock cognate 70 kDa (Hsc70) and co-chaperones BAG11, HIP, HOP and HSP40 and transported to lysosome for degradation [50, 67]. Knock down of *HSF1*, the key regulator of HSP, enhances basal and induced autophagy; exogenous expression of *HSP70* reverses the effect of *HSF1* depletion, indicating HSP70 protects cells by induction of autophagy. Role of HSF and other proteins induced in response to thermal stress in autophagy has been reviewed [31, 32].

In summary, HS transiently induces genes coding for HSP with chaperone activity and other genes including non-coding RNAs. Only a small fraction of these genes are direct targets of HSF1. Global chromatin remodelling and deregulation of non-coding genes may contribute to the deregulation of genes not directly regulated by HSF1. HSP and their interacting protein partners together with many other deregulated genes may contribute to diverse biological processes observed in repose to thermal stress as illustrated in Fig. 1.

In the following sections, we shall review the characteristics of a relatively unknown and uncharacterised protein HYPK, which was identified in 1998. HYPK (gene ID: 25764), also known as HSPC136/C15orf63, localized at 15q15.3, is one of the 13 N-terminal HTT protein interacting partners, identified in Y2H assay. HYPK, at the time of discovery, did not have any known function [41]. Polymorphic CAG repeats in the exon 1 of *HTT* gene is expanded beyond 36 repeats and causes neurodegenerative autosomal dominant Huntington's disease (HD, OMIM ID 143100) (Huntington's Disease Collaborative Research Group, 1993).

1.3 Characteristics of Intrinsically Unstructured/Disordered Protein (IUP/IDP)

Intrinsically disordered protein (IDP), also known as intrinsically unstructured protein (IUP), is defined as protein that lacks a stable tertiary structure when determined under reasonable physiological conditions *in vitro*. IDPs/IUPs have following biophysical characteristics:

(a) IDP/IUPs have relatively low proportion of hydrophobic and aromatic residues and a relatively high proportion of charged and polar residues. Thus, these regions are depleted in order-promoting residues (Trp, Cys, Tyr, Ile, Phe, Val, Asn, and Leu) and enriched in disorder-promoting residues (Arg, Pro, Gln, Gly, Glu, Ser, Ala, and Lys) [125, 127]
(b) IDP/IUPs have low secondary structure content
(c) IDPs/IUPs tend to have low sequence complexity and commonly contain repeats [66]
(d) Apparent molecular weight determined in SDS-PAGE gel electrophoresis is 1.2–1.8 fold higher than their empirical molecular weight [115]
(e) Hydrodynamic volume as determined by size-exclusion chromatography is also higher than that of the estimated volume of a globular protein of same size [124]

Among bacterial proteins, 16–45% had long IDP/IUP regions; while in archaea and eukaryotic proteins these values are 26–51% and 52–67% (reviewed in Uversky [126]. Stretch of disordered/unstructured region with $\geq$30 amino acid residues is significantly higher among transcription factors [78, 114, 120], signalling molecules like kinases [36, 114, 116, 143] and HSP, especially chaperones [136].

1.4 Huntingtin Yeast Two-Hybrid Protein K (HYPK) Is an Intrinsically Unstructured/Disordered Protein (IUP/IDP)

HYPK (NP_057484) shares no significant sequence homology to previously reported genes/proteins. Molecular weights estimated from SDS-PAGE of 6XHN tagged HYPK and tag free HYPK were 22 kDa and 20 kDa respectively. Theoretical molecular weights of the same proteins were 17.1 kDa and 14.7 kDa respectively. Molecular weight determined by mass spectrometry reveals that molecular weight of the tagged HYPK is 16.9 kDa, similar to theoretical calculation of the molecular weight. This unusual mobility of HYPK in polyacrylamide gel indicates that it might be an intrinsically unstructured protein [115]. Stoke's radius of HYPK determined by size exclusion chromatography was higher than expected from a globular protein of similar mass. In size exclusion chromatography, HYPK was eluted in three fractions; mobility of the protein was similar in SDS-PAGE. These results suggest that HYPK could be a non-globular protein and might exist either in three different

oligomeric forms or alternative conformations in solution; the largest one being some kind of soluble aggregates. Hydrodynamic radii of HYPK determined by dynamic light scattering were higher than expected from globular proteins of similar mass. This result further indicates that HYPK may exist in multiple conformations and likely to be an intrinsically unstructured protein [124]. Solution structure determined by circular dichroism revealed that HYPK had 20.5% α-helical, 16.2% β-sheet, and 63.3% random coil secondary structures. Indirect evidence for lack of tertiary structure was also provided. Limited proteolysis of HYPK further revealed absence of specific domain and had significant disordered structure. Theoretical analysis of the amino acids reveals that HYPK has low mean scaled hydropathy and high net charge at neutral pH. HYPK is significantly enriched in glutamine, lysine, arginine and other charged amino acids and is depleted in tryptophan, phenylalanine, and cysteine like hydrophobic or order promoting residues. This analysis along with theoretical prediction from two online resources show that HYPK could be an intrinsically unstructured protein [89, 96, 125, 127]. Solution structural studies by CD indicate that HYPK *in vitro* may remain as pre-molten globule like structures. Result obtained with DLS further indicates that HYPK may have conformation with high propensity of aggregation formation in vitro [97]. Determination of molecular mass of recombinant HYPK by SDS-PAGE, size-exclusion chromatography, mass spectrometry and conformation by dynamic light scattering (DLS), circular dichroism (CD), limited proteolysis and theoretical analysis of HYPK thus revealed that HYPK is a natively unfolded protein, also known as IUP. Experimental evidence that HYPK is an intrinsically unstructured protein was also provided. It has been shown by several investigators that most of the heat-resistant soluble proteins in a cell extracts are IUPs while structured proteins precipitate by heat treatment [26, 44]. Following this approach HYPK was identified in soluble fraction of the heat-treated cell extract showing that HYPK is an IUP/IDP (unpublished result, Kamalika Roy Choudhury, Ph.D. thesis, Calcutta University, 2013).

1.5 In Vitro *and* In Vivo *Chaperone Activity of HYPK*

Chaperone like activity of HYPK is initially shown *in vitro* and in cultured cells. Purified HYPK reduces the aggregates of alcohol dehydrogenase and malate dehydrogenase induced by heat. In these in vitro assays, ATP was not required; indicating that observed chaperone activity of HYPK was independent of ATP. Renaturation of unfolded bovine carbonic anhydrase is enhanced in presence of HYPK. Luciferase reporter assay in cultured cells in presence exogenous HYPK or reduced endogenous HYPK in response to heat stress confirms chaperone activity of the protein. Finally, HYPK was able to modulate the formation of aggregates by the mutant N-terminal HTT with 40 glutamine (40Q) coded by exon1 of the gene. Exogenous expression of *HYPK* decreased the number of aggregates; knock down of *HYPK* increased number of aggregates. Live cell microscopy further reveals that GFP-HYPK not only

suppresses the aggregates of mutant N-terminal HTT but also reduces the kinetics of mutated N-terminal HTT-mediated aggregate formation. Taken together, HYPK exhibits chaperone activity *in vitro* and in *vivo* [97].

1.6 HYPK Binds with Newly Synthesized Peptides/Proteins

Appropriate folding of newly synthesized proteins requires chaperones for preventing protein misfolding and aggregate formation. Various chaperones/HSP like Hsp70 bind co-translationally with newly synthesised protein on ribosomes and stabilize elongating chain preventing misfolding of the newly synthesised peptides. Release of the stabilizing factors including Hsp70 finally folds the newly synthesised proteins by downstream chaperones like chaperonins [56, 69]. Alignment of 105 orthologs of HYPK from plants, lower vertebrates to mammals identified a conserved nascent polypeptide-associated alpha subunit (NPAA) domain of 40 amino acids (90–129) at the C-terminus of HYPK. This region experiences highest selection pressure, signifying its importance in the structural and functional evolution of the domain. This domain of human HYPK has unique amino acid composition preferring glutamic acid and is likely to be more stable with higher α-helices content than the rest of the protein. In cultured cells, C-terminal human HYPK (61st to 129th amino acid) co-localizes with aggregates of mutant N-terminal HTT and reduces the aggregates of mutant HTT as well as the toxic effects of the aggregates indicating that C-terminus of HYPK possesses chaperone activity *in vivo*. This C-terminus HYPK containing the NPAA domain binds the nascent polypeptides [98]. HYPK was co-purified with ribosome-associated complex of MPP11/DNAJC2/Hsp70L1 [90]. Association of HYPK together with NAA10, and NAA15 with polysome fractions was reported [4]. This result together with observation that HYPK with chaperone activity [97] directly binds to the newly synthesized peptides [98] shows that HYPK might be involved in folding of newly synthesized proteins.

1.7 HYPK Interacting Proteins

Initial observation of interaction of HYPK with HTT in Y2H assay [41] was further confirmed by immunoprecipitation and co-localization in cultured cells. It has been shown that HYPK interacts both wild type N-terminal HTT and mutant N-terminal HTT [97]. More precisely, HYPK is shown to interact with first 17 amino acids of N-terminal mutant HTT and prevents aggregate formation by mutant N-terminal HTT. Similar results of bindings of HSPA1A, HSPB1 and DNAJB3/HCG3 with first 17 amino acids have also been reported [20]. HYPK was co-purified with ribosome-associated MPP11/DNAJC2/Hsp70L1 complex along with ARD1/NAA10 and NAA15 indicating that HYPK may act as a binding partner to one or more of these proteins during protein synthesis [90]. NAA10 and NAA15 are the

catalytic and auxiliary subunits of human Nα-terminal-acetyltransferase (NatA) complex. NatA complex is involved in co-translational N-terminal acetylation of proteins. HYPK together with NAA10 and NAA15 is associated with polysome. HYPK is essential for efficient N-terminal acetylation of NatA substrates. Such interaction with NAA10 and NAA15 stabilizes HYPK. Knock-down of NAA10, NAA15 or HYPK increases the aggregates formed by mutant HTT [4]. These observations indicate a possible function of HYPK during or immediately after protein translation on the ribosome. Many proteins are identified in Y2H assays to interact with HYPK (viz., CHD3, GC, MDFI, PSME3, QKI, RBPMS, RHOXF2 and TH1L/NELFCD) [112], but not validated further. High through put assays identified APP, C11orf58, DCTN1, DDI2, EFTUD2, MAGEA1, MFAP1, NAA11, NAA50, PTTG1, RPA2, TRIM28 and TSGA10IP as interacting partners of HYPK. Co-localization of MYL12A, SEPHS1, SPG20, TXLNA, UBQLN1 and ZPR1 with HYPK was observed and catalogued in BioGrid database (https://thebiogrid.org/, accessed August 2018), a curated database of protein-protein interaction repository [91]. Affinity capture followed by mass spectrometry, Western blot analysis and co-localization in cell culture identify 36 proteins to interact with HYPK. Protein partners include several chaperones, HSPA8, HSP90AB1, HSPA1A, HSPB1, DNAJB3 and transcription factors HSF1, TP53, RELA/p65. In several cases HYPK forms high molecular complex with its interacting partners like (HTT, LMNB2), (EEF1A1 and HSPA1A) and (TP53 and RELA), as evident from their mobility in non-denaturing gel [21]. HYPK was further shown to interact with Mortalin/HSPA9, RPL7A, a component of the 60S large ribosomal subunit, BECN1, lysosome-associated protein 2A (LAMP2) and APP with HYPK using affinity capture followed by Western blot analysis and/or co-localization [22]. Combining data from published high confidence interaction data [4, 21, 22, 90], high throughput data [112] and data catalogued in BioGrid [91], it was evident that HYPK interacts with 73 unique proteins.

1.8 Chaperones Are in Hub of Protein-Protein Interaction Network

In protein-protein interaction network (PPIN), proteins are in “nodes” and “edges” represent the physical interactions. In PPIN, most proteins are connected with only a small number of proteins, while few proteins interact with many proteins. These small numbers of proteins which are connected with large number of proteins by physical interaction are defined as “Hub”. Characteristics features of Hub proteins are (a) highly connected to network components (b) have pleiotropic functions (c) have rapid turnover and regulation and (d) have large number of phosphorylation or other post translational modification sites [7, 9]. HSP/chaperones bind to a large variety of unfolded proteins or folding intermediates to prevent their non-specific protein aggregation and facilitate appropriate folding of the proteins. Stretch of disordered/unstructured region with $\geq$30 amino acid residues is significantly higher

among HSP, especially chaperones [136]. Proteins with chaperone like activity in human e.g. SNCA/α-Synuclein, SNCB/β-Synuclein, Tubulin, PRNP/Prion protein and small heat shock protein CRYAA are reported to be IUP/IDP. Their predicted degree of disorder ranges from 24% to 100% [117, 119]. Multiple distinct conformations of these disordered/unstructured regions allow them to associate with many partners forming protein complexes. It has been shown that average predicted disorder region increases with the number of proteins within the protein complexes [25, 59]. Binding of chaperones may result in conformational rearrangements that modulate interactions with other proteins. Such proteins are likely to be "Hub" in PPIN and involve in diverse functions. There are many examples to show that hub proteins are enriched with IDPs/IUPs in comparison with non-hub proteins [33, 37, 43, 58, 60, 62, 109, 118]. Binding may stabilize both interacting partners [108]. Proteins with unstructured single interface possessing multiple distinct conformations may allow binding with different partners and perform different functions [118].

Heat shock proteins/chaperones are associated with large number of proteins. In current version of BioGrid database (https://thebiogrid.org/, access in August 2018), out of 97 HSP/chaperones of different categories, 93 HSP have 3,962 interacting partners in the database. HSP90AA1, HSPA8, HSPD1, HSPA5, HSPB1, HSP90AB1 and HSPA4 have 871, 813, 556, 551, 449, 422, 402 interacting partners respectively. On the other hand DNAJC24, DNAJC25, DNAJC27, DNAJC5B, and HSPD1P11 have 2, 2, 1, 1 and 1 partner respectively. This shows that few HSP are connected to large number of proteins while most of them are connected to few interacting partners. Thus HSP/chaperones are likely to be in "Hub" of PPIN. As mentioned above "Hub" proteins are enriched with IUPs. HYPK being an IUP and interacts with 73 proteins, is likely to be in hub of PPIN.

1.9 HYPK Is Associated with Aggregate Prone Proteins

HD is one of many other neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Prion disease caused by mutations that result in protein conformation change, misfolding and formation of aggregates of mutant proteins [55]. Structural flexibility maintains by proteins through presence of significant unstructured regions in the protein for interaction with multiple protein partners and carrying out diverse functions of the proteins. However, these unstructured regions provide risk for misfolding and aggregation. Significant unstructured region in a protein makes it prone for aggregate formation [18]. HYPK was identified as a global interacting partner/regulator of aggregate prone N-terminal mutant HTT coded by the exon1, mutant α-Synuclein (A53T) and Superoxide dismutase1 (G93A). C-terminal hydrophobic residues 60–69 and 84–93 of HYPK have been identified to make direct contact with these aggregate prone proteins and also self oligomerization of HYPK. Thus, HYPK may act as an aggregate sensor by existing in seeded amyloid-like state that favours its own self-oligomerization and sequestration of aggregate prone proteins and protects cells from toxic effects of aggregates [46, 47]. Internal ribosome entry site (IRES)

independent translation of full length HYPK can prevent aggregates of the mutant TP53 (R248Q) protein in the nucleus, while IRES dependent translation results in shorter version of HYPK without nuclear localization signals [45]. HYPK interacts with wild type and mutant N-terminal HTT coded by exon 1 [97], specifically with the first 17 amino acid. Other proteins like HSPA1A, HSPB1, HCG3/DNAJB3 and MLF1 also interact with this region [20]. Several other proteins TPR [24], CRM1/Exportin1 [83], TRiC/CCT [113] and PACSIN1 [13] also interact with first 17 amino acid of HTT having hydrophobic patch. This result shows that, first 17 amino acids of HTT having mostly disordered prone amino acids is target of binding with several chaperones including HYPK.

To find whether other interacting partners of HYPK like N-terminal HTT have disordered regions, it has been observed that out of 49 protein interacting partners of HYPK, 44 proteins had intrinsically unstructured region $\geq$30 amino acids at a stretch (data not shown). For example, ZNF100, an interacting partner of HYPK [21], has 542 amino acids; 377 amino acids at stretch are disordered (Bhattacharyya NP et al., unpublished observation). Intrinsically unstructured proteins are often aggregation-prone. Unstructured-stretch within a protein is also known to seed aggregation [18, 76]. These unstructured regions are prone for aggregate formation; HYPK might have a preference for binding with aggregate prone proteins with disordered regions.

1.10 Thermal Stress, Hypoxia and Proteasome Inhibitor MG132 Enhance Expression of HYPK in Cultured Cells

Human HeLa and mouse Neuro2A cells subjected to heat shock at 42 °C for 1 h followed by recovery at 37 °C for different time points exhibited increased expression of *HYPK* continually up to 4 h and then declined at 8 h. Pattern of expression of well-known heat shock protein Hsp70 at different time points was similar. However, increase in the level of *HYPK* in response to heat shock was lower than that of *Hsp70*. Hypoxic condition induced by cobalt chloride treatment and proteotoxic stress induced by proteasome inhibitor MG132, also increased *HYPK* expression. Exogenous expression of *HYPK* protects HeLa cells from acute heat induced cell death, while knockdown of *HYPK* sensitized cells. This result shows that *HYPK* is regulated by diverse stress and protects cells from heat stress induced death [27].

1.11 HSF1 Binds to the Promoter Sequence of HYPK *and Regulates the Expression*

Based on the observation that HYPK has *in vitro* and *in vivo* chaperone activity, it has been hypothesized that like genes code for classical HSP, *HYPK* could also be

regulated by the stress-responsive transcription factor HSF1. Subsequently it has been identified that (a) *HYPK* expression is induced by thermal stress at transcript as well as protein level in human (HeLa) and mouse (Neuro2A) cells and the kinetics is similar to *hsp70*, a bona fide target of HSF1. (b) Ectopic expression of HSF1 induced *HYPK* expression following HS in HeLa and Neuro2A cells. (c) Using bioinformatics tool, we identified putative HSF1-binding site in human *HYPK* promoter ($^{+69}$TGAAGCTTCTAGAAC^{+83}) and mouse HYPK promoter (^{208}ATTCGGGATCATTCG^{-198}). By luciferase reporter assay and chromatin immunoprecipitation (ChIP), we confirmed increased occupancy of HSF1 and RNA polymerase II in these sites in response to heat shock. We further showed that heat shock-driven recruitment of HSF1 and RNA polymerase II in *HYPK* promoter is accompanied by acetylation of histone H4 which is an epigenetic marker of inducible HSF1 binding to its cognate sites across the genome [51]. (d) Knocking down endogenous *HSF1* expression in HeLa cells reduces *HYPK* expression at transcript and protein level. Furthermore, HSF1-knocked down cells were deficient in inducing *HYPK* expression in response to elevated temperature. (e) Effect of HSF1 on HYPK expression was further confirmed by using two deletion mutants of HSF1. A constitutively active HSF1 that lacks part of the regulatory domain (amino acids 203–315) is able to *trans*-activate its downstream target genes in the absence of stress [139, 146]. It has been shown that when expressed in HeLa cells, constitutively active HSF1 can induce *HYPK* expression in the absence of heat shock. On the contrary, another deletion mutant lacking part of the transactivation domain of HSF1 (amino acids 454–522) is able to interact with endogenous HSF1 and inhibit its trans-activation ability [139, 146]. It has been observed that when expressed in HeLa cells, this dominant negative HSF1 can inhibit HS-induced expression of *HYPK* showing that *HYPK* is a target of HSF1. Taken together, these results show that *HYPK* is a heat shock responsive gene regulated by HSF1 by binding to the putative promoter [27]. Binding of HSF1 in the promoter of *HYPK* [84] and regulation of the expression of the gene have also been reported by other investigators [101].

1.12 HYPK Negatively Regulates Heat Shock Response

In unstressed cells, HSF1 expresses constitutively and remains as an inactive monomer. Activation of HSF1 and increase expression of HSP in response to HS is transient in nature. In response to HS, HSF1 is activated transiently by moving to the nucleus, trimer formation, phosphorylation and other post translational modifications. Activated HSF1 binds to HSE and upregulates HSP for restoration of damaged/unfolded proteins [94, 137]. Activation of HSP by HSF1 is arrested upon restoration of proteostasis. One of the mechanisms by which activated HSF1 attenuates is through negative feedback relation with HSP. Multi-chaperone complexes of HSP and co-chaperones have been shown to regulate HSF1 activity. Complexes of HSP are not only associated with HSF1, but also with non-native misfolded/damaged clients proteins induced by heat shock and either fold back the misfolded

proteins or degrade the non-native protein through polyubiquitination mediated proteasomal degradation [131]. In response to heat shock, HSP/chaperones release HSF1 to bind with misfolded/unfolded proteins. Translocation of free HSF1 to nucleus and modification by phosphorylation or other post translation modification activates HSF1 to induce the genes coding for HSP/chaperones. After homeostasis is achieved, excess free chaperones again bind to HSF1 and deactivate HSF1. Biochemical, pharmacological and genetic evidences are available to show that HSP90 [35, 145], HSP70 [1, 5, 6], their co-chaperones [35, 88] and others [52, 106] are involved in HSF1 in a negative feedback loop. A simple model for feedback loop of HSP/chaperones in regulating their expressions is shown in the Fig. 2. Heat shock dependent phosphorylation at different positions of HSF1 has also been shown to activate, repress or fine tune the transcription ability of HSF1 in yeast and mammalian cells [142].

Similar negative regulation of HSF1 activity by HYPK has been shown. In presence of exogenous *HYPK*, expression of several HSP like *HSPB1*/HSP27, *DNAJB1*/HSP40, *HSPD1*/HSP60, *HSPA1A*/HSP70, *HSP90AA1*/*HSP90* and its own expression are reduced. Heat shock-driven increase in expression of all

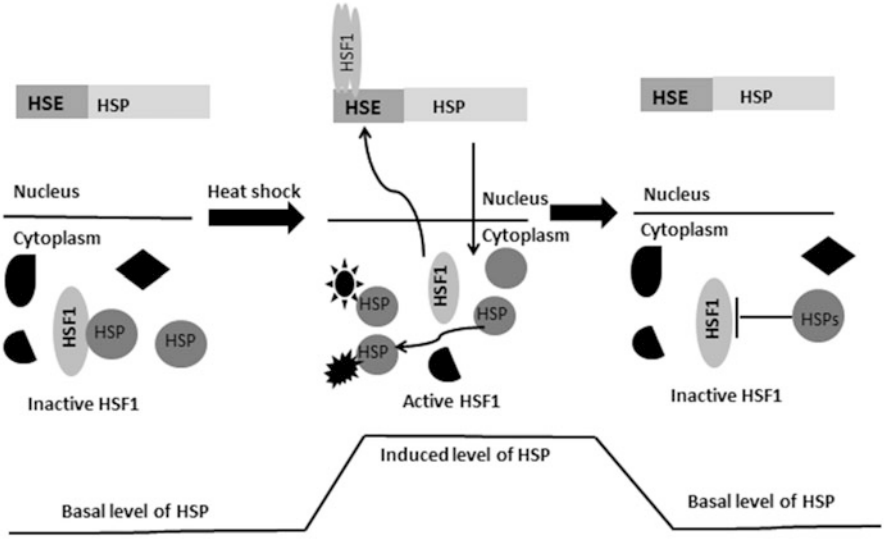

Fig. 2 Simple negative feedback loop of HSF1 activation. In absence of heat shock, HSF1 remains in the cytoplasm in inactivated state bound with other HSP. In response to heat shock, HSP/chaperones are engaged in refolding misfolded proteins (shown in different shapes with rough surfaces) which allows free HSF1 to move to nucleus, undergoes multiple post translational modifications, trimerization and binds to HSE inducing HSP/chaperones. HSP/chaperones engaged in misfolded/damaged protein to functionally folded proteins or degrade the misfolded proteins. Excess HSP/chaperones now bind with HSF1 preventing movement of HSF1 to nucleus and inhibit further expression of HSP/chaperones. Thus, in response to heat shock, expressions of HSP are under regulation of a negative feedback loop. Expression of a hypothetical HSP in basal level, induced after heat shock and after recovery is shown at the bottom

5 HSP-coding genes was arrested in presence of exogenous *HYPK* expression. Out of the 5 genes studied, *HYPK*-transfected cells showed significant increase in *Hsp70* expression; however, fold change was less compared to that of in cells with endogenous HYPK. It has also been shown that HYPK inhibits inducible binding of HSF1 to cognate HSE at *Hsp70* promoter. Similar decreased inducible binding of HSF1 to promoter of *HYPK* has also been shown. This result shows that HYPK may negatively regulate heat shock response [29]. Negative regulation of heat shock response by HYPK could be due to its ability to interact with HSF1 [21]. However, whether such interaction retrains HSF1 in the cytoplasm is unknown.

1.13 HSF1 Also Regulate HYPK Conjoined SERF2 Gene

HYPK is linked to small EDRK-rich factor 2 (SERF2) through a Conjoined Gene (CG), defined as a gene which gives rise to transcripts by combining at least part of one exon from each of two or more parent genes, which lies in same orientation on the same chromosome and may translate independently into different proteins [95]. SERF2 (hg38 chr15:43,792,347–43,800,279) is localized about 8Kb upstream to HYPK (chr15: 43,800,599–43,801,806). We have shown that SERF2 can also be regulated by HSF1 by binding to the promoter of the gene in transcription level [30]; functional significance of two nearby genes regulated by HSF1 by binding two independent HSEs is unknown.

1.14 Functions of HYPK

Function of HYPK is initially inferred from the enrichment analysis with interacting partners of the protein as described [21]. We carried out the same procedure with additional interacting partners of HYPK from BioGrid (https://thebiogrid.org/) [91]. Some of the significantly enriched processes are tested in cell culture systems expressing exogenous *HYPK* or knocking down endogenous *HYPK*. Reduction of endogenous *HYPK* resulted in the impairment of refolding of heat-denatured reporter luciferase protein. Exogenous expressions of either *HYPK* or *HSPA8* in the same cells could recover such defects. We have shown similar result earlier where overexpression of HYPK increases refolding of denatured proteins [97] . EEF1A1 and CALM1, interacting partners of HYPK, could enhance the ability of endogenous HYPK to refold the heat-denatured luciferase protein. Thus, the interacting partners of HYPK, especially EEF1A1 and CALM1, are likely to be involved in response to unfolded proteins. This was also inferred from the enrichment analysis for GO terms. HYPK binds to DNAJC2 and HSPA14 [21] . These two proteins are associated with ‘de novo’ co-translational protein folding (GO: 0051083) and significantly enriched

biological process. HYPK interacting proteins RPL7A, EEF1A1, EEF1A1, APP, QKI, HSPB1 and HSPA14 are also associated with biological process translation. Identification of binding of C-terminal conserved predicted α-helical structure of HYPK to newly synthesised proteins/peptides [98] shows that like many other chaperones, HYPK may be involved in folding newly synthesized proteins/peptides. Ability of HYPK to interact with NatA catalytic subunits NAA10, NAA11, NAA15, NAA16, and NAA50 indicates that HYPK may involve in post translational acetylation of proteins. It has been shown that knockdown of HYPK decreases N-terminal acetylation of NatA substrates. This result shows that HYPK is involved in post-translational modification of target proteins [4]. Many HYPK interacting proteins are associated with cell cycle arrest (MLF1, TP53, CALR and KIF20B), G2 phase of mitotic cell cycle (APP and DNAJC2), mitotic cell cycle (CEP290, PSME3, PTTG1, DCTN1 and RPA2) and cell cycle checkpoint (PSME3, TP53 and RPA2). Exogenous expression or knock down of endogenous *HYPK* resulted in cell cycle abnormalities [4, 21]. Altered proliferation by influencing DNA synthesis due to change in *HYPK* level was observed. Exogenous expression or knock down of endogenous *HYPK* also alters cell death [21]. Involvement of HYPK in cell growth and protein synthesis was observed earlier [4, 90]. Among HYPK interacting partners, several proteins EEF1A1, TP53, RELA/p65, LAMP2, APP, ARCN1, BECN1, HSP90AB1, HSPA8 and HTT are associated with autophagy (Table 2, [22]). Secondary interactors of the HYPK interacting proteins also have many autophagy associated proteins (Table 3, [22]). Exogenous expression of *HYPK* enhanced basal autophagy, while knocked down of the gene reduced autophagy indicating the HYPK in collaboration with its interacting partners involve in autophagy. Reversal of reduced autophagy in cells expressing mutant N-terminal HTT by HYPK was also observed [22]. Similar result of activation of autophagy by HYPK through interaction with Nedd8 and LC3 proteins has been reported [48]. Plant homologue of HYPK has been identified to interact with ATG8 and involves in autophagy in plants [80]. Summary of the involvement of HYPK in different biological processes by interacting with different partners is shown in the Fig. 3.

HSP/chaperones are involved in diverse cellular functions other than folding unfolded proteins in stressed cells or folding newly synthesized protein during translation and degradation of damaged/misfolded/unfolded proteins and maintain protein homeostasis. Besides, chaperones have been involved in diverse biological functions like cell growth, cell cycle, apoptosis, autophagy, transcription and many others as discussed in earlier section. Enrichment analysis with HSP and their interacting partners together revealed that 1,457 biological processes, defined by Gene Ontology terms, were significantly enriched. Top 20 the most significantly enriched biological processes include protein phosphorylation (GO: 0006468), gene expression (GO: 0010467), mitotic cell cycle (GO: 0000278), protein folding (GO: 0006457), translation (GO: 0006412), RNA metabolic process (GO: 0016070), cell cycle (GO: 0007049), apoptotic process (GO:0006915) and protein ubiquitination (GO:0016567) (data not shown).

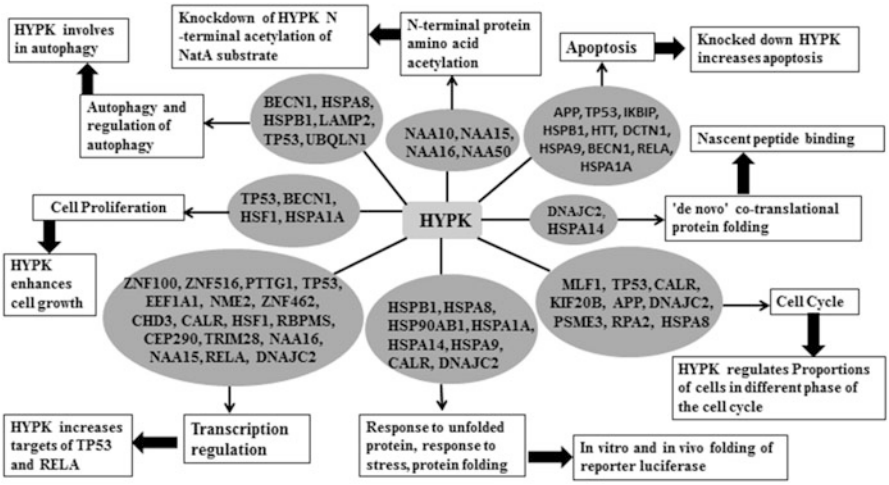

Fig. 3 Enrichment analysis of HYPK interacting proteins described earlier [21]. Additional HYPK interacting partners were used for reanalysis. HYPK interacting proteins (filled oval shape) significantly enriched with biological processes (related processes are clubbed) and shown in boxes and connected by arrows. Filled arrows connecting the boxes indicated the experimental determination of the processes [4, 21, 22]. Various processes associated with autophagy obtained from Gene Ontology database was clubbed together and were not significantly enriched. Details are discussed in the text

1.15 Does HYPK Modify Expression of Other Genes?

Enrichment analysis with the primary HYPK interacting proteins and their interacting partners (secondary interacting proteins) revealed that among many other biological processes and molecular functions, negative regulation of transcription, DNA-dependent (GO: 0045892), positive regulation of transcription, DNA-dependent (GO:0045893), positive regulation of transcription from RNA polymerase II promoter (GO:0045944), DNA binding (GO:0003677) and transcription factor binding (GO:0031625) were significantly enriched with HYPK interacting proteins. This result indicates that HYPK in collaboration with its interacting partners directly or indirectly may modulate gene expression [21, 22]. Intrinsically unstructured protein HYPK with a pre-molten globule like conformation [97] lacks any conventional DNA binding domain and there has so far been no evidence of HYPK binding to nucleic acid. However, since HYPK has been shown to interact with TFs like TP53, p65/RelA and HSF1, ZNF100 and ZNF516 as shown in Fig. 3, the possibility that HYPK modulates transcription of their target genes cannot be ruled out. Chaperones maintain appropriate functional folding of kinases, caspases and other signalling molecules including hormone receptors and heat shock factors. In response to hormones and stress, molecular chaperones regulate activities of intracellular hormone receptors and heat shock transcription factor tightly to ensure rapid reversible transcriptional response [86]. It has been

observed that HS induced Hsp70 interacts with RELA/p65 and supresses NFkB activity by sequestering the complex in cytoplasm in lymphoma cells [53]. Hsp72 and other chaperones binds TP53 and sequesters it in cytoplasm to bypass senescence [105]. Role of Hsp90, Hsp70 and Hsp40 in maintaining native conformation of wild type TP53 during thermal stress as well as for recovery from the stress have been reported. It has been shown that binding of wild type TP53 to the promoter of its target WAF1 is sensitive to Hsp70. Hsp90 stabilizes the binding of TP53 to the promoter sequence at physiological temperature, but under thermal stress, Hsp70-Hsp40 system and its cooperation with Hsp90 maintains the stability [132]. Role of various chaperones in regulation oncogenic and tumour suppressive function of TP53 by directly interacting with TP53 have recently been reviewed [135]. In a mouse neuronal cell line, HYPK was shown to stabilize RELA/p65 and enhance nuclear fraction of TP53 and RELA/p65. In such condition, occupancy of TP53 at the promoter of CASP1, a known target gene of TP53 was increased. Similar result was also obtained with RELA/p65 and its target gene CCL5 (Joyeeta Ghose, Ph.D. thesis, Calcutta University, 2014, unpublished observation). Several transcription factors are known to regulate autophagy. SREBF2/SREBP2 and TFEB are known to enhance autophagy [102, 103], ZKSCAN3/ZNF306 represses autophagy [14] while TP53 can either enhance or repress autophagy depending on nuclear or cytoplasmic localization [68, 140]. Exogenous expression of *HYPK* decreased expression of sterol regulatory element binding transcription factor 2 (*SREBF2/SREBP2*), while expression of transcription factor EB (*TFEB*) and zinc finger with KRAB and SCAN domains 3 (*ZKSCAN3/ZNF306*) in neuronal mouse cells [22]. It is worthy of mentioning that TP53 can bind to the putative promoters of all three genes in the first exons [TFEB at 538 nt chromosomal positions (Chr 6:41,735,680–41,735,763), ZKSCAN3 at 178 nt (Chr 6: 28,350,072–28,350,111) and SREBF2 49 (Chr22: 41,833,097–41,833,158)] (http://rna.sysu.edu.cn/chipbase/, accessed in December, 2019). It remains to be identified whether in presence of HYPK or thermal stress alters the expression of the genes and occupancy of TP53 increases by HYPK or thermal stress.

1.16 Does HYPK Play Any Role Human Disease?

Direct role of HYPK in human disease, if any, remains unknown. Recently, missense mutations in NAA10 and NAA15 have been identified in patients with variable levels of intellectual disability, delayed speech and motor milestones and autism spectrum disorder. HYPK, NAA10, and NAA15 are component of human NatA complex; whether such mutations in NAA10 alter interactions/association with HYPK and contribute to the pathogenesis remain to be identified [16]. Increased expressions of HSP, mainly chaperones, in various solid tumours and hematological malignancies have been reported. Increased chaperone activity in tumour cells have

been implicated in self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis and increased replicative potential, some of the hall marks of cancers [12]. Significantly increased expression of *HYPK* was identified in Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC), Esophageal carcinoma (ESCA), Stomach adenocarcinoma (STAD) and Thymoma (THYM) and decreased expression in Testicular Germ Cell Tumors (TGCT) and Thyroid carcinoma (THCA) was identified analysing RNA sequencing data in The Cancer Genome Atlas (TCGA) using GEPIA, an interactive web server for analyzing the data (http://gepia.cancer-pku.cn/detail.php?gene=HYPK#%23%23, accessed in December, 2019). Significance of this result is yet to be evaluated. It can be speculated like other chaperones, HYPK may contribute to the pathogenesis of cancer by interacting with TP53 and modulating its target genes.

Endogenous expression of HYPK at both transcript as well as protein level in STHdhQ111/HdhQ111 cells, a cell model of HD compared to control STHdhQ7/HdhQ7 cells. These immortalized striatal cell lines exhibit many molecular, cellular and phenotypic characteristics of HD and have been extensively used to detect early transcriptional changes, molecular defects and therapeutic screening in HD [121]. Decreased occupancy of HSF1, RNA polymerase II and acetylation of histone H4 in HYPK promoter encompassing HSF1-binding motif in STHdhQ111/HdhQ111 cells compared to STHdhQ7/HdhQ7 cells have also been observed. Level of *HYPK* was also decreased in 2-month old R6/2 mice which, expresses exon 1 of *HTT* gene with 150 CAG repeats and age-matched control mice [29]. All experimental evidences suggest that transcriptional downregulation of *HYPK* in HD cell and animal models could be a consequence of reduced occupancy of HSF1 and other necessary factors in *HYPK* promoter in presence of mutant HTT and indicate that defects in HYPK could modify the toxic effects of mutant HTT.

2 Conclusions

Few years' back HYPK was relatively an unknown protein identified in Y2H assay to interact with HTT. Throughout the years, it has been observed that *HYPK* is regulated by HSF1 by binding to its putative promoter in response to heat shock, hypoxia, and proteasome inhibition and possesses chaperone activity *in vitro* and *in vivo*. For *in vitro* chaperone activity, ATP is not required. It has also been shown that HYPK binds with newly synthesized peptides/proteins. Like many HSP/chaperone, it is an intrinsically unstructured protein and interacts with more than 70 proteins including transcription factors. Besides, HYPK has been shown to regulate negatively the heat shock response. All these results are summarized in Fig. 4. Based on these results, we conclude that HYPK is a heat shock protein and involves in proteostasis.

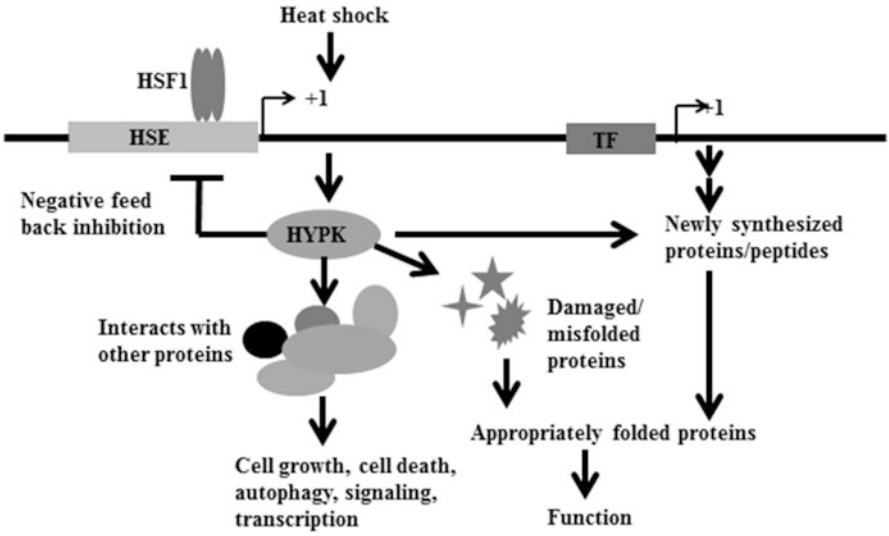

Fig. 4 In response to heat shock, activated HSF1 binds to the putative promoter of *HYPK*. HYPK negatively regulate the heat shock response possibly by binding to HSF1. It interacts with different proteins and likely o involve in diverse functions. It helps in folding the damage or misfolded proteins or newly synthesized peptides/proteins for appropriate folding and functions

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Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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Roles of HSP on Antigen Presentation

Kazuyuki Furuta and Taka Eguchi

Abstract

Introduction Antigen presentation to T cells by dendritic cells is an essential response for the initiation of acquired immunity to eliminate pathogens that have invaded. The pathogen-derived antigens incorporated by dendritic cells are processed into peptides and presented by MHC molecules. There are also mechanisms by which cytoplasmic antigens are presented by MHC molecules. However, it has not been recognized how the HSP family involves antigen presentation. In here, we summarize the current knowledge about the roles of HSP family proteins in antigen presentation.

Methods We review; (i) mechanisms of antigen presentation by dendritic cells, (ii) roles of HSP in antigen presentation by MHC-I, and (iii) roles of HSP in antigen presentation by MHC-II.

Results Recently, the involvement of the HSP family has been revealed at several steps in the process of antigen presentation. In particular, the functions of HSP90 in the MHC-I pathway and the functions of HSP70 in the MHC-II pathway are being elucidated. However, several unsolved questions have still remained. For example, does the same mechanism function in all antigen-presenting cells? Is there specificity for antigen proteins in the HSPs? In addition, the involvement of the other HSPs in antigen presentation is still unclear.

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Conclusions Since acquired immunity is important for the elimination of pathogens and tumors, antigen presentation should be a promising target for immunotherapy for infectious diseases and cancers. HSPs may be a potential target for manipulation of antigen presentation.

Keywords Antigen presentation · Autophagy · Dendritic cells · HSP · MHC-I · MHC-II

Abbreviations

CMA	chaperon-mediated autophagy
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ER	endoplasmic reticulum
MHC	major histocompatibility complex
MIIC	MHC-II compartment
TAP	Transporter associated with antigen processing

1 Introduction

Mammals possess two types of immune responses, that is, innate immunity and acquired immunity. Innate immunity is activated against broad rages of pathogens nonspecifically by recognizing pathogen patterns. On the other hand, acquired immunity responds specifically to invaded pathogens. To recognize pathogens in the acquired immune response, dendritic cells (DCs) take pathogens by phagocytosis, and pathogen-derived proteins are fragmented to peptides by intracellular proteases. As a result, the antigen peptides bind to major histocompatibility complex class I or class II molecules (MHC-I or MHC-II) inside the cells, and the antigen-bound MHCs are presented on the cell surface. Moreover, DCs also have a mechanism to present cytoplasmic antigens by MHCs. Many researches have been conducted to elucidate the transport pathways of the antigens to both MHC-I and MHC-II. Recently, the involvement of HSP family proteins in these pathways has also been found.

1.1 Mechanisms of Antigen Presentation by Dendritic Cells

In the acquired immune response, pathogen-derived antigen peptides are presented from DCs to T cells using cell surface MHC molecules (MHC-I or MHC-II). MHC-I presents antigens to $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs), whereas MHC-II presents antigens to $CD4^+$ T cells, also called helper T cells.

Classically, MHC-I was thought to bind and present intracellular cytoplasmic antigens such as an infected virus-derived peptide. Cytoplasmic antigens are processed to peptides by proteasomes, and the generated peptides enter the ER through a peptide transporter, TAP, where the peptides bind to MHC-I [3]. DCs also present antigens that are taken up from outside the cells by MHC-I. This mechanism is called cross-presentation and is necessary for the activation of CTLs to kill virus-infected cells and tumor cells [3, 11]. There are two pathways for the antigen cross-presentation by MHC-I. One is a cytoplasmic pathway, which is TAP-dependent, and antigens are once transferred in the cytosol and internalized in the ER through TAP. The other is the vacuolar pathway, which is TAP independent. The antigens bind directly to MHC-I within the phagosomes or the endosomes. ([2, 11]; Fig. 1).

MHC-II binds and presents exogenous antigens such as extracellular bacterium-derived proteins. The antigens are taken into cells by uptake mechanisms such as phagocytosis or endocytosis and then transported to the intracellular vesicle, MHC-II compartment (MIIC), where MHC-II is localized [17]. Furthermore,

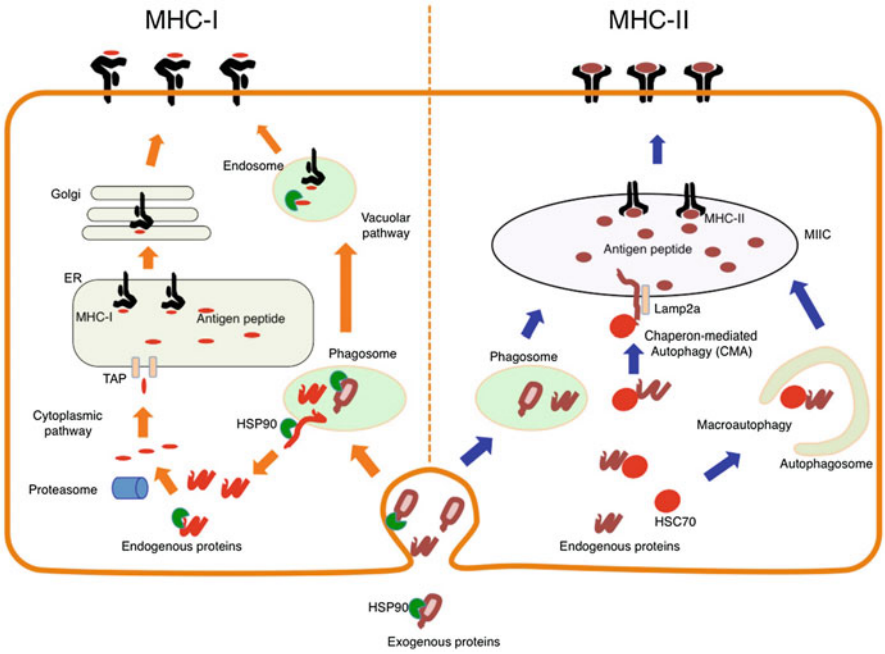

Fig. 1 Role of HSP in antigen presentation. Dendritic cells present antigen through the MHC-I or MHC-II pathway. HSP assists in the antigen presentation in various ways. In the MHC-I pathway, extracellular HSP90 assists in the uptake of exogenous antigens into the cells. In the cytoplasm, HSP90 assists in the transfer of antigen from the phagosome to the cytoplasm. In the MHC-II pathway, HSP70 binds to the antigen in the cytoplasm and assists in the translocation of MHC-II to MIIC by chaperone-mediated autophagy. HSC70 also assists in the transfer of cytoplasmic antigens into autophagosomes for macroautophagy

MHC-II also presents intracellular antigens through another mechanism by which cytoplasmic antigens enter MIIC by autophagy. This mechanism is thought to be important for the presentation of cytoplasmic pathogens-derived antigens [18]. This mechanism of cytoplasmic antigen presentation is also required for the presentation of self-antigens to $CD4^+$ T cells to induce immune tolerance [6, 15].

1.2 Roles of HSP in Antigen Presentation by MHC-I

HSP has been reported to regulate antigen presentation by binding to antigen proteins as a molecular chaperone protein. The role of HSP in MHC-I-mediated antigen presentation has been well studied. Extracellular HSP90 binds to exogenous antigens outside the cell and assists in the uptake of the antigen to the cross-presentation pathway. It has been observed that extracellular HSP90-ovalbumin (OVA) protein complexes are transported to the early endosome and enter the cross-presentation pathway [19]. Since the internalized HSP90-OVA complex is colocalized with proteasomes in the cytosol, HSP90 could be involved in the TAP-dependent cross-presentation pathway and play a key role in promoting the transition of the antigen from the endosomes to the cytosol [16]. HSP90 has been reported to assist in the cross-presentation of tumor-derived antigens. However, in this case, the cross-presentation pathway has shown to be a TAP-independent vacuolar pathway [14]. The cytoplasmic HSP90 is also involved in cross-presentation. HSP90α was shown to be required for the cytoplasmic transition of antigens by using HSP90 inhibitors. In addition, cross-presentation was reduced in HSP90α-deficient DCs, although the presentation of cytosolic antigens by MHC-I was not affected [7, 9, 10, 20]. HSP other than HSP90 is also involved in cross-presentation. Hsp70 and gp96 (also known as Grp94 or Hsp90B) were reported to assist in the uptaking of exogenous antigens [1].

1.3 Roles of HSP in Antigen Presentation by MHC-II

The role of HSP in antigen presentation by MHC-II is not as well understood as MHC-I. HSP90 has been reported not to promote MHC-II-mediated antigen presentation of exogenous antigens derived from OVA protein [16]. On the other hand, it has also been reported that MHC-II-mediated presentation of extracellular glutamate decarboxylase (GAD)-derived peptides was promoted by HSP90 [8]. Therefore, the role of extracellular HSP90 on MHC-II-mediated antigen presentation may depend on the antigen proteins.

The role of cytoplasmic HSP in MHC-II-mediated antigen presentation has also been reported. One of the roles of cytoplasmic HSC70 is chaperone-mediated autophagy (CMA). In CMA, proteins are transported into the autophagosomes by a lysosomal transmembrane protein Lamp2a [12]. HSC70 assists in the association

of the antigens to Lamp2a during CMA [21]. Several endogenous antigens are reported to be transferred into the autophagosome by CMA and presented by MHC-II [4, 21]. HSP70 has also been reported to involve in macroautophagy-mediated antigen presentation by MHC-II. HSC70 assists in the transition of proteins into autophagosomes [5, 13].

2 Conclusions

Many studies have revealed the roles of HSPs in antigen presentation. In particular, the functions of HSP90 in the MHC-I pathway and the functions of HSP70 in the MHC-II pathway are being elucidated. However, several unsolved questions have still remained. For example, does the same mechanism function in all antigen-presenting cells? Is there specificity for antigen proteins in the HSPs? In addition, the involvement of the other HSPs in antigen presentation is still unclear. Since acquired immunity is important for the elimination of pathogens and tumors, antigen presentation should be a promising target for immunotherapy for infectious diseases and cancers. HSPs may be a potential target for manipulation of antigen presentation.

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Ethical Approval for Studies Involving Animals This article does not contain any studies with animals performed by any of the authors.

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Heat Shock Proteins Mediate Anastasis and Plasticity of Thermotolerant Cells

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Abstract

Introduction We recently showed the thermotolerance of animal somatic cells to a lethal temperature and this tolerance was associated with morphological and molecular changes. Notably, heat shock proteins (Hsp) play an important role in the process of thermotolerance. In this chapter, we briefly highlight the essential roles of Hsp in resisting lethal temperatures and regaining the cellular vitality during the recovery from heat shock.

Methods A literature-based collection of articles in the available search engines (PubMed and Google Scholar).

Results We show the critical roles of Hsp in antagonizing the pathways of apoptosis and the involvement of several other proteins in the cellular resilience or anastasis.

Conclusions Modulating the pathways of anastasis through Hsp would permit therapeutic targets for either promoting (such as female folliculogenesis) or hindering anastasis for tumor therapy.

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Keywords Anastasis · Apoptosis · HSP · Hyperthermia · Mitochondria · Plasticity

Abbreviations

CAD	caspase-activated DNase
Cyt *c*	cytochrome c
Hsp	heat shock proteins
MOMP	mitochondrial outer membrane permeabilization
PS	phosphatidylserine
PSM	peptide spectrum match
TNF	tumor necrosis factor
XIAP	X-linked inhibitor of apoptosis

1 Introduction

Thermotolerance is a phenomenon in which cells become resistant to elevated temperatures. Thermotolerance might develop rapidly after the first heat treatment or during the thermal treatment at ~43.0 °C. Studies disclosed that thermotolerance developed in tumors and normal tissues as well [11, 26] and it is well correlated with enhanced synthesis of heat shock proteins [2, 8, 11, 27].

The kinetics of thermotolerance can be affected by various factors [4, 26]. For instance, thermotolerance is found physiologically in certain species as a form of estivation [5, 21]. It might be varied among certain cells of the same species [15, 17–19]. Cells showed variability in thermotolerance because of the way of cell culture; cells grown in 3D compared with 2D culture showed a reduced incidence of apoptosis and necrosis and a higher level of HSP70 expression in response to heat shock [20].

Heat shock proteins (Hsp) are chaperones that cause partial unfolding or protein aggregation that protect cells from the detrimental thermal and oxidative stress [9]. Hsp bind to hydrophobic residues exposed by stress and prevent partially denatured proteins from aggregation and allow them to refold [13]. Furthermore, Hsp stabilizes and protects actin microfilaments and cytoskeleton when organized in small, phosphorylated oligomers [10].

2 Apoptosis

Programmed cell death is a fundamental physiological process. In the last decades, studies have elucidated the functions and consequences of cellular demise and explored several key factors involved in cell death pathways.

2.1 The Role of Mitochondria in Apoptosis

The mitochondrial (or intrinsic) pathway is evoked by cellular stresses or developmental signals that increase the expression Bax, Bak, and Bok protiens to cause mitochondrial outer membrane permeabilization (MOMP) that lead to the release of mitochondrial inter-membrane space proteins into the cytosol. From these proteins, holocytochrome c (Cyt *c*), that binds to the cytosolic protein APAF1 enhancing its oligomerization to form an apoptosome. Moreover, APAF1 hierarchically activates caspase-9 through enforced dimerization which initiates a subsequent activation of caspase-3 and caspase-7 (Fig. 1). These activated caspases (Caspase-9, -3, and -7) can be inhibited by an X-linked inhibitor of apoptosis (XIAP). Paradoxically, some other proteins, such as Smac and Omi, are released upon MOMP and antagonize XIAP. The proteolytic caspases hydrolyzes and activates hundreds of cellular substrates and enzymes, such as inhibitor of caspase-activated DNase (iCAD) that cut inter-nucleosomal DNA. Moreover, cleaving and hydrolysis of cellular substrates cause the common features of apoptotic cell death such as chromatin condensation and membrane blebbing. Furthermore, caspases disturb the plasma membrane phospholipids and expose phosphatidylserine (PS) on the cell surface that promotes phagocytosis of the dying cell.

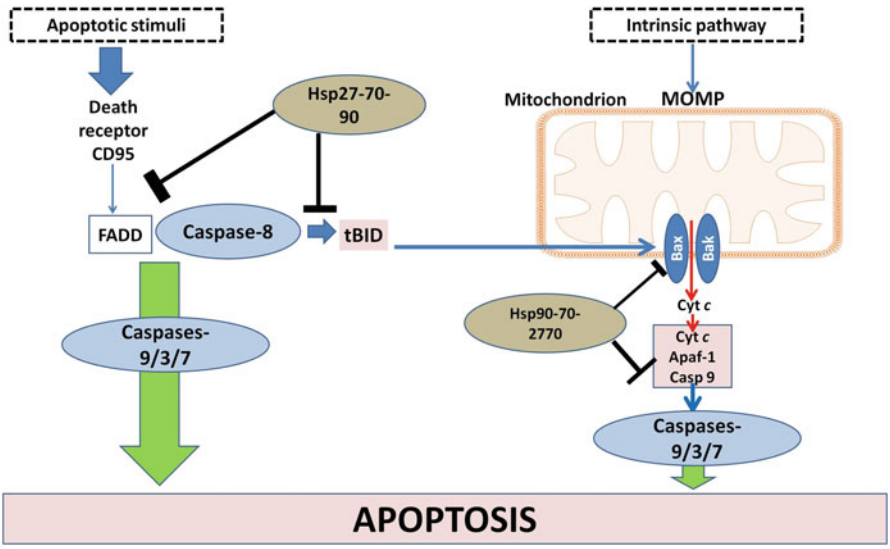

Fig. 1 The pathways of apoptosis and the possible roles of HSP in anastasis or preventing apoptosis

2.2 *The Role of Death Receptors in Apoptosis*

Apoptosis can be provoked with the binding of tumor necrosis factor (TNF) with TNF receptors (the death receptors), that refered as the extrinsic pathway of apoptosis. For example, apoptotic stimuli bind with the death receptor CD95 hierarchically activates FADD and subsequently activates a cascade of caspase-8 molecules and then caspase-3 and caspase-7 (Fig. 1) [14, 28].

3 Thermotolernce and Anastasis

Anastasis is a term coined to outline the process of cell recovery, plasticity, resilience, or cellular resurrection from the brink of cell death [23] and might be a reason for cancer cells thermotolerance [12]. The mechanism invoked to reverse apoptosis has been termed anastasis and poses challenges for the development and utilization of chemotherapeutic agents [3]. Anastasis has also been identified as a mechanism by which cells can recover from apoptotic lesions and revert to their previous functioning state. In heat-shocked cells, we showed morphological changes in the cells (camel, cow, and sheep) associated with exposure to lethal heat shock and these morphological alterations were abolished when the cell recovers at the physiological temperatures [15–19]. Time-lapse imaging clearly showed that cells became rounded shaped and lost the spindle morphology after exposing to 45 °C for 20 h then by the first hours of recovery at 38 °C cells eventually return spindle morphology, gained motility and migration, and remarkably increased motility after 48 h from the recovery [16]. The common feature for the thermotolerant cells was the increase in Hsp70 and Hsp90 in these cells that suggest a critical role for Hsp in combating the apoptotic and lethal stimuli. Similar morphological changes were reported in rat intestinal cells exposed to 42 °C for 4 h [30] and amoeba cells exposed to acute heat shock at 34 °C for 30 min [29].

4 Role of HSP in Anastasis

Due to the essential role of HSP in thermotolerance, we and others propose the phenomenon “anastasis” to illustrate the survival response of thermotolerant cells [6, 16, 18, 19, 22, 23, 25]. As shown in Fig. 1, Hsp inhibits both pathways of apoptosis (the intrinsic and extrinsic pathways) by blocking MOMP and caspases formation. Our study showed expression of several Hsp in cells showed the signs of cell death but recovered at normal temperatures (37 °C) (Table 1) such as heat shock cognate protein, heat shock cognate protein HSP 90-beta, DnaJ subfamily B member 4-like protein, guanine nucleotide-binding protein subunit beta-2-like 1, heat shock cognate protein HSP 90-beta-like isoform 3, heat shock protein 105 kDa, heat shock

Table 1 Differential expression of heat shock proteins and ubiquitins in control, heat-shocked, and anastatic cells

Control	Chronic heat shock	Anastatic	Shared
Heat shock protein isoform 5	Heat shock protein 70 1 L	Heat shock cognate protein	Heat shock protein 70 A1B
Small ubiquitin-related modifier	Heat shock protein 90	Heat shock cognate protein HSP 90-beta	Stress-70 protein, mitochondrial
Ubiquitinyl hydrolase 1	Heat shock cognate protein	DnaJ subfamily B member 4-like protein	Ubiquitin
Ubiquitin carboxyl-terminal hydrolase	Heat shock protein 6	Guanine nucleotide-binding protein subunit beta-2-like 1	Ubiquitin-conjugating enzyme E2 variant 2
Ubiquitin carboxyl-terminal hydrolase 14	Heat shock protein 90	Heat shock cognate protein HSP 90-beta-like isoform 3	
	Heat shock protein beta-1	Heat shock protein 105 kDa	
	Heat shock protein HSP 90-alpha	Heat shock protein 6	
	Heat shock protein isoform 5	Heat shock protein 70 1 L	
	Heat shock protein, mitochondrial (Hsp60) isoform 4-like protein	Heat shock protein 90	
	Small ubiquitin-related modifier	Heat shock protein beta-1	
	Ubiquitin-like modifier-activating enzyme 1 Y-like protein	Heat shock protein HSP 90-alpha	
		Heat shock protein, mitochondrial (Hsp60) isoform 4-like protein	
		Small ubiquitin-related modifier	
		Ubiquitin carboxyl-terminal hydrolase	
		Ubiquitin-like modifier-activating enzyme 1 Y-like protein	

protein 6, Heat shock protein 70 1 L, heat shock protein 90, heat shock protein beta-1, heat shock protein HSP 90-alpha, heat shock protein 60 (mitochondrial), small ubiquitin-related modifier, ubiquitin carboxyl-terminal hydrolase, and ubiquitin-like modifier-activating enzyme 1 Y-like protein. Moreover, heat shock protein 70 A1B, and stress-70 protein (mitochondrial), ubiquitin, ubiquitin-conjugating enzyme E2 variant 2 were also expressed with higher values [16]. Table 2 provides detailed

Table 2 Differential expression, peptide spectrum match (PSM), and gene ontology of commonly altered proteins in control, heat-shocked, and anastatic cells

Protein names	Control	Chronic HS	Anastatic	HS/ Control	Ana/ Control	Ana/ HS	Gene ontology (biological process)	Gene ontology (cellular component)	Gene ontology (molecular function)
Actin, cytoplasmic 1 (Beta-actin)	0.018	0.006	0.011	0.36	0.61	1.69		Actin cytoskeleton [GO:0015629]; cytoskeleton [GO:0005856]; dense body [GO:0097433]; focal adhesion [GO:0005925]; nucleus [GO:0005634]; plasma membrane [GO:0005886]; protein-containing complex [GO:0032991]	ATP binding [GO:0005524]
Aldo-keto reductase	0.0063	0.0038	0.0038	0.6	0.6	1			Oxidoreductase activity [GO:0016491]
ATP synthase subunit a	0.0067	0.0084	0.008	1.25	1.30	1.03	ATP synthesis coupled proton transport [GO:0015986]	Integral component of membrane [GO:0016021]; mitochondrial inner membrane [GO:0005743]; proton-transporting ATP synthase complex, coupling factor F (o) [GO:0045263]	Proton transmembrane transporter activity [GO:0015078]

Endoplasmin (HSP90B1)	0.003	0.004	0.001	1.34	0.46	0.34	Protein folding [GO:0006457]; response to stress [GO:0006950]		ATP binding [GO:0005524]; unfolded protein binding [GO:0051082]
Glyceraldehyde-3-phosphate dehydrogenase	0.023	0.0163	0.014	0.69	0.6	0.87			Glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity [GO:0004365]
Heat shock protein 70 1A	0.0097	0.006	0.008	0.62	0.87	1.40			ATP binding [GO:0005524]
Heat shock protein 70 1 L	0.009	0.006	0.008	0.62	0.87	1.40			ATP binding [GO:0005524]
Heat shock protein 70 A1B	0.0059	0.0087	0.0064	1.45	1.07	0.73			ATP binding [GO:0005524]
Heat shock protein 90	0.0022	0.0035	0.0045	1.54	1.98	1.28	Protein folding [GO:0006457]; response to stress [GO:0006950]		ATP binding [GO:0005524]; unfolded protein binding [GO:0051082]
Heat shock protein beta-1	0.0337	0.0576	0.0612	1.70	1.81	1.06	Protein folding [GO:0006457]; response to stress [GO:0006950]		Molecular chaperone maintaining denatured proteins in a folding-competent state; actin organization
Integrin beta	0.0019	0.0023	0.0007	1.18	0.39	0.33	Cell-matrix adhesion [GO:0007160]; integrin-mediated signaling pathway [GO:0007229]	Integrin complex [GO:0008305]	Signaling receptor activity [GO:0038023]

(continued)

Table 2 (continued)

Protein names	Control	Chronic HS	Anastatic	HS/ Control	Ana/ Control	Ana/ HS	Gene ontology (biological process)	Gene ontology (cellular component)	Gene ontology (molecular function)
Ubiquitin	0.042	0.048	0.048	1.12	1.12	1		Cytoplasm [GO:0005737]; nucleus [GO:0005634]	
Proteins that altered by heat shock									
Protein names	**Control**	**Chronic-20H**	**Anastatic**	**HS/ control**	**Ana/ control**	**Ana/ HS**	**Gene ontology (biological process)**	**Gene ontology (cellular component)**	**Gene ontology (molecular function)**
Proteins that are absent due to chronic heat shock & regained after recovery									
ATP synthase protein 8	0.022	No	0.014	NA	0.66	NA	ATP synthesis coupled proton transport [GO:0015986]	An integral component of the membrane [GO:0016021]; mitochondrial proton-transporting ATP synthase complex, coupling factor F (o) [GO:0000276]	Proton transmembrane transporter activity [GO:0015078]
Estradiol 17-beta-dehydrogenase 1-like protein	0.013	No	0.004	NA	0.31	NA	Estrogen biosynthetic process estrogen metabolic process steroid biosynthetic process		Estradiol 17-beta-dehydrogenase activity;estradiol binding
Glutathione S-transferase mu	0.011	No	0.004	NA	0.41	NA	Metabolic process [GO:0008152]		Glutathione transferase activity [GO:0004364]
Glutathione S-transferase P1–1 class	0.022	No	0.0091	NA	0.4	NA	Metabolic process [GO:0008152]		Glutathione transferase activity [GO:0004364]

Integrin alpha V	0.0016	No	0.0011	NA	0.66	NA	Cell adhesion [GO:0007155]; integrin-mediated signaling pathway [GO:0007229]	Integrin complex [GO:0008305]	
Lysosome-associated membrane glycoprotein 2 isoform 1	0.006	No	0.003	NA	0.5	NA			
Na/K ATPase	0.004	No	0.001	NA	0.31	NA	Metal ion transport [GO:0030001]		Copper ion binding [GO:0005507]
Moesin	0.008	No	0.001	NA	0.12	NA	Connections of major cytoskeletal structures to the plasma membrane, regulation of cell shape, regulation of cell size		Actin-binding, structural constituent of the cytoskeleton,
Clathrin heavy chain	0.0015	No	0.001	NA	0.66	NA	Autophagy, membrane organization, transcytosis, clathrin-dependent endocytosis, intracellular protein transport, receptor internalization, receptor-mediated endocytosis		
Dynein, cytoplasmic, heavy polypeptide 1-like protein	0.00034	No	0	NA	0.1	NA	ER to Golgi vesicle-mediated transport, regulates apoptotic activities of BCL2, a motor for the		

(continued)

Table 2 (continued)

Protein names	Control	Chronic HS	Anastatic	HS/ Control	Ana/ Control	Ana/ HS	Gene ontology (biological process)	Gene ontology (cellular component)	Gene ontology (molecular function)
							intracellular retrograde motility of vesicles and organelles along microtubules. May play a role in changing or maintaining the spatial distribution of cytoskeletal structures.		
Plastin-2	0.00086	No	0.00087	NA	1	NA	Actin crosslink formation, actin filament bundle assembly, actin filament network formation, animal organ regeneration, extracellular matrix disassembly		
Fermitin (family 3)	0.002	No	0.0008	NA	0.4	NA	Involved in cell adhesion. Contributes to integrin activation. When coexpressed with Talin, potentiates activation of ITGA2B. Required for normal keratinocyte proliferation. Required for normal polarization of basal keratinocytes in skin, and normal cell shape. Required for		

							normal adhesion of keratinocytes to fibronectin and laminin, and for normal keratinocyte migration to wound sites. May mediate TGF-beta 1 signaling in tumor progression.		
Proteins that disappeared after recovery									
DNA-(apurinic or apyrimidinic site) lyase	0.012	0.0047	No	0.39	NA	NA	DNA repair [GO:0006281]	MITOCHONDRION [GO:0005739]; NUCLEUS [GO:0005634]	Class I DNA-(apurinic or apyrimidinic site) endonuclease activity [GO:0140078]; DNA binding [GO:0003677]; endonuclease activity [GO:0004519]; metal ion binding [GO:0046872]
Proteins that were heat-shock induced and maintained in recovery									
Alpha-crystallin A chain	NA	0.009	0.02			3.19		Cytoplasm [GO:0005737]; nucleus [GO:0005634]	Metal ion binding [GO:0046872]; structural constituent of eye lens [GO:0005212]
Heat shock protein 6	NA	0.0035	0.0031			0.87			ATP binding [GO:0005524]

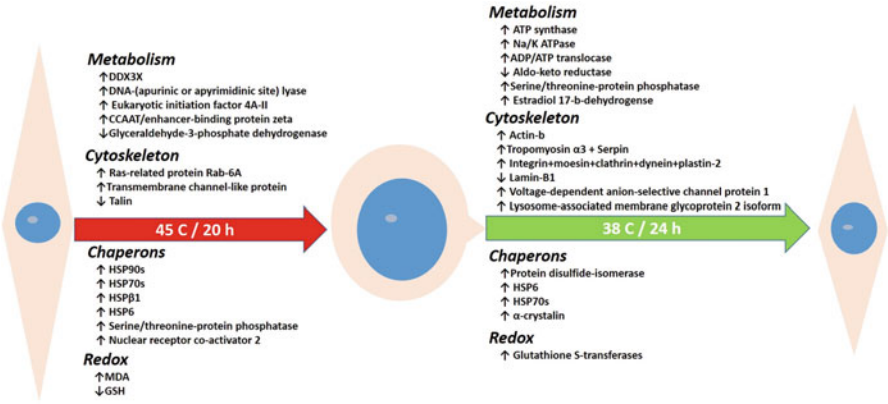

Fig. 2 Morphological and common changes associated with thermotolerance and anastasis. Modified after Saadeldin et al. [16]

proteomic changes in the cells that showed the thermotolerance or what we called anastasis after a lethal heat shock. Notably, Hsp70 elevated dramatically during the cellular recovery or anastatic. This result indicates that Hsp70 is the major cell machinery essential for protein refolding and for misfolding corrections that were caused by the damaging effects of chronic heat shock [16]. Interestingly, Hsp70 inhibits apoptosis through blocking the complex binding of procaspase-9 to the cytochrome *c* apoptosome [1] (Fig. 1). Furthermore, Hsp70 enhances the activity of glutathione reductase that mediates the reduction of glutathione disulfide (GSSG) into the sulfhydryl form of glutathione (GSH), which was found in the thermotolerant cells (Fig. 2, Table 2). Moreover, Hsp 70 suppresses DNAase activity that is associated with heat shock [23–25]. Our results also showed an increase in the level of DNA-(apurinic or apyrimidinic site) lyase which is a DNA repair enzyme during the chronic heat shock and after recovery or anastatic stage [16]. This interplay augments the cytoprotective effects of Hsp70, DNA repair, and the cellular redox environment in response to lethal stresses [7].

5 Conclusions and Perspectives

There are numerous gaps in our current knowledge of anastasis and several challenges must be overcome to define the machinery of anastasis and the critical roles of HSP in promoting it. Balancing cell death and survival through anastasis is essential for attaining the homeostasis. Anastasis can be targeted for cancer therapy (i.e. to suppress anastasis in cancer cells, however, to promote anastasis in normal damaged cells that are affected by chemotherapy or radiotherapy such as gonads and hair follicles). Moreover, anastasis can be promoted for female folliculogenesis to

overcome the early menopause or to prolong the reproductive life of the female where apoptosis is the default pathway of the ovarian follicular cells. Additionally, promoting anastasis may be a beneficial mechanism of preserving differentiated cells that are difficult to replace, such as neurons, photoreceptors, hepatocytes, and cardiomyocytes.

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Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

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