

## RESEARCH ARTICLE

# Evidence of Multimeric Forms of HSP70 with Phosphorylation on Serine and Tyrosine Residues – Implications for Roles of HSP70 in Detection of GI Cancers

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### Abstract

**Background:** Heat-shock protein70 (HSP70) are intracellular protein chaperones, with emerging evidence of their association with various diseases. We have previously reported significantly elevated plasma-HSP70 (pHSP70) in pancreatic cancer. Current methods of pHSP70 isolation are ELISA-based which lack specificity due to cross-reactivity by similarities in the amino-acid sequence in regions of the protein backbone resulting in overestimated HSP70 value. **Materials and Methods:** This study was undertaken to develop a methodology to capture all isoforms of pHSP70, while further defining their tyrosine and serine phosphorylation status. **Results:** The methodology included gel electrophoresis on centrifuged supernatant obtained from plasma incubated with HSP70 antibody-coupled beads. After blocking non-specific binding sites, blots were immunostained with monoclonal-antibody specific for human-HSP70, phosphoserine and phosphotyrosine. **Conclusions:** Our novel immunocapture approach has distinct advantages over the commercially available methods of pHSP70 quantification by allowing isolation of molecular aggregates of HSP70 with additional ability to precisely distinguish phosphorylation state of HSP70 molecules at serine and tyrosine residues.

**Keywords:** HSP70 - multimeric forms - phosphorylation - serine - tyrosine - GI cancer detection

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### Introduction

Heat-shock protein70 (HSP70) are intracellular protein chaperones, with emerging evidence of their association with various diseases. We have previously reported significantly elevated plasma-HSP70 (pHSP70) in pancreatic cancer. Current methods of pHSP70 isolation are ELISA-based which lack specificity due to cross-reactivity by similarities in the amino-acid sequence in regions of the protein backbone resulting in overestimated HSP70 value. This study was undertaken to develop a methodology to capture all isoforms of pHSP70, while further defining their tyrosine and serine phosphorylation status. The methodology included gel electrophoresis on centrifuged supernatant obtained from plasma incubated with HSP70 antibody-coupled beads. After blocking non-specific binding sites, blots were immunostained with monoclonal-antibody specific for human-HSP70, phosphoserine and phosphotyrosine. Our novel immunocapture approach has distinct advantages over the commercially available methods of pHSP70 quantification by allowing isolation of molecular

aggregates of HSP70 with additional ability to precisely distinguish phosphorylation state of HSP70 molecules at serine and tyrosine residues.

Ductal adenocarcinoma of the pancreas remains a leading cause of cancer deaths in the United States. In an effort to devise an early detection system, we recently reported evidence to show that plasma soluble heat-shock protein 70 (HSP70) is significantly elevated in pancreatic cancer (Dutta et al., 2012). In this study, we demonstrated a novel approach involving the immunocapture of plasma HSP70 and the subsequent demonstration by Western blot of the existence of several isoforms of phosphorylated HSP70 on serine and tyrosine residues. Heat shock proteins (HSPs) are ubiquitous, highly conserved proteins that are found in both prokaryote and eukaryote organisms. These proteins are cytoprotective and are involved in intracellular regulatory pathways, functioning as molecular chaperones for other cell proteins (Rappa et al., 2012). They were first described by Ritossa in 1962, and were thought to be elaborated only in response to thermal stress (Ritossa, 1962). However, this family of proteins is now known to be up-regulated in response to various

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other stimuli including oxygen-derived free radicals, amino-acid analogs, ethanol, heavy metals, ischemia-reperfusion injury and several inflammatory states, thus participating in wide array of functions in humans (Kiang et al., 1998; Rakonczay et al., 2003; Shamaei-Tousi et al., 2007; Söti et al., 2007; Murphy, 2013). HSPs are classified according to their molecular weight, and the most well understood members of this diverse family fall between the 60 and 110 kD range. These HSPs are essential to normal cellular function and response to stress, and perform diverse chaperoning functions in cells, including protein folding and peptide aggregation (Smith et al., 1998; Sikora et al., 2007; Bottoni et al., 2009; Baird et al., 2012). HSP70 is the most temperature sensitive and highly conserved group of HSPs and is generally regarded as the intracellular molecule that is released into the extracellular environment during pathological states (Sikora et al., 2007; Kaul et al., 2011). However, there is increasing evidence that under several circumstances these proteins can be released from cells in the absence of cellular necrosis. Furthermore, evidence also suggests that extracellular HSP70 may have a range of poorly understood immune-regulatory activities (Sharp et al., 2001; Joly et al., 2010). It is now clear that soluble HSP70 is present in human serum and has been independently associated with a number of disease states including colorectal cancer, peripheral artery disease and hepatitis C virus-related hepatocellular carcinoma (Takashima et al., 2003; Kocsis et al., 2010; Krepuska et al., 2010). Current methods for isolation of plasma HSP70 include commercially available ELISA based kits. However, these ELISA methods lack specificity because the anti-HSP70 antibody that is used in the assay captures HSP70, and other closely related molecular forms of HSP70. This cross-reactivity is caused by similarities in the amino acid sequence in regions of the protein backbone (sequence homology). As a result, the values obtained by ELISA is a reflection of the entire HSP70 family of cross reacting proteins and may be invalid as a tool for distinguishing different pathological conditions.

During the course of our work on HSP70, we recognized that the native secreted form of this family of proteins exhibit a considerable degree of pleiomorphism resulting in multimeric aggregates that are recognized by antibodies raised against HSP70. Other members of this family, with their distinctive molecular weights, are also detected by the cross-reacting antibody that recognizes segments of the protein backbone that bear sequence homology. In addition, HSP70 is known to undergo post-translational modification through phosphorylation of serine and tyrosine residues (Dutta et al., 2000). Therefore, in order to distinguish these different molecular forms, we proposed to use electrophoretic mobility as a tool. This work was initiated to develop a procedure that would allow us to distinguish the multimeric forms of HSP70 and also provide us with an estimate of the specificity of the phosphorylation on serine and tyrosine residues. Reliable identification of these additional discriminants may shed light on the pathophysiology of disease processes.

## Materials and Methods

This study was undertaken at Sinai Hospital of Baltimore after approval from the Institutional Review Board (IRB). The aims of the study were: *i*) To develop an immunocapture technique to isolate HSP70 from human plasma; *ii*) To characterize the nature of immunoreactive HSP70 and its molecular aggregates; and *iii*) To assess the degree of phosphorylation on serine and tyrosine residues in each species of immunoreactive HSP70.

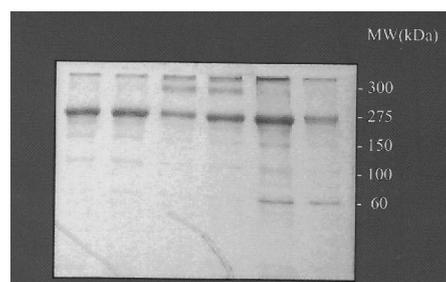
### Patient selection

Six subjects were enrolled into the study after obtaining consent. 10 ml of blood was drawn into collection tubes from each person in a sterile fashion. The plasma was separated and combined with protease and phosphatase inhibitors. Plasma HSP70 was isolated based on a novel approach previously described (Dutta et al., 2000). The steps of plasma HSP70 extraction are summarized below:

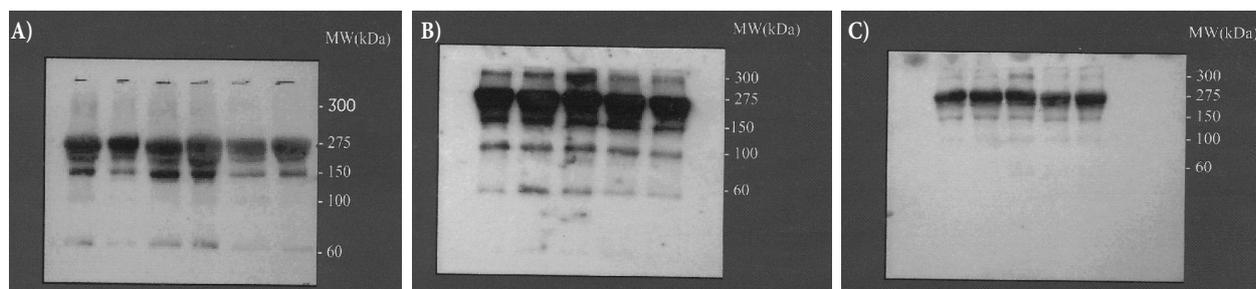
*i) HSP isolation and extraction from serum:* 250  $\mu$ l of plasma mixed with 12.5  $\mu$ l pre-made HSP70 antibody-coupled Protein A sepharose beads was incubated at 4° C overnight. Samples were suspended in 50  $\mu$ l of laemlli buffer and boiled for 8 minutes (in order to elute protein off the coupled beads), centrifuged and the supernatants stored for western blotting.

*ii) Preparation of HSP 70 antibody-coupled protein a sepharose bead:* 50 $\mu$ l of HSP70 antibody (Sigma Chemicals USA) was bound to 100 $\mu$ g Protein A sepharose beads (Sigma Chemicals USA) for 2 hours with gentle rocking at room temperature. The beads were washed twice with 10 volumes (1 ml) of 0.2M Sodium Borate (ph=9) by centrifuging at 3000g for 5 minutes and re-suspended in 0.2M sodium borate buffer. Enough dimethylpilmidate was added for a 20mM final concentration. The beads were then mixed for 30 minutes at room temperature on a shaker. The reaction was stopped by washing the beads in 0.2M ethanolamine (pH 8.0) and then incubated for 2 hours at room temperature with gentle mixing. After the final wash, the beads were re-suspended in 1ml PBS 0.1% merthiolate for long term storage.

*iii) Western blotting:* aliquots, 10  $\mu$ l of protein sample were loaded onto 4-20% gradient polyacrylamide gel (Bio-rad, Hercules, CA) and electrophoresis performed at 75V for 2 hours. After gel electrophoresis, the proteins were blotted onto nitrocellulose membranes using mini-trans-blotcell (Bio-Rad, Hercules, CA) at 40 mA for 18 hours. Non-specific binding sites were blocked by incubating blots with 7% non fat dry milk in phosphate buffered



**Figure 1. Coomassie stain of Immunoreactive HSP-70 (6 subjects)**



**Figure 2. Western Blot Using A) HSP-70 Antibody (6 subjects); B) A Phosphoserine Antibody (5 subjects); and C) Phosphotyrosine Antibody (5 subjects)**

saline containing 0.1% TWEEN (PBS-T). Blots were then immunostained with monoclonal antibody specific for human HSP-70 (Sigma Chemical, St. Louis, USA), phosphoserine and phosphotyrosine. Protein bands were detected using a chemiluminescence kit using anti-mouse immunoglobulin horseradish peroxidase linked whole antibody (Amersham Pharmacia NJ, USA). HSP70 bands were quantified by densitometric analysis using the Stratagene Eagle Eye II software (Stratagene La Jolla, CA). In addition to western blotting, duplicate gels were stained with Coomassie blue (Imperial chemicals) which is used for staining proteins in analytical biochemistry.

## Results

We report here a novel approach of isolation of plasma HSP70, using an immunocapture method.

Figure 1 shows a Coomassie blue stain of all immunoreactive HSP70 species eluted from the coupled beads. Five distinct protein bands are visible at 300, 275, 150, 100 and 60 kD. This suggests presence of multimeric forms of HSP70. Five similar bands are observed on Western blot using highly specific anti-HSP70 antibody (Figure 2A).

Western blotting using an anti-phosphoserine antibody (Figure 2B) showed strong bands at 300, 275, 150, 100 and 60kD locations, which indicate the presence of phosphorylated serine residues on immunoreactive HSP70 proteins. However, Western blot using an anti-phosphotyrosine antibody revealed strong protein bands at 300, 275 and 150kD, and less so at 100 and 60kD (Figure 2C).

## Discussion

There are well-established techniques to isolate and quantify HSP70 from cellular tissue (Phillips et al., 2007). However, plasma HSP70 isolation is mostly based on commercially available ELISA kits. Current methods of determination of plasma HSP70 using ELISA-based kits lack specificity because of cross-reactivity of anti HSP70 antibody with other closely related forms of HSP70 with regions in the protein backbone showing sequence homology. We describe here a novel approach of isolating HSP70 from human plasma using a Western blot based method that allows immunoelectrophoretic distinction of the molecular forms of HSP70, as well as an estimate of the state of phosphorylation of serine and tyrosine residues. The present approach allows us to capture all isoforms

of HSP70 that recognize anti-HSP70, especially the two distinct forms including the constitutive HSP73.

and inducible HSP72. In addition to being able to separate them by Western blot, we have also demonstrated a pleiomorphism based on different degrees of phosphorylation on the tyrosine and serine residues. This technique opens a new window into the biochemical mechanisms of HSP70 expression in its multimeric incarnations as well as defines the nature of the posttranslational modifications by way of phosphorylation on tyrosine and serine residues.

HSPs account for 2-5% of the total protein content of cells under physiological conditions. In response to stress, there is rapid shutdown of the synthesis of most cellular proteins, with a dramatic increase in the expression of HSPs (Rakonczay et al., 2003; Söti et al., 2007; Bottoni et al., 2009; Baird et al., 2012). During periods of physiologic stress, many cellular proteins become partially, or completely, denatured or malformed (Sikora et al., 2007; Söti et al., 2007). HSPs bind to these damaged proteins, stabilize and refold them, thereby preventing irreversible aggregation (Smith et al., 1998). HSPs also play an important role in the protection of cells against stress induced apoptosis (Dudeja et al., 2009). By virtue of this protective function, HSPs have been shown to prevent acinar cell injury in acute pancreatitis (Bhagat et al., 2000; 2002). HSPs have been shown to be markedly elevated in various cancers when compared with non-transformed cells and their inhibition can induce apoptotic cell death in cancer cells (Aghdassi et al., 2007; Phillips et al., 2007). Furthermore, there is recent data to suggest that soluble HSP70 detected from human plasma can be used as a prognostic marker in patients with colorectal cancer without distant metastases (Kocsis et al., 2010).

HSPs are sub-classified based on their molecular weight into large HSPs, HSP90, HSP70, HSP60, HSP40 and small HSPs. Apart from differences in their molecular weights, they are known to demonstrate polymorphism, a phenomena well described in HSP70 (Dutta et al., 2000). This accounts for several of HSP70s structural and functional differences compared with other members of the HSP family. There is emerging evidence that HSPs can form large multimeric structures by creating stable complexes with folding intermediates of their protein substrates (Pockley et al., 1998; Rea et al., 2001). In turn, these qualities make the quantification of plasma HSP a potential diagnostic or prognostic tool in clinical disease states.

Phosphorylation of HSP70 at serine and tyrosine

residues is required for the protective role this protein plays within the cell. Evidence suggests that HSPs undergo phosphorylation at specific serine and tyrosine residues which influences their oligomerization into larger, multimeric aggregates (Dutta et al., 2000). This phosphorylation can occur during several stress states, including thermal and oxidative stress. HSP70 indicates a single band in sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), but shows multiple bands in native- polyacrylamide gel electrophoresis (N-PAGE) (Kim et al., 1992; Udono et al., 1993; Liu et al., 1994). Our results also show multimeric forms of HSP70 at 300, 275, 150, 100 and 60kD regions, demonstrating the pleomorphic nature of this protein in circulating plasma. The 60kD band most likely represents an HSP70-like component that is recognized by the anti-HSP70 antibody. Western blots probed with anti-phosphoserine and anti-phosphotyrosine showed marked differences in their degree of substitution on serine and tyrosine residues. When comparing the results shown in Figures 2B and 2C, the protein bands in the anti-phosphoserine blot are stronger and more prominent than the corresponding protein bands in the anti-phosphotyrosine blot. This may suggest a greater degree of phosphorylation on serine residues compared with tyrosine residues in immunoreactive HSP70 species in human plasma.

Our method of detecting HSP70 in plasma by using an immunocapture technique may have a few additional advantages over the existing ELISA method. In the ELISA test, the commercially available antibody to HSP70 has potential to cross-react with multimeric forms of HSP70 and hence can give an overestimate of HSP70 present in plasma. For example, the 60kD band that we identified using electrophoretic mobility is probably not an HSP70, and in an ELISA test, this protein would be included in the overall estimate of HSP70. The spuriously elevated value of HSP70 by ELISA test can then have potential implications for the clinical application of this test as a marker for pancreatic and other diseases. Our method isolates various molecular aggregates of HSP70 which may be advantageous over the commercially available ELISA test for plasma HSP70 detection.

The significance of multimeric forms in different clinical disorders is currently unclear. In most malignancies, there are distinct small molecular weight tumor-associated antigens (in the 2-5kD range) that get secreted into the blood stream but are missed because of a lack of techniques to detect them. The HSP family of proteins are known as chaperons, in that they can capture and transport other, newly synthesized abnormal proteins and will show up in the blood as variants (HSP70, but appearing in the electrophoretic field with molecular weights like 72, 75 kD, etc.).

An additional overriding benefit of our technique is in its ability to precisely distinguish the state of phosphorylation of HSP70 molecules at serine and tyrosine residues. The precise clinical significance of phosphorylation is under evolution, but it is an important function that assigns vital roles in cellular homeostasis, through post-translational modifications. Cytoplasmic HSP70 plays an important role in mitochondrial protein targeting, particularly in

unfolding the newly synthesized protein in the cytosol, maintaining it in an import-competent state and presenting the protein to mitochondrial contact points (Matouschek et al., 2000; Nollen et al., 2002; Robin et al., 2003). Speculatively, phosphorylation of HSP70 at serine and tyrosine residues may enhance mitochondrial targeting by increased affinity for binding to cytoplasmic HSP70. Although the specific clinical relevance of serine and tyrosine phosphorylation is currently unclear, there is data to suggest that the phosphorylation status of chaperone proteins like HSP70 in human breast cancer tissue may help identify a specific phosphoproteomic signature suggesting a common signaling pathway in tumor progression (Lim et al., 2004). In addition, the possibility of a direct role for tyrosine phosphorylation of heat shock proteins in the prometastatic and antiapoptotic phenotypes has been suggested (Xie et al., 2003; Berger et al., 2005). In the current methodology, not only are we able to distinguish HSPs of different molecular weights, but also define their status of phosphorylation, an important insight into the organismal responses of any aberrant pathophysiological processes.

In summary, we describe a novel method of isolation of HSP70 from plasma. The immunoreactive forms of HSP70 are widely distributed in plasma obtained from healthy human subjects, and appear to exist in heterogeneous forms. We have successfully demonstrated the presence of 5 distinct protein bands of HSP70, indicating multimeric forms of this protein. Increased phosphorylation on serine residues as compared with tyrosine residues appears to be a distinct characteristic of immunoreactive HSP70 in human plasma. Apart from describing a new approach for isolation of plasma HSP70, our observations will facilitate further investigation into the nature of secreted HSP70, particularly their states of aggregation necessary for oligomeric assemblies, which will advance the research of pancreatic cancer biomarkers.

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