RESEARCH ARTICLE

Heat Shock Protein Association with Clinico-Pathological Characteristics of Gastric Cancer in Jordan : HSP70 is Predictive of Poor Prognosis

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Abstract

Gastric cancer (GC) is a major health problem worldwide and is one of the ten most commonly diagnosed cancers in Jordan. GC is usually diagnosed at late aggressive stages in which treatment options are limited. Recently, heat shock proteins (HSPs) were found to be overexpressed in a wide range of malignancies have been considered as promising candidate biomarkers for GC. The aim of this study was to investigate pathogenic roles of a panel of cytosolic HSPs including HSP90, HSP70, HSP60 and HSP27 in GC. Immunohistochemistry was used to assess the level of expression of these proteins in archived tumor samples (N=87) representing various pathological characteristics of GC. HSP90, HSP60 and HSP27 were expressed abundantly in gastric tumors. On the other hand, HSP70 was reduced significantly and also found to be associated with *Helicobacter pylori* infection in tissues collected from GC patients. Furthermore, HSP27 was found to be associated with the level of differentiation. Our findings indicate a role of HSP70 as a potential prognostic biomarker, patients harboring positive HSP70 expression displaying worse disease free survival than those with negative HSP70 expression. Differential expression of HSPs may play crucial roles in the initiation and progression of GC, and could be exploited as future therapeutic targets.

Keywords: Gastric cancer - heat shock proteins - immunohistochemistry - prognosis

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Introduction

Gastric cancer (GC) is the fifth most commonly diagnosed type of cancer worldwide and the second cause of cancer-related mortality and morbidity (Bray et al., 2012; IARC, 2012). Approximately, 723,000 deaths occur annually due to GC which accounts for about 10.4% of cancer related deaths worldwide (IARC, 2012). The distribution of GC worldwide shows significant geographical, ethnic, and socioeconomic variations. Countries such as Japan, Central and Eastern Europe, and parts of South America have the highest incidence rates (approximately 70% of the cases) whereas most Western European and North American have low incidence rates (Layke and Lopez, 2004; Ferro et al., 2014). In the Middle East, GC prevalence is 5-15 times lower than countries with high incidence rates (Abbasi et al., 2010). In Jordan, GC is one of the ten most commonly diagnosed cancers accounting for approximately 3.1% of newly diagnosed cancer cases with an overall crude incidence rate of 2.5/100,000 and an age-specific incidence rate of 4.5/100,000 (Abbasi et al., 2010).

GC is more frequent in males than in females with a male to female ratio of 1.5:1 and the median age at diagnosis of both sexes was estimated to be 61.5 years old (63 years for males and 56 years for females) (JCR, 2010). The etiology of GC is still a matter of debate however, *Helicobacter pylori* infection is considered to be a major risk factor in the pathogenesis of the disease in addition to chronic atrophic gastritis, dietary habits, family history, and socioeconomic status (Layke and Lopez, 2004; Ferro et al., 2014).

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The most common type of gastric cancer is gastric adenocarcinomas accounting for around 95% of the cases. Other rare types include; adeno-squamous carcinomas, squamous carcinomas, and undifferentiated carcinomas (Kumar et al., 2010; Hu et al., 2012; Nagini, 2012). According to the Lauren's classification gastric adenocarcinomas are divided into two types: diffuse and intestinal (Lauren, 1965). The intestinal type is more frequent than the diffuse type, more common in men and older individuals and usually associated with intestinal metaplasia and *H. Pylori* infection. Patients with this subtype often display a better prognosis than those with the diffuse type. On the other hand, the diffuse type is commonly seen in females, younger individuals, patients with blood group A (Lastraioli et al., 2012; Nagini, 2012). Treatment options for GC are limited and there are no internationally accepted standards of care. Up to date, radi¬cal surgery offers a chance of a cure from gastric tumors that invade the muscular layer, yet, the overall prognosis of GC patients remains dismal due to delayed diagnosis and the occurrence of metastasis (Kocevar et al., 2012). New methods of treatment based on the identification of genetic changes and new molecular biomarkers are currently under investigation to improve GC patient's treatment and prognosis.

Previous studies have demonstrated that heat shock proteins (HSPs) are remarkably over-expressed in almost all types of cancers including GC and are involved intumor cell proliferation, differentiation, metastasis and resistance to treatment (Ciocca and Calderwood, 2005; Lianos et al., 2015).

HSPs are ubiquitous and evolutionary conserved proteins among species (Seigneuric et al., 2011). Mammalian HSPs are classified according to their molecular mass into five families; Large HSPs, HSP90, HSP70, HSP60 and small HSPs including; HSP27 (Wang et al., 2013). Intracellular HSPs are either stress-repressible proteins expressed constitutively under normal physiological conditions or stress-inducible (Galluzzi et al., 2009). Inducible HSPs are expressed in response to a wide variety of physiological and environmental stresses where they play a crucial role in the protection of cellular integrity and maintenance of protein homeostasis (Wang et al., 2013; Lianos et al., 2015). These proteins act as molecular chaperones; they assist in protein folding, refolding of misfolded proteins, preventing aggregation, assembly and disassembly of large protein complexes and translocation of newly synthesized polypeptides across membranes. At the molecular level, HSPs, particularly HSP70 and HSP27, were shown to play a crucial role in the suppression of apoptosis and cellular senescence, hence, the chaperoning activity of HSPs were found indispensable in the context of cancer cells progression and aggressiveness (Sherman and Multhoff, 2007). Furthermore, they might provide the cancer cell with an opportunity to alter protein activities, mainly those of the cell cycle machinery and kinases (Schmitt et al., 2007). The phenomenon of elevated HSPs expression in cancer cells has provided the rationale for exploiting them as therapeutic targets as well as prognostic indicators. Recently, it was suggested that targeting HSP90 with or without HER-2, the most studied molecular target for GC therapy, might provide an additional option for the treatment of GC (Berezowska et al., 2013). However, little is known about the pathogenic role of HSPs in GC.

In this study, a retrospective investigation of gastric adenocarcinomas from 87 Jordanian patients was carried out to examine the expression level of a panel of intracellular HSPs including; HSP90, HSP70, HSP60 and HSP27 using immunohistochemistry and to determine their prognostic significance in GC.

Materials and Methods

Patients Selection

Eighty seven patients diagnosed with GC and who underwent gastric resection between the years of 2004 and 2014 were randomly selected for a retrospective study. Archived tissue samples and their corresponding patients' pathological records were provided by the pathology department of four public, military and university hospitals in Jordan including; King Abdullah University Hospital (KAUH), Princess Basma Hospital, Al-Basheer Hospital and the Royal Medical Services. Approval for this work was obtained from the Faculty of Medicine Research Ethics Committee at Jordan University of Science and Technology (JUST) and the Ministry of Health (MOH).

This study included 48 Males (55.2%) and 39 Females (44.8%). At the time of diagnosis, the median age of all patients included was 60.0 years (Range from 25-98 years). Clinicopathological characteristics to be examined in this study included; Lauren's histological type, *H. Pylori* infection, tumor size, tumor location, depth of invasion, Lymph Node (LN) metastasis, stage, grade and Lymphovascular invasion (LVI). Tumor staging was carried out according to the American Joint Committee on Cancer (AJCC)/Union International Cancer Control (UICC) Tumor, Node, Metastasis (TNM) system, 7th edition. Accordingly, tissues were categorized on a scale of I – IV in which tumors with stage I/ II were considered as early GCs (EGC) and tumors with stage III/ IV were considered as advanced GCs (AGC). Grading of tumors was carried out based on four levels of differentiation. Accordingly, all samples were classified into two groups; low grade for well to moderately differentiated tumors (G1/G2) and high grade for poorly to undifferentiated tumors (G3). Additionally, complete clinical data was provided for 73 patients who were selected for further analysis.

In addition to tumor specimens, 16 specimens comprising normal gastric epithelium were randomly selected from the pathological archives of KAUH. All specimens were formalin-fixed and paraffin-embedded for pathological examination. This study included a total of 103 tissue samples processed and prepared for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical analysis was carried out according to the dual link method for immunohistochemistry as described by bodoor et al (Bodoor, 2010; 2012). All procedures were performed manually following the

regulations provided by the pathology department at KAUH. Briefly, formalin-fixed paraffin embedded tissue sections of 4μm thickness loaded on poly-lysine coated slides were deparaffinized in xylene and brought to water gradually through a series of graded alcohol. Antigen retrieval was done in heated citrate buffer (pH=9) for 30 minutes using the pretreatment (PT) Link platform from DAKO. Sections were then treated with freshly prepared hydrogen peroxide (3%) for 10 minutes at room temperature in order to remove endogenous peroxidase activity. Primary antibodies were applied to all specimens (200μl) and incubated for 45 minutes at room temperature. The following antibodies were used; anti-HSP90 antibody (1:200 dilution, #4877, Cell Signaling Technology, US), anti-HSP70 antibody (1:200 dilution, #4872, Cell Signaling Technology, US), anti-HSP60 antibody (1:200 dilution, ab45134, Abcam, UK) and anti-HSP27 antibody (1:100 dilution, #2402, Cell Signaling Technology, US). Immunoreactivity was then evaluated for each marker using horseradish-peroxidase (HRP) conjugated secondary antibodies with an incubation time of 20 minutes at room temperature (EnVesion FLEX/HRP, DAKO), and 3, 3'-diaminobenzidine (DAB) as a chromogen (EnVesion FLEX DAB+Chromogen, DAKO). Sections were washed thoroughly with 1× phosphate buffer saline (PBS) prior to each step. After that, sections were briefly counterstained with Mayer's hematoxyline, dehydrated through a series of graded alcohol and xylene, and mounted in DPX using an automated mounting device (Elica CV5000 LEICA, Germany).

Evaluation of immunohistochemical staining results

For microscopic examination, at least 1000 tumor cells were scored semiquantitatively in well-preserved areas of each slide by a specialized pathologist blinded from any knowledge regarding the patients' clinical data. Discrepant cases were discussed on a multihead microscope by two specialized pathologists simultaneously until a consensus was reached. The scoring criteria used in this study were described previously (Xu et al., 2011). The extent of expression was evaluated as follows: 10-20% positive cells, 20-50% and >50% positive cells were given the scores +1, +2 and +3, respectively. Intensity of staining was estimated as weak (1), moderate (2) and strong staining (3). The final score was obtained by multiplying the extent of expression and intensity of staining. Scores of ≤1 were considered as negative expression and >1 were considered as positive expression. The same scoring criteria were followed for the evaluation of immunhistochemical staining in normal gastric epithelium. Negative controls were used by which all primary antibodies were omitted from the procedure and substituted with 1× PBS. Suitable positive controls were done simultaneously.

Statistical analysis

Pearson's χ^2 test of independence and Fisher's exact probability test were used to analyze the relationship between categorical variables including; age, gender, Lauren's histological type, tumor location, depth of invasion, TNM stage, grade, tumor-size, *H. Pylori* infection, lymph node and venous metastasis along with the various patterns of HSPs expression. To investigate the correlation between HSPs expression amongst each other, the spearman rank correlation test was used. Kaplan-Meier survival curves were used to represent disease-free survival (DFS) of patients. Accordingly, DFS was defined as the period from the time of diagnosis to death from any cause or recurrence of the disease or last contact. The log rank test was used to evaluate the prognostic value of protein expression on survival curves. A p-value of ≤0.05 was considered statistically significant. All data were analyzed using the Statistical Package for the Social Sciences program (IBM SPSS Statistics 21, USA).

Results

Patients

A retrospective analysis of the pathological records from four different hospitals in Jordan identified 87 GC patients who underwent gastric resections between the years 2004 and 2014. Out of 87 patients, 48 patients were males (55.2%) and 39 were females (44.8%). The median age of all patients at the time of diagnosis was 60 years (68 years for males and 52 for females) with a range from 25-98 years. In brief, 49 patients exhibited the intestinal type (56.3%) while 38 patients exhibited the diffuse type (43.7%). Regarding the pathological stage of tumors, this study included 23 patients with EGC (27%) and 62 patients with AGC (73%). Of these, 30 cases (36.6%) showed high level of differentiation (G1/G2) while 57 cases (69.5%) showed poor differentiation (G3). Moreover, LN invasion was identified in 67 patients (78.8%). As a major risk factor of GC, the prevalence of *H. Pylori* infection was investigated. *H. Pylori* infection was identified in 53 out of 70 (76%) patients whose data were available.

Follow-up Data:Complete clinical data was available for 73 patients with follow-up periods up to 88 months. During this period, 29 patients developed recurrence while 44 patients remained free of recurrence until their last contact with the clinician. To our knowledge, none of the patients had received chemotherapy or radiotherapy prior to surgery and only one patient had received postoperative chemotherapy (5-FU and Leucovorin regimen).

HSPs expression and differential localization in GC tissues

Immunohistochemical analysis of 87 GC tissue samples was carried out to identify differential expression of HSPs in GC. Expression of HSP90, HSP70, HSP60 and HSP27 has been investigated. Our results have shown that HSPs exhibit variable levels and different patterns of expression in all GC tissues examined when compared to tissues from normal individuals. Regarding HSP90, All samples examined have shown high expression levels of the protein (>50% positive cells in all cases examined) and high staining intensity (moderate to strong intensity) in GC tissues. HSP60 and HSP27 have shown similar levels of expression in GC tissues where positive staining has been observed in 75.9% of the cases. Positive expression of HSP70 was observed in 20.7% with very weak intensity in most cases. In summary, gastric tumors displayed

100% expression of hsp90 (76.5% in normal tissuses), 20.7% expression of hsp70 (56.3% in normal tissues), 75.9% expression of hsp60 (75% in normal tissues), and 75.9% expression of hsp27 87.5% in normal tissues). It is noteworthy to mention that some tumors with positive HSPs expression showed heterogeneous patterns of staining intensity (i.e., the tumor tissue was comprised of both moderately stained and strongly stained colonies). HSP90, HSP70 and HSP27 displayed a diffused pattern of expression with occasional granular expression for HSP90 and HSP27. In the case of HSP60, most of the samples examined (72.4%) displayed the granular expression pattern as expected for a mitochondrial protein.

Table 1. HSP70 Expression in Association with Various Clinicopathological Characteristics of GC

Characteristics	N	Negative	Positive	p-value
Age				
≤60	48	39	9	
>60	39	30	9	0.408
Gender				
Male	48	38	10	
Female	39	31	8	0.592
Histological type				
Intestinal	49	38	11	
Diffuse	38	31	7	0.426
Tumor size				
≤5	40	31	9	
>5	45	37	8	0.392
Location				
Distal	37	30	7	
Proximal	13	10	3	
Body	37	29	8	0.935
Depth of Invasion (T)				
T1/T2	6	6	0	
T3/T4	80	62	18	0.233
LN invasion (N)				
Negative	18	14	4	
Positive	67	53	14	0.566
TNM stage				
I/II	23	18	5	
III/IV	62	49	13	0.577
Grade				
Low	30	21	9	
High	57	48	9	0.102
LVI				
Negative	15	12	3	
Positive	53	40	13	0.506
H. Pylori				
Negative	34	29	5	
Positive	36	23	13	0.037

Moreover, inflammatory cells infiltrating the tumor with strong cytoplasmic HSP70 expression have been observed. Representative images from well preserved areas in GC tissues are demonstrated in Figures 1. All HSPs mainly located to the cytoplasm of cancer cells with focal nuclear expression (Figure 2A, 2B) whereas HSPs expression in normal gastric epithelium was mainly cytoplasmic. Interestingly, as illustrated in Figure 2A, confined nuclear expression of weak intensity has been observed for HSP70 in GC tissues.

Correlation between HSPs expression and various clinicopathological characteristics of GC

To further understand the implication of the variable

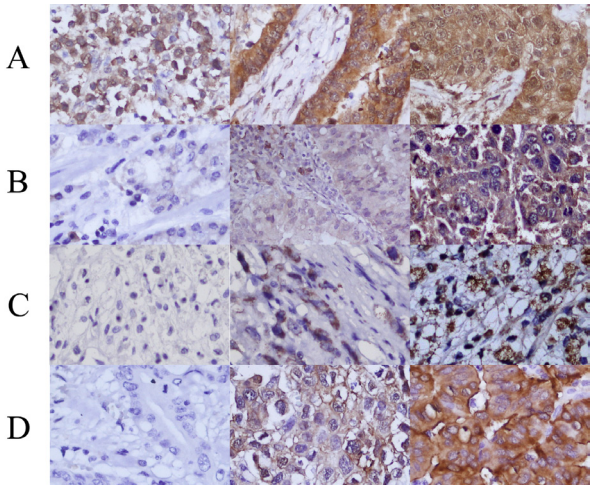

Figure 1. HSP Expression in GC tissues. A: moderate (left panel) and strong (middle and right panel) HSP90 expression. B-D: HSP70, HSP60 and HSP27 expression, respectively. Negative expression is shown in the left panel whilemoderate expression and strong expression are shown in the middle and right panels, respectively. Original magnification: 400X.

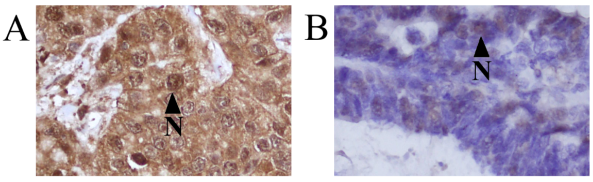

Figure 2. Nuclear Expression of HSPs. A: Strong nuclear expression of HSP90 in GC tissues as indicated by arrow heads. B:Confined nuclear expression of HSP70 in GC tissues is indicated by arrow heads. N: Nuclear expression, original magnification: 400X

Table 2. Predictive Clinicopathological Variables for Disease Free Survival of 73 GC Patients

Characteristics	N	Mean ±SE (months)	95% CI	Median ±SE (months)	95% CI	P-Value
DFS (All patients)	73	39.7 ± 4.7	30.4-48.9	44.0 ± 8.3	27.6-60.3	
LN Invasion (N)	73					
Negative	15	58.4 ± 9.9	39.1-77.8	55.0 ± 6.5	42.3-67.7	
Positive	58	30.7 ± 3.6	23.6-37.8	33.0 ± 8.5	16.4-49.6	0.014*
TNM Stage	73					
I/II	19	53.4 ± 8.6	36.7-70.2	55.0 ±10.3	34.8-75.2	
III/IV	54	30.7 ± 3.9	22.9-38.5	33.0 ± 9.3	14.8-51.2	0.032*
H. Pylori	70					
Negative	34	47.4 ± 5.4	36.8-57.9	49.0 ± 7.6	34.1-63.8	
Positive	36	30.1 ± 5.3	19.7-40.6	30.0 ± 10.9	8.7-51.3	0.028*

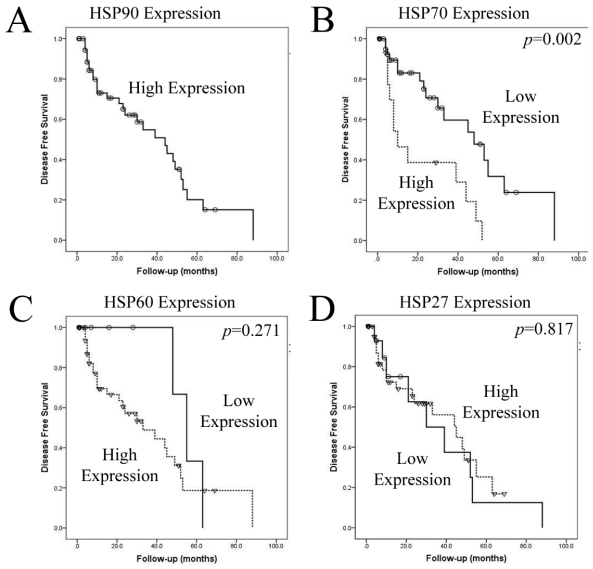

Figure 3. Kaplan-Meier Disease Free Survival (DFS) Analysis of 73 GC Patients According to HSP Expression. Disease Free Survival (DFS) of all patients stratified according to HSP90 expression (A), HSP70 expression (B), HSP60 expression (C) and HSP27 expression (D)

HSPs expression in GC tissues, the expression of HSPs was tested for its association with various clinicopathological characteristics of GC. HSP90 was found to be expressed abundantly in all tumor tissues examined showing moderate to strong staining intensities. Nuclear localization of HSP90 was shown to be significantly associated with earlier presentation of the disease (p=0.054), diffuse type of GC (p=0.004) and higher grade (p=0.045).

Regarding HSP70, our results have shown a significant association between HSP70 expression and *H. Pylori* infection (p=0.037) but not with other clinicopathological characteristics of GC (Table 1). Stratification according to the extent of expression alone revealed a significant association between HSP27 expression and higher grade (p=0.045) and also a marginal significant correlation between HSP60 expression and higher TNM stage (p=0.063).

Spearman's rank correlation test was used to evaluate the linear relationship between HSPs expression amongst each other. The results have shown a significant positive correlation between the expression of HSP70 and HSP60 (rs= 0.288. p=0.003). Similar expression for each of HSP70 and HSP60 was shown in 39 out of 87 cases (positive expression in 18 cases and negative expression in 21 cases). The remaining cases showed only positive HSP60 expression.

HSP70 is a potential prognostic indicator in GC patients

Kapaln-Meier survival analysis was performed to investigate the predictive value of HSPs in GC patients. DFS of 73 patients with GC was analyzed. Variables included in the univariate analysis are; age, gender, Lauren's histological type, tumor location, tumor size, depth of invasion, LN involvement, TNM stage, tumor grade, *H. Pylori* infection, LVI, and HSPs expression. All patients included in the study had a mean disease free survival (DFS) of 39.7 ± 4.7 month and a median of 44.0 ± 8.3. Higher TNM stage (p=0.032), lymphatic invasion (p=0.014), *H. Pylori* infection (p=0.028) showed a significant impact on DFS of GC patients. A trend has been also observed for larger tumor size (p=0.07) and the intestinal type of GC (p=0.08). The results are illustrated in Table 2. The prognostic value of HSPs expression and localization has also been examined. HSP70 expression have shown significant impact on the DFS of patients (p=0.002) while other HSPs failed to predict patients DFS (Figure 3). Postoperative mean DFS was 22.5 months for patients with high HSP70 expression while a mean DFS of 47.6 months was observed in patients with low HSP70 expression.

Since the cohort of patients mostly had very short follow-up periods and further clinical data couldn't be obtained for these patients, a multivariate regression analysis couldn't be performed.

Discussion

AGC remains a major health problem worldwide. Late detection and lack of standard treatment strategies results in high levels of mortality and poor prognosis in GC patients. Chemotherapeutic agents including; 5-FU, taxane and platinum have been used for treatment with only partial responses and poor survival rates in most cases that reflects sensitivityto treatment. In order to overcome the problem of chemoresistance in patients with GC, new methods based on thorough understanding of the molecular mechanisms underlying the pathogenesis of the disease are required. Many agents have been identified including Trastuzumab, a monoclonal antibody against HER-2, which have shown survival benefits for GC patients. However, the overall survival of these patients remains dismal. HSPs have been identified recently as candidate biomarkers and therapeutic targets for a wide range of human malignancies when sufficiently expressed. These proteins were shown to contribute in malignant transformation and progression and to be associated with poor survival (Ciocca and Calderwood, 2005). However, studies regarding the role of HSPs in GC are limited. In the present study, Expression levels of HSP90, HSP70, HSP60 and HSP27 were investigated in a cohort of 87 Jordanian patients diagnosed with GC using Immunohistochemistryin order to identify the pathogenic role of these proteins in GC and their clinical utility.

In the present study, all examined GC cases displayed positive expression for at least one member of the HSPs family with variable expression in each case. HSP90 exhibited the highest expression level and intensity while on the other hand HSP70 exhibited the lowest. These findings are consistent with earlier reports investigating HSPs expression in bladder cancer and squamous cell carcinoma of the esophagus (Kawanishi et al., 1998; Lebret et al., 2003; Capello et al., 2006). However, Giaginis and colleagues have shown that in GC, HSP60 harbors the highest expression amongst other members of the HSPs family (Giaginis et al, 2009).Nevertheless, HSP90 was previously demonstrated to harbor increased expression in tumors from various origins and to be

associated with more aggressive tumors and poor survival of patients. Additionally, it is believed that HSP90 plays a key role in facilitating tumor cells transformation, survival and metastasis by virtue of its client proteins which are involved in multiple pro-survival pathways (Ciocca and Calderwood, 2005; Whitesell and Lindquist, 2005; Seigneuric et al., 2011). An expanding interest for investigating the expression of HSP90 in different types of malignancies has begun upon the identification of inhibitors of this protein which exert noticeable anti-tumor activities with minimal effects on normal cells (Whitesell and Lindquist, 2005; Koga et al., 2009). However, few studies have investigatedthe expression of HSP90 in GC in which they have shown considerable variations in their results. In the present study, we have shown that HSP90 is overexpressed in GC tissues compared to normal tissues of the gastric epithelium. These findings are consistent with earlier reports investigating the expression of the protein in GC (Zuo et al., 2003; Gianginis et al., 2009; Berezowska et al., 2013; Wang et al., 2013). Despite the fact that similar detection methods and scoring criteria were used, we have shown the highest expression level of the protein in GC tissues compared to previous studies. This might be related to differences in the ethnic backgrounds of patients or different choice of antibodies used in these investigations (Zuo et al., 2003; Gianginis et al., 2009; Berezowska et al., 2013).

Regarding HSP70, previous studies indicate that this protein exhibits a reduced level of expression in GC tissues which is consistent with our findings. Lee et al. reported a positive HSP70 expression in 44% of the cases in a cohort of 172 GC patients (Lee et al., 2013). Similarly, in a cohort of 458 patients, only 34% of the cases exhibited positive HSP70 expression (Kang et al., 2013). As an explanation of this phenomenon, we suggest that the attenuation of HSP70 expression in GC might be an effort of cancer cells to evade antitumor immunity exerted by HSP70 as itis known to play a crucial role in the recruitment and differentiation of inflammatory cells which exhibit antitumor activities (Canöz et al. 2002; Bonorito and Souza, 2007). In addition to these findings, our results have shown that HSP70 expression increased significantly in pre-neoplastic lesions of the gastric mucosa (Data not shown). This observation has been reported previously by a number of studies which might indicate a role of HSP70 in oncogenic transformation (Jiancheng et al., 1999; Canöz et al., 2002; Wang et al. 2007). Nevertheless, some studies have shown a significant increase in HSP70 expression in GC tissues where >50% of the cases displayed immunoreactivity towards the protein (Jiancheng et al., 1999; Maehara et al., 2000; Isomoto et al., 2003).In fact, HSP70 was hypothesized to be favorable in the context of oncogeneic transformation and progression by virtue of its cytoprotective activities. However, controversial results have been obtained regarding the role of HSP70 in the pathogenesis of GC.

Furthermore, a positive correlation between *H. Pylori* infection and HSP70 expression has been observed in this study. *H. Pylori*is a highly prevalent pathogen that has been strongly associated with increased risk of developing gastric carcinoma, particularly, distal gastric tumors of the intestinal type where it was shown to trigger a cascade of events that promote the sequential progression of gastric lesions into malignant tumors (Correa, 1992; Wroblewski et al., 2010). *H. Pylori* associated carcinogenesis in the gastric mucosa has been attributed to a spectrum of bacterial virulence factors including; the cytotoxin associated gene pathogenicity island (cag PAI) and the vacuolating cytotoxin (VacA) through which *H. Pylori* were shown to promote the synthesis and secretion of inflammatory cytokines (IL-8), inflammatory enzymes such as cyclo-oxygenase-2, inducible nitric oxide synthase and to interfere with cellular signal transduction systems that disrupt the equilibrium between apoptosis and proliferation (Shibata et al., 2005; Targosz et al., 2012). Recent evidence has demonstrated that pathogenic strains of *H. Pylori* may induce apoptosis and predispose the gastric epithelium to carcinogenesisin vitro through the attenuation of HSP70 which otherwise could modulate the expression of enzymes involved in inflammation and cellular damage, hence, attenuating gastric mucosal injury associated with the pathogen (Tsan and Gao, 2004; Pierzchalski et al. 2006; Targosz et al., 2012). Complete ablation of HSP70 was shown to enhance the anti-proliferative effects of *H. Pylori* in infected cells, render the cells more susceptible to cytotoxicity exerted by various bacterial components and to induce apoptosis by shifting the equilibrium towards the apoptotic state (Liu et al., 2011). On the other hand, it was suggested that HSP70 expression in AGC is inversely linked to *H. Pylori* infection where the decrease in the pathogen prevalence allows the upregulation of HSP70 expression in injured cells that exert cytoprotective activities to enhance cancer cells survival (Tokunaga et al., 2000; Canöz et al., 2002; Axsen et al., 2009).

Moreover, our results have shown an association between the expression of HSP70 and HSP60. Giaginis et al. previously reported similar associations between different HSPs in a series of 66 GC patients (HSP60 and HSP90 with HSP27) (Gianginis et al., 2009). Similarly, Kawanishi et al. have shown a simultaneous loss of HSP70 and HSP27 in tissue samples of squamous cell carcinoma of the esophagus. In that study, the authors suggested common signals regulating the expression of HSPs during carcinogenesis (Kawanishi et al., 1998). Moreover, conflicting results regarding HSP60 expression in urothelial carcinomas of the urinary bladder implied that the expression of HSP60 might be dependent on other proteins involved in pro-apoptotic pathways. However, molecular mechanisms underlying the expression of HSP60 are not fully elucidated (Capello et al., 2006).

As for HSP27, we have shown that HSP27 expression was significantly associated with poorly differentiated tumors. Previous reports have shown similar results in GC (Nagata et al., 2012). It was suggested by previous studies on human HaCat cells that HSP27 activity is not a basic effect related to cell differentiation but rather aimed to protect the cell from drastic changes in their protein content, structural organization and localization (Arrigo, 2005). Recently, the role of HSP27 in cancer cell differentiation was shown to be related to the epithelial-mesenchymal transition (EMT) process (Wei et al., 2011;

Wettstein et al., 2013). In GC, the role of HSP27 in EMT-related dedifferentiation is not well established. In a study conducted by Nagata et al., HSP27 was found to induce EMT in MKN-1 GC cell line but not in MKN-74 cell line (Nagata et al., 2012). This was partially explained by the heterogeneous nature of cancer cells. It is worth noting that, 73% of patients with poorly differentiated tumors that positively expressed HSP27, displayed nuclear localization of the protein. Under normal conditions, Sub-cellular localization is a well known mechanism where HSPs function to make decisions whether the cell must die or differentiate (Lanneau et al., 2007). In support of our results, Kapranos et al. have also indicated nuclear localization of HSP27 in diffuse type GC (poorly differentiated) in particular (Kapranos et al., 2002). Our findings show that HSP27 expression in GC tissues is only related to differentiation but not to the malignant potential of GC.

In addition to linking the expression of HSPs to various clinicopathological features of adverse outcome in GC patients, numerous studies have been carried out to investigate the prognostic significance of HSPs expression in this type of cancer. Up to date, discrepant results have been observed where some studies reported a strong association between some HSPs with poor survival whereas others have shown an association with prolonged survival (Gianginis et al., 2009; Berezowska et al., 2013). On the other hand, some groups have shown no prognostic value of HSPs expression in GC patients (Maehara et al., 2000; Isomoto et al., 2003). In the present study, we have evaluated the DFS in patients with GC postoperatively and demonstrated a significant correlation between HSP70 expression and poor prognosis. HSP70 maintained its predictive value when patients were stratified into subgroups where it was shown to predict poor DFS in patients with both intestinal and diffuse histological types with patients bearing the diffuse type showing the worst survival rate. However, no statistical analysis was conducted for the group of patients with EGC because all of these patients have shown negative HSP70 expression whereas for AGC patients HSP70 expression maintained its predictive value. Previously, a significant correlation between HSP70 and characteristics associated with adverse clinical outcomes and unfavorable prognosis in patients with GC has been reported (Canöz et al., 2002; Wang et al., 2007; Lee et al., 2013). Lee et al. have shown that HSP70 was a prognostic marker for GC patients bearing the intestinal histological type in which it predicted worse overall survival in these patients (Lee et al., 2013). Similarly, a study conducted by Kang et al. indicated that HSP70 predicted poor overall survival in patients with EGC (Kang et al., 2013). In contrast, Isomoto et al. and Maehara et al. haven't demonstrated any influence of HSP70 expression on the survival of patients (Maehara et al., 2000; Isomoto et al., 2003). Nevertheless, few studies have been conducted to evaluate the prognostic impact of HSP70 in GC patients and earlier findings have shown that the role of HSP70 expression in the prognosis of GC remains inconclusive. In this context, larger studies with better distribution of groups are warranted to determine the role HSP70 as a prognostic marker for GC patients.

Collectively, our study has set a starting point for the investigation of HSPs expression and their role in the pathogenesis of GC in Jordanian patients. We have shown that HSP70 might play an essential role as a prognostic indicator in GC patients. However, contradicting results from earlier reports refers to the importance of more extensive and large scale investigations regarding HSPs expression and regulatory mechanisms in GC. Moreover, we have shown that HSPs expressed abundantly in GC tissues including; HSP90, HSP60 and HSP27 might be potential therapeutic targets. Recent evidence has shown that inhibition of HSPs, particularly HSP90, may be beneficial in the treatment of gastrointestinal tumors (Dudeja et al., 2009). Furthermore, abrogation of HSPs activities in vitro and in vivo have shown to induce apoptosis, decrease tumor growth and vascularization and elevate tumors chemosensitivity in tumors of different origins (Zhao and Shen, 2005; Kaul et al., 2006; Lang et al., 2007; Xiang et al., 2008; Liu et al., 2011; Zoubeidi and Gleave, 2012; Murphy, 2013; Howe et al., 2014). Currently, many efforts have been employed to develop anti-HSPs compounds for cancer therapy which so far have shown promising results (Howe et al., 2014). Additionally, clinical trials of phase I/II using HSP90 inhibitors including; Geldanamycin (GA) and its semisynthetic derivatives (17-AAG), and (17-DMAG) alone or in combination with other chemotherapeutic agents are under way for the treatment of several solid tumors including GC (Solit and Rosen, 2006). However, further investigation using HSPs inhibitors in GC cell lines is warranted to determine their exact utility as therapeutic agents in this type of cancer.

In conclusion, understanding of the molecular mechanisms underlying the pathogenesis of GC is required to achieve better patient outcomes. Here, we report overexpression of HSP90, HSP60 and HSP27 in gastric tumors. On the other hand, HSP70 expression was reduced significantly and also found to be associated with *H. Pylori* infection in tissues collected from GC patients. Patients harboring positive HSP70 expression in their tissues were shown to display worse disease free survival than those with negative HSP70 expression. Furthermore, HSP27 was found to be associated with the level of differentiation in GC tissues. The role of HSPs in GC initiation and progression highlights their use as potential future targets for prognosis and treatment.

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