ANTICANCER RESEARCH 30: 2539-2544 (2010)

Heat-shock Protein 27 Is Phosphorylated in Gemcitabine-resistant Pancreatic Cancer Cells

KUMIKO TABA[1,2], YASUHIRO KURAMITSU[1], SHOMEI RYOZAWA[1,2], KANAKO YOSHIDA[1,2], TOSHIYUKI TANAKA[1], SHIN-ICHIRO MAEHARA[3], YOSHIHIRO MAEHARA[3], ISAO SAKAIDA[2] and KAZUYUKI NAKAMURA[1]

Departments of [1]Biochemistry and Functional Proteomics, and [2]Hepatology and Gastroenterology, Yamaguchi University Graduate School of Medicine, Ube, 755-8505, Yamaguchi, Japan;
[3]Department of Surgery and Science, Graduate School of Medical Sciences, Kyusyu University, Fukuoka, Japan

Abstract. *Background: Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine: Gemzar) (GEM) appears to be the only effective anticancer drug for pancreatic cancer, but it has little impact on outcome due to a high level of inherent and acquired tumor resistance. Our previous proteomic study demonstrated that the expression of three spots of heat-shock protein 27 (HSP27) was increased in GEM-resistant pancreatic cancer cells and could play a role in determining the sensitivity of pancreatic cancer to GEM. Materials and Methods and Results: In the present study, using one-dimensional and two-dimensional Western blotting, we elucidated that these three spots of HSP27 were phosphorylated in GEM-resistant pancreatic cancer cell line, KLM1-R. Conclusion: Phosphorylated HSP27 may play an important role in the resistance to GEM, and it could also be a possible biomarker for predicting the response of pancreatic cancer patients to treatment with GEM.*

The prognosis of patients with pancreatic cancer is still very poor because of the disease aggressiveness and lack of early diagnosis and effective therapies (1, 2). Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine: Gemzar) (GEM) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. Although at the moment GEM appears to be the only clinically effective drug for pancreatic cancer, intrinsic or acquired resistance of pancreatic cancer impacts the therapeutic effect of GEM (3). Our previous studies investigated protein expression in GEM-resistant and -sensitive human pancreatic adenocarcinoma cell lines by proteomics. Two-dimensional gel electrophoresis (2-DE) showed up-regulated and down-regulated protein spots in GEM-resistant cell lines compared with GEM-sensitive cell lines, and they were identified by liquid chromatography-tandem mass spectrometry and Western blotting. Three isoform spots of heat-shock protein 27 (HSP27) on 2-DE were found to be increased in the resistant cell lines compared with sensitive cell lines. The knock-down analysis for HSP27 in KLM1-R cells restored sensitivity to GEM, and increased HSP27 expression in tumor specimens was related to higher resistance to GEM in patients with pancreatic cancer (4, 5). Further experiments showed the treatment of KLM1-R with interferon-γ down-regulated HSP-27 and increased the cytotoxic effect of GEM on GEM-resistant KLM1-R cells (6). The aim of our present study was to clarify the three isoforms identified as HSP27 in GEM-resistant pancreatic cancer cells.

Correspondence to: Yasuhiro Kuramitsu, MD, Ph.D., Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836222213, Fax: +81 836222212, e-mail: climates@yamaguchi-u.ac.jp

Key Words: Two-dimensional gel electrophoresis, 2-DE, LC-MS/MS, pancreatic cancer, gemcitabine, GEM, proteomics, heat-shock protein 27.

Materials and Methods

Tumor cell lines and culture conditions. Two human pancreatic cancer cell lines, GEM-sensitive KLM1 cells and GEM-resistant KLM1-R cells, were provided by the Department of Surgery and Science at the Kyushu University Graduate School of Medical Science. KLM1-R was established by exposing KLM1 cells to GEM, as described previously (7). The human pancreatic adenocarcinoma cell lines, MiaPaCa-2 and BxPC-3 were purchased from the American Type Culture Collection, and Panc-1 was kindly provided by the Institute of Development, Aging and Cancer at Tohoku University. Panc-1 and MiaPaCa-2 cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% fetal bovine serum (FBS). BxPC-3, KLM1 and KLM1-R cells were grown in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C (5).

Table I. *Identification of proteins which are differentially expressed between KLM1and KLM1-R cells.*

Spot no.	Protein name	Accession no.	Theoretical pI	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score
1	Heat-shock 27 kDa protein	P04792	5.98	22782.6	7	56	107.74
2	Heat-shock 27 kDa protein	P04792	5.98	22782.6	7	37	95.36
3	Heat-shock 27 kDa protein	P04792	5.98	22782.6	2	16	26.38

Sample preparation. Cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4˚C, centrifuged at 21,500 ×*g* for 30 min at 4˚C, and the supernatants were stored at –80˚C until use (8).

Two-dimensional gel electrophoresis (2-DE). Eighty micrograms of protein were used for each 2-DE. For the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip. Samples were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed using the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. In the second dimension, SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V(9).

Fluorescent gel staining. Agitation was carried out at all stages. After 2-DE, the gels were subjected to fixing solution with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight. Stained gels were washed with Milli-Q water three times.

In-gel digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The procedures of in-gel digestion and LC-MS/MS are described at length elsewhere (4).

Western blot analysis. Fifteen micrograms of protein were used for both 1-DE and 2-DE Western blotting, respectively. After electrophoresis, gels were transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked for 1 h at room temperature with TBS containing 5% skimmed milk. Primary antibodies were anti-HSP27 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-phospho-HSP27 rabbit polyclonal antibody (Ser15, Ser78, Ser82) (Cell Signaling Tech., Beverly, MA, USA). Membranes were incubated with the primary antibody overnight at 4˚C, washed three times with TBS containing 0.05% Tween-20 and once with TBS, and then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution range 1:5,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1.5 h at room temperature, and developed with a chemifluorescence reagent (ECL Plus Western Blotting Detection Reagents; GE Healthcare). The immunoreactive protein bands were then obtained by using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA) (10, 11).

Results

Up-regulation of HSP27 spots in GEM-resistant KLM1-R compared with GEM-sensitive KLM1 cell. Protein spots on the 2-DE gels of KLM1-R and KLM1 were visualized, and three spots of 27 kDa (spot 1, 2, and 3 in Figure 1 A, B) were identified as heat-shock protein 27 (HSP27) (Table I).

Up-regulation of phosphorylated HSP27 in GEM-resistant KLM1-R compared with GEM-sensitive lines. Western blotting analysis of HSP27 and phosphorylated HSP27 (Ser15, Ser78 and Ser82) was performed in pancreatic cancer cell lines. Total HSP27 expression tended to be higher in GEM-resistant KLM1-R compared with the other GEM-sensitive lines (Figure 2A). The level of phosphorylation of serine 78 and serine 82 of HSP27 was increased (Figure 2C, D respectively), but serine 15 of HSP27 was not phosphorylated (Figure 2B).

2-D Western blotting showed up-regulation of phosphorylated HSP27 spots in GEM-resistant KLM1-R cells. By 2-D Western blotting for phosphorylated HSP27 (Ser78), three phosphorylated HSP27 spots were observed in KLM1-R cells (Figure 3 B), but they were very faint in KLM1 cells (Figure 3 A). Furthermore, by 2-D Western blotting for phosphorylated HSP27 (Ser82), three phosphorylated HSP27 spots were observed in KLM1-R cells (Figure 4 B), but they were also very faint in KLM1 cells (Figure 4 A).

Discussion

In the present study, Western blotting analysis revealed that phosphorylation of HSP27 was increased in a GEM-resistant pancreatic cancer cell line. This finding suggests that not only the total protein level of HSP27, but also the level of phosphorylated HSP27 might contribute to GEM resistance.

HSP 27 is a molecular chaperone that is involved in the unfolded protein response and that plays a role in facilitating the assembly of multimeric protein complexes inside the

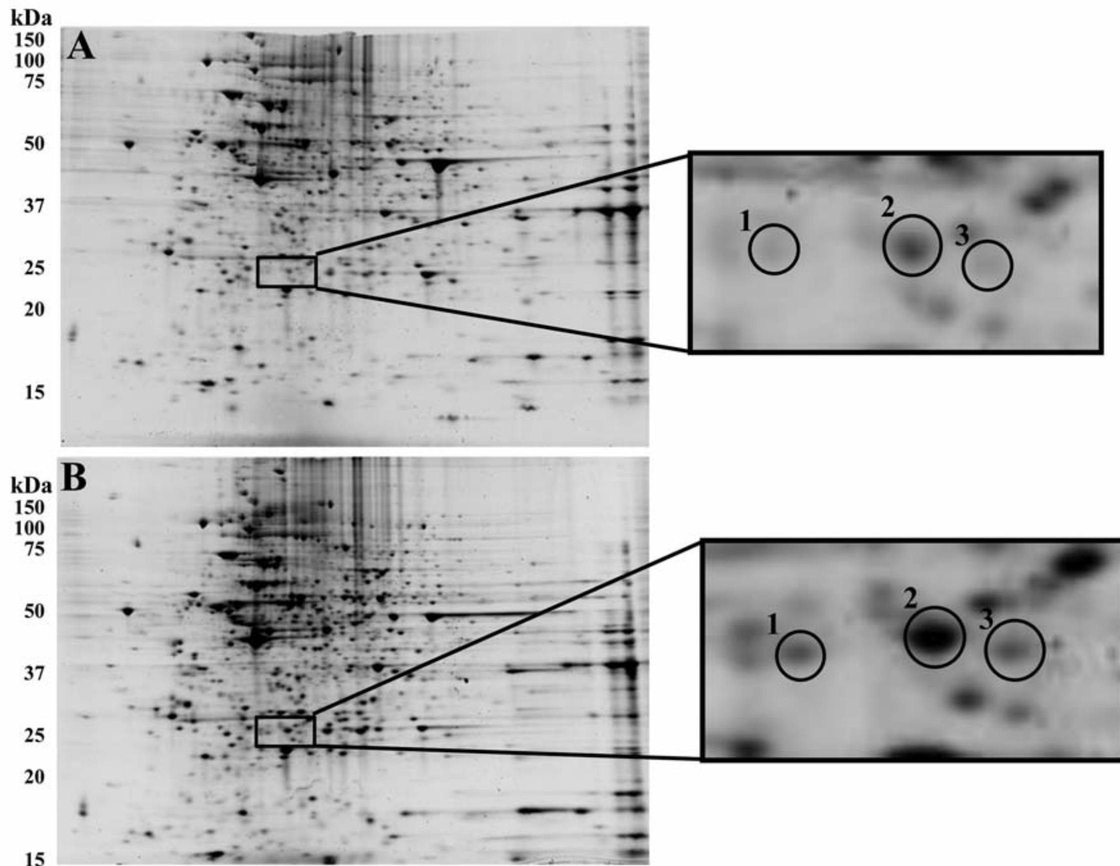

Figure 1. *2-DE patterns of KLM1 and KLM1-R cells when stained with Flamingo gel stain. Three protein spots of HSP27 isoforms whose expression levels were different between GEM-sensitive pancreatic cancer cell line KLM1 (A) and GEM-resistant pancreatic cancer cell line KLM1-R (B) are indicated by circles. Three protein spots (spot 1-3 in Figure 1B) appeared to be stronger in KLM1-R.*

endoplasmic reticulum, and it can prevent a wide variety of apoptotic agents from causing cell death by interacting with key components of the apoptotic signaling pathway (12). Previous studies reported that HSP27 inhibited doxorubicin, etoposide-, diethylmaleate-, cycloheximide- or radiation-induced apoptosis of cancer cells (13-15). HSP27 protein levels are elevated in gastric, liver, breast, prostate carcinomas and osteosarcoma, and overexpression of this protein correlates with a poor prognosis and response to therapy (16-18). Our previous proteomic studies also showed that HSP27 protein levels are elevated in GEM-resistant pancreatic cancer cell lines and the sensitivity to GEM in HSP27-knocked down cells was restored. Furthermore, we clarified that increased HSP27 expression in tumor specimens was related to higher resistibility to GEM in patients of pancreatic cancer (4, 5).

Phosphorylation of Ser15, Ser78, or Ser82 activates HSP27 (19, 20). Our Western blotting study showed that the phosphorylation level of Ser15 was extremely faint, but the phosphorylation level of Ser78 and Ser82 was elevated in GEM-resistant pancreatic cancer cells compared with GEM-sensitive pancreatic cancer cells. Furthermore, 2-D Western blotting study showed that at least three spots of phosphorylated HSP27 were detected. The level of the most acidic isoform (spot 1 in Figure 3 and 4) was found to be weaker than the other two spots. It is likely that three serine residues were phosphorylated in this isoform. Spot 2 may be the isoform whose two serine residues were phosphorylated, and spot 3 may be the isoform whose one serine residue was phosphorylated.

In conclusion, phosphorylated HSP27 may be involved in the mechanism of resistance to chemotherapy with GEM, and could also be indicators of response to pancreatic cancer therapy.

Acknowledgements

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (No. 20659091 to Yasuhiro Kuramitsu) and Grants-in-Aid from Japan Science and Technology Agency (No. 12-143 to Yasuhiro Kuramitsu).

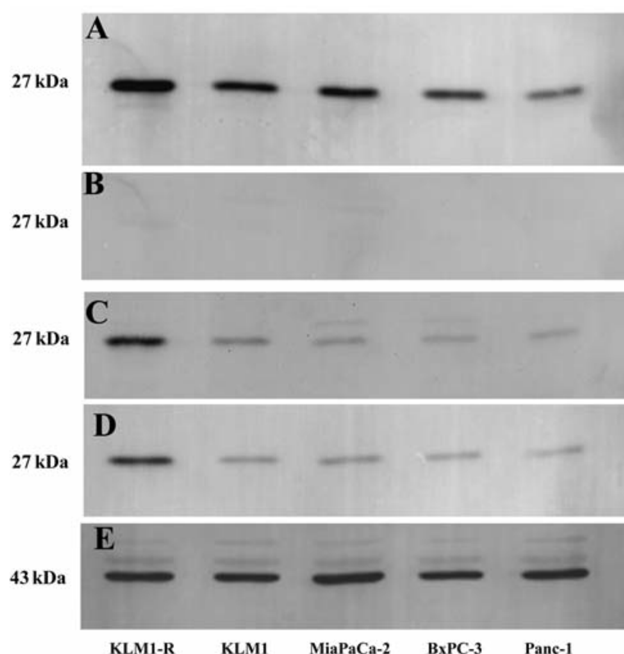

Figure 2. *Western blot analysis for total HSP27 and phosphorylated HSP27 (Ser15, Ser78 and Ser82) in KLM1-R, KLM1, MiaPaCa-2, BxPC-3 and Panc-1 cells. A: Total HSP27 expression level was up-regulated in KLM1-R cells compared with these in KLM1, MiaPaCa-2, BxPC-3 and Panc-1 cells. B: Phosphorylated HSP27 (Ser15) expression level was the same in all cell lines. C: Phosphorylated HSP27 (Ser78) expression level was up-regulated in KLM1-R cells compared with these in KLM1, MiaPaCa-2, BxPC-3 and Panc-1 cells. D: Phosphorylated HSP27 (Ser82) expression level was up-regulated in KLM1-R cells compared with these in KLM1, MiaPaCa-2, BxPC-3 and Panc-1 cells. E: Actin expression level was the same in all cell lines. Fifteen micrograms of protein were used.*

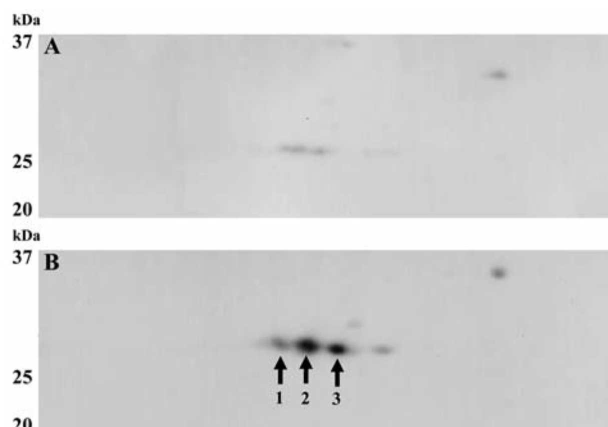

Figure 3. *2-D Western blot analysis for phosphorylated HSP27 (Ser78) in KLM1 (A) and KLM1-R (B) cells.*

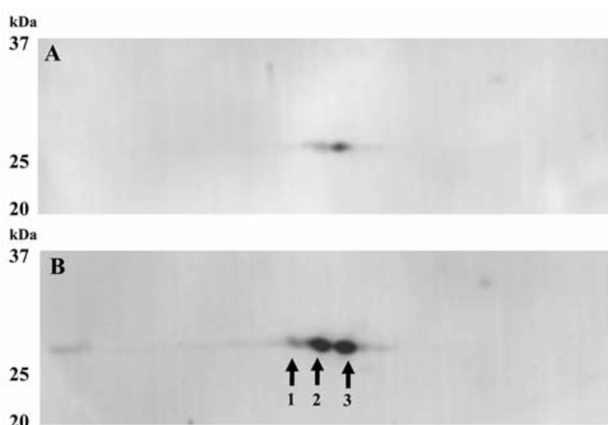

Figure 4. *2-D Western blot analysis for phosphorylated HSP27 (Ser82) in KLM1 (A) and KLM1-R (B) cells.*

References

1 Williamson RC: Pancreatic cancer: the greatest oncological challenge. Br Med J (Clin Res Ed) *13*: 445-446, 1988.

2 Gordis L and Gold EB: Epidemiology of pancreatic cancer. World J Surg *8*: 808-821, 1984.

3 Carmichael J, Fink U, Russell RC, Spittle MF, Harris AL, Spiessi G and Blatter J: Phase II study of gemcitabine in patients with advanced pancreatic cancer. Br J Cancer *73*: 1-5, 1996.

4 Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Mikuriya K, Fujimoto M, Maehara S, Maehara Y, Okita K, Nakamura K and Sakaida I: Proteomics finding heat-shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine. Int J Oncol *31*: 1345-1350, 2007.

5 Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Taba K, Fujimoto M, Okita K, Nakamura K and Sakaida I: A proteomic profiling of gemcitabine resistance in pancreatic cancer cell lines. Mol Med Rep *1*: 429-434, 2008.

6 Mori-Iwamoto S, Taba K, Kuramitsu Y, Ryozawa S, Tanaka T, Maehara S, Maehara Y, Okita K, Nakamura K and Sakaida I: Interferon-γ down-regulates HSP27 of pancreatic cancer cells, and helps the cytotoxic effect of gemcitabine. Pancreas *38*: 224-226, 2009.

7 Maehara S, Tanaka S, Shimada M, Shirabe K, Saito Y, Takahashi K and Maehara Y: Selenoprotein P, as a predictor for evaluating gemcitabine resistance in human pancreatic cancer cells. Int J Cancer *112*: 184-189, 2004.

8 Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis *29*: 2651-2659, 2008.

9 Kuramitsu Y, Miyamoto H, Tanaka T, Zhang X, Fujimoto M, Ueda K, Tanaka T, Hamano K and Nakamura K: Proteomic differential display analysis identified up-regulated astrocytic phsphoprotein PEA-15 in human malignant pleural mesothelioma cell lines. Proteomics *9*: 5078-5089, 2009.

10 Kuramitsu Y, Harada T, Takashima M, Yokoyama Y, Hidaka I, Iizuka N, Toda T, Fujimoto M, Zhang X, Sakaida I, Okita K, Oka M and Nakamura K: Increased expression and phosphorylation of liver glutamine synthetase in well-differentiated hepatocellular carcinoma tissues of patients infected with hepatitis C virus. Electrophoresis *27*: 1651-1658, 2006.
11 Tamesa M, Kuramitsu Y, Fujimoto M, Maeda N, Nagashima Y, Tanaka T, Yamamoto S, Oka M and Nakamura K: Detection of autoantibodies against cyclophilin A and triosephosphate isomerase in sera from breast cancer patients by proteomic analysis. Electrophoresis *30*: 2168-2181, 2009.
12 Arrigo AP: Hsp27: novel regulator of intracellular redox state. IUBMB Life *52*: 303-307, 2001.
13 Hansen RK, Parra I, Lemieux P, Oesterreich S, Hilsenbeck SG and Fuqua SA: Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. Breast Cancer Res Treat *56*: 187-196, 1999.
14 Gibbons NB, Watson RW, Coffey RN, Brady HP and Fitzpatrick JM: Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. Prostate *45*: 58-65, 2000.
15 Urbani A, Poland J, Bernardini S, Bellincampi L, Biroccio A, Schnölzer M, Sinha P and Federici G: A proteomic investigation into etoposide chemo-resistance of neuroblastoma cell lines. Proteomics *5*: 796-804, 2005.
16 Ciocca DR, Oesterreich S, Chamness GC, McGuire WL and Fuqua SA: Biological and clinical implications of heat-shock protein 27,000 (Hsp27): a review. J Natl Cancer Inst *85*: 1558-1570, 1993.
17 Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke Y and Foster CS: Heat-shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res *60*: 7099-7105, 2000.
18 Romanucci M, Marinelli A, Sarli G and Della Salda L: Heat-shock protein expression in canine malignant mammary tumours. BMC Cancer *6*: 171, 2006.
19 Landry J, Lambert H, Zhou M, Lavoie JN, Hickey E, Weber LA and Anderson CW: Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. J Biol Chem *267*: 794-803, 1992.
20 Döppler H, Storz P, Li J, Comb MJ and Toker A: A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. J Biol Chem *280*: 15013-15019, 2005.

Received March 26, 2010
Revised June 11, 2010
Accepted June 16, 2010