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*Original article*

# Immunohistochemical evaluation of expression of heat shock proteins HSP70 and HSP90 in mammary gland neoplasms in bitches

A.M. Badowska-Kozakiewicz<sup>1</sup>, E. Malicka<sup>2</sup>

<sup>1</sup> Department of Biophysics and Human Physiology, Medical University of Warsaw, Zwirki i Wigury 61, 02-091 Warsaw, Poland

<sup>2</sup> Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159c, 02-766 Warsaw, Poland

## Abstract

Heat shock proteins have essential roles in a number of pathophysiologic conditions including carcinogenesis and represent a group of novel molecular markers in cancer management.

The aim of this study was to investigate heat shock protein expression in correlation with other neoplasm traits such as: histological type, differentiation grade, proliferative activity, estrogenic receptor expression, and cyclooxygenase-2 and p53 proteins. Material for the investigation comprised 133 tumors of the mammary gland collected from bitches. In total 14 adenomas, 66 complex carcinomas, 47 simple carcinomas and 6 solid carcinomas were collected. Evaluations were conducted with histopathological and immunohistochemical methods using suitable antibodies. Expression of heat shock protein 70 was observed in all types of evaluated neoplasms. A higher average number of cells undergoing expression of heat shock protein 70, which was statistically insignificant, was established in complex and simple cancers and in cancers with the 1<sup>st</sup> and the 2<sup>nd</sup> degree of histological malignancy. Expression of heat shock protein 90 was observed in all studied neoplasms; it was very insignificant in adenomas, compared to cancers, and the highest expression was established in the solid cancers, as well as in cancers with the 2<sup>nd</sup> degree of histological malignancy. This high expression of heat shock protein 90 was correlated with proliferative activity. The results suggest that heat shock protein 90 is involved in canine mammary gland carcinogenesis. The results also suggest that heat shock protein 90 may be a prognostic factor, but this requires detailed clinical confirmation.

**Key words:** heat shock proteins (HSP70, HSP90), neoplasms, mammary gland, dogs

## Introduction

Mammary gland tumors are the most frequently diagnosed neoplasms in female dogs. Roughly 80% of canine mammary tumors are diagnosed in bitches older than 7 years. About 30% of the cases are malignant mammary carcinoma. The major risk factors associated with canine mammary tumors include: age of the animal, sentinel lymph node status, histological type of tumor and the differentiation grade of histological malignancy (Welch 1992). Heat shock proteins were first discovered as a cohort of proteins that are powerfully induced by heat shock and other chemical and physical stress in a wide range of species (Georgopolis and Welch 1993, Lindquist and Craig 1999). Heat shock proteins have been subsequently characterized as molecular chaperones, proteins which have in common the property of modifying the structures and interactions of other proteins (Beckmann 1990). Heat shock proteins, also known as "stress proteins", are a large class of proteins that have been highly conserved throughout evolution and are expressed by prokaryota and eukaryota organisms. Heat shock proteins can be classified according to their molecular weight, expressed in kDa: HSP15-30, HSP40, HSP60, HSP70, HSP90 and HSP100. The level of heat shock proteins molecular chaperones is elevated in many cancers, and heat shock protein overexpression signals a poor prognosis in terms of survival and response to therapy in specific cancer types (Ciocca et al. 1992, Ciocca et al. 1993, Ciocca et al. 1998). Elevated heat shock proteins expression in malignant cells plays a key role in protection from spontaneous apoptosis associated with malignancy, as well as the apoptosis generated by therapy, mechanisms which may underline the role of heat shock proteins in tumor progression and resistance to treatment (Ciocca et al. 1993, Gyrd-Hansen et al. 2004). In human oncology, Hsp27 expression was found in colon cancer (Tsuruta et al. 2008), and urinary bladder carcinomas (He et al. 2005, Hadaschik et al. 2008, Kamada et al. 2007). Hsp 70 expression was found in human gastric carcinoma (Xiang et al. 2008). Hsp70 and Hsp90 expression were found in human breast carcinoma cells (Havik and Bramham 2007). In dogs, the expression of heat shock proteins was found in squamous cell carcinoma of the skin (Romanucci et al. 2005, Bongiovanni et al. 2008), cervical cancer (Chu et al. 2001) and mammary gland tumors (Kumaraguruparan et al. 2006, Romanucci et al. 2006). Romanucci (2006) demonstrated the expression of Hsp90 in all examined tumors (simple and complex carcinomas) and found that these proteins are associated with poor outcome and are involved in carcinogenesis in the same way as heat shock protein 27.

Available published data (Chen et al. 1999) show that in breast cancer cells, the overexpression of heat shock protein 70 is also associated with poor prognosis. The mechanisms of the heat shock protein protective effect in apoptosis are not fully recognized, but by using the high immunogenicity of these proteins, scientists are working on their application in vaccine production, which may in future play an important role in the field of transplantology, in the prevention and treatment of autoimmune diseases, infectious diseases and cancer.

The aim of the study was to investigate heat shock protein expression in correlation with other neoplasm traits such as: histological type, differentiation grade, proliferative activity, estrogenic receptor expression, and cyclooxygenase-2 and p53 proteins.

## Materials and Methods

Material for this investigation comprised 133 tumours of the mammary gland collected from bitches during surgical procedures performed in Warsaw veterinary clinics and the Small Animal Clinic of the Department of Clinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW. Tumour samples were fixed in 8% formalin buffered with phosphates. After 24h fixing, material was dehydrated in a number of alcohols of increasing concentrations and embedded in paraffin. Paraffin blocks were cut into serial sections of 4 µm in thickness. They were then stained using standard methods. In the sections stained with the routine H&E method, the following determinations were carried out: type of neoplasm (WHO classification, Misdorp 1999), tumour grade including tubule formation, intensity of division and degree of neoplastic cell differentiation (Misdorp and Meuten 2002), and mitotic index as a mean number of mitoses in neoplastic cells counted in 10 fields of vision at an objective magnification 400x (surface field 0.17 mm<sup>2</sup>). Paraffin sections on the slides covered with 2% saline solution in acetone at temperature of 42°C were used in immunohistochemical methods. In the immunohistochemical reactions, the following antibodies, properly diluted in 1% BSA (Sigma), were used: mouse monoclonal against human nuclear antigen Ki-67 (Dako) diluted 1:75 (Nieto et al. 2005), mouse monoclonal antibodies against p53 (Dako) human protein, diluted in 1:25 proportion (Gamblin et al. 1997, Rodo 2007), mouse monoclonal antibodies against alpha (Dako) human estrogenic receptor, diluted 1:35 (Mulas et al. 2005), mouse monoclonal antibodies against cyclooxygenase -2 (Dako) human receptor, diluted 1:100 (Soslow et al. 2000, Doré et al. 2003, Queiroga et al. 2007), and mouse

monoclonal antibodies against heat shock protein 70 and heat shock protein 90 (Novocastra) human heat shock proteins, diluted 1:40 (Romanucci et al. 2006). Sections were deparafinized in xylene and dehydrated in graded alcohols. Then they were placed in a buffer of pH 6 (Dako). In order to uncover the antigen epitope, sections were warmed in a microwave oven (1x5 min at 600 W, 2x5 min at 300 W). They were then cooled for 20 min. After two washings in distilled water and 5 min incubations they were then washed in TRIS buffered saline pH 8 (Sigma) and incubated with the primary antibody for 1h at room temperature. The preparations were then washed in TRIS buffer for 10 min. The En Vision + <sup>TM</sup>system (Dako) was used for visualization. After 30 min of incubation with reagent, the slides were washed in TRIS buffer and a solution of diaminobenzidine (DAB, Dako) prepared according to the procedure supplied by the producer. The degree of slide staining was checked, they were then washed in drinking water and stained with Ehrlich hematoxylin for 5 min, contrasted in 1% acid alcohol and washed again in drinking water. The slides were then dehydrated through graded alcohols, passed through xylene and closed in DPX mounting medium (Gurr<sup>®</sup>). Computer image analysis and Lucia v. 4.21 application were used for interpretation of the results of heat shock protein 70 and heat shock protein 90 expression; using these facilities the number of neoplastic cells featuring stained cytoplasm per 1000 neoplastic cells were counted. The results were analyzed using the statistical pack SPSS v. 12.0 program. To determine the significance of differences for a few independent traits the Kruskal-Wallis test was used. This test is an equivalent to the test of variance for traits with no normal distribution. Two sided correlations were performed using the Spearman correlation test. The differences were deemed statistically significant at  $P \leq 0.05$ .

## Results

The investigated material consisted of 14 adenomas, 66 complex carcinomas (adenocarcinomas), 47 simple carcinomas (adenocarcinomas) and 6 solid carcinomas (Table 1). The number of cancers with a defined grade amounted, respectively, to: 1<sup>st</sup> – 48, 2<sup>nd</sup> – 39 and 3<sup>rd</sup> – 32 (Table 2). The mammary gland neoplasms originated from bitches belonging to 18 breeds aged between 9 and 12 years. Heat shock proteins were found in the cytoplasm and nuclei of cancer cells. The largest group of tumors exhibiting heat shock protein 70 and heat shock protein 90 expression included simple and complex cancers whereas solid tumors were the least numerous group. Grades 1 and

2 of histological malignancy cancers constituted the biggest group for both heat shock protein 70 and heat shock protein 90. Immunohistochemical analysis

Table 1. Number of tumors showing positive expression of heat shock protein 70 and heat shock protein 90 depending on the type of tumor.

Type of tumor	Heat shock protein 70	Heat shock protein 90
Adenoma (14)	12 (10.4%)	11 (12.5%)
Solid carcinoma (6)	6 (5.2%)	3 (3.4%)
Simple carcinoma (47)	43 (37.4%)	34 (38.6%)
Complex carcinoma (66)	54 (47.0%)	40 (45.5%)

Table 2. Number of tumors showing positive expression of heat shock protein 70 and heat shock protein 90 depending on tumor grade.

Tumor grade	Heat shock protein 70	Heat shock protein 90
I <sup>o</sup> (48)	39 (37.9%)	32 (41.5%)
II <sup>o</sup> (39)	33 (32.0%)	23 (29.9%)
III <sup>o</sup> (32)	31 (30.1%)	22 (28.6%)

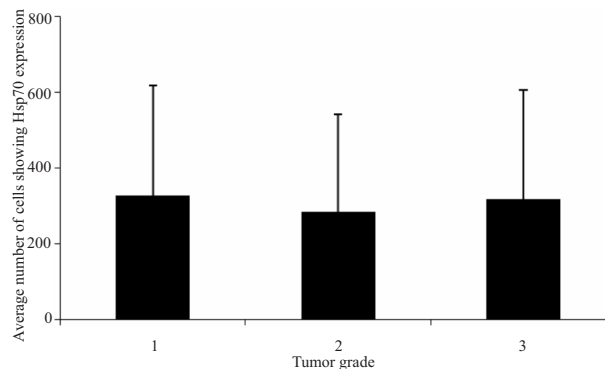


Fig. 1. Average number of cells showing Hsp70 expression depending on tumor grade. Data are presented as means SEM.

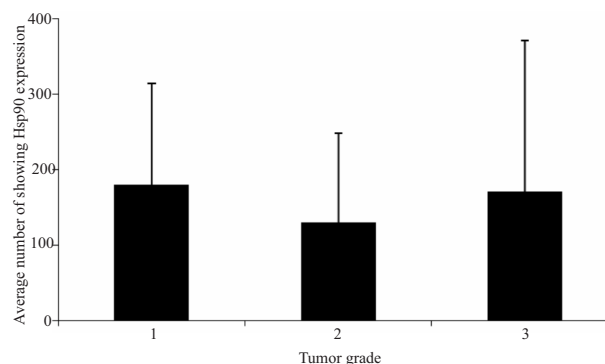


Fig. 2. Average number of cells showing Hsp90 expression depending on tumor grade. Data are presented as means SEM.

showed a high expression of heat shock protein 90 in simple and complex cancers, but no significant difference was found between the investigated types of tumors,  $P = 0.443$ . High expression of heat shock protein 90 was confirmed in solid cancers and in this particular group a significant difference was found between tumors,  $P = 0.032$ . As far as grading was concerned, no statistically significant difference was found between the mean number of cells showing heat shock protein 70 and heat shock protein 90 protein expression and the grade (Fig. 1, Fig. 2). When comparing the expression of heat shock protein 70 to the expression of heat shock protein 90 in particular types of cancer, we found a highly significant statistical difference,  $P = 0.005$  between the expression of both proteins. The results of this study of the expression of heat shock proteins (HSP70 and HSP90) were compared to nuclear antigen Ki-67 expression. Based on this comparison it was found that the expression of nuclear antigen Ki-67 was most pronounced in solid tumors, as was also the expression of heat shock protein 90. Also, in neoplasms of the highest grade, the expression of nuclear antigen Ki-67 and protein heat shock protein 90 was at the highest level (Fig. 3). A high expression of heat shock protein 70 was found in tumors with grades 1 and 3 of cyclooxygenase-2 expression, whereas the lowest heat shock protein 70 expression was observed in tumors of grade 2 of cyclooxygenase-2 expression. Statistical significance was found between the investigated features of neoplasms,  $P = 0.009$ . Increased expression of cyclooxygenase-2 was observed in tumors with a low mean number of cells showing a positive immunohistochemical reaction of protein heat shock protein 90. The highest level of expression of this protein was confirmed in grade 1 tumors of cyclooxygenase-2 expression (Fig. 4). Between the mean number of cells showing positive heat shock protein 90 reaction and the level of cyclooxygenase-2 expression, there was a statistical

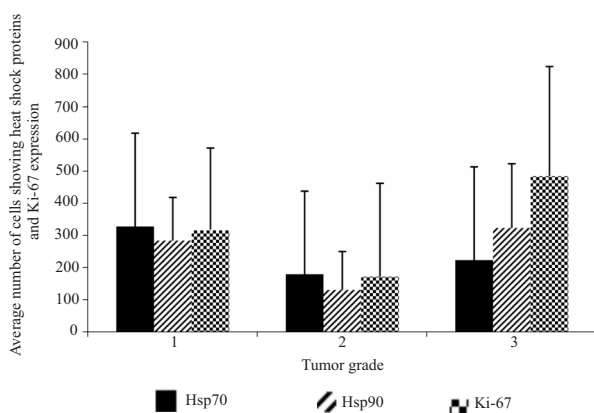


Fig. 3. Average number of cells showing heat shock proteins expression and Ki-67 nuclear antigen expression depending on tumor grade. Data are presented as means SEM.

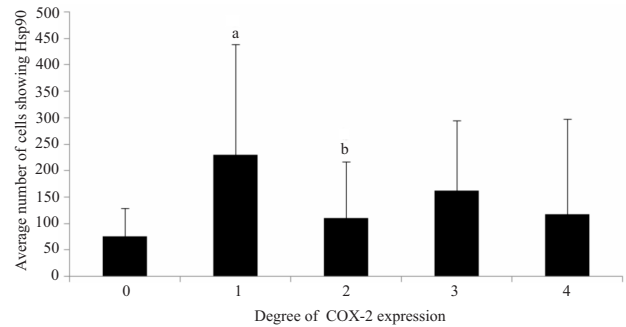


Fig. 4. Average number of cells showing heat shock protein 90 expression depending on the degree of cyclooxygenase-2 expression. Data are presented as means SEM. Various letters (a, b) above the columns show that the difference between means was statistically significant ( $p \leq 0.005$ ).

significance observed for grades 1 and 2 of cyclooxygenase-2 expression,  $P = 0.039$ . A statistical analysis found a correlation between the expression of protein heat shock protein 70 and expression of p53 in tumors of epithelial origin. A high significance was shown between the investigated neoplastic features,  $P = 0.002$ . Taking into account the type of tumor, the expression of both proteins was highest in complex carcinoma tumors with the lowest histological grade. High expression level of heat shock protein 90 protein and Ki-67 nuclear antigen was shown in the case of solid carcinomas and in carcinomas featuring the 3<sup>rd</sup> histological malignancy degree, which are described by low apoptotic index.

## Discussion

According to studies in humans, heat shock proteins may be an important predictor of breast cancer (Morino et al. 1997). There is little published literature data on heat shock protein expression in mammary gland tumors of bitches. Nor has the role of these proteins in carcinogenesis been clearly defined. Studies have shown only that there is expression of heat shock proteins in mammary gland tumors in bitches, but correlation between these proteins and other tumor markers was not confirmed. Seymour et al. (1990) studied endometrial cancer in women and found that heat shock proteins were a useful marker in the diagnosis of these tumors. The expression of heat shock protein 27 was also investigated in breast cancer in women (Ciocca et al. 1990). A relationship between the expression of this protein and the degree of differentiation of the tumor cells was found. Similar studies were conducted by Storm et al. (1996) who stated that tumors with heat shock protein 27 expression showed a higher histological grade than tumors with negative expression of this protein.

Kumaraguruparan et al. (2006) studied the expression of heat shock protein 70 and heat shock protein 90 in breast cancer in women and found a correlation between the expression of both proteins and proliferative activity. He suggested that the heat shock proteins are an important prognostic factor. The presence of heat shock protein 70 was also reported in normal gastrointestinal tissues, but an increased expression of this protein was observed by Isomoto et al. (2003) in gastrointestinal tumors. It is believed that heat shock protein 70 plays an important role in the degree of cellular differentiation in tumors, which would indicate that heat shock 70 is an important prognostic factor. Similar studies have been conducted in veterinary medicine, but to a lesser extent. In dogs, the expression of heat shock proteins was studied in normal skin and squamous cell carcinoma of the skin (Romanucci et al. 2005), as well as in canine transmissible venereal tumor (CTVT) (Beckmann et al. 1990). A similar study was conducted by Romanucci et al. (2006) in mammary gland tumors in bitches. This study found heat shock protein 70 and heat shock protein 90 expressed in simple and complex, as well as solid-type adenocarcinomas. Expression of these proteins was observed in the cytoplasm, but also in the nuclei of tumor cells. Romanucci et al. (2006) did not attempt to verify the possible correlations between heat shock proteins and other tumor markers. The focus was on establishing the degree of protein expression and their location in tumor cells. Based on the study results, it was suggested that these proteins may play an important role in the process of carcinogenesis. In our study, heat shock protein 70 expression was found in 86.4% of tumors, while the expression of heat shock protein 90 was observed in 66.2% of tumors. The aim of our study was to demonstrate the relationship between the expression of heat shock proteins and other prognostic factors. When comparing the expression of heat shock protein 70 to the expression of heat shock protein 90 in different types of tumors, we found a high statistical significance between the expression of both proteins. The analysis of the relationship between the expression of nuclear antigen Ki-67 and the expression of heat shock proteins showed that the highest expression of Ki-67 as well as heat shock protein 90 was present in solid tumors. The highest expression of heat shock protein 90 and Ki-67 was also found in cancers of the highest histological grade. Based on these data it can be concluded that heat shock protein 90 is an important factor which could be considered a marker of malignancy and which may be useful in the diagnosis of cancers. Analysis of the results showed a high expression of heat shock protein 70 in cancers with grade 1 and 3 of cyclooxygenase-2 expression; the lowest expression of heat shock protein 70 was found

in cancers with grade 2 of cyclooxygenase-2 expression. A statistical significance of  $P = 0.009$  was found between the expression of heat shock protein 70 and expression of cyclooxygenase-2. An increased expression of cyclooxygenase-2 was observed in tumors with a low mean number of cells showing positive reaction of heat shock protein 90. Correlation between the expression of heat shock protein 70 and p53 protein in tumors of epithelial origin was also confirmed. Based on the analysis of expression of both proteins, and taking into account the mean apoptotic index, it was found that in cancers with the third (the highest) histological grade, the expression of p53 protein was significantly lower than the expression of heat shock protein 70, and the value of apoptotic index in these cancers was the lowest.

## Conclusions

The expression of heat shock protein 90 can be considered a marker of the degree of differentiation and of histological grade of mammary gland tumors in bitches, since the highest level of expression was found in solid carcinomas and in cancers featuring the highest histological malignancy degrees. Heat shock protein 70 expression was confirmed, but no correlation with other factors was found. This may suggest that heat shock protein 70 is not a useful marker in the diagnosis of breast cancers in female dogs.

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