

Overexpression of Heat Shock Protein 90 α in Canine Mammary Tumors.

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Abstract

Canine mammary gland tumor is the most patronize and dangerous type of tumor diagnosed by histopathology and requires a surgical approach. Hsp90 α , a member of the heat shock protein-90 family that found to be over-expressed in breast cancer. However, biological function is unknown in the canine mammary tumor. So, we focused on studying the expression of *hsp90AA1* and protein in canine mammary tumor (CMT) tissue. Expression of *hsp90AA1* mRNA and protein were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC), respectively. High levels of *hsp90AA1* mRNA expression (4.613 ± 0.49 times) were observed in CMT compared to age and breed-matched healthy controls. Further, expression of hsp90 α protein was detected in paraffin-embedded mammary tumor tissues from the same subjects by IHC. A high level of hsp90 α expression was scored in 75% of the CMT subjects. Moreover, no significant differences in IHC score and qRT-PCR score with respect to CMT histotypes were observed, indicating that *Hsp90AA1* over-expression occurred irrespective of CMT types or grades.

Introduction

Geriatric and unspayed bitches have the most common, dangerous, and frequently diagnosed neoplasm, i.e., mammary tumor. It is also life-threatening to bitches due to distant metastasis development affecting other organs of the body (Kumar et al. 2018; Misdorp 2002). The incidence of mammary neoplasia in female dogs (bitches) is approximately three times more than that in women, followed by cats and other pet animals (Birdi et al. 2019; Kumar et al. 2018). Early diagnosis of neoplastic growth at an early stage saved the life of a female dog. However, in later stages, once the tumor gets metastasized to other organs, it shows overlapping signs and symptoms with other diseases (Kumar et al. 2018; Pandey et al. 2018). Multiple reports suggested that the instigation of mammary tumors leads to the overexpression of various proteins.

These proteins get into the circulation, are quantified, and are further used to diagnose the malady (Kumar et al. 2018). Therefore, heat shock proteins, which play a pivotal role in moderating cellular stress, represent a group of molecular markers of cancer (Kumar et al. 2018; Pandey et al. 2018).

Heat shock proteins (Hsps) are members of the molecular chaperone family that play essential roles in folding a large number of cellular proteins apart from acting as mediators of resistance to hyperthermia in all cellular organisms (Calderwood 2010). They also play an important part in cell proliferation and drug resistance, making them proteins of special clinical interest. Elevated levels of Hsps are seen in a wide spectrum of malignant tumors, including mammary carcinoma and some members viz. Hsp27, Hsp70, and Hsp90 are thought to play roles in breast cancer (Wang et al. 2009; Liu et al. 2019).

Hsp90 is one of the most abundant proteins in mammalian cells and has four major isoforms: Hsp90A (cytosolic inducible form), Hsp90B1 constitutive form (endoplasmic reticulum/ER form or endoplasmin/Grp94), and Trap (mitochondrial form). The cytosolic form can again be sub-classified into

Hsp90AA (Hsp90 α) and Hsp90AB (Hsp90 β) (Kumar et al. 2018) isoforms. The aim of research on mammary neoplasms in female dogs should be to extend the panel of tumor markers by adding new ones that could help in diagnostics and therapy. One such marker is a subtype of HSP90, Hsp90 α encoded by *hsp90AA1*, which is induced by stress. *Hsp90AA1* is overexpressed in different types of human cancers (Wang et al. 2009). There are no data on *hsp90AA1* expression in canine mammary tumors.

Methods

Ethical Permission and Sample sources

The Institutional Animal Ethics Committee (IAEC), GADVASU approved the collection of tissue samples via memo no. GADVASU/2020/IAEC/53/17; dated 25/01/2020.

In the present study, all the mammary gland tumor samples were collected from unsprayed female dogs (n=20) of the 6-12 years old age group with a median of 8.5 years during surgeries performed in the Department of Veterinary Surgery and Radiology, GADVASU, Ludhiana, India. Healthy canine mammary tissues were also used from age and breed-matched dogs (n=20). After removing tumors and post-operative care, the animals were discharged from the clinics and allowed to go with their owners. No treatment of any type is given to dogs prior to surgery.

For total RNA extraction, tissue samples were collected in RNA/aterTM solution and stored at -20°C. Similarly, tissue samples were collected in 10% neutral buffered formalin for histopathological and immunohistochemical analyses.

Histopathology

H&E staining of tissue paraffin sections (5 μ m thick) was done using the HE stain kit (Vector laboratories). Tissue sections were also sliced and mounted over clean poly-L lysine (PLL) coated glass slides for IHC. According to WHO standards, the classification and grading of the tumors were carried out by pathologists unknowing to subject history (Goldschmidt et al. 2011).

RNA isolation and cDNA synthesis

Extraction of RNA from mammary tissues were done using TRIzolTM reagent (Life Technologies, USA). About 1 μ g of the isolated RNA was used for cDNA synthesis using oligo dT primers and Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, USA). After that, the synthesized cDNA was confirmed using specific β -actin primers.

Real-Time PCR (qRT-PCR)

Relative expression of *HSP90AA1* mRNA in tumorous and healthy canine mammary tissues was assessed by SYBR green-based qRT-PCR. The specific primers targeting the *HSP90AA1* gene (ACCCTGACGACATCACCAAC and GGGATCAGCTCTTCGCAGTT), along with endogenous housekeeping

control genes, *RPS-19* (CCTTCCTCAAAAAGTCTGGG and GTTTCATCGTAGGGAGCAAG) (Kumar et al. 2018) and *β-actin* (CCGCGAGAAGATGACCCAGA and GTGAGGATCTTCATGAGGTAGTCGG) (Timmermans-Sprang et al. 2015) and 2x SYBR Green qPCR Mix (Real Gene, US) was used for the expression profiling. The efficiency of these housekeeping genes had previously been tested in cancer research (Kumar et al. 2018). Primers of all three genes were used at 0.25μM final concentration. Annealing and extension of all the three genes were carried out at 53°C, and 72°C and dissociation curves were generated between 65°C to 95°C to assess the specificity of the amplicons.

The percent efficiencies of the PCR amplification for each gene were calculated as $E = (10^{-1/\text{slope}-1}) \times 100$, where the slope was calculated for the semi-log regression curve plotted between log cDNA (serially diluted cDNA samples) versus their threshold cycle (Ct) values (Kumar et al. 2018). For evaluating the fold change in *HSP90AA1* mRNA expression between canine mammary tumors and healthy mammary tissues, the Ct values of the *HSP90AA1* gene and the geometric mean of the Ct values of *RPS-19* and *β actin* after 40 cycles of amplification were utilized (Kumar et al. 2018; Pandey et al. 2018). Non-template control was kept to check for non-specific amplification.

The statistical analyses were made according to Livak and Schmittgen (2001) using SAS version 9.3. Further, the qPCR score ($2^{-\delta Ct}$) was also calculated for each sample to analyze the relationship between HSP90α expression at mRNA and protein level.

Immunohistochemistry (IHC)

IHC was performed as standardized earlier in the lab (Pandey et al. 2018) to assess the expression of Hsp90α in various CMT histotypes and previously mounted tissues on slides coated with poly-L lysine.

Scoring of HSP90α positive cells

Semi-quantitative scoring of Hsp90α positive cells was done by a pathologist without considering the history of the patients (Rizzardi et al. 2012). This method considered the intensity of developed brown color and the percentage of the cells showing positive staining (Pandey et al. 2018). H or SI score (staining index), and percentage of the positive cells were calculated according to Pandey et al (2018).

Statistical Analysis

Statistical analyses were checked using Statistical analysis software (SAS ver. 9.3). Pearson correlation coefficient (r) was calculated between H-score and $2^{-\delta Ct}$ (qRT-PCR score) to determine any association between them. The q-scores and H-scores were also correlated using Spearman's rank correlation. Statistical association of tumor histotypes with tumor grades, qRT-PCR score, and H score was assessed using the Kruskal Wallis test.

Results

Histopathological analysis of CMT of the 20 tumor samples, 7 were classified as complex carcinoma, three each were of anaplastic carcinoma, papillary carcinoma, and early carcinoma, respectively, two of

mixed tumors and one of hemangiosarcoma and mast cell tumor. About 45% (n=9) were designated as grade II (moderately differentiated), 35% of tumors (n=7) were found to be of grade I (well-differentiated), and only 20% of tumors (n=4) belonged to grade III (poorly differentiated).

Expression analysis of HSP90AA1 mRNA using Real-Time PCR

In the present study, *HSP90AA1*, *RPS19*, and β -*actin* genes had amplification efficiencies of 89.15%, 94.24%, and 95.65%, respectively, suggesting that the exponential efficiencies of the *HSP90AA1* gene along with the internal controls were suitable for real-time PCR. *HSP90AA1* gene was found to be 4.613 ± 0.49 times significantly ($P \leq 0.05$) overexpressed in CMT as compared to the healthy tissue glands.

Immunohistochemical detection of HSP90 α expression in canine mammary neoplasia

The IgG purified from the hyperimmune sera of rabbit distinctly reacted with native HSP90 α isolated from the tumor tissue homogenate upon Western blotting. A distinct band corresponding to 64 kDa was spotted on the nitrocellulose membrane (Fig. 1), which confirmed that the purified IgG specifically reacted with the native cellular HSP90 α (Bhardwaj 2022). Upon IHC, the HSP90 α immunopositive cells were stained brown. The negative controls, in which unimmunized rabbit serum was used, did not show any immunostaining with the raised HSP90 α antibodies. Immunoreactivity against cellular HSP90 α was judged based on the pathologist's staining index (SI). It has been reported that in the current study, a mild cytoplasmic expression of Hsp90 α (SI<6) was detected in the majority of healthy canine mammary tissue (95%). In contrast, a varied Hsp90 α expression was evident in most CMT histotypes under study. 75% of CMT tissues showed a strong cytoplasmic immunoreactivity (SI ≥ 6) for Hsp90 α (Fig. 2a-2h), which correlated with the aggressiveness of CMT. Weak immunostaining (SI<6) against Hsp90 α was detected in two sections of papillary carcinoma and one section in hemangiosarcoma. At the same time, a strong immuno-staining (SI ≥ 6) was seen in one section of mixed mammary tumors, mast cell tumors, and three sections of anaplastic carcinomas. 100% sections of complex carcinomas showed strong immunoreactivity against the protein. Moreover, a strong Hsp90 α protein expression was detected in 33.33% of grade-1, 80% of grade-2, and 100% of grade-3 CMT.

Relationship between HSPD1 expression at mRNA and protein level in various types of CMT

The statistical analysis between the IHC score (H-Score) and qRT-PCR score revealed that a positive and high ($P<0.005$) correlation between *HSP90AA1* transcript and protein expression with a Pearson Correlation coefficient of 0.6289, as evident by the Scatter plot (Fig. 3). Also, a spearman's rank correlation of 0.69702 ($P<0.005$) was found between H-score and qPCR score, indicating that rankings for *HSP90AA1* mRNA and Hsp90 α protein expression were highly similar among various CMT samples. Though, the Kruskal Wallis test revealed no significant differences in the H scores with respect to

different CMT histotypes indicating that *Hsp90AA1* was overexpressed in CMT irrespective of the histotypes.

Discussion

Canine mammary tumors (CMTs) are a dangerous condition in the modern era and are becoming more prevalent globally. Nevertheless, with the advent of advanced technology, it is possible to diagnose most of these tumors at an early stage (Pandey et al. 2018). Diagnosis of mammary tumors at an early stage is as important as in later stages, and they may metastasize. Diagnosis, if done via histopathology, involves invasive techniques of tissue collection. Milder steps like fine needle aspirate (FNA) are less invasive but less accurate also. As it is difficult to determine the biological behavior of mammary tumors by microscopic examination, markers are important tools in its diagnosis. With the advancement of research in cancer, various biomarkers have been successfully discovered for a different type of human neoplasia. Heat shock proteins are one of the potential biomarkers in human carcinogenesis (Kumar et al. 2018). HSP90 α is a heat shock protein that also plays an indispensable role in immune responses and apoptosis.

Several reports advocate that HSP90 α is upregulated in the different types of human cancer like breast cancer, liver, head, and neck cancer. Given the tight relationship between CMT and human breast cancer, we decided to investigate HSP90 α expression in canine mammary cancers. Like our present study, many reports also observed that complex carcinoma, among all other canine mammary tumors, is the most persistent (Birdi et al. 2019; Kumar et al. 2018, Lopes-Neto et al. 2017; Mulligan 1975; Mitchell et al. 1974; Pandey et al. 2018).

Likewise, overexpression of the *HSP90AA1* gene has been found in human breast cancer patients, related to poor prognosis and less survivability. (Klimczak et al. 2019). Yano M et al (1999) investigated the expression level of hsp90 α , hsp90 β , and cyclin D1 in human breast cancer. Breast cancer tissues were found to have significantly higher levels of mRNAs coding for hsp90 α and cyclin D1 than non-cancer tissues. Previous studies have revealed over-expression of hsp90 α also found in the different types of tumor-like in pancreatic carcinoma cells (Gress et al. 1994; Yano et al. 1999) and leukemic cells (Yano et al. 1999; Yufu et al. 1992). Detection of Hsp90 α has been considered a poor prognostic marker of human breast cancer (Jameel et al. 1992; Yano et al. 1999). Also, one recent finding showed increased hsp90 α in head and neck cancer (Fan et al. 2020). Our outcomes were consistent with these results, and we also reported overexpressed hsp90 α in canine mammary tumors.

Different types of HSPs were also upregulated during the malignant alteration of mammary glands in pets. A recent study showed that Cytoplasmic Hsp90 protein level was significantly ($p < 0.0001$) higher in ductal carcinoma in situ and aggressive breast carcinomas than normal breast tissue. (Diehl et al. 2009). It has been reported that with a higher stage of mammary gland tissue malignancies, HSP90 expression has been correlated in dogs. The expression was highest in solid and complex carcinomas (Badowska-Kozakiewicz and Malicka 2012). Immunohistochemistry has also revealed that this isoform of HSP90 is

overexpressed in distinct histotypes of canine mammary cancers. Grp94 was overexpressed in all forms of canine mammary cancers examined, regardless of histological types and grades (Kumar et al. 2018), which was similar to our present study in which Hsp90 α , another isoform of heat shock protein, overexpressed in CMT histotypes. We are first to report an elevated level of Hsp90 α expression in various histotypes of canine mammary tumors. Hence, adding Hsp90 α to the current panel of CMT biomarkers will certainly improve the sensitivity and specificity of CMT diagnosis. However, owing to the lesser sample size, the data need to be confirmed in a study employing a large number and types of CMT in the future.

Declarations

Authors' contribution statement:

Himalaya Bhardwaj: Investigation, Writing- Original draft preparation.; BV Sunil Kumar: Supervision, Conceptualization, Writing- Reviewing and Editing.; Chanchal Singh: Planning, Writing- Reviewing and Editing.; Kuldip Gupta: IHC, Classification, and grading of tumors, Formal analysis.; Ashwani Kumar for helping in collecting tumor samples; Digvijay Singh: Guidance.

Conflict of interest:

None of the authors of this paper has a financial or personal relationship with other people or institutions that could inappropriately influence or bias the content of the paper.

Ethics Approval:

The Institutional Animal Ethics Committee (IAEC), GADVASU approved the collection of tissue samples via memo no. GADVASU/2020/IAEC/53/17; dated 25/01/2020.

Consent to participate:

Not Applicable

Consent for publication:

Not Applicable.

Code or Data Availability:

Not applicable.

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Figures

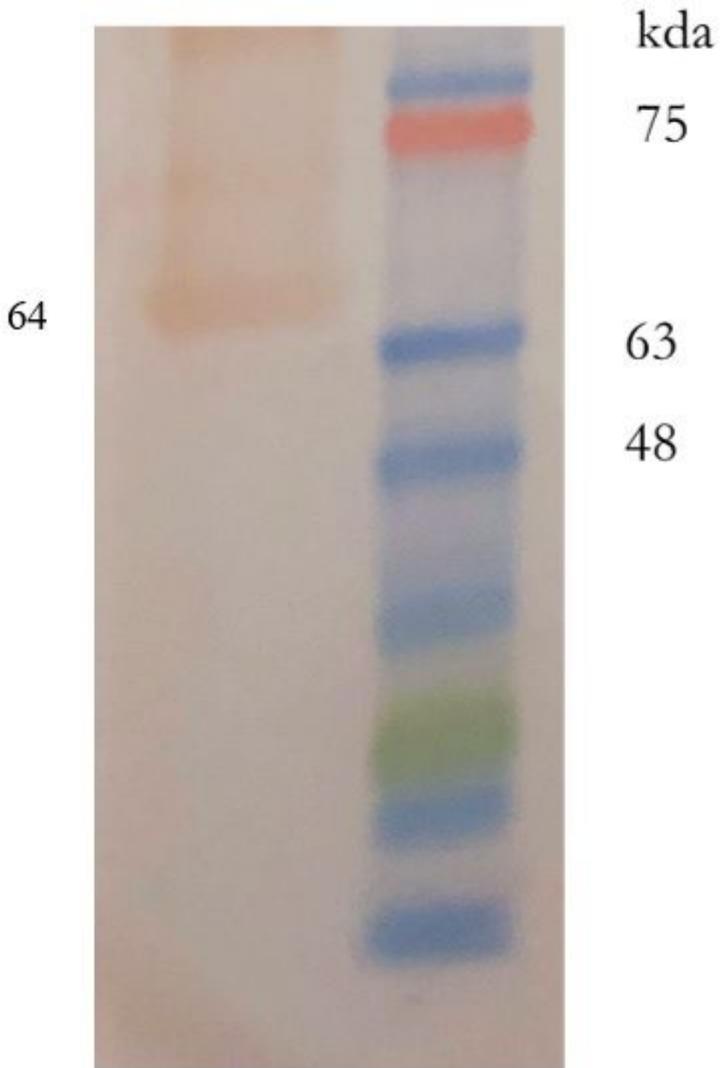


Figure 1

Western blot analysis: Using hyperimmune sera (HIS) of rabbit to check reactivity against native Hsp90 α in the CMT tissue homogenate.

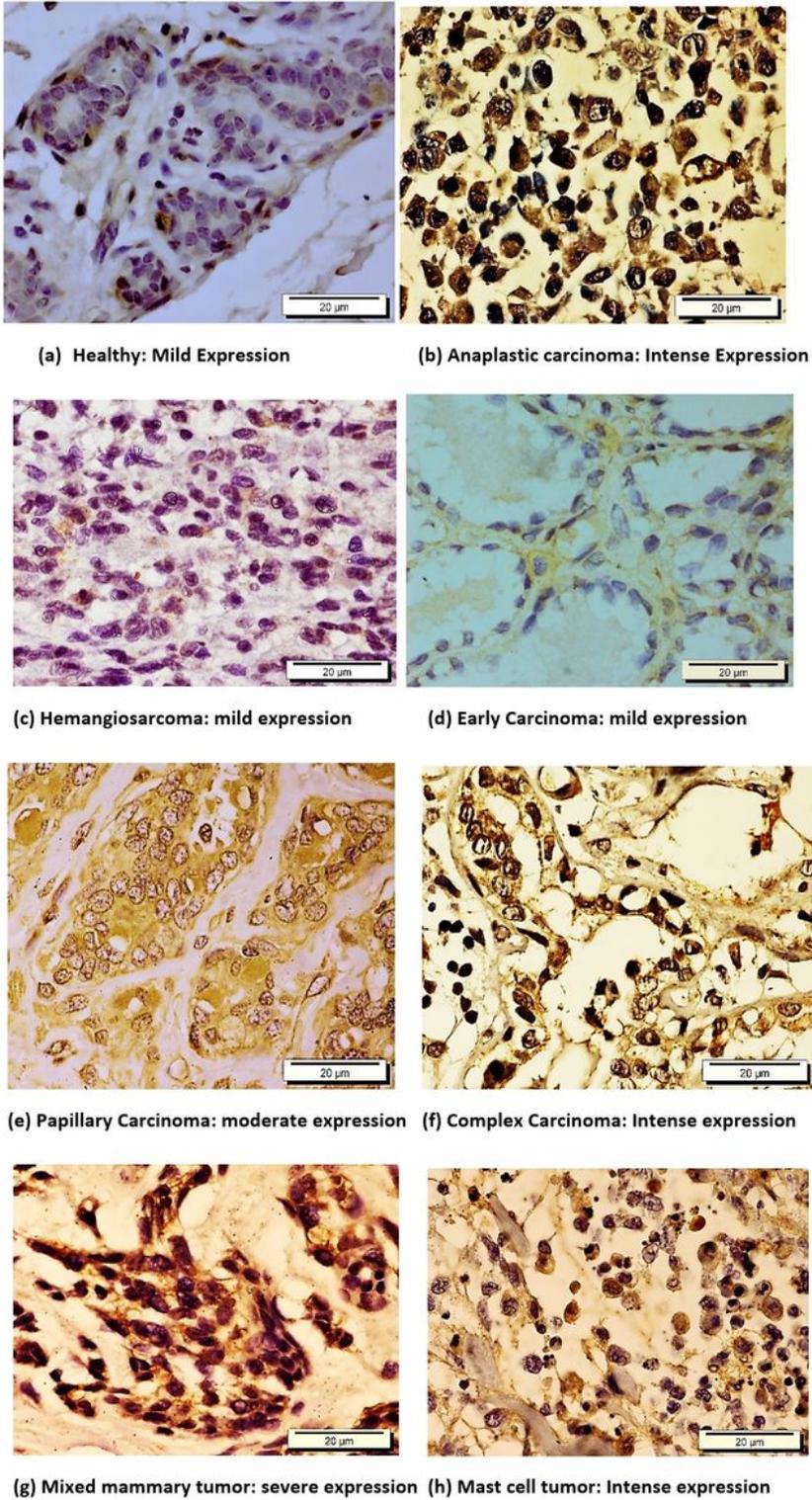


Figure 2

(a-h) Immunolocalization of Hsp90 α showing Variable expression of Hsp90 α in the cytoplasm of the neoplastic epithelial cells (IHC, 20 μ m).

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Figure 3

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