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Original Article

Heat shock protein D1 is up-regulated in various types of canine mammary tumors

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Abstract

Background: Mammary tumors are the most common tumors in female dogs. An early diagnosis makes the treatment easier. **Aims:** The present study aimed to assess heat shock protein D1 (HSPD1) expression in canine mammary tumors. **Methods:** Canine mammary tumor (CMT) samples were collected from clinics after surgery. Expression of *HSPD1* transcript in CMT and apparently healthy mammary tissues was analyzed by SYBR green based real-time PCR (qRT-PCR). Further, gene encoding the immuno-dominant region of HSPD1 was cloned using the expression vector pPROEX-HTa and expressed in a prokaryotic system and recombinant HSPD1 (rHSPD1) was purified by affinity chromatography. Hyperimmune serum was raised against rHSPD1 in mice, and immunohistochemistry was standardized to assess the expression of this protein in various histotypes of canine mammary tumors. **Results:** An elevated *HSPD1* mRNA expression (5.973 ± 0.862 folds) was observed in canine mammary tumors. Upon purification, a 60 kDa recombinant protein was obtained and confirmed by Western blotting. In 83.3% of healthy mammary tissues, a mild/feeble HSPD1 expression was observed whereas, a strong expression of HSPD1 was seen in 80% CMT samples. **Conclusion:** The findings suggested that HSPD1 could be used as a molecular marker for canine mammary tumors.

Key words: Biomarker, Canine mammary tumor, Heterologous expression, HSPD1, Immunohistochemistry

Introduction

Mammary gland tumors are commonly seen in humans, dogs, and cats but infrequently seen in other animal species (Goldschmidt *et al*., 2016). Canine mammary tumors (CMT) are the most common neoplasia that are prominently seen in unspayed female dogs (Sleeckx *et al*., 2011). There is a plethora of factors that contribute to the threat of developing mammary tumors which include breed, age, ovariectomy, exogenous hormonal exposure, diet, and obesity (Schneider *et al*., 1969). Conventionally, CMT is diagnosed by histopathological examination of the stained tumor sections. However, differentially expressed biomarkers can also be used for more specific diagnosis. Thus, identification and characterization of CMT specific/associated biomarkers are greatly encouraged for the diagnosis of this malady (Pandey *et al*., 2018).

Heat shock proteins (HSP) are highly conserved proteins that serve as mediators of hyperthermia resistance (Lindquist, 1986; Calderwood, 2010; Kumar *et al*., 2018). These proteins act as molecular chaperones that help in the correct folding of the nascent polypeptide chains, translocation of proteins across the membranes, and marking the denatured ones for degradation (Weibezahn *et al*., 2005; Tutar and Tutar, 2010). Apart from the canonical functions, HSP also plays an important role in carcinogenesis, immune response, and apoptosis (Badowska-Kozakiewicz and Malicka, 2012; Kumar *et al*., 2018). Tumorigenesis is associated with a cascade of molecular and physiological events, and each cascade is led by some receptors/enzymes/growth factors with their clientship to one or more classes of HSP (Kumar *et al*., 2020). These HSPs prevent oppression of growth signals during tumorigenesis by their tendency to bring out proteostasis (Calderwood and Gong, 2016). Similarly, some HSPs (small HSP, HSP70, and HSP90) chaperone the mutated P53, preventing cancer cell apoptosis (Kumar *et al*., 2020). HSP also regulate the binding of telomerase with DNA complex, thereby preventing apoptosis and promoting tumor progression (Toogun *et al*., 2008).

In animal cells, the 60 KDa HSPD1, also known as HSP60, primarily resides in the mitochondria. However,

certain studies have elucidated that HSPD1 is also located in the cytosol, cell surface, extracellular space, and biological fluids (Jindal *et al*., 1989; Cappello *et al*., 2008). In human beings, the *HSPD1* gene is situated on chromosome 2q33.1 (Bukau and Horwich, 1998). It is abundantly expressed in normal cells but its expression is further induced by stress factors (Hansen *et al*., 2003; Nakamura and Minegishi, 2013).

Higher HSPD1 mRNA and corresponding protein levels have been significantly associated with breast carcinogenesis in human beings and auto-antibodies against this chaperone have been found in the serum of breast cancer patients (Bini *et al*., 1997; Li *et al*., 2006; Desmetz *et al*., 2008; Seigneuric *et al*., 2011). Elevated HSPD1 expression is also observed in astroglioma cells (Bajramović *et al*., 2000), colorectal cancer (Mori *et al*., 2005), cervical cancer (Castle *et al*., 2005), ovarian cancer (Schneider *et al*., 1999) and prostate cancer (Johansson *et al*., 2006). Moreover, HSPD1 over-expression has also been reported in cellular infiltrate of canine mixed mammary carcinoma (Lopes-Neto *et al*., 2017). Scanty reports are available on the expression of HSPD1 in various other histotypes of CMT. Therefore, this study aimed to compare the HSPD1 mRNA and corresponding protein expression in different histotypes of CMT.

Materials and Methods

Sample sources

CMT samples (n=20) were collected from 6-12 years old dogs with a median of 8.5 years, during surgeries executed in the Department of Veterinary Surgery and Radiology, GADVASU, Ludhiana, India. For the extraction of total RNA, tissue samples were collected in RNA*later*™ and stored at -20°C. For histopathological and immunohistochemical analyses, tissue samples were collected in 10% Neutral Buffered formalin. Additionally, six apparently healthy mammary tissue samples were also included in the study. The Institutional Animal Ethics Committee (IAEC), GADVASU approved the collection of tissue samples and experimentation on mice *via* memo no. GADVASU/ 2020/IAEC/53/14; dated 25/01/2020. Moreover, approval from the Institutional Biosafety Committee (IBSC) was also procured via memo No. IBSC/20/9 dated 14/01/2020 for recombinant DNA technology work.

Histopathology

Tissue samples collected in 10% neutral buffered formalin (NBF) were processed and stained with Hematoxylin and Eosin (H&E). Classification and grading of the tumors were carried out according to WHO standards of classification and grading (Karayannopoulou *et al*., 2005; Goldschmidt *et al*., 2011).

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol™ reagent (Life Technologies, USA) following the manufacturer's instructions. Genomic DNA contamination, if any, was removed using DNase I. About 1 µg of the isolated RNA was used for cDNA synthesis using oligo dT primers and Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, USA). Further, the synthesized cDNA was confirmed using specific *GAPDH* primers (Thermoscientific, USA).

Real-time RT-PCR (qRT-PCR)

Relative expression of HSPD1 mRNA in tumorous and healthy canine mammary tissues was assessed by SYBR green based qRT-PCR. The specific primers targeting *HSPD1* gene (5´-CGG GAA CTA GCC TAA GCC-3´ and 5´-GTT CTT CCC TTT GGC CCC AT-3´), along with endogenous housekeeping normalizer genes, RPS-19 (5´-CCT TCC TCA AAA AGT CTG GG-3´ and 5´-GTT CTC ATC GTA GGG AGC AAG-3´) (Wang *et al*., 2007) and β-actin (5´-CCG CGA GAA GAT GAC CCA GA-3´ and 5´-GTG AGG ATC TTC ATG AGG TAG TCG G-3´) (Timmermans-Sprang *et al*., 2015) were used. iTaq universal SYBR® green supermix (BioRad, USA) was used for the expression profiling. The validity of these housekeeping genes had been previously checked in cancer studies (Kumar *et al*., 2018). Primers of all three genes were used at 0.25 µM final concentration. Annealing and extension of all the three genes were carried out at 60°C and dissociation curves were generated between 65°C to 95°C to assess the specificity of the amplicons.

The efficiencies of the qRT-PCR for each gene were calculated as:

$$E = (10^{-1/slope} - 1) \times 100$$

Where, slope was calculated for the semi-log regression curve plotted between log cDNA (serially diluted cDNA samples) versus their threshold cycle (Ct) values (McNeill *et al*., 2007). For evaluating the fold change in *HSPD1* mRNA expression between canine mammary tumor and healthy mammary tissues, the Ct values of *HSPD1* gene and the geometric mean of the Ct values of *RPS-19* and *β actin* after 40 cycles of amplification were used (Abasht *et al*., 2009). The statistical analyses were made according to Livak and Schmittgen (2001) using SAS version 9.3. Further, qRT-PCR score ($2^{-\delta Ct}$) was also calculated for each sample to analyze the relationship between HSPD1 expression at mRNA and protein levels.

Cloning and sequencing of the canine *HSPD1* gene

Using the primer BLAST program of NCBI, primers were designed to amplify the gene encoding *HSPD1* (Spanning 1643 bp). Further, to proceed with directional cloning, the sites for *NcoI* and *XhoI* restriction enzymes were added at the 5´ ends of both the primers (5´-AAC CAT GGA TAT GCT TCG ATT ACC CGC AGT-3´ and 5´-AAC TCG AGA ACG TGG TTA ACA GAG AGG CCA-3´). The primers were custom synthesized from Integrated DNA Technologies (IDT). Herculase II

fusion DNA polymerase kit (Agilent, USA) was used to amplify the *HSPD1* gene as per the manufacturer's instructions. 0.8 µM of each primer was used in 25 µL reaction, which annealed with the template at 56°C. The product thus obtained was resolved on agarose gel (1.5%) and was purified using Quick gel extraction and PCR purification kit (Thermo Fisher Scientific, USA) following user's guide.

The restriction enzyme double digestion reaction was initiated separately for the purified PCR product and the circular pPROEX-HTa expression vector, using *NcoI* and *XhoI* (New England Biolabs, UK) restriction enzymes. Both the digested vector and insert were ligated using T4 DNA ligase at 22°C and the ligated product was transformed into competent BL21DE3 cells and plated onto LB agar plates containing ampicillin (100 µg/ml). Following 16 h incubation, off-white colonies containing the recombinant plasmids were collected and allowed to grow in LB broth supplemented with the ampicillin (100 µg/ml). Subsequently, plasmids were isolated by alkaline lysis method (Sambrook and Russell, 2001) and restriction double digestion was performed to confirm the accurate orientation of insert into the vector using *NcoI* and *XhoI* (New England Bio Labs, UK) restriction enzymes. Stabs containing the positive clones were forwarded to the University of Delhi, South Campus (UDSC), New Delhi for custom sequencing. The deduced sequence was then deposited to NCBI and an accession number was obtained.

Heterologous expression of the recombinant HSPD1 (rHSPD1) and its purification

A positive clone was cultured in bulk and subjected to IPTG induction (1 mM) and pelleted down after 6 h of induction. Purification of recombinant protein was performed upon Ni-NTA based affinity chromatography under denaturing conditions (Kumar *et al*., 2018). Further, the purified HSPD1 recombinant protein was dialyzed in Snake Skin Dialysis tubing, 10 K MWCO (Thermo-Scientific, USA) for the removal of any residual urea. Expression of rHSPD1 was assessed by SDS-PAGE (Laemmli, 1970) and to confirm the expressed recombinant HSPD1 protein, western blotting (Towbin *et al*., 1979) was carried using commercially available anti-HSPD1 antibody.

Raising hyperimmune sera against canine rHSPD1 in mice

To raise hyperimmune sera against rHSPD1, 10 Swiss Albino mice (4 months old) were used. Firstly, mice were acclimatized for 1 week and later used for immunization. Purified recombinant HSPD1 protein was mixed with an equivalent amount of Freund's Complete Adjuvant (FCA) and was subcutaneously injected in mice (50 µg protein/mice) for the priming. Booster doses were injected on 7th, 14th, 21st, and 28th day post priming with half the initial quantity of the protein emulsified in Freund's incomplete Adjuvant (FIA). On the 29th day, test bleeding was done from the tail veins of mice to examine the titer of antibodies (Pandey *et al*., 2018). The final bleeding was accomplished on the 30th day by cardiac puncture and sera were collected. IgG purification kit (Thermo Scientific, USA) was used to purify immunoglobulin G from the harvested hyper immune sera. Western Blotting (Towbin *et al*., 1979) was carried out using the purified IgG raised to detect HSPD1 in mammary tumor tissue homogenate.

Immunohistochemistry (IHC) to analyze the tissue level expression of HSPD1

IHC was carried out as per the protocol standardized in the lab (Pandey *et al*., 2018). Briefly, the tissue samples stored in 10% NBF were processed and paraffin wax blocks were prepared. Using rotary microtome ~ 5 µm thick sections of tissues were cut and mounted on slides coated with poly-L lysine. Slides were firstly deparaffinized by 2 changes of xylene and then rehydrated by immersing them in graded alcohols. Further, heat induced retrieval of epitope was done by dipping the slides in citrate buffer and heating in a microwave for varied times and watts (3 min at 850 W, 7 min at 450 W). Slides were cooled down to room temperature and washed with 3 changes of PBST. Subsequently, endogenous blocking was carried out by mixing 35 µL of H_20_2 in 1000 ml of methanol and incubated in humidify chamber for 30 min. In order to minimize non-specific binding 2.5% Horse Serum (Vector laboratories, USA) was added and incubated for 40 min. After the completion of the incubation period, primary antibody (purified IgG from hyperimmune serum) was poured in 1:250 dilution, and in the negative control unimmunized mice serum was added and slides were incubated at 4°C overnight.

On the subsequent day, secondary HRP conjugate antibody (ImmPRESS Universal polymer kit, peroxidase, Vector Laboratories, USA) was poured and the slides were incubated at room temperature for 30 min in humidify chamber. Impact DAB substrate kit (Vector Laboratories, USA) was used for color development. Then, slides were counterstained with Gill's hematoxylin for 1 min and were furthered dehydrated with graded alcohols and were finally mounted.

Scoring of HSPD1 positive cells

IHC analysis was carried out following a semi-quantitative approach. In this method, the intensity of developed brown color together with percentage of the cells showing positive staining was considered (Pandey *et al*., 2018). Scoring was carried out by a pathologist without considering the history of the patients (Rizzardi *et al*., 2012). To evaluate the H or SI score (staining index), the assigned score for the percentage of HSPD1 positive cells were multiplied with score provided for staining intensity. In order to detect the strong (SI>6) or weak (SI<6) HSPD1 expression at tissue level, SI score was used. Percentage of the positive cells were calculated by randomly allotted scores as 1 for 0-25% positive cells, 2 for 26-50% positive cells, 3 for 51-75% positive cells, and 4 for 76-100% positive cells. Staining intensity was scored as follows; no staining: 0, mild

staining: 1, moderate staining: 2, and intense staining: 3, respectively.

Statistical analysis

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

Results

Histopathological analysis of CMT

Out of the 20 tumor samples, 11 were classified as complex carcinoma, 4 were anaplastic carcinoma, 3 were papillary carcinoma, and 2 were mixed mammary tumors. About 55% (n=11) were designated as grade II (moderately differentiated), 30% tumors (n=6) were found to be of grade I (well differentiated) and only 15% tumors (n=3) belonged to grade III (poorly differentiated).

Expression analysis of *HSPD1* mRNA using qRT-PCR

In our study, *HSPD1*, *RPS19* and *β-actin* genes had amplification efficiencies of 86.78%, 95.63%, and 96.44%, respectively. This suggests that the efficiencies of qRT-PCRs on *HSPD1* gene and normalizer genes were suitable for the calculation of expression ratio using Livak method. A significantly elevated expression of 5.973 ± 0.862 folds ($P<0.05$) for HSPD1 mRNA was observed in canine mammary tumors as compared with the healthy tissue samples (Fig. 1).

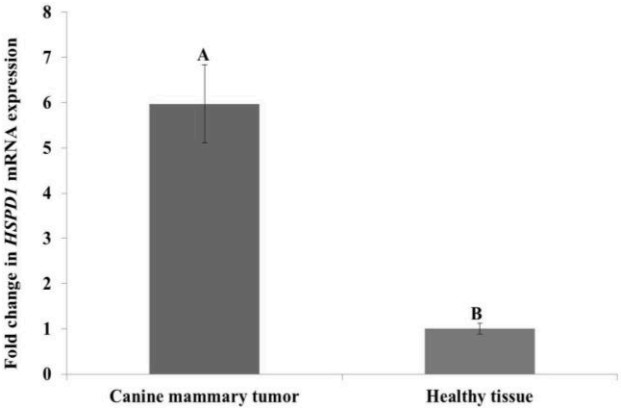

Fig. 1: A graph depicting fold change in *HSPB1* mRNA expression in canine mammary tumor tissue as compared with the healthy mammary tissue

Cloning, expression of *HSPD1* gene and purification of recombinant HSPD1 protein

A single specific band corresponding to 1643 bp (Supplementary Figure 1 (SF1)) was resolved upon the PCR amplification using specific primers targeting the *HSPD1* gene. The isolated plasmids from the cultured positive clones, upon *NcoI* and *XhoI* double digestion released a specified insert of 1643 bp (Fig. 2), which confirmed proper cloning of the gene. The obtained positive clones were forwarded for custom sequencing and the deduced sequence was deposited to GenBank of National Center for Biotechnology information (NCBI) with an accession number of OK484370.

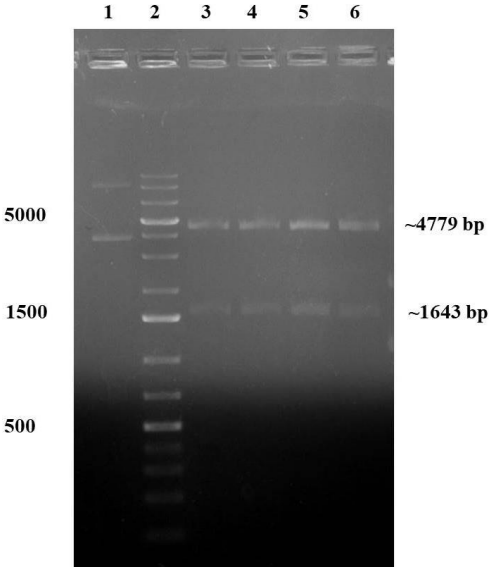

Fig. 2: Restriction enzyme double digestion of pPROEXHTa-*HSPD1* plasmid. Lane 1: Undigested plasmid, Lane 2: 1 kb plus DNA ladder, and Lanes 3-6: *Nco1* and *Xho1* digested plasmids (4779 bp) with released inserts of *HSPD1* (1643 bp)

Upon induction with 1 mM IPTG, the expression kinetics revealed that after 6 h post IPTG induction, the expression level of rHSPD1 was optimum. Upon Ni-NTA based affinity chromatography, under denaturing conditions, the purified recombinant protein was resolved as a ~ 60 kDa protein on SDS-PAGE analysis (Fig. 3). The total yield of the rHSPD1 protein was 6.2 mg/L of the induced culture. A specified immune-reactivity corresponding to 60 kDa on the nitrocellulose membrane post Western blotting confirmed the recombinant protein (Fig. 4).

The IgG purified from the hyperimmune sera also distinctly reacted with native HSPD1 in the tumor tissue homogenate upon Western blotting, which confirmed that the purified IgG specifically reacted with the native cellular HSPD1 (Supplementary Figure 2 (SF2)).

Immunohistochemical detection of HSPD1 expression in canine mammary neoplasia

The HSPD1 immunopositive cells were stained brown, which was restricted to the cytoplasm of the cells alone. The negative controls, in which unimmunized mice serum was used, did not show any immunostaining with the raised HSPD1 antibodies. Immunoreactivity against cellular HSPD1 was judged based on the staining index (SI) obtained by multiplying the number of immunopositive cells in random fields with the staining

intensity score of the pathologist. Tissue expression of HSPD1 was judged as strong when SI was greater than/equal to 6 and it was referred to as mild when SI was less than 6 (Pandey *et al*., 2018). Based on this convention, a weak expression of HSPD1 was detected in 83.3% of the healthy mammary glands (Fig. 5) while the rest showed no reactivity. A varied HSPD1 expression was evident in most of the CMT histotypes under study. The immunoreactivity for HSPD1 was found to be strong in 80% of the CMT tissues (Figs. 6A-D). Further, a weak HSPD1 expression was indicated in both the specimens of mixed mammary tumor, while a strong HSPD1 expression was seen in all four specimens of anaplastic carcinomas, two papillary carcinomas and 10 complex carcinomas (Fig. 7). When, HSPD1 expression was compared with different grades of CMT, a strong HSPD1 expression was discerned in 50%, 72.73%, and 85.57% of grade-1, grade-2, and grade-3 CMT, respectively.

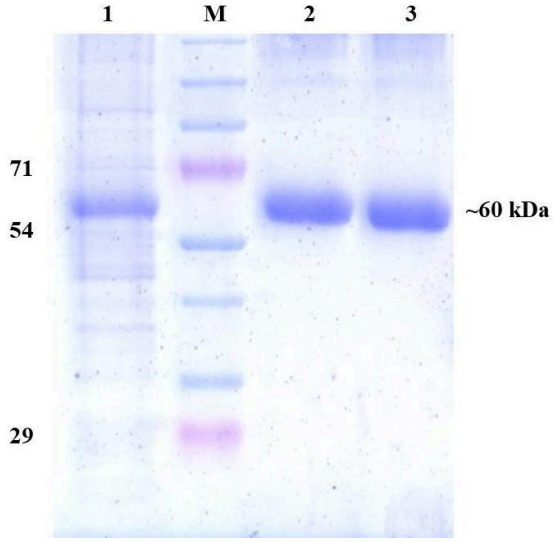

Fig. 3: SDS PAGE depicting purified recombinant HSPD1 protein. Lane M: Pre-stained protein ladder (Puregene). Lane 1: Unpurified cell lysate, and Lanes 2 and 3: Purified protein

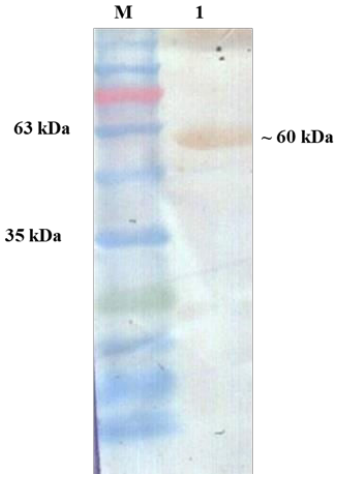

Fig. 4: Western blotting analysis of recombinant HSPD1 protein. Lane M: Pre-stained protein ladder. Lane 1: Immunoreactive rHSPD1

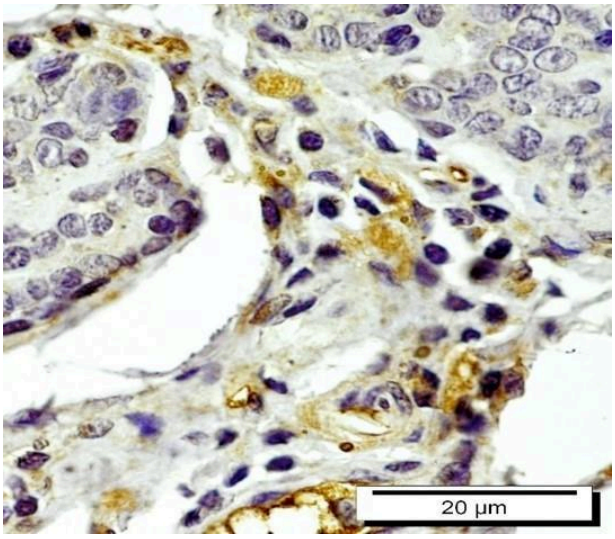

Fig. 5: Immunolocalization of HSPD1 showing mild expression of HSPD1 within the cytoplasm in healthy mammary tissue (IHC, 20 µm)

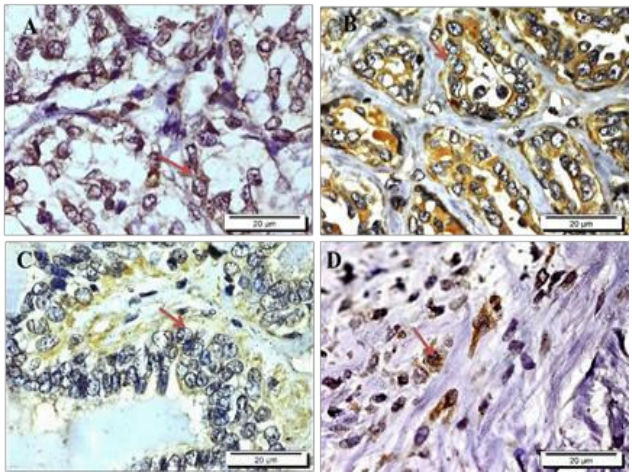

Fig. 6: HSPD1 expression in various histotypes of CMT. (**A**) Anaplastic carcinoma: Immunolocalization of HSPD1 showing strong expression of HSPD1 within the cytoplasm and mostly perinuclear area of the neoplastic epithelial cells (IHC, 20 µm), (**B**) Complex carcinoma: Immunolocalization of HSPD1 showing strong expression of HSPD1 in the cytoplasm of the neoplastic epithelial cells which are arranged in nest (IHC, 20 µm), (**C**) Papillary carcinoma: Immunolocalization of HSPD1 showing weak expression of HSPD1 in the cytoplasm of the neoplastic epithelial cells (IHC, 20 µm), and (**D**) Mixed mammary tumor: Immunolocalization of HSPD1 showing moderate immunoreactivity of HSPD1 in the cytoplasm of the epithelial cells and fibroblast (IHC, 20 µm)

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

The statistical analysis between the H score and qRT-PCR score revealed no significant correlation between the HSPD1 transcript and corresponding protein expression. However, the Kruscal Wallis test showed significant differences ($P<0.05$) in the H scores alone concerning different CMT histotypes. The Wilcoxon mean rank for H score was highest in anaplastic

carcinoma and least in mixed mammary tumors indicating that HSPD1 expression was least in mixed mammary tumors and was highest in anaplastic carcinoma among the CMT histotypes under study. Moreover, one-way ANOVA was also carried out to check for the significant differences in HSPD1 expression (if any) in various histotypes of CMT based on the H scores. It was found that the expression of the protein (H-score) in anaplastic carcinoma (10.00±0.91) was significantly higher ($P<0.05$) than that in mixed mammary tumors (1.68±1.05).

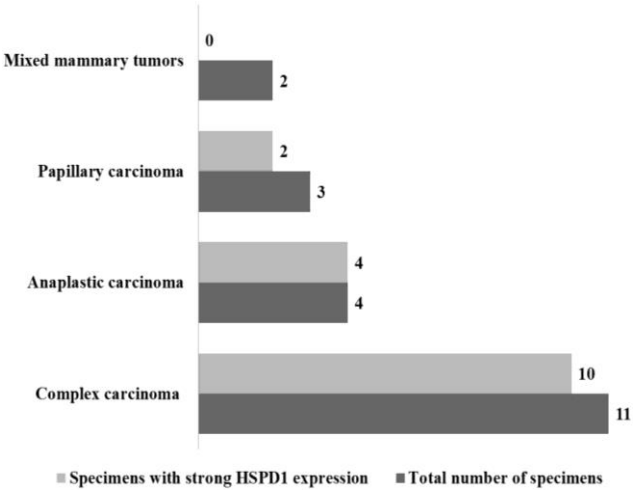

Fig. 7: Strong HSPD1 expression in different histotypes of canine mammary tumors

Discussion

In this contemporary epoch, there are reports on increased incidence of canine mammary neoplasia, globally. With the advent of advanced technology, it is possible to diagnose a majority of these tumors at an early stage. For the diagnosis of CMT, the identification of certain biomarkers to diagnose this malady is absolutely crucial. The progressive research in cancer biology has depicted the significance of HSP as a potential biomarker of mammary carcinogenesis (Kumar *et al*., 2018).

In the present study, we report an upregulation of HSPD1 in CMT at mRNA and protein levels. HSPD1 is a heat shock protein that also plays an indispensable role in immune responses has anti-apoptotic characteristics and promotes tumor growth, progression, invasion, and metastasis. It is also accountable for treatment resistance and worse survival rates. During carcinogenesis, HSPD1 is deposited on the outside of mitochondria, in the cytoplasm, plasma membrane, and secretory vesicles, protecting tumor cells from external environmental stress and promoting cell proliferation. HSPD1 also plays a role in the permeabilization of the mitochondrial membrane by interacting with cyclophilin D, a protein that regulates the permeability transition pore in mitochondria (Tang *et al*., 2022). HSPD1 controls the action of p53 and causes a cytoprotective cascade by stabilizing the quantum of survivin. The mitochondrial survivin stores are depleted as a result of acute HSPD1 extraction. Survivin is known for inhibiting apoptosis, as well as increasing p53 expression and triggering p53-dependent apoptosis in tumor cells (Hu *et al*., 2021). These cytoprotective characteristics of HSPD1 have been thoroughly investigated *in vivo* in malignancies, where HSPD1 is selectively up-regulated in contrast with normal cells, while HSPD1 deficiency in normal cells is not connected to mitochondrial malfunction or cell death.

Moreover, HSPD1 not only stabilizes the amount of survivin in the mitochondria, but it also provides cyto-protection through another method, which involves the formation of a complex by HSPD1's interaction with p53, which inhibits the function of p53 tumor cells (Asea and Kaur, 2019). Several reports advocate that HSPD1 is up-regulated in human breast cancer. Provided that, CMT and human breast cancer share a close association, we chose to study the expression of HSPD1 in CMT subjects.

In our study majority of the tumors were complex carcinomas. Many reports in the past also advocate those complex carcinomas, among all other canine mammary tumors, are the most pervasive (Mitchell *et al*., 1974; Mulligan, 1975; Badowska-Kozakiewicz and Malicka, 2012; Pandey *et al*., 2015; Lopes-Neto *et al*., 2017; Kumar *et al*., 2018; Birdi *et al*., 2019). The *HSPD1* mRNA was 5.973 ± 0.862 folds overexpressed in CMT. In the past, Desmetz *et al*. (2008) reported an elevated expression of HSPD1 in early breast cancer. Apart from breast cancer, an increased HSPD1 mRNA expression has also been reported in ovarian (Abu-Hadid *et al*., 1997) and colorectal cancer (Campanella *et al*., 2015) in human beings. However, a downregulation of *HSPD1* mRNA expression has also been reported in hepatocellular carcinoma (Zhang *et al*., 2016).

In human breast pathology, IHC is routinely used in the diagnosis and prognosis of breast neoplasia. In the present study, we found a strong expression of HSPD1 in the majority of CMT tissues and the HSPD1 expression was associated with the aggressiveness of CMT. In the past, a moderate to high cytoplasmic expression of HSPD1 has been reported in cellular infiltrate of mixed canine mammary carcinomas (Lopes-Neto *et al*., 2017) and triple negative human breast cancer (Bodoor *et al*., 2018) which was associated with an advanced stage of tumor. It has also been reported that the elevated expression of HSPD1 in the initial stages of breast carcinogenesis has a significant correlation with tumor growth and progression (Desmetz *et al*., 2008). In our study, we found a strong HSPD1 expression in a majority of CMT histotypes. Therefore, HSPD1 may be included in the panel of IHC-based biomarkers of CMT. This could help in specific diagnosis of the tumor and also may help in assessing the response to tumor treatment.

We reported an elevated level of HSPD1 expression in four different histotypes of CMT. The HSPD1 protein expression was maximum in anaplastic carcinoma whereas in the mixed mammary tumors, the expression

was minimum. However, due to the smaller sample size, the data need to be confirmed in a study employing a large number and types of CMT in future.

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Conflict of interest

All the authors declare that they have no conflict of interest.

References

Abasht, B; Kaiser, MG; Van der Poel, J and Lamont, SJ (2009). Genetic lines differ in toll-like receptor gene expression in spleens of chicks inoculated with *Salmonella enterica* serovar Enteritidis. Poult. Sci., 88: 744-749.

Abu-Hadid, M; Wilkes, JD; Elakawi, Z; Pendyala, L and Perez, RP (1997). Relationship between heat shock protein 60 (HSP60) mRNA expression and resistance to platinum analogues in human ovarian and bladder carcinoma cell lines. Cancer Lett., 119: 63-70.

Asea, AA and Kaur, P (2019). *Heat shock protein 60 in human diseases and disorders*. 1st Edn., Vol. 18, Switzerland, Springer Nature. PP: 135-143.

Badowska-Kozakiewicz, AM and Malicka, E (2012). Immunohistochemical evaluation of expression of heat shock proteins HSP70 and HSP90 in mammary gland neoplasms in bitches. Pol. J. Vet. Sci., 15: 35-39.

Bajramović, JJ; Geutskens, SB; Bsibsi, M; Boot, M; Hassankhan, R; Verhulst, KC and van Noort, JM (2000). The stress kit: a new method based on competitive reverse transcriptase-polymerase chain reaction to quantify the expression of human αB-crystallin, Hsp27, and Hsp60. Cell Stress Chap., 5: 30-36.

Bini, L; Magi, B; Marzocchi, B; Arcuri, F; Tripodi, S; Cintorino, M; Sanchez, JC; Frutiger, S; Hughes, G; Pallini, V and Hochstrasser, DF (1997). Protein expression profiles in human breast ductal carcinoma and histologically normal tissue. Electrophoresis. 18: 2832-2841.

Birdi, R; Kumar, BVS; Gupta, K; Kashyap, N and Kumar, A (2019). Circulating level of heat shock protein 27 is elevated in dogs with mammary tumors. 3 Biotech., 9: 1-9.

Bodoor, K; Abu-Sheikha, A; Matalka, I; Alzou'bi, H; Batiha, O; Abu-Awad, A; Jalboush, SA; Fayyad, LM; Qadiri, E; Jarun, Y and Albatayneh, K (2018). Immunohistochemical analysis of heat shock proteins in triple negative breast cancer: HSP60 expression is a marker of poor prognosis. Eur. J. Gynaecol. Oncol., 39: 926-934.

Bukau, B and Horwich, AL (1998). The Hsp70 and Hsp60 chaperone machines. Cell. 92: 351-366.

Calderwood, SK (2010). Heat shock proteins in breast cancer progression-a suitable case for treatment? Int. J. Hyperther., 26: 681-685.

Calderwood, SK and Gong, J (2016). Heat shock proteins promote cancer: it's a protection racket. Trends Biochem. Sci., 41: 311-323.

Campanella, C; Rappa, F; Sciumè, C; Marino Gammazza, A; Barone, R; Bucchieri, F; David, S; Curcurù, G; Caruso Bavisotto, C; Pitruzzella, A and Geraci, G (2015). Heat shock protein 60 levels in tissue and circulating exosomes in human large bowel cancer before and after ablative surgery. Cancer. 121: 3230-3239.

Cappello, F; Conway de Macario, E; Marasà, L; Zummo, G and Macario, AJ (2008). Hsp60 expression, new locations, functions, and perspectives for cancer diagnosis and therapy. Cancer Biol. Ther., 7: 801-809.

Castle, PE; Ashfaq, R; Ansari, F and Muller, CY (2005). Immunohistochemical evaluation of heat shock proteins in normal and preinvasive lesions of the cervix. Cancer Lett., 229: 245-252.

Desmetz, C; Bibeau, F; Boissiere, F; Bellet, V; Rouanet, P; Maudelonde, T; Mangé, A and Solassol, J (2008). Proteomics-based identification of HSP60 as a tumor-associated antigen in early stage breast cancer and ductal carcinoma *in situ*. J. Prot. Res., 7: 3830-3837.

Goldschmidt, M; Peña, L; Rasotto, R and Zappulli, V (2011). Classification and grading of canine mammary tumors. Vet. Pathol., 48: 117-131.

Goldschmidt, M; Peña, L and Zappulli, V (2016). Tumors of the mammary gland. Tumors Dom. Anim., 15: 723-765.

Hansen, JJ; Bross, P; Westergaard, M; Nielsen, M; Eiberg, H; Børglum, AD; Mogensen, J; Kristiansen, K; Bolund, L and Gregersen, N (2003). Genomic structure of the human mitochondrial chaperonin genes: HSP60 and HSP10 are localised head to head on chromosome 2 separated by a bidirectional promoter. Human Genet., 112: 71-77.

Hu, X; Li, H; Ip, TK; Cheung, YF; Koohi-Moghadam, M; Wang, H; Yang, X; Tritton, DN; Wang, Y; Wang, R; Ng, KM; Naranmandura, H; Tse, EW and Sun, H (2021). Arsenic trioxide targets Hsp60, triggering degradation of p53 and survivin. Chem. Sci., 12: 10893-10900.

Jindal, S; Dudani, AK; Singh, BHAG; Harley, CB and Gupta, RS (1989). Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. Mol. Cell Biol., 9: 2279-2283.

Johansson, B; Pourian, MR; Chuan, YC; Byman, I; Bergh, A; Pang, ST; Norstedt, G; Bergman, T and Pousette, Å (2006). Proteomic comparison of prostate cancer cell lines LNCaP-FGC and LNCaP-r reveals heatshock protein 60 as a marker for prostate malignancy. Prostate. 66: 1235-1244.

Karayannopoulou, M; Kaldrymidou, E; Constantinidis, TC and Dessiris, A (2005). Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. J. Comp. Pathol., 133: 246-252.

Kumar, BVS; Bhardwaj, R; Mahajan, K; Kashyap, N; Kumar, A and Verma, R (2018). The overexpression of Hsp90B1 is associated with tumorigenesis of canine mammary glands. Mol. Cell Biochem., 440: 23-31.

Kumar, BVS; Gopal, PK; Gurao, A and Verma, R (2020). Binary role of heat shock proteins in cancer immunotherapy: A detailed perspective. In: Asea, AAA and Kaur, P (Eds.), *Heat shock proteins in inflammatory diseases*. *Heat shock proteins*. 1st Edn,, Vol. 22, Springer, Cham. PP: 387-405. https://doi.org/10.1007/7515_2020_34.

Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Li, DQ; Wang, L; Fei, F; Hou, YF; Luo, JM; Zeng, R; Wu, J; Lu, JS; Di, GH; Ou, ZL and Xia, QC (2006).

Identification of breast cancer metastasis-associated proteins in an isogenic tumor metastasis model using two-dimensional gel electrophoresis and liquid chromatography-ion trap-mass spectrometry. Proteomics. 6: 3352-3368.

Lindquist, S (1986). The heat-shock response. Ann. Rev. Biochem., 55: 1151-1191.

Livak, KJ and Schmittgen, TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25: 402-408.

Lopes-Neto, BE; Souza, SCB; Bouty, LM; Santos, GJL; Oliveira, ES; de Freitas, JCC and Nunes-Pinheiro, DC (2017). CD4+, CD8+, FoxP3+ and HSP60+ expressions in cellular infiltrate of canine mammary carcinoma in mixed tumor. Acta Sci. Vet., 45: 1-8.

McNeill, RE; Miller, N and Kerin, MJ (2007). Evaluation and validation of candidate endogenous control genes for real-time quantitative PCR studies of breast cancer. BMC Mol. Biol., 8: 1-13.

Mitchell, L; De la Iglesia, FA; Wenkoff, MS; Van Dreumel, AA and Lumb, G (1974). Mammary tumors in dogs: survey of clinical and pathological characteristics. Can. Vet. J., 15: 131-138.

Mori, D; Nakafusa, Y; Miyazaki, K and Tokunaga, O (2005). Differential expression of Janus kinase 3 (JAK3), matrix metalloproteinase 13 (MMP13), heat shock protein 60 (HSP60), and mouse double minute 2 (MDM2) in human colorectal cancer progression using human cancer cDNA microarrays. Pathol. Res. Pract., 201: 777-789.

Mulligan, RM (1975). Mammary cancer in the dog: a study of 120 cases. American J. Vet. Res., 36: 1391-1396.

Nakamura, H and Minegishi, H (2013). HSP60 as a drug target. Curr. Pharm. Des., 19: 441-451.

Pandey, M; Kumar, BVS; Gupta, K; Sethi, RS; Kumar, A and Verma, R (2018). Over-expression of mammaglobin-B in canine mammary tumors. BMC Vet. Res., 14: 1-8.

Pandey, M; Kumar, BVS and Verma, R (2015). Mammaglobin as a diagnostic serum marker of complex canine mammary carcinomas. Res. Vet. Sci., 103: 187-192.

Rizzardi, AE; Johnson, AT; Vogel, RI; Pambuccian, SE; Henriksen, J; Skubitz, AP; Metzger, GJ and Schmechel, SC (2012). Quantitative comparison of immuno-histochemical staining measured by digital image analysis versus pathologist visual scoring. Diag. Pathol., 7: 1-10.

Sambrook, J and Russell, DW (2001). *Molecular cloning: a laboratory manual*. 2nd Edn., Vol. 1, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. PP: 112-138.

Schneider, R; Dorn, CR and Taylor, DO (1969). Factors influencing canine mammary cancer development and postsurgical survival. J. Natl. Cancer Ins., 43: 1249-1261.

Schneider, J; Jimenez, E; Marenbach, K; Romero, H; Marx, D and Meden, H (1999). Immunohistochemical detection of HSP60-expression in human ovarian cancer. Correlation with survival in a series of 247 patients. Anticancer Res., 19: 2141-2146.

Seigneuric, R; Mjahed, H; Gobbo, J; Joly, AL; Berthenet, K; Shirley, S and Garrido, C (2011). Heat shock proteins as danger signals for cancer detection. Front. Oncol., 1: 37-47.

Sleeckx, N; de Rooster, H; Veldhuis Kroeze, EJ; Van Ginneken, C and Van Brantegem, L (2011). Canine mammary tumors, an overview. Rep. Dom. Anim., 46: 1112-1131.

Tang, Y; Zhou, Y; Fan, S and Wen, Q (2022). The multiple roles and therapeutic potential of HSP60 in cancer. Biochem. Pharmacol., 201: 115096-115109.

Timmermans-Sprang, EP; Gracanin, A and Mol, JA (2015). High basal Wnt signaling is further induced by PI3K/mTor inhibition but sensitive to cSRC inhibition in mammary carcinoma cell lines with HER2/3 overexpression. BMC Cancer. 15: 1-12.

Toogun, OA; DeZwaan, DC and Freeman, BC (2008). The hsp90 molecular chaperone modulates multiple telomerase activities. Mol. Cell. Biol., 28: 457-467.

Towbin, H; Staehelin, T and Gordon, J (1979). Electrophoresis transfer of protein from polyacrylamide gels to nitrocellulose sheets. Procedure and applications. Proc. Natl. Acad. Sci. USA., 76: 4350-4354.

Tutar, L and Tutar, Y (2010). Heat shock proteins: an overview. Curr. Pharm. Biotechnol., 11: 216-222.

Wang, YS; Chi, KH and Chu, RM (2007). Cytokine profiles of canine monocyte-derived dendritic cells as a function of lipopolysaccharide-or tumor necrosis factor-alpha-induced maturation. Vet. Immunol. Immunopathol., 118: 186-198.

Weibezahn, J; Schlieker, C; Tessarz, P; Mogk, A and Bukau, B (2005). Novel insights into the mechanism of chaperone-assisted protein disaggregation. Biol. Chem., 386: 739-744.

Zhang, J; Zhou, X; Chang, H; Huang, X; Guo, X; Du, X; Tian, S; Wang, L; Lyv, Y; Yuan, P and Xing, J (2016). Hsp60 exerts a tumor suppressor function by inducing cell differentiation and inhibiting invasion in hepatocellular carcinoma. Oncotarget. 7: 68976-68989.

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