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Research Article

Expression Profiling of *Hspb1* and *Tp53* Genes through RT-qPCR in Different Cancer Types of *Canis familiaris*

Rashid Saif *[1], Ali Raza Awan [2], Muhammad Tayyab [2], Masroor Ellahi Babar [1], Asim Khalid Mahmood [3], Zia Ullah [3], Saeeda Zia [4], Muhammad Wasim [2]

[1] Department of Biotechnology, Virtual University of Pakistan, Lahore 54000, Pakistan
[2] Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Outfall Road, Lahore 54000, Pakistan
[3] Pet center, University of Veterinary and Animal Sciences, Outfall Road, 5400, Lahore, Pakistan
[4] Department of Sciences and Humanities, National University of Computer and Emerging Sciences, Lahore, Pakistan

*Correspondence author: Rashid Saif, Department of Biotechnology, 1-Davis Road Campus, Virtual University of Pakistan, Lahore 54000, Pakistan
Phone: +92-321-7107501, E-mail: rashid.saif@vu.edu.pk

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Background: Diagnostic molecular marker studies are in vogue to have insight of most prevalent animal diseases including cancer.
Objectives: Gene expression profiling of pro and anti-apoptotic genes was conducted in dog Lymphoma, CTVT, SCC, granuloma, perianal adenocarcinoma and mammary tumors.
Materials and Methods: Cancerous tissues of 21 affected animals were obtained. Total RNA was extracted followed by cDNA synthesis. Comparative Ct method via *Taqman* assay (RT-qPCR) was used to quantify corresponding mRNA molecules, *Tp53* and *Hspb1*, as normalized by *GAPDH* as the reference gene .
Results: *Hspb1* showed ectopic expression in lymphoma, CTVT and mammary tumors; its down-regulation was observed in granuloma and oral SCC with fold difference (FD) of ±35. Similarly, *Tp53* as the tumor suppressor gene with pro-apoptotic properties, showed up-regulation in all tumor types, notably 80% of mammary tumors and 60% of CTVT. The FD values were 33.31 and 2.27, respectively.
Conclusion: Altered transcriptomic response of *Hspb1* and *Tp53* was observed in all cancer types of *Canis familiaris*. The resulting profile depicts the involvement of the genes in cancer pathways. Thus, the data might be helpful for diagnosis, prognosis, identification and classification of these widespread neoplasms in this species.

Keywords: Dog neoplasias, *Hspb1*, Livak method, RT-qPCR, *TaqMan* probes, *Tp53*

1. Background

Among the companion animals, dogs have played a considerable role for the mental peace of households and cancer is one of the major confronting diseases in small pet species. Common types of cancer in *Canis familiaris* are mammary gland adenocarcinoma, transmissible venereal tumor (TVT), lymphocytic lymphomas (nodal, mediastinal, multicentric, atypical form), perianal adenocarcinoma and squamous cell carcinoma (SCC) (1).

Cancer diagnosis is important especially at early stages to opt the best immediate treatment modality. Cancer-associated mutation may serve as diagnostic markers in different neoplasias in animals species (2). However, differential expression profiling may also serve as confirmatory, supportive and tumor classification method for better diagnosis and prognosis in conjunction to mutation markers (3).

Heat shock proteins (Hsps) are one of the key indicators in presumed biotic (oxidative) and abiotic stresses (heat, irradiation, chemotherapy) (4, 5). *Hsp* expression profiling is informative and useful biomarker for tumorigenesis in neoplastic tissues (6). Furthermore, detection of Hsp and antibodies in the blood circulation of cancer patients may also be a helpful indicator to diagnose and to fight against

different disease conditions (7). Among these proteins, Hsp27 is an interesting biomarker in molecular diagnostic oncology and renowned as chaperone with anti-tumor suppression properties. This protein has its role in cell differentiation, proliferation, invasion, apoptosis, metastasis and recognition of tumorous cells by the immune system (8). However, *Hspb1* does not fall under the major category of cancer associated genes. It is hypothesized that diagnostic and prognostic value of this gene has the same function as the *Bcl-2*, which comes under the anti-apoptosis gene category. So, ectopic expression of *Hspb1* might be helpful to predict the response to anticancer treatments viz. chemotherapy (9).

Similarly, the role of Tp53 protein is to protect the whole genome from external and internal stresses. Cell cycle arrest is one of the significant functions in conjunction with other associated proteins e.g. P21. This protein also acts as cell brakes at G1 stage, respond to DNA/spindle fiber damage, hypoxic conditions (10,11) and performs the cytoprotective role to maintains the whole genome integrity (12). Gene expression profiling of *Tp53* may help to have an insight of its tumor suppression characteristics in different cancers.

Seven cancer types were analyzed in this study, including mammary adenocarcinoma, sticker's sarcoma (sexually transmitting tumor of the genitalia) (13-15), perianal adenocarcinoma (cancer of hairless skin around the anus, circumaural and anal sack glands on the 4 and 8 o'clock position of the anus may also involve) (16), lymphosarcoma (lymphocytes and lymphoid tissues e.g. spleen, bone marrow and lymph nodes, especially in the head and neck region. one of the inherited cancers in dogs and highly prone to certain breeds e.g. Boxer, Bull Mastiff, Saint Bernard, Scottish Terrier and Airedales), squamous cell carcinomas, melanoma and granulomas (17).

2. Objectives

The purpose of the current study is to ascertain the involvement of *Hspb1*and *Tp53* genes in cancer-related pathways in *Canis familiaris.* Whether, the ectopic expression of these target genes may convey substantial information for the diagnosis, prognosis and classification of the subject cancer cases.

3. Materials and Methods

3.1. Sample Collection

Tumorous tissue samples (n=21) along with peripheral blood from cancerous animals were collected from the Pet Centre, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan and other private pet clinics through standard methods. Five CTVT samples, four mammary adenocarcinoma, three samples from each perianal adenocarcinoma and SCC, two from each lymphocytic lymphoma (mostly necropsy sample of the neck region lymph node) and granuloma, one from each melanoma and pelvic-warts were collected and analyzed. All collected samples were excisional biopsies, resected tissues, which were stored immediately at -86 ˚C for downstream considerations. Five healthy tissues were also collected as control (Fig. 1).

Figure 1. (A) Granuloma (B) CTVT (C) SCC and (D) Mammary tumor in dogs.

3.2. Histopathological Examination

Histopathological examination was performed on formalin-fixed paraffin embedded (FFPE) cancerous tissues. Formalin-filled (10%) sample collection tubes were used to preserve the neoplastic tissues after grossing and isolation of core tumorous masses. The tissues were used for hematoxylin and eosin staining to confirm the malignancy, grading and staging (18) (Fig. 2).

3.3. Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from cancerous and normal tissues using Thermo Scientific GeneJet RNA purification kit (19). For neoplastic tissues with insufficient tissues TriZol reagent (20, 21) was used. RNA integrity was confirmed by agarose gel electrophoresis and concentrations were measured by NanoDrop 2000 (Thermo Fisher Scientific, Pittsburg, PA, USA).Total RNA was reverse transcribed using

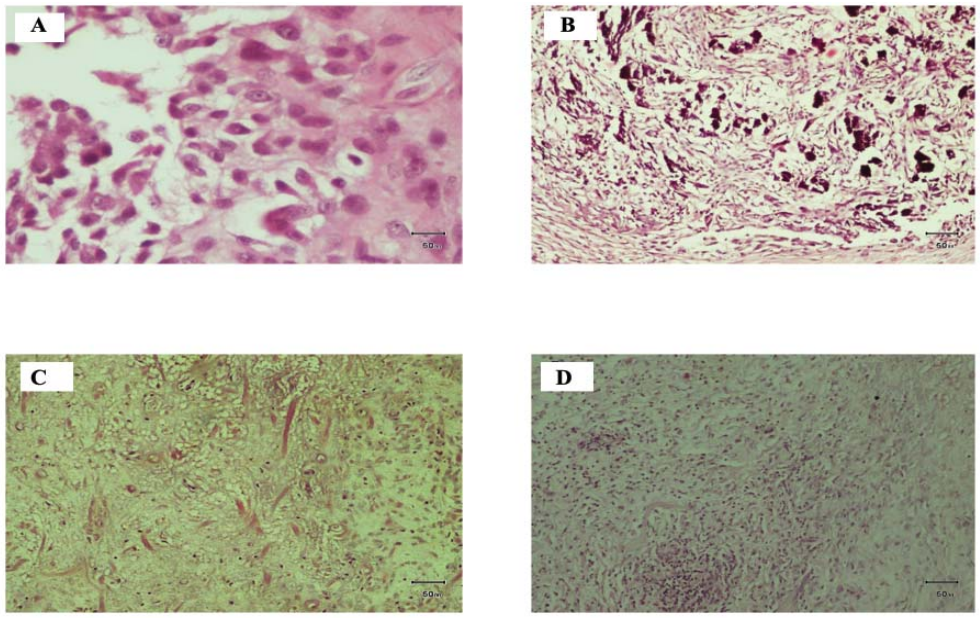

Figure 2. Mammary tumor A (400× 10 × 40), B (100× 10 ×10) and granuloma (C, D) in dogs

Revert Aid first strand cDNA Synthesis Kit (Thermo Fisher Scientific, Pittsburg, PA, USA) (22). Synthesis of first strand cDNA was performed with oligo (dT) 18 primer and random hexamer primers simultaneously. Oligo (dT) 18 primers synthesize cDNA from the poly (A) tail mRNA, while random primers initiate cDNA synthesis from rest of the RNA population.

3.4. TaqMan Detection Chemistry

TaqMan primer-probe hydrolysis based assays detection chemistry was adopted by using Applied Biosystems 7500 real-time *q*PCR system. Primer Express software (Applied Biosystem, USA) (23) was used to design the primer-probe of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) reference gene for normalization of target genes (24). *Hspb1* and *Tp53* primer-probe assays were selected and ordered from *Canis familiaris* genome assembly. Both target assays were designed with FAM, while *GAPDH* was designed with VIC reporter dye on its 5' end and TAMRA dye as quencher on its 3' end.Standard recipe (Applied Biosystem, USA) was used in which 20 µL reaction volume contain 1 µL 20× TaqMan gene expression assay, 10 µL of 2× TaqMan gene expression master mixes, 4 µL of cDNA template of 5 ng.µL^{-1} and 5 µL of RNase-free DEPC treated water. Singleplex two-step qPCR with 40 cycles was performed in which both targets and internal reference were amplified in triplicate in all cases. Cycle threshold (Ct) values were obtained at the end of the reaction and fold changes (FD) were calculated through ΔΔCt method using the following formulae (25).

ΔCt (Test)	= Ct (Target) – Ct (Reference)
ΔCt (Calibrator)	= Ct (Target) – Ct (Reference)
Δ ΔCt	= ΔCt (Test) – ΔCt (Calibrator)
Fold Change	= $2^{-\Delta\Delta Ct}$

4. Results

Histopathological slides of mammary tumor showed mixed population of cells with massive nucleus and prominent mitotic figures (Fig. 2A). Multiple focal areas were evident in different magnification, where deposition of fibroblast and collagen fiber can be seen (Fig. 2B). One of the granuloma cases showed small fibers of skeletal muscles scattered across the whole tissue, while its larger cell population had clear nucleus with cytoplasmic components displacing alongwith mixed population of inflammatory cells including lymphocytes and neutrophils (Fig. 2C). Maximum populations of cells, including lymphocytes and neutrophils, are larger with less basophilic nucleus (Fig. 2D).

4.1. Differential Expression of Hspb1

Ct values of *Hspb1* were obtained from RT-qPCR experiment and fold differences were calculated through comparative Ct method. Mean ΔCt (0.72) of the normal tissues (calibrator) was obtained by subtracting mean

Ct target (*Hspb1)* from the average Ct of endogenous (*GAPDH*) from normal dog tissues. All tumor samples showed altered expression as compared to normal tissues (Supplementary Table 1, Fig. 3).

4.1.1. Lymphoma Cases

Hspb1 proved informative in dog's cancer cases and its up-regulation was observed in lymphoma sample (Lymph-1) with FD of 3.70, and down-regulated in (Lymph-2) with FD = 0.21. These opposite FD might be due to different mutational landscape and/or variant cancer stage (Supplementary Table 1, Fig. 3).

4.1.2. Mammary Adenocarcinoma Cases

Similarly, three mammary tumor samples (MT-1, MT-3, MT-5) showed down-regulation of *Hspb1* with FD of 0.89, 0.03 and 0.18, respectively. In contrast, MT-2 and MT-4 were up-regulated with FD of 10.23 and 5.54, respectively. The up- and down-regulation of *Hspb1* might be due to the differences in mutational landscape.

4.1.3. Granuloma and Squamous Cell Carcinoma (SCC) Cases

Both granuloma samples (Granu-1, Granu-2) and pelvic-wart sample (Pel rts) showed down-regulation, while SCC (SCC-1, SCC-2) showed up-regulation for *Hspb1*.

4.1.4. Canine Transmissible Venereal Tumor (CTVT) Cases

As far as CTVT cases are concerned, up-regulation of *Hspb1* in three samples (CTVT-1, CTVT-2, CTVT-4) was observed. The higher expression is supposed to be associated with sequence variations and stage of the cancer. Meanwhile, two other samples (CTVT-3, CTVT-5) showed down-regulation (Fig. 3).

4.1.5. Perianal Adenocarcinoma Cases

Higher expression of *Hspb1* was also observed in two perianal adenocarcinoma cases (PAC-1, PAC-2), while one sample (PAC-3) showed down-regulation. Up and down-regulation within same type of cancer might be due to different types of mutations and/or variant stage of the cancer.

Overall 57% (n=12) of cancer samples showed down-regulations for *Hspb1*, while the remainder (n=9; 43%) of samples showed up-regulation. This down-regulation might be associated with loss of function mutations in the genomic landscapes of the gene. As for as single type of cancers are concerned, 40% of CTVT, 60% of mammary tumor, 50% of lymphoma and 100% of granuloma samples showed down-regulations, while 100% of SCC samples showed up-regulation.

4.2. Differential Expression of Tp53

Two-step singleplex RT-qPCR experiment was conducted on all cancer and normal samples with *Tp53* target and fold differences were calculated from the Ct values. Average ΔCt of (1.3) was obtained using the same approach with normal tissues (calibrator) by subtracting mean Ct of target *Tp53* from the average

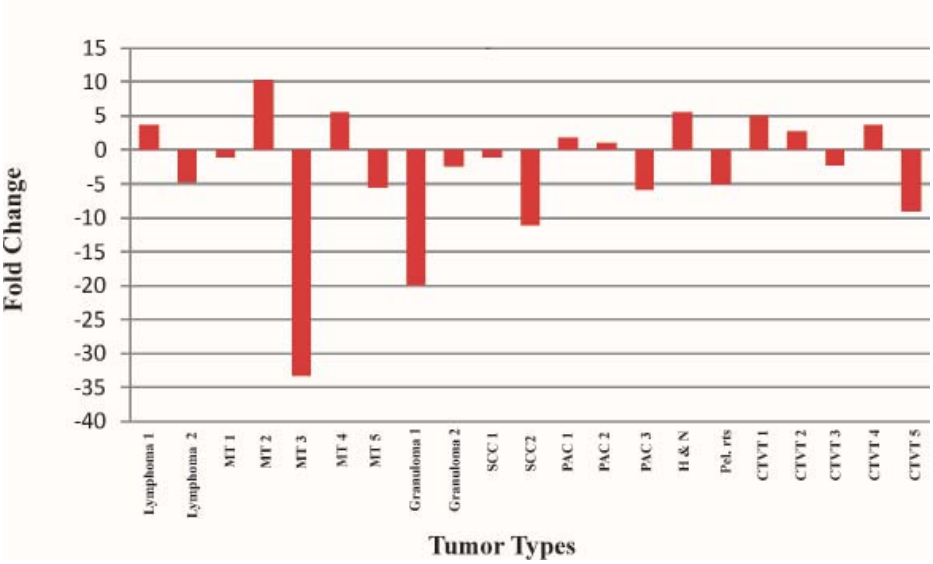

Figure 3. Expression of *Hspb1* gene different dogs tumors. Graphical representation of *Hspb1* expressions in *Canis familiaris*. X-axis = different types of cancer cases, Y-axis = differential expression (Fold change).

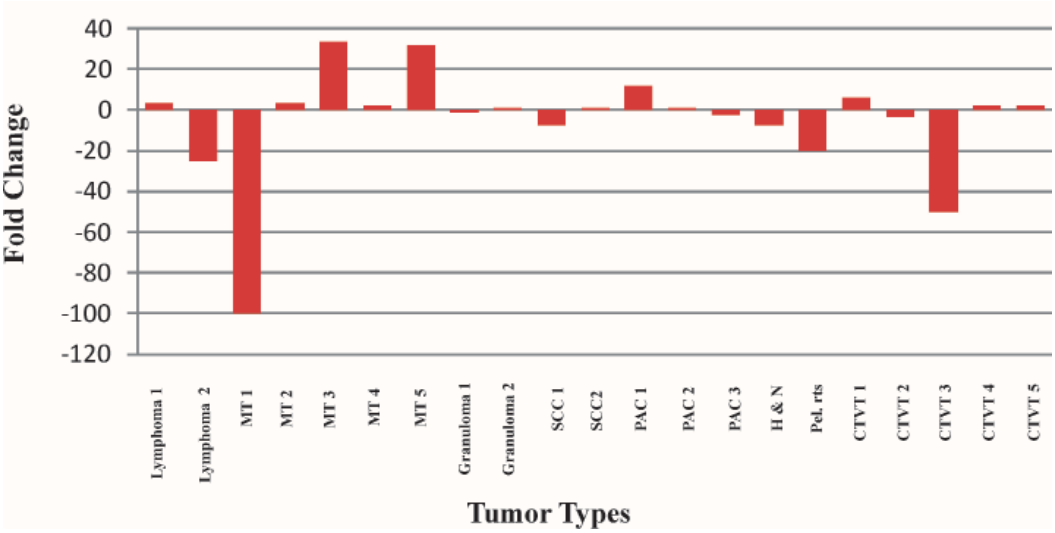

Figure 4. Expression of *Tp53* in different dogs tumors. Graphical representation of *Tp53* expression in *Canis familiaris*. X-axis = different types of cancer, Y-axis = differential expression (Fold change).

Ct of reference/endogenous *GAPDH* of normal dog tissues. All tumor samples showed ectopic expression of *Tp53*. This might be due to tumor growth stage or mutational changes in the genomic structure of the gene (Supplementary Table 2, Fig. 4).

4.2.1. Lymphoma Cases

Lymph-1 showed up-regulation, while Lymph-2 was substantially down-regulated. This might be due to different cancer stage and/or mutational chaos in this gene.

4.2.2. Mammary Adenocarcinoma Cases

Four mammary tumor samples (MT-2, MT-3, MT-4, MT-5) showed over expression with FD of 3.07, 33.31, 2.10 and 31.68, respectively as compared to MT-1 with FD of 0.01.

4.2.3. Granuloma Cases

One granuloma sample (Granu-2) showed up-regulation of *TP53* with FD of 1.32, while Granu-1 showed down-regulation with FD of 0.76.

4.2.4. Squamous Cell Carcinoma (SCC) Cases

Two SCC samples, (SCC-1) and H&N-SCC demonstrated down-regulation with FD of 0.13 and 0.11, respectively. In contrast, SCC-2 showed up-regulation of this gene with FD of 1.19.

4.2.5. Perianal Adenocarcinoma (PAC) Cases

Perianal adenocarcinoma samples (PAC-1, PAC-2) showed up-regulation of *TP53* expression with fold change of 11.88 and 1.04, respectively. While PAC-3 sample was found down-regulated with a fold change of 0.48. Head and neck SCC (H&N-SCC) and pelvic-warts samples were observed with down-regulated expression of *TP53*.

4.2.6. Canine Transmissible Venereal Tumor (CTVT) Cases

As far as the CTVT samples are concerned, three samples (CTVT-1, CTVT-4, CTVT-5) showed up-regulation of this gene with fold difference of 6.39, 2.27, 1.98, while CTVT-2, CTVT-3 showed down-regulation of this gene with FD of 0.28, 0.02, respectively.

Overall, 43% of cancers cases (n=9) showed down regulation of *TP53*, while 57% showed up-regulations of this gene. Ectopic expression of *Tp53* in all cancer samples proved the involvement of TP53 gene in dog cancers. (Supplementary Table 2, Fig. 4).

Generally, *TP53* was up-regulated in 57% of the cancers, possibly to suppress tumor formation. In contrast, *Hspb1* with anti-apoptotic properties was down-regulated in 43% of the cancers.

5. Discussion

Cancer is a disease of adversity in tissue growth regulation. Genes which regulate cell growth and differentiation must be altered to transform a normal cell into neoplastic tissue (26). Gene expression profiles can be used to predict actively dividing cells and how these cells are involved in different pathways. For instance, if breast cancer cells express higher levels of mRNA associated with a particular transmembrane receptor than normal cells do, it may indicate this receptor may play a role in breast cancer. A drug that interferes with this receptor

may prevent or treat breast cancer. Likewise in the process of drug development, one may perform gene expression profiling experiments, which may help to assess the drug's toxicity, perhaps by looking at changing levels in the expression of cytochrome *P450* genes, which may be a biomarker of drug metabolism (27). Gene expression profiling has become an important diagnostic prediction in cancer metastasis as well (28).

Expression of *Hsp27* is considered to be reliable predictive biomarkers of aggressive human cancers (29-32). However, few reports are available regarding their role in animal cancers. Here, *Hspb1* and *Tp53* genes were selected to test our hypothesis to assess the putative functions of these genes as cancer markers in dogs.

Hspb1 is one of the great chaperones that alters its expression during many physiological and diseased conditions. This gene was found up and down-regulated in different dog tumors in this study. This protein has anti-apoptotic and tumorigenic properties; its expression would be higher with tumor progression, because it has to provide the support to the newly dividing cancerous cells. It also resists to the regression of the cancerous cells. Thus, its expression may be higher at the regression stage of the tumor as compared to the initial or exponential phases, which augments its anti-apoptotic activity. Moreover, if *Hspb1* expression is high, it will sustain the cancerous tissues. So, this protein may also be used as the therapeutic target. On the other hand, if its expression being ceased in cancers, that can regress the tumor growth as well (33). A study was conducted on *Hspb1* to RP101 (Brivudine) showed an improved efficacy in chemotherapy of human pancreatic and rat cancers (34). In this study, both lymphoma samples (Lymph-1, Lymph-2) revealed altered expression of *Hspb1* (Fig. 3). Lymph-1 showed up-regulated expression of both *Hspb1* and *TP53*, while Lymph-2 showed down-regulation of both genes. Another interesting phenomenon of expression-dependency in both of these markers was observed that, *Tp53* gene is pro-apoptotic, while *Hspb1* gene harbors reciprocal functions. *Tp53* has the ability to repress the expression of anti-apoptotic genes (40), if its expression is being up-regulated due to any reason, So, this phenomena of gene expression dependency was observed in two of mammary tumor samples (MT-3, MT-5), which showed down-regulation of *Hsbp1* (anti-apoptotic) and up-regulation of *TP53*. Two of the CTVT samples (CTVT-2, CTVT-5) and SCC-2 and Granu-2 also showed the same trend.

Where both genes were upregulated in a sole sample, it may indicate cancer regression. However, its down-regulation may happen due to initial or exponential phase of the tumor which is quite rationale. Altered expression of *Hspb1* was also reported in neurodegenerative diseases, myopathies, asthma, cataracts and cancers (35).

As far as p53 protein expression is concerned, exclusion of this protein and loss of function was observed in all observed melanoma cell lines and almost 72% of canine melanoma tumors (38). Similarly, up-regulation of *Tp53* product was observed in the canine mammary tumor (39). Similarly in our study, four out of total five mammary tumor samples showed up-regulation for *Tp53*.

In case of other cancer samples where down-regulation of *TP53* gene was observed, loss of function and down-regulation coupled phenomenon can be speculated . To date, more than 15,000 tumor-associated *Tp53* alleles exist in human tumor cell genomes (40). Similarly, mutations in the error-correcting machinery of a cell might also cause that cell and its progenitor to pile errors up more rapidly. Furthermore, a mutation in signaling machinery of the cell might send error-causing signals to proliferate cells rather than to suppress cancerous cells and may be one of the reasons to lowering down the gene expression (41). *Tp53* and *Hspb1* combined ectopic gene expression might be useful as potential diagnostic and prognostic markers in the subject of cancers.

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References

1. Chu R, Sun T, Yang H, Wang D, Liao K, Chuang T, *et al*. Heat shock proteins in canine transmissible venereal tumor. *Vet Immunol Immunop*. 2001;**82**(1):9-21. DOI: 10.1016/s0165-2427(01)00327-0
2. Liu W, Ma Y, Huang L, Peng J, Zhang P, Zhang H, *et al*. Identification of HSP27 as a potential tumor marker for colorectal cancer by the two-dimensional polyacrylamide gel electrophoresis. *Mol Biol Rep.* 2010;**37**(7):3207-16. DOI:10.1007/s11033-009-9903-x
3. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek

M, Mesirov JP, *et al*. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*. 1999;**286**(5439):531-7. DOI: 10.1126/science.286.5439.531
4. Ciocca DR, Vargas-Roig LM. Hsp27 as a prognostic and predictive factor in cancer. Small Stress Proteins. In: *art of the Progress in Molecular and Subcellular Biology* book series (PMSB, volume 28) Springer; 2002. p. 205-18.
5. Attila Lorincz, Cancer markers. EP Patent 2,505,665; 2012.
6. Zoubeidi A, Gleave M. Small heat shock proteins in cancer therapy and prognosis. *Int J Biochem Cell Biol*. 2012;**44**(10):1646-56. DOI:10.1016/j.biocel.2012.04.010
7. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperone.* 2005;**10**(2):86-103. DOI:10.1379/csc-99r.1
8. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle.* 2006;**5**(22):2592-601.DOI: 10.4161/cc.5.22.3448
9. Romanucci M, Bastow T, Della Salda L. Heat shock proteins in animal neoplasms and human tumours-a comparison. *Cell Stress Chaperone.* 2008;**13**(3):253-62. DOI:10.1007/s12192-008-0030-8
10. Wahl G, Linke S, Paulson T, Huang L. Maintaining genetic stability through TP53 mediated checkpoint control. *Cancer Surv.* 1997;29:183-219.
11. Bensaad K, Vousden KH. p53: new roles in metabolism. *Trends Cell Biol*. 2007;**17**(6):286-91. DOI:10.1016/j.tcb.2007.04.004
12. Suzuki K, Mori I, Nakayama Y, Miyakoda M, Kodama S, Watanabe M. Radiation-induced senescence-like growth arrest requires TP53 function but not telomere shortening. *Radiat Res.* 155(1):248-253. 2001, DOI:10.1667/0033-7587(2001)155[0248:rislga]2.0.co;2
13. Vermooten M. Canine transmissible venereal tumor (TVT): a review. *J S Afr Vet Assoc*. 1987;**58**(3):147. DOI: 10.5580/a6a
14. Eze C, Anyanwu H, Kene R. Review of canine transmissible venereal tumour (TVT) in dogs. Nigerian Veterinary Journal. 2008;**28**(1):54-70. DOI:10.4314/nvj.v28i1.3544
15. Ganguly B, Das U, Das A. Canine transmissible venereal tumour: a review. Veterinary and comparative oncology. 2013.DOI: 10.5580/a6a
16. North SM, Banks TA. *Small Animal Oncology: An Introduction*.Saunders/Elsevier; 2009.
17. Withrow SJ, Vail DM. Withrow and MacEwen's small animal clinical oncology: WB Saunders Company; 2007.
18. Lester SC. *Manual of Surgical Pathology:* Elsevier Health Sciences; 2010.
19. Boom, Sol C, Salimans M, Jansen C, Wertheim-van Dillen P, Van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clinic Microbiol*. 1990;**28**(3):495-503.
20. Chomczynski p. RNA/DNA/Proteins extraction throug TRIzole reagents. 1987. Availablefrom:http://tools.lifetechnologies.com/content/sfs/manuals/trizol_reagent.pdf.
21. Hummon AB, Lim SR, Difilippantonio MJ, Ried T. Isolation and solubilization of proteins after TRIzol® extraction of RNA and DNA from patient material following prolonged storage. *Biotechniques*. 2007;**42**(4):467. DOI: 10.2144/000112401
22. Malek JA, Shatsman S, Akinretoye B, Gill J. Irreversible heat inactivation of DNase I without RNA degradation. *BioTechniques*. 2000;**29**(2):252-6. DOI: 10.2144/000112401
23. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. 2002;**148**(1):257-66.DOI: 10.1099/00221287-148-1-257
24. Murthi P, Fitzpatrick E, Borg A, Donath S, Brennecke S, Kalionis B. GAPDH, 18S rRNA and YWHAZ are Suitable Endogenous Reference Genes for Relative Gene Expression Studies in Placental Tissues from Human Idiopathic Fetal Growth Restriction. *Placenta*. 2008;**29**(9):798-801. DOI: 10.1016/j.placenta.2008.06.007
25. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*. 2001;**25**(4):402-8. DOI: 10.1006/meth.2001.1262
26. Croce CM. Oncogenes and cancer. New England Journal of Medicine. 2008;**358**(5):502-11.DOI:10.1056/nejmra072367
27. Magic Z, Radulovic S, Brankovic-Magic M. cDNA microarrays: identification of gene signatures and their application in clinical practice. *J BUON: J Balkan Union Oncol.* 2007;**12**:S39-44.
28. Nagaraja G, Kaur P, Asea A. Role of human and mouse HspB1 in metastasis. *Curr Mol Med*. 2012;**12**(9):1142-50. DOI: 10.2174/156652412803306701
29. Gress TM, Müller-Pillasch F, Weber C, Lerch MM, Friess H, Büchler M, *et al*. Differential expression of heat shock proteins in pancreatic carcinoma. *Cancer Res.* 1994;**54(**2):547-51.
30. Foster C, Dodson A, Ambroisine L, Fisher G, Møller H, Clark J, *et al*. Hsp-27 expression at diagnosis predicts poor clinical outcome in prostate cancer independent of ETS-gene rearrangement. *British J Cancer*. 2009;**101**(7):1137-44.DOI: 10.1038/sj.bjc.6605227
31. Zeng G-Q, Zhang P-F, Deng X, Yu F-L, Li C, Xu Y, *et al*. Identification of candidate biomarkers for early detection of human lung squamous cell cancer by quantitative proteomics. *Mol Cell Proteom.* 2012;**11**(6):M111. 013946.DOI: 10.1074/mcp.m111.013946
32. Norton JA, Weinberger PM, Waller JL, Merkley MA, Jackson LL, Dynan WS. Significance of HSPB1 expression in head and neck squamous cell carcinoma: a meta-analysis of published literatures. *Laryngoscope*. 2010;**120**(Suppl 4):S172. DOI: 10.1002/lary.21636
33. Lukacs KV, Pardo OE, Colston MJ, Geddes DM, Alton EW. Heat shock proteins in cancer therapy In: *Cancer Gene Therapy.* Springer; 2002. p. 363-8.
34. Heinrich J-C, Tuukkanen A, Schroeder M, Fahrig T, Fahrig R. RP101 (brivudine) binds to heat shock protein HSP27 (HSPB1) and enhances survival in animals and pancreatic cancer patients. *J Cancer Res Clinic Oncol.* 2011;**137**(9):1349-61.DOI: 10.1007/s00432-011-1005-1
35. Garrido, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle*. 2006;**5**(22):2592-601.DOI: 10.4161/cc.5.22.3448
36. Arrigo A-P, Simon S, Gibert B, Kretz-Remy C, Nivon M, Czekalla A, *et al*. Hsp27 (HspB1) and αB-crystallin (HspB5) as therapeutic targets. *FEBS letter*. 2007;**581**(19):3665-74. DOI: 10.1016/j.febslet.2007.04.033
37. Arnouk H, Merkley MA, Podolsky RH, Stöppler H, Santos C, Álvarez M, *et al*. Characterization of molecular markers

indicative of cervical cancer progression. *Proteom Clinic Appl.* 2009;**3**(5):516-27.DOI: 10.1002/prca.200800068
38. Koenig A, Bianco S, Fosmire S, Wojcieszyn J, Modiano J. Expression and significance of p53, rb, p21/waf-1, p16/ink-4a, and PTEN tumor suppressors in canine melanoma. *Vet Pathol Online*. 2002;**39**(4):458-72. DOI: 10.1354/vp.39-4-458
39. Haga S, Nakayama M, Tatsumi K, Maeda M, Imai S, Umesako S, *et al*. Overexpression of the p53 gene product in canine mammary tumors. *Oncol Rep.* 2001;**8**(6):1215-9. DOI: 10.3892/or.8.6.1215
40. Weinberg. *The biology of cancer*: Garland Science; 2013.
41. Merlo LM, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. *Nat Rev Cancer.* 2006;**6**(12):924-35. DOI: 10.1038/nrc2013