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Expression Profiling of microRNAs *hsa-222-3p, hsa-let-7b-5p, hsa-let-7f-5p* and their Putative Targets *HMGA1* and *CDKN1B* Genes in Canine Mammary Tumor

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MW, HMFY presented the concept and designed the study. HMFY conducted experiments and collected data. HMFY, RS and MW analysed and interpreted data. HMFY, SF, ARA, MT, RS, MR and MW wrote, reviewed and edited the manuscript.

Key words
Expression profiling, miRNAs, Canine, Mammary tumor, Target gene

ABSTRACT

Cancer is unregulated growth of cells that can spread to other part of body via blood circulation and lymphatic system. Mutations in genetic material can alter cell physiology, ultimately resulting in tumor. Like human cancers, dogs have relatively high incidence of cancers, relatively large body size and responses to cytotoxic and other therapeutics. Small noncoding RNA having length of 22 base pair (bp) are called micro-RNA (miRNA), that are processed by Dicer from precursors with a characteristic hairpin secondary structure. miRNAs can regulate gene expression at the post-transcriptional level by binding to the 3′ untranslated region (UTR) of target mRNAs. In this study dog mammary tumor samples were collected to investigate the expression level of miRNAs *hsa-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* and effect of their expression on the target genes (*HMGA1* and *CDKN1B*) of these miRNAs. Qiagen miScript Primer Assay based expression analyses revealed the over-expression of all three miRNAs in most of the studied canine mammary tumor samples compared to normal mammary tissue. Furthermore, this over-expression of tested miRNAs, down regulated their target genes in tumor samples compared to normal samples.

INTRODUCTION

MicroRNA (miRNA) was first discovered in *Caenorhabditis elegans* in 1993. It is also found in most eukaryotes, chief among them are humans (Perron and Provost, 2008). miRNAs are around 22 nucleotides long and perform as regulatory non coding RNAs. Discovery of RNA interference (RNAi) drastically increased the number of studies on miRNAs which was further sped up by the search for small endogenous RNAs of a similar type in various species (Fire *et al*., 1998; Treiber *et al*., 2019).

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miRNAs also play a major role in the regulation of post transcriptional gene expression via a mechanism of complimentary sequences and repression of target RNAs. These studies have established the importance of miRNAs in development, physiology and disease (Perron and Provost, 2008; Croce, 2009)

The miRNA let-7 (MIRLET7) is a family that controls growing effectiveness and diversity. Generally, the loss of let-7 is seen as a major contributor in oncogenesis through increase of target oncogenes and stemness factors, its targets include cell signaling pathways, the cell cycle and cell variation. It is characterized as a tumor suppressor. It was suggested that let-7 family take part in metastasis. Let-7a was seen to influence down regulation of CCR7 by targeting its 3' UTR that resulted in the down regulation of breast cancer cells capacity for invasion and migration. Other studies confirmed similar results as let-7a acted as a tumor suppressor in zebrafish embryo models via regulating the expression of RAS and HMGA2 oncogenes. Furthermore, decreased let-7a levels were related to increased RAS levels in lung squamous Carcinoma

(Johnson *et al*., 2005; Cunningham *et al*., 2010).

However, in certain albeit rare cases the Let-7 are known to be oncogenic in nature showing increasing route, attack, chemoresistance and also increased genes expression that are related with the development and metastasis of cancer. This makes this family a major potential target as a diagnostic and prognostic marker and even a candidate for cancer therapy (Chirshev *et al*., 2019). *hsa-let-7b-5p* and *hsa-let-7f-5p* are two such miRNAs that are part of the let-7 family which are seen to be fatal for humans as they are major contributors in cancer pathogenesis. *hsa-mir 222-3p* was seen to be overexpressed in patients with carcinoid lung tumors like *hsa-let-7b-5p* (Di Fazio *et al*., 2017).

Over the decades, many similarities have been identified between human and canine mammary tumors at molecular level. These similarities include incidence of tumor, age of onset and course of disease. From a clinical perspective tumor size, stage and lymph node invasion are seen to be mostly identical (Queiroga *et al*., 2011). As is the case in human females, the most commonly occurring spontaneous malignancy in female canine is mammary neoplasia (Tavasoly *et al*., 2013), and premalignant lesions are prevalent in canine mammary glands (Antuofermo *et al*., 2007).

In this study, our aim was to study the expression pattern of three microRNAs, *hsa-222-3p, hsa-let-7b-5p*, *hsa-let-7f-5p*, in canine mammary tumors and further to investigate the effect of their expression on two of their putative target genes (*HMGA1* and *CDKN1B*).

MATERIALS AND METHODS

Sample collection

Dog mammary tumor and normal tissues samples (10 each) were collected from University of Veterinary and Animal Sciences (UVAS) Pet Center, after informed consent of the pet owners, and were preserved in absolute ethanol and 10% formalin solution for expression and histopathological analyses, respectively.

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 (H and E) staining to confirm the malignancy, grading and staging as described earlier (Manzoor *et al*, 2017).

Total RNA isolation and quantification

Total RNA was extracted from tumor and normal tissue samples by using RNeasy tissue mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Quantification of RNA was conducted by Nano Drop 2000 (Thermo Fisher Scientific, Pittsburg, PA, USA).

RT-qPCR

Complementary DNA (cDNA) was amplified by using miScript Primer Assays (Qiagen, Hilden, Germany) and commercially available primers for *miR-222-3p* (MS00007609), *hsa-let-7b-5p* (MS00003122), *hsa-let-7f-5p* (MS00006489). *RNU6B* was run as reference miRNA using miScript Primer Assays (Qiagen, Hilden, Germany) for *RNU6B* (MS00029204). miScript II RT Kit (Qiagen, Hilden, Germany) was used to reverse transcribed miRNA-enriched RNA lysate. Primer 3 software was used to design primers for target genes (*HMGA 1* and *CDKN 1B*) of these miRNAs. Sequences of these genes were taken from ENSEMBLE Genome Browser (https://asia.ensembl.org/index.html) and *GAPDH* was used as a reference gene. All experiments were performed using RotorGene-Q (5-plex) instrument (Qiagen). For relative expression, *RNU6B*/ *GAPDH* normalized data of cancer (DMT) vs normal tissues (DNS) for each target miRNA/gene, was used to calculate ΔCt [ΔCt (Cancer) = Ct (*miRNAs*/*genes*) – Ct (*RNU 6B/GAPDH*)]. For fold change calculations, data from three technically replicated measurements were averaged and normalized to the internal *RNU 6B* control. Log 2-fold change values were calculated using following statistics:

ΔCt (Test) = Ct (Target) – Ct (Reference)

ΔCt (Cancer) = Ct (*miRNAs*/*genes*) – Ct (*RNU 6B/GAPDH*)

ΔCt (Calibrator) = Ct (Target) – Ct (Reference)

ΔCt (Normal) = Ct (*miRNAs*/*genes*) – Ct (*RNU 6B/GAPDH*)

ΔΔCt = ΔCt (Cancer) – ΔCt (Normal)

Fold Change $=2^{-\Delta\Delta Ct}$

Statistical analysis

Student t-test was applied on *RNU6B*-normalized relative expression data (cancer vs normal) on each of the target miRNAs and similarly on *GAPDH*-normalized relative expression data on both target genes as well. *P* value <0.05 was considered as significant.

GraphPad prism analysis

Expression data of *hsa-mir-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* was analyzed by using GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/) for dog tumor and normal mammary tissue samples.

RESULTS

Histopathological examination

Histopathological analyses of representative dog mammary tumors showed varied population of cells with enormous nucleus and prominent mitotic characteristic (Fig. 1a). Numerous key regions were marked by using different magnifications, indicating the presence of fibroblasts and collagen fibers as shown in Figure 1.

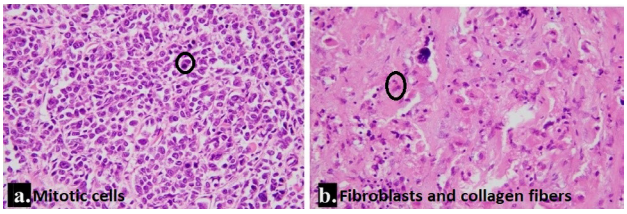

Fig. 1. Histopathological analysis of dog mammary tumor. (a) Representative dog mammary tumors showed varied population of cells with enormous nucleus and prominent mitotic characteristic. (b) Numerous key regions indicating the presence of fibroblasts and collagen fibers.

Expression of miRNAs

miRNAs *hsa-mir-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* were amplified to check their expression in dog mammary tumors. *hsa-mir-222-3p* miRNA showed up-regulation in five tumor samples (DMT01, DMT02, DMT04, DMT06 and DMT09) while remaining five (DMT03, DMT05, DMT07, DMT08 and DMT10) did not show statistically significant up-regulation (Fig. 2A) so a not-significant *p*-values of 0.059 was obtained (Fig. 2B), *hsa-let-7b-5p* miRNA showed eight samples (DMT02, DMT03, DMT04, DMT05, DMT06, DMT07, DMT09 and DMT10) as up-regulated while remaining two (DMT01 and DMT08) were not significantly up-regulated (Fig. 2C) with a significant *p*-value of 0.039 (Fig. 2D), similarly, *hsa-let-7f-5p* miRNA showed up-regulation in six samples (DMT02, DMT04, DMT05, DMT06, DMT08 and DMT09) whereas, remaining four samples (DMT01, DMT03, DMT07 and DMT10) were not significantly up-regulated (Fig. 2E) with not-significant *p*-values of 0.06 (Fig. 2F) which were calculated by student t-test applied on all. Figures 2A, 2C and 2E show the expression of miRNAs in terms of fold change in dog mammary tumor samples (DMT).

Figure 3 shows comparison of miRNAs expression level, in terms of fold change, in tumor samples (DMT). Overall, all three types of miRNA show upregulation in most of the studied dog mammary tumor samples (Fig. 4).

Comparative expression analysis of subjected miRNAs in dog normal (DNS) and mammary tumor (DMT) samples is shown in Figure 5. *hsa-let-7b-5p* has overall more expression in mammary tumor tissues as well

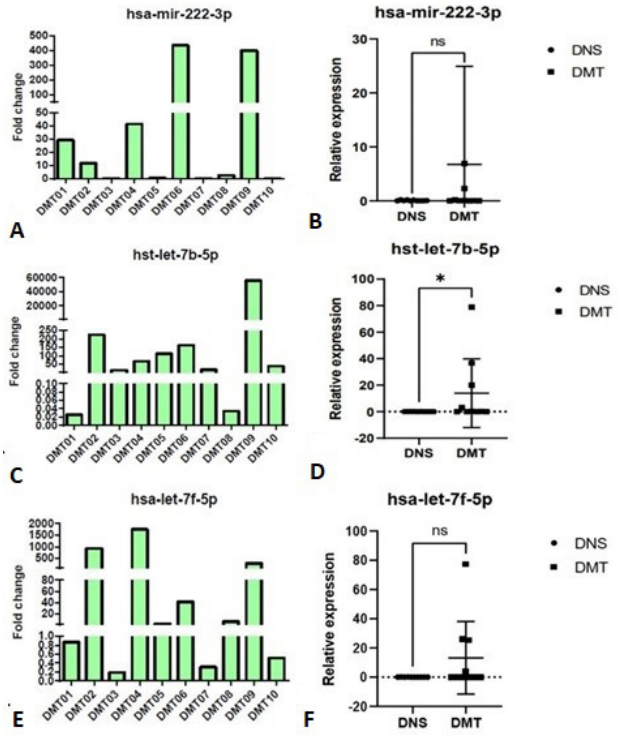

Fig. 2. Expression level of *hsa-mir-222-3p* (A, B), *hsa-let-7b-5p* (C, D) and *hsa-let-7f-5p* (E, F) miRNA in canine mammary tumor was measured using RT-qPCR. C, E data from three technically replicated measurements were averaged and normalized to the internal *RNU6B* control. Log 2-fold change values were calculated for 10 dog mammary tumor samples (DMT). D, F, Relative expression of for all the three *hsa-mir-222-3p* miRNA using *RNU6B* normalized data of cancer (DMT) vs normal tissues (DNS) to calculate ΔCt.

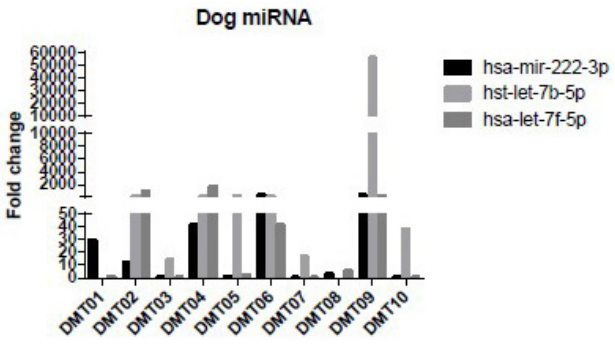

Fig. 3. Comparison of *hsa-mir-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* expression (fold change) in dog mammary tumor samples (DMT) using RT-qPCR.

as supported by the statistical hypothesis testing with significant *p*-value but with caveat emptor of the small sampled populations and of not very strongly association *p*-value.

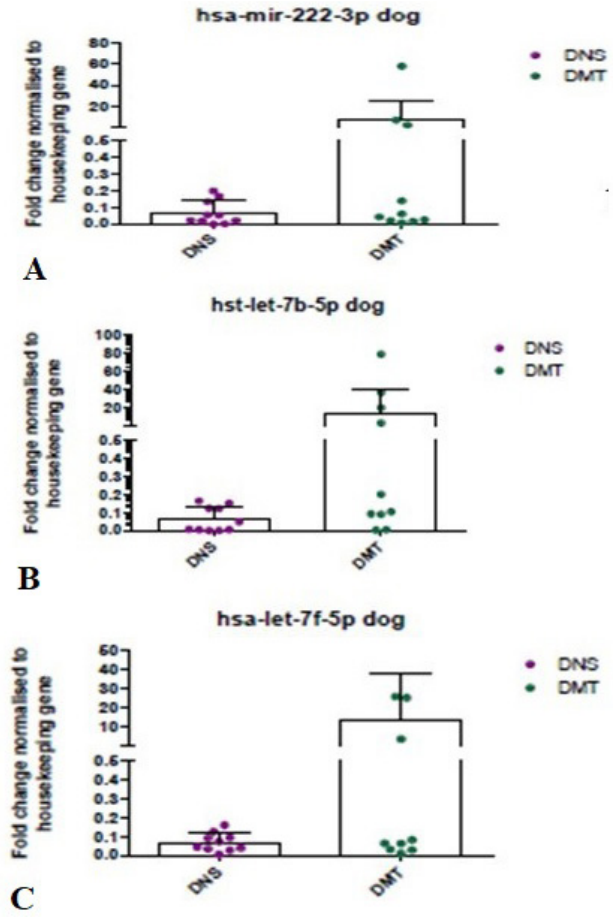

Fig. 4. Expression data of miRNAs (fold change), in dog tumor (DMT) and normal mammary tissue (DNS) samples, was analyzed using GraphPad Prism software. (a) *hsa-mir-222-3p*, (b) *hsa-let-7b-5p* and (c) *hsa-let-7f-5p*.

Similarly, Table I shows the expression (fold change) of different miRNAs in analyzed mammary tumor samples and their expression intensity. *hsa-let-7b-5p* has highest expression in canine mammary tumor (DMT) samples.

Expression of HMGA1 and CDKN 1B in dog mammary tumors

Expression of *HMGA1* and *CDKN1B* genes was analyzed in dog normal (DNS) and mammary tumor (DMT) samples. The expression of *HMGA1* gene was found down-regulated in all tumor samples (DMT) and strongly supported by the significant *p*-value of 0.000004 (Fig. 6A). Similarly, *CDKN1B* gene expression was also down-regulated in all tumor samples (DMT) and very strongly supported by significant *p*-value of 6.8×10^{-08} (Fig. 6B).

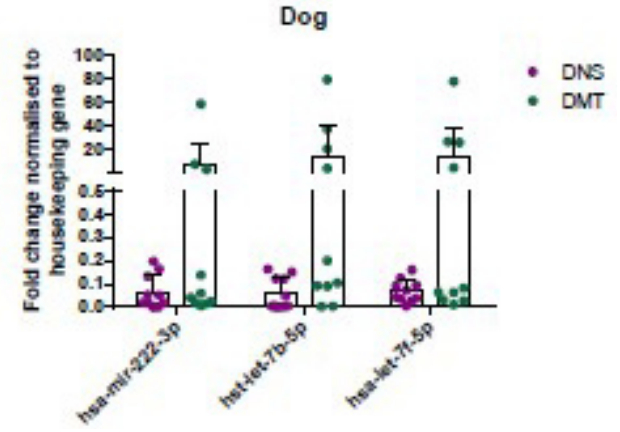

Fig. 5. Comparison of *hsa-mir-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* expression in dog mammary tumor samples (DMT) using using GraphPad Prism software.

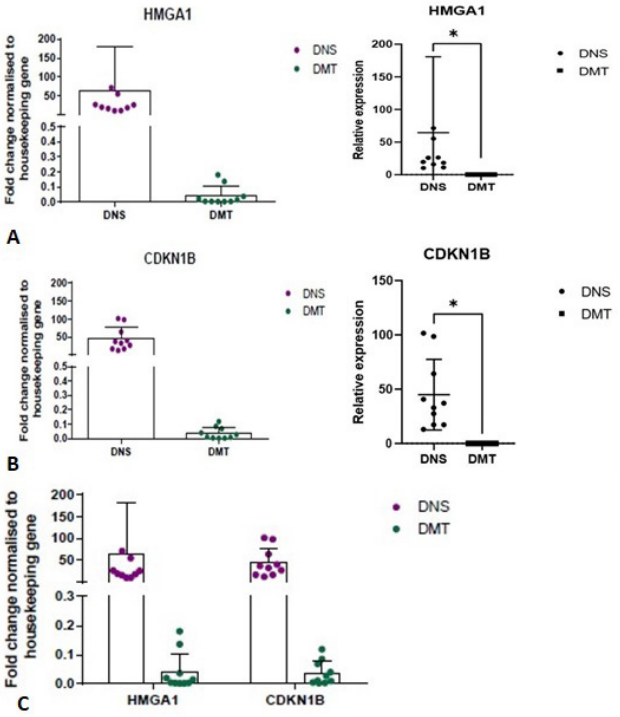

Fig. 6. Expression data of target genes, in dog tumor (DMT) and normal mammary tissue (DNS) samples, was analyzed using GraphPad Prism software. (A) Fold change of *HMGA1* gene in dog mammary tumor. (B) Fold change of *CDKN18* gene in dog mammary tumor. (C) Comparison of *HGMA 1 and CDKN 1B* genes expression in dog mammary tumor samples (DMT) using using GraphPad Prism software.

Table I. Overview of three miRNAs and their fold changes in canine mammary tumor samples.

Tested samples	Fold change*	Up-regulation intensity
hsa-mir-222-3p		
DMT 01	29.13	Moderately
DMT 02	11.66	Moderately
DMT 03	0.37	slightly
DMT 04	41.45	Highly
DMT 05	0.84	slightly
DMT 06	436.55	Highly
DMT 07	0.35	slightly
DMT 08	2.76	Moderately
DMT 09	399.86	Highly
DMT 10	0.30	slightly
hsa-let-7b-5p		
DMT 01	0.03	slightly
DMT 02	224.41	Highly
DMT 03	13.83	Moderately
DMT 04	66.41	Highly
DMT 05	110.41	Highly
DMT 06	162.39	Highly
DMT 07	16.60	Moderately
DMT 08	0.03	slightly
DMT 09	55749.32	Highly
DMT 10	37.88	Moderately
hsa-let-7f-5p		
DMT 01	0.87	slightly
DMT 02	929.30	Highly
DMT 03	0.19	slightly
DMT 04	1742.17	Highly
DMT 05	2.35	slightly
DMT 06	41.16	Highly
DMT 07	0.31	slightly
DMT 08	5.71	slightly
DMT 09	272.48	Highly
DMT 10	0.52	slightly

Highly up-regulated: FC >40, Moderately up-regulated: FC >10 and <40, slightly up-regulated: FC >0 and <10. * Data from three technically replicated measurements were averaged and normalized to the internal *RNU6B* control. Log 2-fold change values were calculated for ten dog mammary tumor samples (DMT).

GraphPad Prism software was used for combined representation of dog normal (DNS) and mammary tumor tissue (DMT) samples for better understanding of data displayed in Figure 6C.

Table II represent the down-regulation values and intensity of down-regulation in miRNA target genes.

Table II. Comparison of expression down-regulation *HGMA 1 and CDKN 1B* genes in dog mammary tumors.

Samples	Fold change*	Down-regulation intensity
HGMA1		
DMT01	0.00224	Slightly
DMT02	0.01706	Slightly
DMT03	0.00022	Slightly
DMT04	0.00017	Slightly
DMT05	0.00001	Slightly
DMT06	0.00029	Slightly
DMT02	0.00769	Slightly
DMT08	0.00040	Slightly
DMT09	0.00044	Slightly
DMT10	0.00756	Slightly
CDKN1B		
DMT01	0.00058	Slightly
DMT02	0.00129	Slightly
DMT3	0.00027	Slightly
DMT04	0.00024	Slightly
DMT05	0.00277	Slightly
DMT06	0.00013	Slightly
DMT07	0.00331	Slightly
DMT08	0.00205	Slightly
DMT09	0.00128	Slightly
DMT10	0.01075	Slightly

Highly down-regulated: FC >40, Moderately down-regulated: FC >10 and <40, Slightly down-regulated: FC >0 and <10. *Data from three technically replicated measurements were averaged and normalized to the internal *GAPDH* control. Log 2-fold change values were calculated for ten dog mammary tumor samples (DMT).

DISCUSSION

One of the most common disease in dogs is cancer although some breeds of dogs have high risk of cancer types and one of them is mammary tumor (Manzoor *et al*., 2019). In dog and human population mammary tumor occurs spontaneously (Egenvall *et al*., 2005). As both human and dog share similar environment hence epidemiology and progression of cancer is similar in both of them. Human and dog mammary tumor initiate from epithelial tissue and in both species are hormone-dependent (Misdorp, 1999). To understand the human breast cancer naturally-occurring canine mammary tumor plays central role because both species have same etiology, histopathologic division and disease pathogenesis (Gray *et al*., 2020). Fish *et al*. (2020) reported that circulating

miRNAs could be used as a biomarker for the detection of canine mammary tumor (CMT). The findings of Kim *et al*. (2020) explain how the cross-species oncogenic similarities help to comprehend pathogenesis mechanisms of breast cancer progression as well as give an insight for exact diagnostics and therapeutics of breast cancer in domestic dogs.

Aim of the present study was to understand the role of *hsa-miR-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* miRNAs in canine mammary tumor and to discover it as potential biomarkers for the early diagnosis of mammary tumor in future by confirming it with next generation sequencing (NGS) and many other advanced techniques. To this end, expression of *hsa-mir-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* miRNAs and their targeted genes (*HMGA 1* and *CDKN 1B*) were analyzed for tumor positive samples which was confirmed by histopathological studied, all miRNAs showed up-regulation and their target genes were down-regulate in tumorous samples. These results are in comparison with a human lungs cancer study which reported the significant overexpression of *hsa-mir-222-3p,* and *hsa-let-7f-5p* and downregulation of *hsa-let-7b-5p* in majority of studied tumor samples, resulting in significant overexpression or stable level of *CDKN 1B* in majority of the samples and stable level or downregulation of *HMGA2* gene (Di Fazio *et al*., 2017). Similarly, another study by Wang and Zhai (2020) validate our results describing the *mir-222-3p*/*p27kip1* axis. They found that high intensity focused ultrasound (HIFU) treatment downregulated the *mir-222-3p* resulting in overexpression of *p27kip1* and consequently apoptosis was activated. Whereas, overexpression of *mir-222-3p* restored cell proliferation and deactivated apoptosis, which was overturned by overexpression of *p27kip1* in breast cancer cells.

The results of the study are correlated to our unpublished results in which expression of same miRNAs as well as their target genes were analyzed in humans (manuscript in preparation). In this study, all these miRNAs were found up regulated and their target genes were found downregulated in breast tumor samples compared to normal tissue samples.

hsa-let-7b-5p and *hsa-let-7f-5p* miRNAs have higher expression in mammary tumor which was calculated by comparing it with its reference gene *RNU 6B* and found fold change. Up-regulation of miRNAs have been observed in both subjected miRNAs in dog mammary tumor. We also checked its target gene response in both tumorous and normal tissues samples; in case of tumorous samples *HMGA 1* gene was down-regulate in all positive samples as mentioned in Figure 6, whereas in case of normal tissues the expression of these subjected miRNAs suppressed and their target gene is upregulating and perform its functioning.

We also studied expression of *hsa-miR-222-3p* miRNA in dog mammary tumor, expression of this miRNA is also up-regulated in tumorous samples like Let-7 family and its target gene *CDKN 1B* is suppressed in all cases whereas in normal samples expression of this miRNA is suppressed and its targeted gene is up-regulated.

CONCLUSIONS

In this study, dog mammary tumor samples were collected to investigate the expression level of *hsa-222-3p, hsa-let-7b-5p* and *hsa-let-7f-5p* miRNAs and effect of their expression on the target genes (*HMGA1* and *CDKN1B*) of these miRNAs. Qiagen miScript Primer Assay based expression analyses revealed the over-expression of all three miRNAs in canine mammary tumor samples compared to normal mammary tissue samples. Furthermore, this over-expression of tested miRNAs, down regulated their target genes in in most of the studied tumor tumor samples compared to normal samples. This study might be helpful to develop biomarkers for diagnosis of dog mammary tumor by using different advance techniques including NGS.

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IRB approval

The study was approved by Advanced Studies and Research Board (ASRB), UVAS (DAS/575-06.03.2019).

Ethical statement

During the samples collection, animals were handled according to the approved guidelines provided by Ethical Institutional Review Board of University of Veterinary and Animal Sciences, Lahore.

Data availability

There is no data submitted to any database and no supplementary files available. All figures and tables are available in the manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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