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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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).

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 oncopgenes 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
Total RNA isolation and quantification

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 (Chirichev 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., 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 tumors 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 RNaseasy 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 (HMGA1 and CDKN1B) 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 (DTC) vs normal tissues (DNS) for each target miRNA/gene, was used to calculate ∆Ct [∆Ct (Cancer) = Ct (miRNAs/gene) – 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:

\[
\Delta Ct = Ct (Target) – Ct (Reference)
\]

\[
\Delta C = Ct (Cancer) – Ct (Normal)
\]

\[
\text{Fold Change} = 2^{-\Delta C}
\]

**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.

![Histopathological analysis of dog mammary tumor.](image)

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.
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.

regulated in all tumor samples (DMT) and very strongly supported by significant \( p \)-value of \( 6.8 \times 10^{-08} \) (Fig. 6B).

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 CDKN1B 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-

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 **CDKN1B** gene in dog mammary tumor. (C) Comparison of **HMGA1** and **CDKN1B** 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.

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

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).

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

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

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 HMGAl2 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 HMGAl 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 (HMGAI 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.

ACKNOWLEDGEMENTS

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Funding

The study received no external funds.

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.
REFERENCES


