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ORIGINAL ARTICLE

CEA, CA 15-3, and miRNA expression as potential biomarkers in canine mammary tumors

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Abstract The most often detected tumor in intact bitches is mammary tumors and represents a significant clinical problem throughout the world. Mammary neoplasms in canine have heterogeneous morphology, so the choice of the most appropriate biomarker is the biggest challenge in CMT detection. We performed a retrospective analysis and evaluated the canine cancer antigens and miRNA expression profiles as potential biomarkers. Sixty dogs based on histological examination divided into three groups, viz., dogs with a benign mammary tumor, malignant mammary tumor, and control/healthy. The CA 15-3 was found more sensitive than CEA but detection of both will increase sensitivity. miR-21 expression differed significantly in all three groups. miR-29b expression differed significantly between the control and benign group and control and malignant group. The miR-21 overexpression and miR-29b downregulation with CMT are associated with clinical stage and can be used as non-invasive diagnostic and prognostic biomarkers. Hence, evaluation of CA 15-3 along with CEA would be a non-invasive technique for detecting canine mammary tumors. Evaluation of deregulated circulating miR-21 could be a valuable prognostic marker for early detection of mammary tumors in canines while miR-29b can add sensitivity in the detection of the canine mammary tumors if evaluated with miR-21.

Keywords CEA · CA 15-3 · canine · mammary tumor · miRNA

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Abbreviations

CEA	Carcinoembryonic antigen
CA 15-3	Cancer Antigen 15-3
mRNA	Messenger RNA
miRNA	MicroRNA
CMT	Canine mammary tumor
FNA	Fine needle aspirate
ROC curve	Receiver operating characteristic curve
HIF1a	Hypoxia Inducible Factor 1-Alpha
VEGF	Vascular endothelial growth factor
EMT	Epithelial-mesenchymal transition

Introduction

A canine mammary tumor (CMT) is the most often detected tumor in intact bitches. CMTs are the second most commonly occurring neoplasms in canines after skin tumor; therefore, CMTs represent a significant clinical problem throughout the world (Kaszak et al. 2018). Certain proteins are reported to be differentially expressed and secreted in serum at the onset of mammary tumors. The worldwide-accepted definition of tumor markers was framed which includes all the substances that are synthesized and secreted by tumor cells, which can be determined and quantified by non-invasive techniques (Luthgens, 1989). Thus, tumor markers are potentially pathognostic biomarkers for the diagnosis of early stages of cancer.

CMTs have heterogeneous morphology and biology which makes it difficult to choose the most appropriate biomarker for its diagnosis. The tumors are usually noticed by owners when there are visible macroscopic changes in the mammary gland. Determination of tumor biomarker for CMT can be a milestone for early diagnosis of the disease in dogs which can help evaluate the progress of the disease and its response to chemotherapy (Kaszak et al. 2018). Serum tumor markers play an important role in screening, early diagnosis of the disease, and its recurrence, and help in deciding the treatment of many malignancies (Dai et al. 2013; Incoronato et al. 2014). Canine carcinoembryonic antigen (CEA) and cancer antigen (CA 15-3) are the two most widely used serum tumor markers being used in the clinic for the diagnosis of human breast cancer for more than 30 years (Shao et al. 2015). Studies have shown that preoperative CEA levels combined with CA 15-3 levels may provide useful information for the diagnosis and treatment of breast cancer (Lee et al. 2013; Pedersen et al. 2013). A higher incidence of CMTs is a motivating factor for scientists to develop better screening and diagnostic tools for the disease in dogs. Neoplasms of the canine mammary glands are often considered a late endpoint of carcinogenesis, and treatment is effective only when the diagnosis is made at an early stage (Pandey, 2016). Currently, CMTs are diagnosed mainly based upon histopathological examination of tissue samples which involves a disadvantageous procedure of anesthesia or sedation for the collection of bioptic tissue. Moreover, FNA is much less invasive and usually does not require any sedation or anesthesia but it has less accuracy than surgical biopsy. Non-invasive serological techniques, which quantify tumor-specific serum markers, are available, but a single biomarker may not be sufficient enough for pinpoint diagnosis and prognosis of CMT (Pandey, 2016). Studies showed that some miRNAs can regulate cellular differentiation, proliferation, and apoptosis processes that can be important in cancer intensification. Several miRNAs from the tissues have been reported, and their expression profiles may be used as the potential biomarkers for the diagnosis, prognosis, and therapy. The discovery of the roles of the miRNAs in developing breast cancer may offer new opportunities for the development of novel strategies for diagnosing and treating this type of malignancy (Khalighfard et al. 2018). Thus, research on the mammary tumors in canines needs to be extended to the panel of tumor markers by adding new markers, which helps in diagnostic and therapeutic purposes. Bulkowska et al. (2017) and von Deetzen et al. (2014) have reported more than 300 miRNA expression profiles. Many similar oncogenes were found for human breast cancer and canine mammary carcinoma, for instance, oncogenic microRNAs (Boggs et al. 2008). miRNAs are of two types: oncogenic miRNA (oncomiR) that inhibits the suppressor gene of tumor and tumor suppressor miRNA which inhibits the oncogenic gene expression (Fu et al. 2011). Both types of miRNA function as a biomarker and may be used for miRNA gene therapies. Each miRNA gene has different targets at each phase of carcinogenesis (George and Mittal, 2010). Alteration in miRNA expression is used for the early detection of disease and intervention of the progress of the disease.

miR-21 is one of the best evaluated and most significantly upregulated miRNAs in human breast cancer, and dysregulation in the expression of miR-21 has been associated with tumor advancement and poor prognosis. Potential targets of miR-21 are those genes that code for Bcl-2, tropomyosin 1 (TPM1), phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4), and maspin (Meng et al., 2007, Zhu et al. 2007, Frankel et al. 2008, Zhu et al. 2008).

miR-29b, as a miR-29 family member, is a fundamental regulator of epithelial-mesenchymal transition (EMT) event, which is concerned with cancer metastasis and resistance to chemotherapy. miR-29b tempers many target genes, such as the DNMT family (Sandhu et al. 2012, Sandhu et al. 2014), oncogenes (Park et al. 2009, Wang et al. 2015), and tumor suppressor genes (Langsch et al. 2016, Zhu et al. 2016). The miR-29

family contributes to epigenetic regulation in the development of a tumor and primordial germ cell by targeting TET1, which leads to global DNA hypermethylation (Takada et al. 2009, Taylor et al. 2016). Low expression of miR-29b is positively associated with larger tumor size and advanced cancer stage (Shinden et al. 2015). miR-29b affects breast cancer proliferation and metastasis by its targeting gene TET1, which regulates EMT-related gene ZEB2 by binding to its promoter and demethylating CpG islands (Wang et al. 2017).

An ideal marker for tumor diagnosis should have two characteristics: first, its measurable concentration in blood would be present only after the cells that produce it transform into malignant, and second, the detection of these markers would permit conclusions as to the tumorous site. Despite worldwide constant efforts and after so many years of their discovery, there is no such tumor marker which exists in the strict sense, i.e., marker having a specificity of 100% (undetectable in healthy and benign patients) with 100% sensitivity (always detectable in malignancy even in early stages of a tumor) (Prskalo et al. 2015).

Therefore, this study aimed to evaluate CEA, CA 15-3, and miRNA expression as biomarkers in canine mammary tumors.

Material and methods

Source of experimental animals

The dogs suspected to have CMT were presented to Veterinary Clinical Complex. Criteria applied for inclusion of animals under the study were (a) female dogs of any age or breed, (b) dogs either neutered or unneutered (no estrus signs) with naturally occurring mammary gland tumors, and (c) dogs having mammary tumors either primary or recurred. The dogs having swelling of non-tumorous origin, viz., due to thalitis, abscess, or/and inflammation of the mammary gland were excluded. All dogs included in this study were non-pregnant.

Grouping of experimental animals

A total of 60 female dogs were included in the study. The animals selected were based on the histopathological examination for confirming CMTs. Based on the type of the mammary tumor, the animals were divided into three groups (20 each), viz., group I: dogs with benign tumors and group II: dogs with a malignant tumor. Twenty healthy female dogs were selected as the control group.

Anamnesis and history taking

Information related to the breed, age, sex of the animal, neuter status, reproductive history, and time duration since growth appeared, and clinical signs were recorded from the owner/representative of the animal. Clinical observations, viz., location, size, mammary gland involvement, and healing tendency, were carefully recorded.

Collection, preservation, and processing of clinical samples

Serum samples

Blood was collected in dry sterilized vacutainers without anti-coagulant. The serum was separated on the centrifugation of the clotted blood at 3000 rpm for 15 min. Upon separation, serum samples were stored in RNase and DNase free microcentrifuge tube in duplicate at − 80 °C until further processing with only one freeze-thaw cycle of each sample.

Tumor tissue samples

CMTs were surgically excised and collected aseptically immediately. The excised CMTs were observed for size, location, teat (s) involvement, etc. and the observations were recorded. The representative pieces (1.5 × 1.5 cm) of the CMTs were fixed in 10% neutral buffered formalin (NBF) for 48 h and then were subjected to histopathological study.

Histopathology of tumor tissues

The formalin-fixed CMTs were then paraffin-embedded (58–60 °C) using a routine dehydration process using alcohol and xylene. The paraffin-embedded CMT tissues were sectioned at 5-μm thickness, and the slides were then subjected to standard hematoxylin and eosin staining (H & E staining). Prepared slides were examined under the microscope.

Estimation of CEA

The serum CEA was estimated by using Canine Carcinoembryonic Antigen (CEA) ELISA kit (Bioassay Technology Laboratory, Shanghai, China, Cat. # E0157Ca). The sensitivity of the CEA assay was 5.18 ng/L with a standard curve range from 10 to 4000 ng/L. The intra-assay and inter-assay precision (CV) was < 8% and < 10%, respectively.

The mean optical density (OD value) for each standard, control, and an unknown sample was calculated. The CEA concentration of unknown samples was determined by matching their mean OD readings with the corresponding CEA concentration. The mean absorbance of standard, control, and unknown samples was utilized after blank subtraction.

Estimation of CA 15-3

The serum CA 15-3 was estimated by using Canine Carbohydrate Antigen 15-3 ELISA kit (Bioassay Technology Laboratory, Shanghai, China, and Cat. # E0156Ca). The sensitivity of the CA assay was 0.021 kU/L with a standard curve range from 0.05 to 19 kU/L. The intra-assay and inter-assay precision (CV) was < 8% and < 10%, respectively.

The mean optical density (OD value) for each standard, control, and an unknown sample was calculated. The CA 15-3 concentration of unknown samples was determined by matching their mean OD readings with the corresponding CA 15-3 concentration. The mean absorbance of standards and unknown samples were utilized after blank correction.

miRNA (miR-21 and miR-29b) expression

Isolation of miRNA

Isolation of miRNA from serum was done using miRNeasy® serum/plasma kit (QIAGEN GmbH, Hildane, Germany, Cat.#217184) and the instructions provided by the manufacturer. Frozen serum samples were thawed before DNA isolation and centrifuged for 3 minutes at ≥ 11,000×*g* to remove residual cells, cell debris, and particulate matter. The supernatant was used for miRNA isolation.

Reverse transcription of miRNA

The synthesis of cDNA from miRNA was done by using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio Inc., Shiga, Japan, Cat.# 6110A) and the instructions provided by the manufacturer.

qRT-PCR

Quantitative expressions of miRNAs, namely, miR-21 and miR-29b, were studied using SYBR green real-time PCR (qRT-PCR) assay. RNU6b was used as a reference gene for the miRNA expression studies. The selection of appropriate reference genes as normalizers for the relative quantification of miRNA expression levels is required to avoid erroneous results and to improve the comparability of miRNA expression level data among studies. The primer sequences for respective miRNAs published elsewhere (Yan et al. 2008; Hou et al. 2017) were used.

SYBR green real-time PCR (qRT-PCR) was performed using CFX96™ real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and commercially available TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara BIO INC., Shiga, Japan, Cat. #RR820A) master mix kit. The assay was selected as a comparative experiment between a control and a test group of dogs.

Standardization of qPCR

The miRNA concentration variability per sample was adjusted to the same concentration for all the samples for accurate predictions of its expression. The optimization parameters like concentration of miRNA input and housekeeping reference gene were carried out. Initially, constant serum volume (200 μL) was taken for isolation of miRNA and downstream volume adjusted to 5 μL for cDNA synthesis in qRT-PCR.

miR-21

The cycling conditions were 95 °C for 10 min followed by a cycling program of 95 °C for 15 s, and 65 °C for 60 s for 40 cycles. RT-negative PCR reactions were also performed for the respective miRNA to ensure the complete absence of genomic DNA that contains no cDNA.

miR-29b and RNU6b

The cycling conditions for both the miRNAs were 95 °C for 3 min followed by a cycling program of 95 °C for 15 s, and 72 °C for 60 s for 40 cycles. RT-negative PCR reactions were also performed for the respective miRNA to ensure the complete absence of genomic DNA that contains no cDNA.

Expression assay

Gene expression in the serum samples used in this study was determined concerning RNU6B as a reference gene (Boggs et al. 2008). The changes in gene expression in the serum samples of dogs having tumor were compared with gene expression in the serum samples of normal/healthy dogs. C_T values of technical duplicates of each sample pulled for data analysis and the average C_T value for each sample was taken for the calculation. For the control group, the average C_T value was taken of all 20 samples for further ΔΔCT calculation. The relative difference in the expression level of a target miRNA in test samples compared to the control samples was determined using the $2^{-\Delta\Delta C}{}_T$ method (Yuan et al. 2006).

First, the C_T of the target gene was normalized to the C_T of the reference gene for both the test sample and control sample as follows:

$$\Delta C_{T(test)} = C_{T(target,test)} - C_{T(ref,test)}$$

$$\Delta C_{T(control)} = C_{T(target,control)} - C_{T(ref,control)}$$

Second, the ΔC_T of the test sample was normalized to the ΔC_T of the control as follows:

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(control)}$$

Finally, the expression ratio was calculated as follows:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

Statistical analysis

The data were analyzed by a completely randomized design (CRD) using WASP 2.0 (Web Agri Stat Package), ICAR, Goa. For calculation of sensitivity and specificity of CEA and CA 15-3, AUC-ROC curve analysis was used. The gene expression level of control samples was compared to cancerous samples using ANOVA. To study the relationship between serum CEA, CA 15-3, miR-21, and miR-29b, the correlation coefficients were calculated.

Results

Out of the 40 dogs with CMTs, 10% were below 5 years of age, 70% were in between 5 and 10 years of age, and 20% of dogs were above 10 years of age. The average age of animals in the control and test group was 6.75 ± 1.80 and 8.25 ± 2.34 years, respectively. Thus, the majority of animals bearing mammary tumors belonged to 5–10 years of age. A total of 72.50% of the animals included in the study belonged to non-descript breed, 17.5% of Labrador Retriever, and 2.5% each of Pomeranian, Beagle, Lhasa Apso, and Boxer. The higher percentages of mammary tumors observed in crossbreeds or ND are similar to those reported by Malicka et al. (1996).

Out of the total 40 dogs with the mammary tumor, the duration of suffering in 13 animals was less than 3 months and 20 animals were suffering from 3 to 6 months whereas the duration of illness in 7 of the animals was more than 6 months. The higher incidence of animals under the 3–6 months category could be because of the insignificance in the size of the tumor at an early stage of development. Mostly in long hair/furry dogs as compared to short hair/nonfurry dogs, the mammary tumors go unnoticed, as they do not cause major symptoms in the early stages. About 90% of animals were found intact in contrast to 10% of animals in which ovariohysterectomy has been performed before the incidence of mammary gland tumors. The mammary gland tumors are hormone-dependent. Most of the affected animals were intact. The higher incidence of mammary gland tumors in intact dogs is due to the presence of hormones, i.e., estrogen and progesterone, which is an attributing factor (Sorenmo, 2003).

Twenty-three out of 40 dogs presented tumors in left-sided mammary glands whereas 17 dogs presented tumors in the right-sided mammary glands. Three-fourth of dog populations included in the study presented the tumor in the last two pairs of mammary glands (caudal abdominal and inguinal). The higher tumor incidence in the posterior mammary gland pairs is correlated with the higher gland volume and abundant secretion during the lactation period (O'Keefe, 1995). The size of the tumor

was above 6 cm in 37.50% of animals and 52.50% of animals presented the tumor size between 3 and 6 cm whereas 10% of the animals presented the tumor size of less than 3 cm. The skin over the tumor was found ulcerated in 47.5% of animals that showed no tendency to heal which might be due to a change in the condition of the skin over the tumor mass. The ulceration on the skin over the tumor mass could be due to a highly malignant tumor that generates tremendously, inflamed locally, and ultimately become ulcerated.

Histopathology of mammary tumors

Benign tumors were identified as complex adenoma (n=8), simple adenoma (n=4), benign mixed tumor (n=4), fibroadenoma (n=2), ductal adenoma (n=1), and basaloid adenoma (n=1). Among the malignant tumors, the most frequently represented tumor types were cystic papillary carcinoma (n=7) followed by solid carcinoma (n=4), malignant mixed tumor (n=4), squamous cell carcinoma (n=3), tubular carcinoma (n=1), and intraductal carcinoma (n=1).

Carcinoembryonic antigen concentration

The mean serum carcinoembryonic antigen (CEA) concentration (ng/L) is presented in Table 1. The range in control/healthy bitches, and bitches with benign and malignant mammary tumors is 129.58 to 285.08, 181.94 to 459.17, and 255.30 to 438.69 ng/L, respectively. For CEA, upper cut-off value determined by ROC curve analysis (95% CI) was 247.65 ng/L (Fig. 1). The sensitivity and specificity of CEA for detecting CMTs were 77.50% and 80%, respectively.

The analysis of data revealed that the mean concentration of CEA in control/healthy bitches, and bitches with benign and malignant mammary tumors differed significantly (p<0.01). The serum CEA concentration was found to be lowest in healthy bitches, higher in bitches with benign tumors, and highest in bitches with malignant CMTs. A significant increase in the serum CEA concentration was observed in malignant CMTs included in the study.

Table 1 Mean ± SE of serum CEA (ng/L) and CA 15-3 concentration (kU/L) in healthy bitches, bitches with benign and with malignant mammary tumors

Clinical cases	Mean ± SE CEA (ng/L)	Mean ± SE CA 15-3 (kU/L)
Healthy bitches (n = 20)	201.03 ± 48.54[a]	5.02 ± 0.90[a]
Bitches with benign mammary tumor (n = 20)	281.08 ± 83.75[b]	5.91 ± 0.60[b]
Bitches with malignant mammary tumor (n = 20)	377.92 ± 65.80[c]	7.71 ± 0.88[c]

Carbohydrate antigen 15-3/ Cancer antigen 15-3 (CA 15-3) concentration

The mean values of serum CA 15-3 concentration are presented in Table 1. The average value of CA 15-3 concentration in healthy bitches, and with benign and malignant mammary tumor bitches ranged from 3.09 to 6.93, 2.48 to 4.88, and 5.68 to 8.50 kU/L, respectively. Based on ROC curve analysis (95% CI), we found a cut-off value of 5.65 kU/L (Fig. 2). The sensitivity and specificity of CA 15-3 for detecting canine mammary tumors were 85% and 80%, respectively.

The analysis of data revealed that the mean concentration of CA 15-3 in control/healthy bitches, and bitches with benign and malignant mammary tumors differed significantly (p<0.01). The CA 15-3 concentration was lowest in control/healthy bitches, higher in bitches with benign mammary tumors, and highest in malignant mammary tumors bitches. The serum CA 15-3 concentration increased as malignancy increases.

miR-21 expression

miR-21 was upregulated in all the test samples compared to the control samples. The average miR21 expression fold change in healthy bitches and in bitches with benign and malignant mammary tumors were 1.1-, 1.8-, and 3.0-folds, respectively. Expression fold change values ranged from 1.1 to 2.7 in bitches with benign mammary tumors and from 1.7 to 4.6 in bitches with malignant mammary tumors (Fig. 3).

The analysis of data revealed that the upregulation of miR-21 differs significantly (p<0.01) among dogs with benign and malignant mammary tumors compared to control/healthy dogs. Comparatively the expression was higher in malignant tumors than benign tumors.

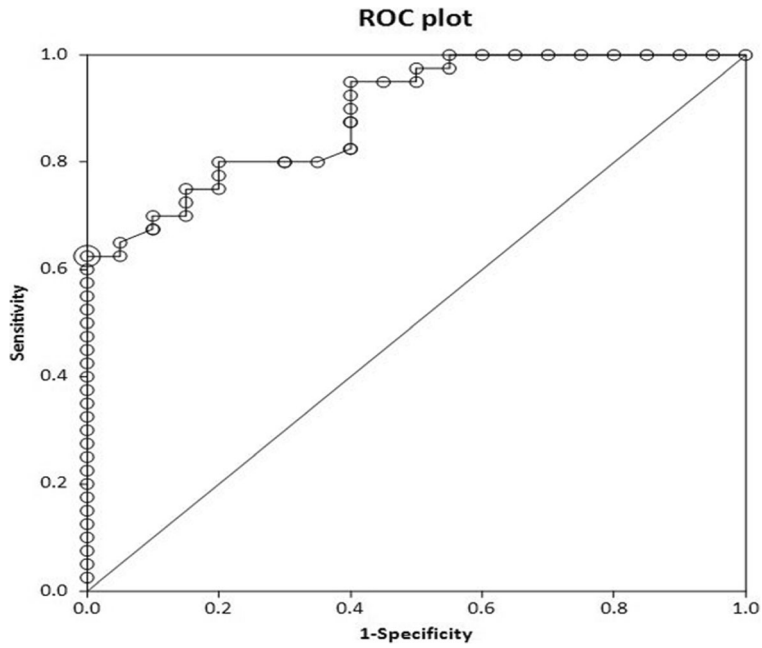

Fig. 1 ROC curve for CEA concentration. The *x*-axis showing 1 – specificity (= false positive fraction = FP/(FP+TN). The *y*-axis showing sensitivity (= true positive fraction = TP/(TP+FN)

miR-29b expression

miR-29b was downregulated in all samples compared to the control samples. Average miR-29b expression fold change in healthy bitches and in bitches with benign and with malignant tumors were 1.1, 0.4, and 0.2, respectively. Expression fold change values were ranging from 0.6 to 0.1 in both dogs with benign tumors and dogs with malignant tumors (Fig. 4).

The miR-29b expression was comparatively lower in malignant tumors than benign tumors. In the present study, there was a significant difference ($p<0.01$) in

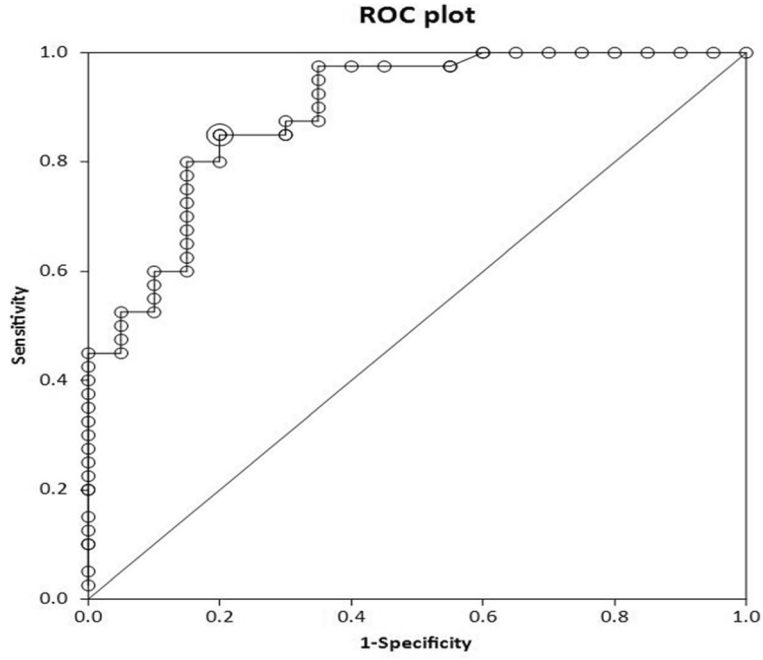

Fig. 2 ROC curve for CA 15-3 concentration. The *x*-axis showing 1 – specificity (= false positive fraction = FP/(FP+TN). The *y*-axis showing sensitivity (= true positive fraction = TP/(TP+FN)

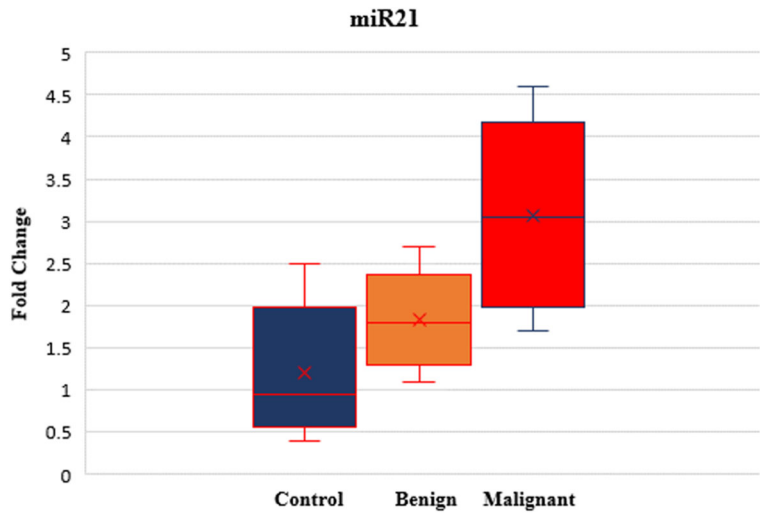

Fig. 3 Box and whisker plots for miR-21 expression by qRT-PCR between control/healthy, benign and malignant mammary tumors of canines. Horizontal lines in box are median values and X are mean values

miR-29b expression among dogs with benign and dogs with malignant mammary tumors compared to control/healthy dogs.

Correlation of serum CEA, CA 15-3, miR-21, and miR-29b in canines

The relationship of serum CEA concerning CA 15-3 and miR-21 was found to be positively correlated while with miR-29b it is negatively correlated (Table 2). CA 15-3 was positively correlated with miR-21 and negatively correlated with miR-29b. A negative correlation was found between miR-21 and miR-29b. All these relationships were statistically non-significant except CA 15-3 and miR-21 which were found statistically significant.

Discussion

The potential of CEA, CA 15-3, miRNA 21, and miRNA 29b expression as biomarkers to diagnose the benign and malignant CMTs was explored in the current experiment.

CEA is one of the first and most widely used tumor markers of human breast cancer (Guadagni et al. 2001). CEA is a part of the immunoglobulin superfamily and as an adhesion molecule; it might play a role in cellular matrix recognition. A significant difference in serum CEA concentration was observed which is similar to that reported in dogs (Balint et al. 2008, Valencakova-Agyagosova et al. 2012) and humans (Moazzezy et al. 2014, Stieber et al. 2015, Di Gioia et al. 2016). However, no significant difference between serum CEA concentrations in healthy dogs and dogs with malignant tumors was reported previously (Campos et al. 2012).

The use of tumor markers in humans is a routine methodology to detect, treat, and prevent oncological diseases. Similar to human medicine, in veterinary practice for bitches, CEA is a neither specific nor sensitive marker for primary diagnosis of CMTs but can be a subsidiary marker to detect CMTs. It can be used as the marker for early detection of metastasis but not for confirmation. About 50% of carcinomas of the canine mammary gland are detected by increased CEA (Valencakova-Agyagosova et al. 2012). The rate and production of CEA by carcinoma cells can influence the concentration of serum CEA (Campos et al. 2012). Besides, the hepatic metabolism and renal elimination rate can influence CEA levels but the combination of CEA, AKP, and LDH can be more reliable for confirmation of metastasis (Pirich et al. 1983). This CEA tumor marker can be useful for the early detection of asymptomatic tumors as well as monitoring the progress of the disease. Serum CEA can be a non-invasive diagnostic approach for mammary tumors in bitches and can be used effectively before clinical signs occur.

Similar to CEA, the serum concentrations of the CA 15-3 also differed significantly between healthy and affected bitches. Our results were similar to those reported in dogs (Balint et al. 2008, Marchesi et al. 2010, Valencakova-Agyagosova et al. 2012) and humans (Pederson et al. 2013, Moazzezy et al. 2014, Stieber

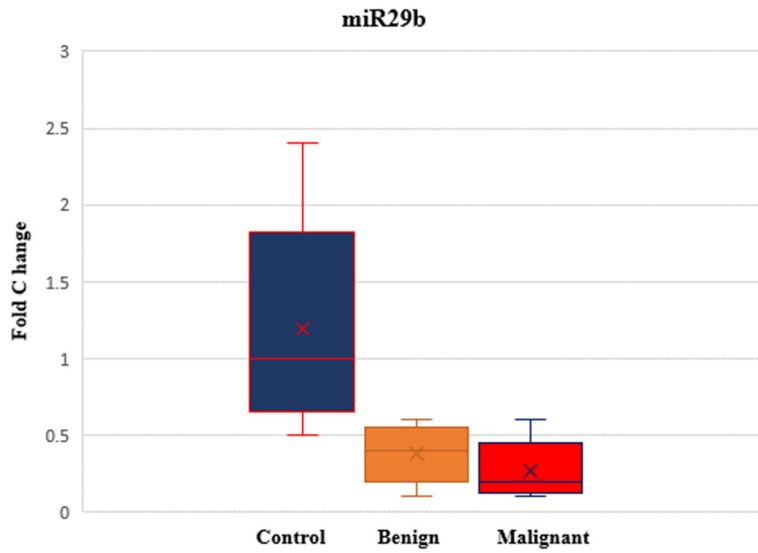

Fig. 4 Box and whisker plots for miR-29b expression by qRT-PCR between control/healthy, benign and malignant mammary tumors of canines. Horizontal lines in box are median values and X are mean values

et al. 2015, Di Gioia et al. 2016, Gupta et al. 2018). In contrast, a lower CA 15-3 concentration than the present study was analyzed (Campos et al. 2012) which may be due to the staging of the disease.

The study observed the higher concentrations of CA 15-3 and malignancy in the larger sized CMTs. Also, the concentration of CA 15-3 was proportional to the clinical stage of the disease (Campos et al. 2012). The CA 15-3 values exceeding 7 kU/L were regarded as pathological which was similar to the report of Valencakova-Agyagosova et al. (2012). Thus, CA 15-3 concentrations were considered more sensitive and specific in CMTs as these values were higher than in healthy bitches. Similarly, in humans, CA 15-3 is considered the main marker for monitoring oncological diseases. Any increase in serum CA 15-3 ectodomain is related to carcinoma (Gupta et al. 2018). Thus, CA 15-3 marker can be used for early detection of mammary tumor recurrence. Similarly, CA 15-3 has prognostic importance for mammary tumors in bitches and can provide an initial prognosis. The preoperative concentration of CA 15-3 along with the existing prognostic factor (CEA) may help in selecting exact adjuvant therapy. Thus, increased preoperative CA 15-3 concentration relates to mammary cancer in bitches.

Table 2 Intercorrelation matrix of serum CEA, CA 15-3, miR-21, and miR-29b in canines

Attributes	CEA	CA 15-3	miR-21	miR-29b
CEA	1	-	-	-
CA 15-3	0.99^{NS}	1	-	-
miR-21	1.00^{NS}	1.00^{S}	1	-
miR-29b	-0.93^{NS}	-0.88^{NS}	-0.90^{NS}	1

^{NS} Non-significant. ^{S} Significant

The upregulated miR-21 observed in our study was similar to those analyzed previously (Boggs et al. 2008, Losiewicz et al. 2014, Kabir et al. 2015, Bulkowska et al. 2017). miR-21 presented 3-fold higher expression changes in sarcoma and benign tumors while in carcinoma its expression increased by 5-folds. The expression analysis was studied by relating the miRNA expressions with that of RNU6 which was used as a reference gene. Similarly, von Deetzen et al. (2014) observed the upregulated expression of miR-21 in the benign and malignant tumors of the dog. The upregulation of miR-21 observed in human breast cancer was found to be associated with the tumor or cancerous nature of the cells (Zhang et al. 2016, Yanwirasti and Arisanty, 2017, Khalighfard et al. 2018).

Higher expression of miR-21 may be a characteristic of cancerous cells and represent a common feature of pathological cell growth or cell stress. miR-21 was distinguished in breast cancer from healthy control, and its expression level was significantly associated with patient's survival (Yan et al. 2008, Asaga et al. 2011).

Many researchers have proved that miR-21 is an oncomiR, which was frequently upregulated in breast cancer and many other types of human cancers. miR-21 plays an imperative role in proliferation, invasion,

angiogenesis, and metastasis of tumors (Si et al. 2007, Yan et al. 2008, Liu et al. 2011, Yan et al. 2011). miR-21 blocks genes that encode tropomyosin 1 (*TPM1*) (Zhu et al. 2007), programmed cell death 4 (*PDCD4*) (Lu et al. 2008), phosphatase and tensin homolog (*PTEN*) (Huang et al. 2009), chromosome condensation protein G (NCAPG), reticulon 4 isoform A (RTN4) (Yang et al. 2009), and tissue inhibitor of metalloproteinase 3 (*TIMP3*) (Song et al. 2010). Blocking miR-21 expression inhibits tumorous cell growth and metastasis (Yan et al. 2011). It has been reported that overexpression of miR-21 induces tumor angiogenesis by increasing the expression of HIF-1a and VEGF in human prostate cancer (Liu et al. 2011). The miR-21 utilizes its oncogenic function mainly through cellular inhibition apoptosis (Medina et al. 2010).

The overexpression of miR-21 is entangled in the early induction of EMT, a process that causes epithelial cells to lose their cell polarity and cell-cell adhesion highly resulting in cancer aggressiveness and metastasis (Bornachea et al. 2012, Han et al. 2012). Similarly, miR-21 targets maspin protein and is encoded by the *SERPINB5* gene which acts as a tumor suppressor gene. Maspin increases tumor promoter activity and thus designs a microenvironment, which is permissive for more tumor invasion (Zhu et al. 2008). The miR-21 expression is influenced by epidermal growth factor receptor activity (Seike et al. 2009), and the binding of oncoprotein c-Jun to miR-21 DNA promoter regions leads to the aberrant increasing of miR-21expression through the c-Jun N-terminal kinases-1/c-Jun pathway (Echevarria-Vargas et al. 2014). Thus, miR-21 overexpression in bitches with mammary tumor is associated with advanced clinical stage and poor prognosis. The sensitivity and specificity of miR-21 can help to differentiate the bitches with mammary tumors (benign/malignant) from normal healthy bitches without mammary tumors. The insinuation of miR-21 in resistance to many anticancer agents highlights the possible clinical application of miR-21 inhibition for reducing the resistance of chemotherapeutic drugs towards cancer along with the potential to use miR-21 inhibitors as targeted therapeutic strategies in addition to conventional cytotoxic agents (Sanchez et al. 2020). The primary finding in this study showing high miR-21 expression in benign and malignant mammary tumors indicates that miR-21 can be a valuable prognostic biomarker in CMTs as in human breast tumors. Therefore, more light should be shed on the potential correction between the expression level of miR-21 and clinicopathological characteristics of CMTs such as age, the pathological grade of the tumor, lymph node metastasis, expression of sex hormone receptors, and patient survival.

The downregulation of miR-29b observed in the present study was in accordance with miR-29b expression in the plasma of dogs with non-metastasizing mammary tumors (Bulkowska et al. 2017, von Deetzen et al. 2014) and human breast cancer tissue and cell lines (Wang et al. 2017). Conversely, Fish et al. (2020) and Boggs et al. (2008) observed upregulated miR-29b expression in CMT serum and tissue samples, while Osaki et al. (2016) found 4.07-fold upregulation in miR-29b expression in canine SNP cell line. miR29b downregulation promotes cellular viability and metastasis by activating p38-STAT1 in breast cancer (Liu et al. 2017). miR29 is regulated by many molecules like GATA3, Myc which control miR29 expressions in the early stages (Jiang et al. 2014). The tumor progression stage does play an important role in downregulation and upregulation. In the present study, the time duration since CMT appeared was 3–6 months when serum was collected, which may be one of the reasons for downregulation in miR29b expressions. Also, the preprocessing steps make a difference when measuring their expression (Tiberio et al. 2015). Out of the miR-29 family microRNAs, miR-29b is the most deregulated miRNA in most human malignancies (Wang et al. 2013). However, the role of miR-29b in cancer has not been clarified yet. Instead, its role in carcinogenesis remains controversial as miR-29b has been claimed to be involved in both tumor-suppressive and tumor-promoting activities (Liu et al. 2014; Yan et al. 2015). The oncogenic effect of miR-29b is the result of the loss of its regulatory activity on DNMT3A and DNMT3B genes that directly targets DNA methylation status (Morita et al. 2013). miR-29b also regulates EMT signaling pathways as miR-21 do, to suppress the metastasis in human prostate cancer (Ru et al. 2012).

miR-29b seems to act as a regulator of several genes in mammary tumors whose product activity is crucial for the creation and development of tumors (Zhao et al. 2015). GATA3 promotes differentiation, suppresses metastasis, and alters the microenvironment in breast cancer by altering *miR-29b* expression (Chou et al. 2013).

miR-29b can be utilized as a non-invasive diagnostic and prognostic biomarker for various types of cancer in dogs including mammary gland tumors. Based on this

concept, further research on miRNA-29b signaling pathways should begin with the aim of elucidating their effects on conventional protein signaling pathways.

Circulating miRNAs might have great potential as novel diagnostic tools for cancer. Given that serum or plasma samples are more feasible and relatively non-invasive than tumor tissues in clinical practice, it is not surprising that some studies have highlighted the potential of circulating miRNAs in the management of cancers. It was demonstrated that miRNAs were resistant to degradation after being subjected to harsh conditions including boiling, low/high pH levels, extended storage, and freeze-thaw cycles (Chen et al. 2008).

Conclusion

In conclusion, CA 15-3 can be useful for early detection of asymptomatic tumors as well as monitoring the progress of the disease. The concentration of CA 15-3 in serum will be towards the higher end in larger size tumors and during malignancies. Detection of CEA along with CA 15-3 will upsurge sensitivity in detecting CMTs. Expression of circulating miRNAs (miR-21 and miR-29b) will be a valuable prognostic biomarker in CMTs. Moreover, the mutual combination of miRs together with CEA and CA 15-3 increases the accuracy of diagnosis.

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Declarations

Competing interests The authors declare no competing interests.

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