MicroRNA-21 expression, serum tumor markers, and immunohistochemistry in canine mammary tumors

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Abstract

Background Canine mammary tumors (CMTs) are one of the most common malignancies in dogs and are associated with significant mortality. Serum tumor markers and non-coding microRNAs have gained widespread popularity in human oncology studies. The present study has two aims, first one is to investigate the miR-21 expression compared with changes in serum tumor markers (CEA and CA15-3) in CMT. The second aim is to detect the immunohistochemistry markers as vimentin, P63, and -SMA in CMT.

Methods This study enrolled 17 female dogs: 10 with mammary tumors and seven controls without tumors. Blood samples were collected to measure miR-21, CEA, and CA 15-3, and histological samples were prepared for histological grading and immunohistochemistry.

Results CA 15-3 was elevated in all animals, whereas CEA levels showed no change compared with controls. miR-21 was upregulated 12.84-fold in animals with CMT. The most frequently recorded CMT was the mixed type. Myoepithelial cells were identified by P63 immunoreactivity, but not SMA. High expression of miR-21 was observed with positive vimentin immunoreactivity, indicating the mesenchymal origin of the tumor cells.

Conclusion The present study showed that miR-21 was elevated to a greater extent than CA 15-3 (12.84-fold vs. threefold). Tumors that was positive for vimentin immunoreactivity was also associated with an elevation in the levels of miR-21, showing that miR-21 is released from mesenchymal cells. These findings support the hypothesis that miR-21 may be a more sensitive, noninvasive indicator for CMT.

Keywords Canine mammary tumor · CEA · CA 15-3 · Immunohistochemistry · miRNA-21

Introduction

Canine mammary tumors (CMTs) are one of the most commonly observed tumors in female dogs and can cause mortality (Bonnett et al. 2005; Manuali et al. 2012; Banerjee et al. 2018). The local invasion of the lymphatic system, grade, and diameter of the tumor are crucial factors in the overall survival rate (Rasotto et al. 2017; Nguyen et al. 2018).

It was demonstrated that up to 81 % of CMTs are malignant (Simeonov and Stoikov 2006). Diagnosis of mammary tumors requires a constellation of tests, laboratory assessments for cancer using fine-needle aspiration, histopathology, and immunohistochemistry. Ultrasonography and radiography may be used for further diagnostic approach (Moris and Dobson 2001; Henry and Higginbotham 2010). Despite the availability of a wide range of diagnostic modalities for cancer, no single modality is foolproof for the identification of cancer (Kumar et al. 2018). The need to add diagnostic tests to mammary tumor panel, especially those that are labeled as minimally invasive, could improve clinical intervention and the decision-making process (Fish et al. 2020). The stability of microRNA (miRNA) and the fact that they are
minimally invasive tests make them a good and practical candidate for assessment in the tumor panel (Mall et al. 2013; Papadaki et al. 2018).

Studies in human medicine have shown characteristic expression profiles of miRNA in tumors (Lu et al. 2005). MicroRNAs (miRNAs) are clusters of small non-coding RNA that play an essential part in posttranscriptional gene expression as imperative regulators (Eman et al. 2018; Ramadan et al. 2019). Several miRNA species have been identified to be involved in human breast cancer (Iorio et al. 2005; Boggs et al. 2008; Hong et al. 2012). Studies about miRNA in human breast cancer are numerous in which several miRNAs were identified in association with breast cancer like miR-21, miR-210, and miR-145 (Iorio et al. 2005). MiR-21 showed different expression levels between normal and neoplastic tissue in CMTs (von Deetzen et al. 2014) and was one of the most upregulated miRNAs in human breast cancer. Furthermore, it was demonstrated that targeting this miRNA might have a future therapeutic potential (Dan et al. 2021).

Tumor markers are products of cellular metabolism that are elevated due to malignant transformation (Eisenberg and Koifman 2001). The most widely used serum markers in breast cancer are cancer antigen 15-3 (CA 15-3) and carcinoembryonic antigen (CEA). Measurement of these markers is relatively inexpensive and requires a less invasive method of sample collection compared with immunohistochemical tests, which have been used in veterinary medicine (Campos et al. 2012).

Many histological classification systems have been proposed for CMTs (Goldschmidt et al. 2011). A histological staging system for CM carcinomas was recently published and provides a strong prognostic factor for CM carcinoma. The histological stage of this system includes disease-free-interval, overall survival, and specific survival of dogs with mammary carcinoma (Chocteau et al. 2019). The types of cells in the carcinoma or malignant myoepithelioma can be identified using immunohistochemistry markers such as CK8, CK18, CK19, and CK7 for epithelial cells and CK5, CK6, CK14, CK17, smooth muscle actin, calponin, vimentin, and p63 for basal/myoepithelial cells. Cell differentiation markers have been investigated to elucidate the histogenesis of tumors, particularly mixed-type mammary carcinoma, which is common in dogs (Sorenmo et al. 2011a).

Serum tumor markers and novel miRNAs could be used as a rapid diagnostic test; the miRNA could be included in the diagnostic panel along with other tumor biomarkers to improve the diagnostic accuracy (Jain et al. 2021). The relation between the origin of tumor cells and immunoreactivity could affect the expression of certain miRNAs. Therefore, the present study aimed to investigate the miR-21 expression, and changes in serum tumor markers (CEA and CA15-3) in classified CMT using immunohistochemistry markers as cytokeratin, vimentin, P63, and α-SMA.

Methods

Animals, examination, and allocation

The study included seventeen (17) non-lactating and non-pregnant female dogs admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Cairo University. Ten (10) dogs had mammary tumors and seven (7) healthy dogs (with similar age range, sex, and breeds) were used as a control group and underwent a complete examination to exclude any underlying diseases (Table 1).

Physical examination of mammary glands was performed via inspection and palpation for masses on all five pairs of glands. Clinical signs were recorded at the time of admission. The control group comprised healthy dogs (T0) free from mammary tumors or any health issue, whereas the disease group comprised dogs with mammary tumor ≥3 cm in diameter and no evidence of metastasis (T2,3, N0–1, M0), as adapted from the World Health Organization guidelines (Owen 1980; Rivera 2010). In cases with more than one mass, the largest was used for tumor size (Senhorello et al. 2020).

Radiographical examination

Radiographical recordings were made using X-ray (Fischer, Berlin, Germany). The radiographical setting factors were 58–70 kVp, 10 mAs, and 90-cm focal spot-film distance. The radiographical exposures were conducted dorsoventrally and right laterally in both healthy and diseased animals. The tumor dimensions were measured using calipers and confirmed with sonar in both healthy and diseased animals.

Routine hematology and serum tumor markers

Blood was collected from the cephalic vein of each dog and then divided into two portions. The first portion was collected in an EDTA-containing tube for hematological analysis to all the animals assigned in this study by an automated veterinary hematology analyzer (IDEXX Veterinary hematology analyzer). The second portion was collected in a sterile tube for serum separation and divided into two aliquots. The first aliquot was used to determine CEA and CA 15-3 levels (MyBioSource, USA). The second aliquot was stored immediately at -20 °C until used to measure miR-21 expression.

Determination of serum miR-21

RNA was extracted from serum samples using the RNeasy Mini kit (catalog no.74104, QIAGEN, Germany) according to the manufacturer’s instructions. Quantitative PCR
was performed using a QuantiTect SYBR Green PCR kit (Cat. No.204141, QIAGEN, Germany). The primers used in SYBR green real-time PCR for miR-21 and U6 (housekeeping gene), primer sequence for U6 (housekeeping gene): 5' -GCT  
TCG  
GCA  
GCA  
CAT  
ATA  
CTA  
AAA  
T-3' and 5'- CGC  
TT  
C  
A  
CG  
A  
AT  
TT  
G  
C  
GT  
G  
TCAT -3' and that for miR-21: 5'- CGG  
CGG  
T  
AG  
CTT  
A  
TC  
A  
GA  
CT  
G  
A  
TG  
T  
-3' and 5'- GTG  
C  
AG  
GG  
T  
CCG  
A  
GGT -3' (Chen et al. 2013; Wu et al. 2017). Cycling conditions for both U6 and miR-21 were as follow: Reverse transcription at 50°C/30min; primary denaturation at 94°C/15min, followed by 40 cycles amplification (secondary denaturation 94°C/15 sec., annealing (optics on) 60°C/30 sec, and extension 72°C/30 sec). Secondary denaturation at 94°C/1 min. Dissociation curve (1 cycle) composed of annealing 60°C/1 min and final denaturation 94°C/1 min. Amplification curves and cycle threshold (Ct) values were determined using Strata-gene MX3005P software. The Ct value of each sample was compared with that of the control group according to the “ΔΔCt” method (Yuan et al. 2006) using the following ratio $2^{-ΔΔ Ct}$ to estimate the variation in gene expression in the different samples.

### Surgical intervention and post-operative care

General anesthesia was applied to animals during the surgical mastectomy (Suppl. 1). Under general injectable anesthesia, each animal was premedicated with atropine sulfate (1 %, 0.05–0.1 mg/kg b. wt.; Adwia Co. S.A.E., Cairo, Egypt) and xylazine (Xyla-Ject 2 %, 1 mg/kg b. wt.; Adwia Co. S.A.E.), and then anesthesia was induced using ketamine HCl (Ketalar, 10–15 mg/kg b. wt.; Sigma-Aldrich Co.) and maintained by ketamine HCl (Abd Elkader et al. 2020). After complete recovery the animals were discharged to owner care, during postoperative care the animal was medicated Cefobid® 500 mg IM twice daily, fluid therapy for 3 days, daily dressing on the wound with normal saline, Fucidin® cream, and Bevatracin® spray follow up after 10 days to remove stitch after 10 days or 2weeks.

### Histopathology, histological grading, and immunohistochemistry of mammary tissue

Mammary tissue specimens were fixed in 10% neutral buffered formalin, processed by paraffin-embedded technique, and sectioned into 3–4-µm thick sections using a microtome (Leica

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**Table 1 Clinical data of each clinical case of mammary tumor**

| Case no | Breed / Age | Tumor location | Size | Lymph node | Metastasis | Pathology | Tumor pathology grade |
|---------|-------------|---------------|------|------------|------------|-----------|----------------------|
| 1       | Rottweiler /9 years | Left caudal abdomen | 4*5 cm | Not metastatic | Tubular carcinoma | I |
| 2       | German shepherd /9 years old | Right caudal abdomen +right inguinal | 7*3 cm | Tubular carcinoma | II |
| 3       | German shepherd / 8 year | Tumor in the inguinal mammary gland with inguinal hernia | 6*2 cm | Not metastatic | Micropapillary carcinoma | II |
| 4       | mongrel dog / 7 years old | Right cranial abdomen | 3*4 cm | Not metastatic | Ductal carcinoma | II |
| 5       | Golden retriever / 9 years | Left inguinal | 5*2 cm | Not metastatic | Mixed carcinoma | I |
| 6       | North American shepherd /15 years | Left inguinal | 5*3.5 cm | Not metastatic | Mixed carcinoma | I |
| 7       | German shepherd /10 years | Left cranial abdomen +caudal abdominal | 7*3 cm with ulceration | Not metastatic | Carcinoma complex | III |
| 8       | German shepherd /7 years old | Right inguinal | 9*7 cm | Enlarged right inguinal lymph node 4.5*4 cm | Not metastatic | Carcinoma complex | III |
| 9       | Labrador /7 years old | Left caudal abdomen +left inguinal | 11*3 cm | Not metastatic | Fibromyxoid sarcoma | II |
| 10      | Griffon /14 year | Right inguinal | 7*6 cm | Enlarged right inguinal lymph node 3*2 cm | Not metastatic | Undifferentiated sarcoma | III |
Tissue sections were stained with hematoxylin and eosin for histopathological examination using a light microscope equipped with a digital camera (Olympus XC30, Tokyo, Japan). CMTs were diagnosed according to a previously proposed histological classification (Goldschmidt et al. 2011).

The tumors were graded according to the Elston and Ellis scoring system (Elston and Ellis 1998), previously verified in dogs (Karayannopoulou et al. 2005; Peña et al. 2013), in which three morphological features were assessed: glandular differentiation and tubule formation, nuclear pleomorphism, and mitotic activity. A score of 1–3 was used to evaluate each parameter (Tavasoly et al. 2013). Tubule formation with clear central lumina was scored 1 when comprising > 75% of the tumor, 2 when comprising 10–75% of the tumor, and 3 when comprising < 10% of the tumor. The nuclear pleomorphism was scored 1 when small uniform regular cells were present, 2 when the nuclear size and variation was moderate, and 3 when the nuclear variation was marked. The number of cells undergoing mitosis per 10 high-power fields (40x) was scored as 1 (0–7 mitosis), 2 (8–16 mitosis), and 3 (≥17 mitoses). The sum of the three scores was used to grade each tumor as follows: grade I, well-differentiated, or low-grade (3–5 points); grade II, moderately differentiated or intermediate-grade (6–7 points); and grade III, poorly differentiated or high-grade (8–9 points).

Cytokeratin, vimentin, α-SMA, and P63 were immunohistochemically stained in paraffin-embedded tissue sections after deparaffinization and hydration of the tissue to differentiate between different types of undiagnosed tumors (Peña et al. 2014). Anti-pan-cytokeratin (PCK) (rabbit polyclonal, 1:100, Dako), anti-vimentin (mouse monoclonal, 1:100, clone V9, Dako), anti-α-SMA (mouse monoclonal, clone 1A4, 1:100, Dako), anti-p63 (mouse monoclonal, clone 4A4, 1:50, Dako, Carpinteria, USA) were applied to tissue sections after antigen retrieval by citrate buffer (pH 6) and blocking of endogenous peroxidase activity with 3% H2O2. A secondary horseradish peroxidase-conjugated anti-species antibody (Envision, Dako, Carpinteria, USA) was then applied for one hour followed by 3’-diaminobenzidine (Dako, Carpinteria, USA) to develop a brown color.

Statistical analysis

Age was presented as median values. All quantitative data of hematology and serum tumor markers (CEA, CA 15-3) were presented as mean ± standard error. Comparison between the control and diseased group were performed using SPSS statistics program version 16.0 (independent samples t-test).

Results

Preliminary patient data and radiological examination

The median age range for affection was 9 years (range, 7–15 years). The German Shepherd breed appeared to be over-represented in the present study, accounting for 40% of all dogs (Table 1). There was no evidence of metastasis at the time of admission. Radiographic examinations showed no signs of lung metastases or thorax chest lesions in any of the animals with CMT (Suppl. 2).

Hematology and tumor markers

The hematological evaluation revealed a non-significant reduction in red blood cells, packed cell volume (PCV), and hemoglobin (Hb), along with a significant reduction in total leucocyte count (TLC) in the disease group compared with the controls. Significant elevation in thrombocytes was recorded in animals in the disease group compared with the controls (Table 2).

CEA showed no significant changes between the normal and diseased groups (range: 0.047-0.24; 0.03-0.051 respectively), in contrast, CA 15-3 showed significant elevation in the CMT group compared with healthy counterpart (range: 1-1.63; 0.85-6.05 respectively). All the CMT patients showed elevation in CA 15-3 except one patient (0.85U/ml).

Table 2 Hematologic findings in bitches with mammary tumor and control group

| Parameters/unit | Control group | Diseased group |
|-----------------|---------------|----------------|
| RBCs (10¹²/L)   | 6.80 ± 0.30   | 6.04 ± 0.21    |
| PCV (l/l)       | 0.43 ± 0.02   | 0.40 ± 0.05    |
| HB (gm/L)       | 144.05 ± 7.54 | 136.66 ± 4.26  |
| MCV (fl)        | 62.81 ± 1.64  | 68.12 ± 2.61   |
| MCH (pg)        | 21.14 ± 0.50  | 22.99 ± 0.80   |
| MCHC (gm/L)     | 337.31 ± 8.47 | 340.91 ± 6.77  |
| Platelets (10⁹/L) | 184.00 ± 7.11 | 271.90 ± 7.26*** |
| N (%)           | 63.14 ± 1.45  | 62.15 ± 4.80   |
| L (%)           | 23.28 ± 1.76  | 26.80 ± 4.76   |
| M (%)           | 7.28 ± 0.74   | 6.78 ± 0.82    |
| E (%)           | 4.85 ± 0.80   | 4.12 ± 0.55    |
| WBCs (10⁹/L)    | 11.18 ± 0.80  | 8.06 ± 0.76**  |

Data are represented as mean ±SE, *P value≤ 0.05 considered significant, SPSS program version16.0 independent sample t test (RBCs: Red blood cells, PCV: packed cell volume, HB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, N: neutrophils, L: lymphocytes, M: monocytes, E: eosinophils, WBCs: white blood cells)
The results of serum tumor markers (CEA and CA 15-3) are shown in Table 3.

### MiR-21 expression

The miR-21 showed a steady pattern in all CMT patients in this study with a mean fold change of 12.84 (Table 4). The ranges of expression fold changes were 9.00 to 19.97 in CMT patients. All the diseased patients showed up-regulation of miR-21 expression when compared to control. The mean and SE of CT for both U6 and miR-21 as well as fold changes are tabulated in Table 4.

### Histology and immunohistochemistry

Microscopy of the CMT revealed two tubular carcinomas, micropapillary carcinoma, ductal carcinoma, two mixed-type carcinomas, two complex-type carcinomas, low-grade fibromyxoid sarcoma, and undifferentiated sarcoma. The histological gradings and immunoexpression of CK, P63, SMA, and vimentin in different CMT are shown in Table 5. In tubular carcinoma, the neoplastic cells appeared large hepatoid cells with eosinophilic cytoplasm. They were pleomorphic with prominent nucleoli, often vacuolated and arranged in an acinar pattern. Neoplastic cells were separated by delicate fibrovascular stroma, and mitosis was frequently observed in these cells (Fig. 1a). Epithelial cells were positive for CK (Fig. 1b) whereas myoepithelial cells were positive for vimentin, SMA, and P63 (Fig. 1c, d, e).

Ductal carcinoma was also diagnosed in one case that showed neoplastic cells arranged in cords and tubules that surrounded slit-like lumina that were often lined by multiple layers of epithelial cells exhibiting significant anisokaryosis and anisocytosis. Focal or multifocal areas of squamous differentiation and keratinization were present, with intracytoplasmic keratohyalin granules within some cells (Fig. 1f). Epithelial cells were positive for CK (Fig. 1g). Neoplastic cells expressed high vimentin and mild P63. SMA was expressed in connective tissue stroma (Fig. 1h, i, j).

In micropapillary carcinoma, neoplastic cells formed a morula-like micropapillary projecting in the mammary lumina. Cells showed pleomorphism, karyomegaly, and mitosis. An extensive necrosed area was seen in the neoplastic tissue, and leukocytic infiltration was observed in

### Table 3 Serum levels of CEA and CA 15-3 in bitches with mammary tumor and control group

| Parameters/unit | Control group | Diseased group |
|-----------------|---------------|----------------|
| CEA (ng/mL)     | 0.14 ± 0.03   | 0.15 ± 0.05    |
| CA 15-3 (U/mL)  | 1.33 ± 0.10   | 3.76± 0.43**   |

Data are represented as mean ±SE, *P value ≤ 0.05 considered significant, SPSS program version 16.00 independent sample t test

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### Table 4 Expression of MiR-21 in bitches with mammary tumor and control group

| Group               | U6 CT | MiR-21 CT | Fold change (2-ΔΔCT) |
|---------------------|-------|-----------|----------------------|
| Control group       | 19.57± 0.04 | 23.58 ± 0.06 | -                     |
| Canine mammary tumor| 20.71± 0.13 | 21.04 ± 0.16 | 12.84***             |

Data are represented as CT mean ± SE

### Table 5 The scores and grades of the neoplasms recorded in this study

| Type of neoplasm    | No. of animals | T | M | N | Score Sum | tumor grade | CK | P63 | SMA | Vimentin |
|---------------------|----------------|---|---|---|-----------|-------------|----|-----|-----|----------|
| Tubular carcinoma   | 2/10           | 1 | 2 | 2 | 5         | I           | +++| ++  | ++  | ++++     |
|                     |                | 2 | 2 | 2 | 6         | II          | ++ | +   | ++  | ++       |
| Micropapillary      | 1/10           | 2 | 3 | 2 | 7         | II          | +++| +   | +   | ++++     |
| carcinoma           |                | 2 | 2 | 2 | 6         | II          | +++| ++  | +   | +++      |
| Ductal carcinoma    | 1/10           | 2 | 1 | 2 | 5         | I           | +++| ++  | +   | ++       |
| Carcinoma-mixed type| 2/10          | 2 | 1 | 2 | 5         | I           | +++| ++  | +   | ++       |
| Carcinoma-complex type | 2/10       | 3 | 3 | 3 | 9         | III         | +++| +   | -   | ++++     |
| Fibromyxoid sarcoma | 1/10           | 3 | 1 | 3 | 7         | II          | -  | -   | -   | -        |
| Undifferentiated Sarcoma | 1/10     | 3 | 3 | 3 | 9         | III         | -  | -   | -   | ++       |

T= score of tubule formation; M= Score of Mitosis; N= Score of nuclear pleomorphism; Ck=cytokeratin; SMA; smooth muscle actin. (+) Mild expression; (++) moderate expression; (+++) high expression
the interstitial tissue of the mammary gland. Interstitial and peripheral fibrosis was also observed (Fig. 1k). Epithelial cells were strongly positive for CK. Myoepithelial cells expressed vimentin moderately, SMA, and P63 mildly (Fig. 1l, m, n, o).

Moderately differentiated complex carcinoma was diagnosed in two cases. Epithelial cells and spindle-shaped myoepithelial cells were observed as infiltrating cords. A layer of myoepithelial cells combined with a small grouping of epithelial cells invaded the adjacent stroma (Fig. 2a). Epithelial cells were strongly positive for pan-cytokeratin. Myoepithelial cells were positive for P63 but not α-SMA (Fig. 2b, c, d). The connective tissue stroma was positive for vimentin (Fig. 2e). Mixed-type carcinoma was reported in two cases and showed irregular tubules lined by a single layer of epithelial cells with scant cytoplasm, fusiform, or stellate myoepithelial cells combined with an abundant fibrous connective tissue and cartilage formation. The cartilage appeared as different-sized plaques of low-to-moderate numbers of chondrocytes and chondroblasts. Epithelial cells were positive for pan-cytokeratin. Myoepithelial cells were positive for P63 and α-SMA. The connective tissue stroma and cartilage were positive for vimentin.

Mammary gland sarcoma was recorded in two cases. Low-grade fibromyxoid sarcoma had fibroblastic spindle-shaped cells arranged in a whirling growth pattern with
myxoid and fibrous stroma. The fibrous areas showed varying degrees of cellularity, including some hypocellular areas formed entirely of dense collagenous tissue. Nuclear pleomorphism was seen and mitotic figures were rare. Prominent capillaries were observed. The tumor was surrounded by a fibrous capsule nevertheless it infiltrated adjacent tissues (Fig. 2f). Only a few spindle-shaped cells in the tumor expressed vimentin but not CK, SMA, or P63 (Fig. 2g, h, i, j). The other undifferentiated sarcoma was composed of polygonal neoplastic cells arranged in solid cellular areas and clusters separated by a fine fibrovascular stroma. The neoplastic cells were undifferentiated with vesicular nuclei and showed frequent mitosis (Fig. 2k). They were moderately positive for vimentin and entirely negative for cytokeratin, α-SMA, and P63 (Fig. 2l, m, n, o).

Discussion

This study showed an elevated CA 15-3 in association with CMT in all patients, except one patient when compared to control data, while miR-21 showed significant upregulation in all patients compared to the control group. Cytokeratin, P63, SMA expression in tumor cells decreased corresponding to loss of differentiation whereas vimentin expression was partially maintained.

CMT is frequently diagnosed, especially in female dogs (Benavente et al. 2016). In the present study, intact female dogs, with a median age of 9 years, were affected. The onset of CMT is believed to be influenced by age, hormones, and genetic predisposition and is therefore rarely seen in young dogs (<3 years of age) (Egenvall...
et al. 2005). An incidence rate of 73% was previously reported in intact female dogs with an approximate age range of 8–13 years (Vascellari et al. 2016), and another study reported a mean age of 9.5 years (Sorenmo et al. 2009). Cocker Spaniels and German Shepherds were reported to be at higher risk compared with other breeds (Sleeckx et al. 2011); however, Labrador Retrievers and Poodles were overrepresented in another study (Egenvall et al. 2005).

CMT diagnosis is relatively feasible using clinical data, and a combination of tumor visualization and palpation provides the practitioner with the necessary information to narrow differential diagnosis lists (Kutzler 2020). In the present study, two females showed multiple masses, and tumor size was taken as the size of the largest tumor (Sorenmo 2011; Senhorello et al. 2020). The radiological examination was conducted to confirm the absence of distant metastasis. It is recommended to conduct the left and right lateral view and ventrodorsal view to confirm or rule out the presence of metastasis (Kutzler 2020). Although the clinical examination associated with radiography could provide the clinician with the needed clues to take the next step, as they are alone can’t confirm or overrule the diagnosis. Till now, the histopathological examination continues to be the keystone for diagnosis/grading CMTs (Canadas et al. 2019). However, to perform these sets of tests, the tumor has to be large enough to warrant the owner’s attention. Tumor markers might provide early identification of the disease (Kaszak et al. 2018). There are numerous serum tumor markers available, however, CEA and Ca 15-3 remain the most widely used in human breast cancer clinics (Shao et al. 2015).

Histological examination showed malignant CMTs that were either carcinoma or sarcoma. The most frequently recorded CMT as recorded in previous studies was mixed-type which agrees with our finding (Misdorp et al. 1999; Cassali et al. 2011). However, tubular carcinoma and complex carcinoma were also recorded in the same number of cases. Histological classification of mammary carcinoma using the human “Elston and Ellis grading method” was useful in CMT where it was correlated with the grade of the tumor (Karayannopoulou et al. 2005). Further immunohistochemical studies are useful to identify the origin of neoplastic cells (Dolka et al. 2013). Keratin immunoreactivity was limited to epithelial cells. Myofibroblasts expressed both α-actin and vimentin, whereas connective tissue cells expressed vimentin. Myoepithelial cells are a major component of carcinomas, which usually express CK14, CK5, SMA, calponin, and p63 (Destexhe et al. 1993). However, proliferating myoepithelial cells show a decreased expression of these markers with an increased expression of vimentin, and eventually become a fibroblast-like cell expressing only vimentin (Sorenmo et al. 2011). Overexpression of vimentin filaments, as part of the process of epithelial-mesenchymal transition (EMT), was significantly associated with tumor size and grade, angiogenesis, and vascular invasion (Rismanchi et al. 2014). Identification of the type of neoplastic cells involved in the tumor was suggested to be important for proper diagnosis and subsequent therapeutic approach. Tumors with or without myoepithelial cells should be identified since it was proposed that myoepithelial cells’ presence in malignant tumors exhibit an inhibitory effect on neoplastic cells (Misdorp et al. 1999; Pandey et al. 2010). In the present study, it was difficult to diagnose myoepithelial cells in some tumors using hematoxylin and eosin-stained tissue sections; therefore, they were identified by P63 immunoreactivity but not SMA which was negative in some tumors especially undifferentiated ones. Therefore, P63 is more specific for myoepithelial cells and shows no cross-reactivity with stromal myofibroblasts compared with SMA and calponin as reported previously (Sorenmo et al. 2011). Remarkably, the expression of CK, P63, SMA was reduced or almost diminished in poorly differentiated tumors which only expressed vimentin supporting the hypothesis of EMT proposed by Rismanchi et al. (2014).

Hematological alterations showed a non-significant reduction in erythrogram and MCV, and MCHC was within the reference intervals. PCV and Hb were reported to be reduced, along with close reference intervals for other hematological parameters in CMTs (Pankaj et al. 2014; Mohapatra et al. 2016; Kumar et al. 2018). Thrombocytes were elevated but were within the reference interval. Thrombocytosis and leukopenia were previously reported to be the most prominent change in CBC in malignant mammary tumors (Lallo et al. 2016). However, leukocytosis and anemia were associated with chronicity and extent of advanced stage (Da Silva et al. 2014). Thrombocyte elevation was associated with a systematic inflammatory response (Alexandrakis et al. 2002). Immune-mediated involvements and non-treated solid neoplasm were postulated as a cause (Lallo et al. 2016).

The increased incidence of CMTs promotes the researchers to improve the screening modalities for better diagnosis (Jain et al. 2021), among those modalities are the serum tumor markers. Our results showed significant elevation in CA 15-3 in CMT compared to normal healthy bitches. This elevation was reported previously (Manuali et al. 2012; Campos et al. 2012; Jain et al. 2021). The stage, size, and malignancy of the tumor are factored in the detected concentration of CA 15-3, as large size and malignancy were associated with higher concentrations of CA 15-3 (Jain et al. 2021). In human medicine, CA 15-3 is considered a specific diagnostic and monitoring tool for mammary tumors. It is elevated in around 70% of advanced cases (Ebeling et al. 2002; Marchesi et al. 2010). Higher levels of CA 15-3 are positively correlated with the tumor stage (Manuali et al. 2012; Kaszak et al. 2018). In the present study, CA 15-3 was elevated in association with CMT in all the diseased cases.
except one patient showed CA 15-3 similar to those of the control range. It was proposed that the elevation in CA 15-3 ectodomain is correlated with carcinoma, also the level of CA15-3 was significantly higher in the pre-treatment group compared to post-treatment and 6-months follow-up (Gupta et al. 2018). In one human study, the highest level of CA15-3 was seen in association with distant metastatic compared to locoregional ones (Wojtacki et al. 2001). In this study, there was no distant metastasis, and these results are on a par with those previously reported (Campos et al. 2012). CA 15-3 and CEA are relatively inexpensive and require a less invasive method of sample collection compared with immuno-histochemical tests, which have been used in veterinary medicine (Campos et al. 2012).

The present study did not detect changes in CEA between the control and diseased groups, as previously reported (Campos et al. 2012). It was hypothesized the CEA is expected to be elevated in 50 % of CM carcinomas (Valencakova-Agyagosova et al. 2012). There was no distant metastasis in this study, and this could explain our results. Moreover, CEA was demonstrated to detect early metastasis but not in a confirmatory way (Jain et al. 2021). However, other studies showed that a change is expected (Balint et al. 2008; Sorenmo 2011). Tumor grading, types, method of analysis, and distant metastasis could explain the difference in the results. Liver metabolism, kidney clearance rate, and method of assay could be implicated (Waisberg et al. 2002; Campos et al. 2012). Moreover, the lower positivity rate of CA15-3 in breast cancer makes it a controversial marker for the diagnosis of this type of tumor (Li et al. 2018). Previous studies (Loprinzi et al. 1986; Nan et al. 2017) reported that CEA lacks predictive ability in metastatic breast cancer, although other studies correlated it with poor prognosis (Lee et al. 2013; Dai et al. 2016). It was reported that CA 15-3 was higher in association with advanced CMT, indicating CA 15-3 has a better diagnostic value than CEA (Campos et al. 2012).

The use of miRNAs as indicators is becoming more common in human medicine as they are stable, non-invasive, and easy to assess markers (Bautista-Sánchez et al. 2020). miR-21, miR-155, and miR-10b are established as oncogenic and metastasis-promoting miRNAs in breast cancer (Ma et al. 2007; Yan et al. 2008). The oncogenes, miR-21 and miR-29b are associated with several cancers, including human mammary cancer, and have consistently been found to be upregulated in mammary cancers using microarray analysis (Blenkiron et al. 2007; Si et al. 2007; Zhu et al. 2007). As miR-21 is one of the most upregulated miRNAs in human breast cancer, it was postulated that it might have a future therapeutic potential (Dan et al. 2021).

In this study, an upregulation in miR-21 was detected in all diseased patients when compared to control. Nowadays, miRNAs gained wide popularity in CMTs as a potential candidate for detection, prognosis, and recently for usage in therapy (Jain et al. 2021). In this study, upregulation of miR-21 occurred in all patients while CA 15-3 was elevated in all patients except one that has CA 15-3 on par with control intervals. Both markers were able to differentiate between healthy and diseased subjects. This data might promote the addition of miR-21 in the diagnostic panel of CMT.

The miR-21 was upregulated in association with CMT in both benign and malignant tumors as reported in previous studies (von Deetzen et al. 2014; Jain et al. 2021). In this study, the upregulation was approximately 12-fold changes in CMT patients compared to the control group. However, previous reports showed miR-21 was upregulated in association with mammary gland neoplasms by 5-folds (Łosiewicz et al. 2014; Jain et al. 2021). Our findings support the notion that miR-21 was able to differentiate between healthy and diseased animals and that further investigations are required to correlate miR-21 overexpression in canine patients and individual tumor types, tumor grade, animal breed, and age (Jain et al. 2021). MiR-21 overexpression is linked to epithelial-to-mesenchymal transitioning (EMT), which can play a role in metastasis (Jain et al. 2021). The expression of miR-21 in our investigation was studied relative to U6 as a reference gene. Different reference genes were used in association with miR-21, among them U6 (Wu et al. 2015), and RNU6b (Boggs et al. 2008; Jain et al. 2021). MiR-21 targets both PTEN and TPM1, and loss of this miRNA results in increased caspase activity and subsequent apoptosis (Zhu et al. 2007; Meng et al. 2007).

In the examined animals, the up regulatory pattern of miR-21 was detected in association with CMT patients but not in their control counterparts which agreed with previous studies (Anwar et al. 2019; Jain et al. 2021). The upregulation of miR-21 in CMTs was suggested to be a valuable prognostic marker (Jain et al. 2021), in which higher expression of miR-21 was correlated with the worst clinical consequences compared with lower expression (Yan et al. 2008). In human breast cancer, miR-21 overexpression was reduced after surgery and chemotherapy completion suggesting that miR-21 may be a useful marker for therapeutic monitoring (Anwar et al. 2019). The overexpression of miR-21 was also linked to radiation resistance, in which a combination of anti-miR-21 and radiation was proposed to overcome the supposed resistance (Anastasov et al. 2012). Therefore, this upregulation makes miR-21 a potential candidate to be employed in therapeutic trials in CMTs (Boggs et al. 2008).

The tumors presented in the present study showed positive vimentin immunoreactivity, either partially or completely suggesting a mesenchymal origin of the tumor cells or occurrence of EMT. In esophageal squamous cell carcinoma, miR-21 expression was predominantly present in fibroblasts associated with cancer (Nouraei et al. 2013). In epithelial-to-mesenchymal transitioning (EMT), the main
alterations in the gene expression are linked to elevated mesenchymal genes (ex, Vimentin, and α-SMA) and reduction in the epithelial gene (ex, E-cadherin) (Bartkowiak et al. 2019). MiRNAs may play a role in the inhibition/initiation of EMT (Ghahhari et al. 2015). In a study conducted by Liu et al. (2015), they reported upregulation of Vimentin in association with elevated miR-21 and concluded that the metastasis and invasion of cholangiocarcinoma were powered by EMT partially induced by miR-21. Therefore, the elevation of miR-21 may be due to mesenchymal cells expressing vimentin.

This study showed that serum CA 15-3 is a minimally invasive tool that aids in detecting CMT. Its coupling with miR-21 up-regulation might improve the diagnostic outcomes. The main limitations of this study were the small sample size examined, the use of U6 as a normalizer for miR-21 as it might not be the perfect normalizer. Using a better technique like in-situ hybridization could elucidate the type of cells with mir-21 overexpression. The correlation between vimentin expression and upregulation of miR-21 should be further studied.

This study employed miR-21 to aid in the diagnosis of CMT in association with other serum biomarkers (CEA and CA 15-3) as well as histopathology. Further studying about the correlation between miR-21 upregulation, overall survival time, prognostic capability, and potential usage to monitor therapeutic response should be performed in dogs.

Conclusions

The present study showed CA 15-3 was elevated and miR-21 was upregulated in association with CMT. Including CA 15-3 and miR-21 in the diagnostic panel of CMT may improve the diagnostic outcomes. All tumors were positive for vimentin immunoactivity but not for CK, P63, SMA. The expression of different tumor markers performed was altered corresponding to cellular differentiation. These findings support the notion that miR-21 could be used as an ancillary test but in no circumstances a sole indicator of CMT. Further studies are still needed to evaluate the prognostic importance as well as the effect of targeting miR-21 as a therapeutic potential for CMT.

Abbreviations  CA 15-3: cancer antigen 15-3; CEA: carcinoembryonic antigen; CMT: canine mammary tumor; miR-21: microRNA-21; MT: mammary tumor

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Author contributions  All authors designed the study. NYS and ESR performed clinical examinations and estimated serum tumor markers and miR-21 expression. NAA, IAE, and HAF performed diagnostic imaging and surgical interference. MSK performed the pathological examination. All authors wrote the paper and read and approved the final manuscript.

Data availability  The data that support the findings of this study are within the manuscript.

Declarations

Ethical approval and consent to participate  This study was granted ethical approval permission of the Institutional Animal Care and Use Committee, Cairo University (Vet CU 2022020128).

Consent for publications  N/A.

Conflict of interest  The authors declare that they have no competing interests.

References

Abd Elkader NA, Emam IA, Farghali HA, M DS, Salem NY (2020) Oesophageal foreign bodies in cats: Clinical and anatomic findings. PLoS One 15(6):e0233983. https://doi.org/10.1371/journal.pone.0233983
Alexanderakis MG, Passam FH, Perisinakis K, Ganotakis E, Marganitis G, Kyriakou DS, Bouros D (2002) Serum pro-inflammatory cytokines and its relationship to clinical parameters in lung cancer patients with reactive thrombocytosis. Respir Med 96:553–558
Anwar SL, Sari DNI, Kartika AI, Fitria MS, Tanjung DS, Rakhmina D, Wardana T, Astuti I, Haryana SM, Aryandono T (2019) Upregulation of circulating MiR-21 expression as a potential biomarker for therapeutic monitoring and clinical outcome in breast cancer. Asian Pac J Cancer Prev 20(4):1223-1228. https://doi.org/10.31557/APJCP.2019.20.4.1223
Anastasov N, Höfig I, Vasconcellos IG, Rappl K, Braselmann H, Ludgya N, Auer G, Aubele M, Atkinson MJ (2012) Radiation resistance due to high expression of miR-21 and G2/M checkpoint arrest in breast cancer cells. Radiat Oncol 7:206. https://doi.org/10.1186/1748-717X-7-206
Balint E, Manolescu N, Branasu D et al (2008) Molecular detection through CEA (carcino-embrionic-antigen) of different types of cancer in animal. Lucr Ştiinţ Med Vet 51:89–93
Banerjee A, Islam MM, Das M, Chakrabarty S, Chowdhury S, Sarkar S (2018) Hemato-biochemical alterations associated with malignant mammary tumours in canine. Environ Ecol 36:860–863
Bartkowiak M, Bartkowiak K, Niderla-Bielińska, Jankowska-Steifer E (2019) The effects of miRNA-21 on the epithelial to mesenchymal transition in cancer. Rev Res Cancer Treat 5:50–56
Bautista-Sánchez D, Arriaga-Canon C, Pedroza-Torres A, De La Rosa-Velázquez I, González-Barrios R, Contreras-Espinoza L, Montiel-Manríquez R, Castro-Hernández C, Fragoso-Onitveros V, Alvarez-Gómez RM, Herrera LA (2020) The Promising role of miR-21 as a cancer biomarker and its importance in RNA-based therapeutics. Mol Ther Nucleic Acids 20:409–420
Benavente MA, Bianchi CP, Aba MA (2016) Canine mammary tumors: risk factors, prognosis, and treatments. J Vet Adv 6:1291–1300
Blenkiron C, Goldstein LD, Thorne NP et al (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumour subtype. Genome Biol 8:R214
Ghahhari NM, Babashah S (2015) Interplay between microRNAs and Fish EJ, Martinez-Romero EG, DeInnocentes P, Koehler JW, Prasad Eisenberg ALA, Koifman S (2001) Breast cancer: Tumor markers. Rev Egenvall A, Bonnett BN, Ohagen P, Olson P, Hedhammar A, von Ebeling FG, Stieber P, Untch M, Nagel D, Konecny GE, Schmitt Dolka I, Sapierzyński R, Król M (2013) Retrospective study and immu-
Dolka I, Sapierzyński R, Król M (2013) Retrospective study and immu-
Destexhe E, Lespagnard L, Degeyter M, Eymann RH, Coignoul F
Canadas A, França M, Pereira C et al (2019) Canine mammary tumors: comparison of classification and grading methods in a survival study. Vet Pathol 56:208–219
Cassali GD, Lavalle GE, De Nardi AB et al (2011) Consensus for the diagnosis, prognosis, and treatment of canine mammary tumors. Braz J Vet Pathol 4:153–180
Campos LC, Lavalle GE, Estrela-Lima A et al (2012) CA15.3, CEA and LDH in dogs with malignant mammary tumors. J Vet Intern Med 26:1383–1388
Chen Y, Sun Y, Chen L, Xu X, Zhang X, Wang B, Min L, Liu W
Campos LC, Lavalle GE, Estrela-Lima A et al (2012) CA15.3, CEA and LDH in dogs with malignant mammary tumors. J Vet Intern Med 26:1383–1388
Dai D, Chen B, Tang H, Wang B, Zhao Z, Xie X, Wei W (2016) Nomograms for predicting the prognostic value of pre-therapeu-
tic CA15-3 and CEA serum levels in TNBC patients. PLoS One 11(8):e0161902. https://doi.org/10.1371/journal.pone.0161902
Dan T, Shastri AA, Palagani A et al (2021) miR-21 plays a dual role in tumor formation and cytotoxic response in breast tumors. Cancers (Basel) 13(4):888
Da Silva AHC, Da Silva DM, Ribas CR, Dittrich RL, Dornbusch PT, Guérios SD (2014) Alterações no hemograma de cadelas com neoplasia mamária. Ciência Anim Bras 15:87–92 (Complete blood count changes in bitches with mammary tumor)
Diestexhe E, Lespagnard L, Degeyter M, Eymann RH, Coignoul F (1993) Immunohistochemical identification of myoepithelial, epithelial, and connective tissue cells in canine mammary tumors. Vet Pathol 30:146–154
Dolk I, Sapierzynski R, Król M (2013) Retrospective study and immuno-
histochemical analysis of canine mammary sarcomas. BMC Vet Res 9:248
Ebeling PG, Steiber P, Untch M, Nagel D, Konecny GE, Schmitt UM, Fateh-Moghadam A, Seidel D (2002) Serum CEA and CA 15-3 as prognostic factors in primary breast cancer. Br J Cancer 86:1217–1222
Egenvall A, Bonnett BN, Ohagen P, Olson P, Hedhammar A, von Euler H (2005) Incidence of and survival after mammary tumors in a population of over 80,000 insured female dogs in Sweden from1995 to 2002. Prev Vet Med 69:109–127
Eisenberg ALA, Koifman S (2001) Breast cancer: Tumor markers. Churchill & Livingstone, London, UK, pp 365–384
Eman SR, Kubesy AA, Baraka TA, Torad FA, Shaymaa IS, Mohamed FF (2018) Evaluation of hepatocyte-derived microRNA-122 for diagnosis of acute and chronic hepatitis of dogs. Vet World 11:667–673
Fish EJ, Martinez-Romero EG, DeInnocentes P, Koehler JW, Prasad N, Smith AN, Bird RC (2020) Circulating microRNA as biomark-
ers of canine mammary carcinoma in dogs. J Vet Intern Med 34:1282–1290
Ghahhari NM, Babashah S (2015) Interplay between microRNAs and WNT/beta-catenin signaling pathway regulates epithelial-mesen-
chymal transition in cancer. Eur J Cancer 51:1638–1649
Goldschmidt M, Peña L, Rasotto R, Zappulli V (2011) Classification and grading of canine mammary tumors. Vet Pathol 48(1):117–131
Gupta SK, Kumar V, Anees A, Goel A (2018) The study of prognostic significance of CA 15-3 in breast cancer. Int Surg J 5:580–583
Henry CJ, Higginbotham ML (2010) Cancer management in small animal practice. 1st edn. Saunders, Philadelphia
Hong L, Yang J, Han Y, Lu Q, Cao J, Syed L (2012) High expression of miR-210 predicts poor survival in patients with breast cancer: a meta-analysis. Gene 507:135–138
Iorio MV, Ferracin M, Liu CG et al (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65:7065–7070
Jain M, Inoge SD, Deshmukh RS et al (2021) CEA, CA 15-3, and miRNA expression as potential biomarkers in canine mammary tumors. Chromosome Res 29:175–188
Karayannopoulos M, Kaldymidou E, Constantinidis TC, Dessiris A (2005) Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. J Comp Pathol 133:246–252
Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M, Jurka P (2018) Current biomarkers of canine mammary tumors. Acta Vet Scand 60(1):66
Kumar VVVA, Kumari KN, Kumar KS, Kumar VG, Lakshman M (2018) Hemato-biochemical changes in mammary tumors affected dogs. J Pharm Innov 7:187–189
Kutzler M (2020) Mammary tumors in dogs and cats. MSD Veterinary Manual. Available from https://www.msdvetmanual.com/reprodu-
Kutzler M (2020) Mammary tumors in dogs and cats. MSD Veterinary Manual. Available from https://www.msdvetmanual.com/reproductive-system/mammary-tumors/mammary-tumors-in-dogs-and-cats.
Lallo MA, Ferrarías TM, Adriane Stravino, Rodriguez JFM, Zucare RLC (2016) Hematologic abnormalities in dogs bearing mammary tumors. R bras Ci Vet 23:3–8
Lee JS, Park S, Park JM, Cho JH, Kim SI, Park BW (2013) Elevated levels of preoperative CA 15-3 and CEA serum levels have independently poor prognostic significance in breast cancer. Ann Oncol 24:1225–1231
Li X, Dai D, Chen B, Tang H, Xie X, Wei W (2018) Clinopathological and prognostic significance of cancer antigen 15-3 and carci-
noembryonic antigen in breast cancer: a meta-analysis including 12,993 patients. Dis Markers 2018:9863092. https://doi.org/10.1155/2018/9863092
Liu Z, Jin ZY, Liu CH, Xie F, Lin XS, Huang Q (2015) MicroRNA-21 regulates biological behavior by inducing EMT in human cholan-
giocarcinoma. Int J Clin Exp Pathol 8:4684–4694
Loprinzi CL, Tormey DC, Rasmussen P, Falkson G, Davis TE, Falkson HC, Chang AY (1986) Prospective evaluation of carcinoembry-
onic antigen levels and alternating chemotherapeutic regimens in metastatic breast cancer. J Clin Oncol 4:46–56
Losiewicz K, Chmielewska-Krzesińska M, Socha P, Jakimiuk A, Wąsowicz K (2014) MiRNA-21, miRNA-10b, and miRNA-34a expression in canine mammary gland neoplasms. Bull Vet Inst Pulawy 58:447–451
Lui J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-
Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Robert Horvitz H, Golub TR (2005) MicroRNA expression profiles classify human cancers. Nature 435:834–838
Ma L, Teruya-Feldstein J, Weinberg RA (2007) Tumour invasion and metastasis initiated by microRNA–10b in breast cancer. Nature 449:682–688
Mall C, Rocke DM, Durbin-Johnson B, Weiss RH (2013) Stability of miRNA in human urine supports its biomarker potential. Biomark Med 7:623–631
Manueli E, De Giuseppe A, Feliziani F et al (2012) CA 15-3 cell lines and tissue expression in canine mammary cancer and the correla-
tion between serum levels and tumour histological grade. BMC Vet Res 8:86

Veterinary Research Communications (2022) 46:377–388
Marchesi MC, Manuelli E, Pacifico E, Ferri C, Romagnoli M, Mangili V, Fruganti G (2010) Cancer antigen 15/3: Possible diagnostic use in veterinary clinical oncology. Preliminary study. Vet Res Commun 34:103–106

Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133:647–658

Misorp W, Else RW, Hellenen E (1999) Histological classification of mammary tumors of the dog and the cat. World Health Organization, Geneva, Switzerland

Mohapatra AK, Das D, Panda SK, Singh J (2016) Spontaneous canine mammary neoplasia: A clinicopathological study. Cell Tissue Res 16:5661–5666

Moris J, Dobson J (2001) Textbook of small animal oncology. Blackwell Science, Hoboken

Nan J, Li J, Li X, Guo G, Wen X, Tian Y (2017) Preoperative serum carcinoembryonic antigen as a marker for predicting the outcome of three cancers. Biomark Cancer 9:1–7

Nguyen F, Peña L, Ibisch C et al (2018) Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. Breast Cancer Res Treat 167:635–648

Nourae N, Van Roosbroeck K, Vasei M et al (2013) Expression tissue distribution and function of miR-21 in esophageal squamous cell carcinoma. PLoS One 8(9):e73009

Owen L (1980) TNM classification of tumors in domestic animals. World Health Organization, Geneva

Papadaki C, Stratigos M, Markakis G et al (2018) Circulating microRNAs in the early prediction of disease recurrence in primary breast cancer. Breast Cancer Res 20(1):7

Pandey PR, Saidou J, Watabe K (2010) Role of myoepithelial cells in breast tumor progression. Front Biosci 15:226–236

Pankaj G, Raghunath M, Gupta AK, Ankur S, Kawardeep K (2014) Clinical study for diagnosis and treatment of canine mammary Neoplasms (CMNs) using different modalities. Indian J Anim Res 48:45–49

Peña L, Andrés PJ, Clemente M, Cuesta P, Pérez-Alenza MD (2013) Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Vet Pathol 50:94–105

Peña L, Gama A, Goldschmidt MH et al (2014) Canine mammary tumors: A review and consensus of standard guidelines on epithelial and prognostic factors. Breast Cancer Res Treat 167:635–648

Rismanchi S, Yadegar O, Muhammadnejad S, Amanpour S, Taghizadeh-Jahed M, Mohammadnejad A (2014) Expression of vimentin filaments in canine malignant mammary gland tumors: A simulation of clinicopathological features of human breast cancer. Biomed Rep 2:725–728

Rivera P (2010) Biochemical markers and genetic risk factors in canine tumors. Doctoral thesis, Swedish university of agricultural sciences

Shao Y, Sun X, He Y, Liu C, Liu H (2015) Elevated levels of serum tumor markers CEA and CA15-3 are prognostic parameters for different molecular subtypes of breast cancer. PLoS ONE 10(7):1–11

Senhorel ILS, Terra EM, Sueiro FAR et al (2020) Clinical value of carcinoembryonic antigen in mammary neoplasms of bitches. Vet Comp Oncol 18:315–323

Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY (2007) miR-21-mediated tumor growth. Oncogene 26:2799–2803

Simeonov R, Stoiko D (2006) Study on the correlation between the cytological and histological tests in the diagnostics of canine spontaneous mammary neoplasms. Bulg J Vet Med 9(3):211–219

Sleeckx N, de Rooster H, Veldhuis Kroeze E, Van Ginneken C, Van Brantegem L (2011) Canine mammary tumours, an overview. Reprod Domest Anim 46:1112–1131

Sorenko NU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, Mongil CM, Grondahl AM, Teige J, Goldschmidt ME (2009) Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. Vet Comp Oncol 7:162–172

Sorenko NU (2011) Canine mammary tumors: clinical features, diagnostics, and staging. World Small Animal Veterinary Association World Congress Proceedings. Available from https://www.vin.com/appuity/content/defaultadv1.aspx?pld=11343&catId=34576&id=5124313

Sorenko NU, Rascotto R, Zappulli V, Goldschmidt MH (2011) Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. Vet Pathol 48:85–97

Tavusoly A, Golshahi H, Rezaie A, Farhari M (2013) Classification and grading of canine malignant mammary tumors. Vet Res Forum 4:25–30

Valencakova-Agyagosova A, Frischova Z, Sekvicova Z et al (2012) Determination of carcinoembryonic antigen and cancer antigen (CA 15-3) in bitches with tumors on mammary gland: preliminary report. Vet Comp Oncol 12(3):205–214

Vascellar1 M, Capello K, Carminato A, Zanardello C, Bai S, Italianini L (2016) Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. Prev Vet Med 126:183–189

Von Deetzen MC, Schmeck BT, Gruber AD, Klopfleisch R (2014) Malignancy Associated MicroRNA expression changes in canine mammary cancer of different malignancies. Int Sch Res Notices 2014, Article ID 148597, 5 pages. https://doi.org/10.1155/2014/14859765

Waisberg J, Landman G, Cha ASH et al (2002) Standard of the histopathological distribution of the CEA in the carcinoma colorectal: relation with the serial level of the CEA and Dukes’ classification. Rev Bras Coloproctol 22:20–26

Wojtacki J, Kruszewski WJ, Sliwińska M et al (2001) Elevation of serum Ca 15-3 antigen: an early indicator of distant metastasis from breast cancer. Retrospective analysis of 733 cases. Przegl Lek 58:498–503. Polish

Wu J, Li G, Wang Z, Yao Y, Chen R, Pu X, Wang J (2015) Circulating microRNA-21 is a potential diagnostic biomarker in gastric cancer. Dis Markers 2015:435656. https://doi.org/10.1155/2015/435656

Wu Y, Song X, Xiong Y et al (2017) MicroRNA-21 (Mir-21) promotes cell growth and invasion by repressing tumor suppressor PTEN in colorectal cancer. Cell Physiol Biochem 43:945–958

Yan LX, Huang XF, Shao Q et al (2008) MicroRNA miR–21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis, and patient poor prognosis. RNA 14:2348–2360

Yuan JS, Reed A, Chen F, Stewart CN Jr (2006) Statistical analysis of real-time PCR data. BMC Bioinf 7:85

Zhu S, Si ML, Wu H, Mo YY (2007) MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 282:14328–14336

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