Determining the expression levels of circulating tumour cell markers in canine mammary tumours

Zeynep Günay Uçmak[1], Özlem Timirci Kahraman[2], Güldal İnal Gültekin[3], Sevgin Değirmencioğlu[4], İlhan Yaylım[2], Kazım Güvenç[1]

[1]Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Istanbul, Turkey
[2]Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Molecular Medicine, Istanbul, Turkey
[3]Istanbul Okan University, Faculty of Medicine, Department of Physiology, Istanbul, Turkey
[4]Istanbul Arel University, Faculty of Medicine, Department of Biochemistry, Istanbul, Turkey

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Abstract

Detection of the circulating tumour cells (CTC) in dogs with a mammary tumour is a useful tool to reveal the micrometastases long before metastases are recognised clinically. The aim of this study was to evaluate the association of the epidermal growth factor receptor (EGFR), claudin 7 (CLND7) and epithelial cell adhesion molecule (EPCAM) with the clinical indices and to reveal the diagnostic importance of these biomarkers in canine mammary tumours (CMTs). Peripheral blood (PB) samples were collected from 45 bitches (group MT) which had single mass with malignant epithelial tumours and 9 healthy bitches (group H). Real time PCR (rt-PCR) was performed to determine the expression levels of EGFR, CLDN7, and EPCAM. Mean values of EGFR and CLDN7 expressions were significantly higher in group MT compared to group H ($P < 0.01$ and $P < 0.001$, respectively). The expression level of CLDN7 was positively correlated with EGFR and EPCAM ($P < 0.001$ and $P < 0.05$, respectively). The EPCAM expression was associated with increased tumour size ($P < 0.05$) and EPCAM tended to decrease in the presence of skin ulceration on tumour ($P = 0.05$). Furthermore, expression levels of EGFR in intact dogs were significantly higher compared to spayed dogs in group MT ($P < 0.01$). The EGFR expression was significantly higher in the presence of metastases ($P < 0.05$). Also, increased EGFR was determined in grade 2 compared to grade 1 ($P < 0.05$). In conclusion, these results show that EGFR, CLDN7, EPCAM markers are measureable in PB and they may provide valuable information about the clinical pathophysiology of CMT.

Claudin 7, epidermal growth factor receptor, epithelial cell adhesion molecule

Metastatic risks in canine mammary tumours (CMTs) are determined depending upon several clinical prognostic factors such as the tumour type, histological differentiation, and tumour size (Ferreira et al. 2009). Metastatic cancer cells named as circulating tumour cells (CTCs) are spread to distant organs by blood and lymph vessels (Sleeckx et al. 2011; Canadas et al. 2019). The CTCs have a prognostic value because these markers are able to detect metastases before they are recognized clinically. The CTC markers are potential mRNA markers that are expressed in mammary carcinoma (da Costa et al. 2013; Lee et al. 2018).

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that plays a role in growth, differentiation, proliferation and anabolism of tumour cells (Tang et al. 2012; Kaszak et al. 2018). Claudins have an important role in the tight junction (TJ) in canine mammary epithelial cells (Jakab et al 2008). Oshima et al. (2008) have claimed that loss or decreased levels of claudin 7 (CLDN7) are a metastatic predictor. Besides, the epithelial cell adhesion molecule (EPCAM) is one of the most common epithelial markers

Address for correspondence:
Assoc. Prof. Kazim Guvenc
Department of Obstetrics and Gynecology
Cerrahpaşa Faculty of Veterinary Medicine
Istanbul University
34320 Avcılar, Istanbul, Turkey

Phone: 0212 4737070/17318
E-mail: kguvenc@istanbul.edu.tr
http://actavet.vfu.cz/

in humans (Alunni-Fabbroni and Sandri 2010). The EPCAM reflects the cancer progression and prognosis and it is used as a diagnostic and prognostic marker in humans with breast carcinomas (Gao et al. 2017). There were several reports on tissue expression of EGFR (Gama et al. 2009; Carvalho et al. 2013; Guimarães et al. 2014) and CLDN7 (Jakab et al. 2008; Hammer et al. 2016) in CMTs. Moreover, e-cadherin was usually expressed in tissue as cell adhesion molecule in CMTs but in human breast cancer EPCAM expression was evaluated as a cell adhesion molecule (Gao et al. 2017).

Detection of the CTC markers from the peripheral blood (PB) of dogs with a mammary tumour in the pre-operative term may be a useful tool for early diagnosis of CMTs. The aim of this study was to investigate the expression levels of EGFR, CLDN7, and EPCAM in PB. The relationship between the clinical indicators (ovariohysterectomy [OVH] status, histology types, histology grade, metastases, ulceration and necrosis) and the circulated tumour markers (EGFR, CLDN7, EPCAM) in dogs with mammary tumor were explored.

Materials and Methods

The animal care protocol and experimental procedures in this study were approved by the Local Ethics Committee of Istanbul University (Approval Number: 2013/14).

Animals and study design

Forty-five bitches that had a single mass with malignant epithelial tumour (group MT) and 9 healthy bitches (group H) were included in this study. Nine bitches in group H were presented to our clinic for OVH section. The mean age and weight of the bitches in group MT and group H were 11.19 ± 0.41 years vs 5.94 ± 1.21 years and 16.26 ± 1.49 kg vs 20.16 ± 2.73 kg, respectively. The breeds in the groups were Golden Retriever, Terrier, German Shepherd, Kangal, Cocker Spaniel, Labrador Retriever and mix breed. Vaginal examination (palpation, cytology, vaginoscopy), abdominal ultrasonography and examination of mammary glands were performed in the clinical examination of all groups. Complete blood count and biochemical analyses were performed in order to evaluate the preoperative anaesthesia risks in both groups. Three-view thoracic radiography was taken by computerized radiography (Orex PcCR 1417 and Viztek diagnostic imaging program, USA) in order to evaluate macrometastases of the lungs. The PB samples for RNA isolation were collected in the pre-operative term into EDTA containing tubes. The 0.5 ml blood from an EDTA containing tube and 1.3 ml RNA-later solution was collected into an Eppendorf tube for each bitch. The blood samples were stored at -20 °C until RNA isolation. The bitches were initially premedicated with atropine sulphate (0.03 mg/kg, s.c.) (Atropin®, Teknovet, Turkey). For induction of anaesthesia, 1% propofol (Lipuro®, Braun, England) was used at 4 mg/kg, i.v.. The anaesthesia was maintained with 3% isoflurane (Forane liquid®, Abbott Laboratories, England) and 0.5–1% oxygen combination. Mastectomy was performed using the method described by Kirsan et al. (2005). Ovariohysterectomy was not performed during mastectomy for the intact bitches in group MT to avoid the contamination of tumour cells into the abdomen. Stages of CMTs were established according to the tumour-lymph node-metastasis (TNM) staging system (Owen 1980). The histopathological classification of CMTs was obtained according to Gundim et al. (2016). Histological malignancy grades of CMTs were classified according to Goldschmidt et al. (2011) as grade 1, 2, and 3.

Total RNA isolation and real time PCR procedure

A commercial total RNA purification kit was used for RNA isolation (Ribo-Pure Blood kit, Ambion, Cat. No. AM1928, USA). Moreover, DNAse I treatment was performed to prevent DNA contamination at the end of the RNA isolation process according to manufacturer's protocol (Ribo-Pure Blood kit, Ambion, Cat. No. AM1928, USA). After the amount and purity of the RNAs were analyzed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), isolated RNAs were stored at -86 °C. A script complementary DNA (cDNA) synthesis kit (Jena Bioscience PCR-511L, Germany) was used to transcribe 1 µg of each total RNA sample into cDNA in accordance with the manufacturer's protocol. Commercial kits (Jena Bioscience, qPCR ProbesMaster UNG/lowROX, Cat. No.PCR-306L, Germany) and validated primer-probe strands belonging to EGFR, CLDN7, and EPCAM were used for rt-PCR which was performed according to the manufacturer's protocol on a Bio-Rad CFX 96 (Bio-Rad, USA). Reference sequence numbers related to EGFR, CLDN7, and EPCAM were XM_533073.2, XM_546484.2 and XM_538481.2, respectively. In addition, ATP5B was used as the housekeeping gene (HKG) because researchers (Bougarn et al. 2011; Hvid et al. 2011) have reported that ATP5B is the most stably expressed HKG in the mammary epithelial cells in animals (Ref. Seq. No. XM_531639.2). Fold changes were evaluated using relative mRNA expression $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The SPSS 22.0 (IBM SPSS Statistics, ABD) program was used for statistical analysis. The relation between fold change values ($2^{-\Delta\Delta Ct}$) of the tumour markers in terms of the groups were evaluated by *t*-test. One-way

Anova and *t*-test were performed for determining the relationship between group MT and clinical indicators (OVH status, histology types, histology grade, metastases, ulceration and necrosis) by evaluating ΔCt values of the tumour markers. Down-regulation percentages of the tumour markers in regard to different mass sizes were evaluated by chi-square test. Correlation between the expression levels of the tumour markers were evaluated with Pearson correlation test. Besides, association between the levels of tumour markers and the presence of metastasis in group MT were evaluated by Pearson correlation test. The receiver-operating characteristics (ROC) curve analysis was performed to determine sensitivity and specificity of EGFR, CLDN7, and EPCAM in group H and MT. The significance level was accepted at $P < 0.05$.

Results

The CTC markers were analysed in the pre-operative term for determining the association between mRNA expression levels of the markers and various clinical indicators (OVH status, tumour size, histology types, histology grade, metastases, ulceration and necrosis). Numbers of the bitches with MT according to clinical indicators are presented in Table 1. Mean values of EGFR, EPCAM, and CLDN7 mRNA expressions in PB of both groups are presented in Table 2. The mRNA expression levels of EGFR and CLDN7 were significantly higher in group MT compared to group H. Also, ROC curve results for EGFR, EPCAM, CLND7 and their significances were noted as 0.777(ΔCt) ± 0.84, 0.647(ΔCt) ± 0.99, 0.931(ΔCt) ± 0.40 and $P < 0.01$, $P > 0.05$, $P < 0.001$, respectively. As a result of ROC curve analysis, cut-off points for EGFR, EPCAM, and CLDN7 were 4.937, 3.395, and 6.002, respectively. Pearson correlation coefficients (PCC)

Table 1. Number of the bitches with MT according to clinical indicators.

Clinical indicator	Number of bitches with MT
OVH status (n = 45)	
(+)	7
(-)	38
Tumor size (n = 45)	
< 5cm	12
5–10cm	12
> 10cm	21
Histological type (n = 45)	
Tubular carcinoma	7
Tubulopapillar carcinoma	9
Solid carcinoma	6
Complex carcinoma	18
Carcinosarcoma and malignant mix tumour	5
Histological grade (n = 45)	
1	13
2	13
3	19
Metastases (n = 45)	
(+)	22
(-)	23

Table 2. Expression levels ($2^{-\Delta\Delta Ct}$) of EGFR, CLDN7 and EPCAM and their significance (*P*) in both groups (*t*-test).

Indicator	Group	Mean (±) SEM	P values
EPCAM ($2^{-\Delta\Delta Ct}$)	MT (n = 45)	4.54 ± 0.22	> 0.05
	H (n = 9)	3.83 ± 0.52	
CLDN7 ($2^{-\Delta\Delta Ct}$)	MT (n = 45)	7.24 ± 0.19[a]	< 0.001
	H (n = 9)	5.04 ± 0.27[b]	
EGFR ($2^{-\Delta\Delta Ct}$)	MT (n = 45)	6.12 ± 0.17[a]	< 0.01
	H (n = 9)	4.84 ± 0.39[b]	

[a,b] Different superscripts in the same column indicate significant differences at $P < 0.01$ and $P < 0.001$.
MT - dogs with a malignant epithelial tumour; H - healthy dogs; SEM - standard error of the mean; EPCAM - epithelial cell adhesion molecule; CLDN7 - claudin 7; EGFR - epidermal growth factor receptor

and significances of Pearson correlation test for the expression levels of EGFR, EPCAM, and CLDN7 are presented in Table 3. The expression level of EPCAM was positively correlated with CLDN7 and EGFR.

Table 3. Pearson correlation coefficients and significances of the Pearson correlation test for the tumour markers.

		EPCAM (ΔCt)	CLDN7 (ΔCt)	EGFR (ΔCt)
EPCAM (ΔCt)	PCC	1	0.302	0.532
	P	-	< 0.05	< 0.001
CLDN7 (ΔCt)	PCC	0.302	1	0.667
	P	< 0.05	-	< 0.001
EGFR (ΔCt)	PCC	0.532	0.667	1
	P	< 0.001	< 0.001	-

EPCAM - epithelial cell adhesion molecule; CLDN7 - claudin 7; EGFR - epidermal growth factor receptor; PCC - Pearson correlation coefficients; *P* - significance

Macroscopic metastases on lungs were diagnosed in 22 bitches in group MT. Although the expression levels of EPCAM and CLDN7 in group MT were not significantly associated *(P* > 0.05*)* with the presence of metastasis, EGFR expression was significantly higher in dogs with metastases *(P* < 0.05*)*. In the presence of metastases in group MT, EGFR levels were positively correlated with EPCAM and CLDN7 (*P* < 0.05; PCC: 0.463 and 0.516, respectively). However, in the absence of metastases in group MT, EGFR levels had a strong correlation with CLDN7 (*P* < 0.01 and PCC: 0.621). Furthermore, 19 dogs had an ulcerated mammary tumour. Whereas the presence of ulceration was not associated with the expression of CLDN7 and EGFR *(P* > 0.05*)*, EPCAM expression tended to decrease in dogs with an ulcerated mammary tumour *(P* = 0.05*)*.

Seven bitches with a mammary tumour had already been spayed after 6 years of age. Effects of OVH on the EGFR, CLDN7, and EPCAM expression levels in group MT are presented in Table 4. The CLDN7 and EPCAM expression levels were not associated with the OVH status of the bitches in group MT. The mRNA expression level of EGFR significantly increased in intact bitches belonging to group MT *(P* < 0.01*)*.

Table 4. Effect of OVH on EGFR, CLND7, and EPCAM expressions in group MT.

Indicators	OVH (+/-)	Mean (±) SEM	*P* value
EPCAM (ΔCt)	OVH (+) (n = 7)	3.83 ± 0.80	> 0.05
	OVH (-) (n = 38)	4.68 ± 0.22	
CLDN7 (ΔCt)	OVH (+) (n = 7)	6.62 ± 0.42	> 0.05
	OVH (-) (n = 38)	7.35 ± 0.21	
EGFR (ΔCt)	OVH (+) (n = 7)	5.07 ± 0.42 [a]	< 0.01
	OVH (-) (n = 38)	6.32 ± 0.17 [b]	

[a,b] Different superscripts in the same column indicate significant differences (*P* < 0.01). EPCAM - epithelial cell adhesion molecule; CLDN7 - claudin 7; EGFR - epidermal growth factor receptor; SEM - standard error of the mean; OVH - ovariohysterectomy

According to the TNM classification system, no significant difference was found between tumour markers with regard to tumour size *(P* > 0.05*)*. However, when the mass size was reclassified into 3 different groups (< 5cm, 5–10 cm, > 10cm) in addition to the TNM system, the EPCAM expressions were significantly up-regulated in tumours up to 10 cm *(P* < 0.05; Table 5). Also, it was indicated that EGFR and CLDN7 were not significantly associated with tumour size in this study *(P* > 0.05*)*.

Table 5. Down-regulation percentages of EPCAM, CLDN7, and EGFR according to mass size in dogs with mammary carcinoma.

	Mass size			*P* value
	>10 cm	5–10 cm	< 5 cm	
EPCAM	0% [a]	42.3% [ab]	66.7% [b]	< 0.05
CLDN7	100%	76.9%	86.7%	> 0.05
EGFR	50%	73.1%	73.3%	> 0.05

[a,b] Different superscripts in the same row indicate significant differences ($P < 0.05$). EPCAM - epithelial cell adhesion molecule; CLDN7 - claudin 7; EGFR - epidermal growth factor receptor

The histopathological subtypes of CMTs were tubular carcinoma, tubulopapillary carcinoma, solid carcinoma, complex carcinoma, carcinosarcoma and malignant mixed tumours. Relationships between the tumour subtypes and EGFR, EPCAM, CLDN7 expression levels are presented in Table 6. The EGFR expression levels were significantly higher in dogs with tubular carcinoma, carcinosarcoma, and malign mix tumours compared to group H *($P < 0.05$)*. Increased levels of CLDN7 were detected in group MT *($P < 0.001$)*. Distinction between group H and group MT could be made by EGFR and CLDN7, however, EPCAM, EGFR, and CLDN7 were not capable enough to make a distinction between histological subtypes of CMTs *($P > 0.05$)*.

Table 6. Effect of tumour types on EGFR, CLDN7, and EPCAM.

Indicator	Tumour type	Mean (±) SEM	*P* value
EPCAM (ΔCt)	Group H (n = 9)	3.83 ± 0.52	> 0.05
	Tubular carcinoma (n = 7)	4.42 ± 0.40	
	Tubulopapillar carcinoma (n = 9)	4.72 ± 0.63	
	Solid carcinoma (n = 6)	4.11 ± 0.85	
	Complex carcinoma (n = 18)	4.59 ± 0.35	
	Carcinosarcoma and malignant mix tumour (n = 5)	4.76 ± 0.47	
CLDN7 (ΔCt)	Group H (n = 9)	5.04^{b} ± 0.27	< 0.001
	Tubular carcinoma (n = 7)	7.89^{a} ± 0.58	
	Tubulopapillar carcinoma (n = 9)	6.55^{a}±0.28	
	Solid carcinoma (n = 6)	7.45^{a} ± 0.66	
	Complex carcinoma (n = 18)	7.31^{a} ± 0.31	
	Carcinosarcoma and malignant mix tumour (n = 5)	7.04^{a} ± 0.49	
EGFR (ΔCt)	Group H (n = 9)	4.84^{b} ± 0.39	< 0.05
	Tubular carcinoma (n = 7)	6.72^{a} ± 0.30	
	Tubulopapillar carcinoma (n = 9)	5.96^{ab} ± 0.28	
	Solid carcinoma (n = 6)	5.55^{ab} ± 0.54	
	Complex carcinoma (n = 18)	6.04^{ab} ± 0.29	
	Carcinosarcoma and malignant mix tumour (n = 5)	6.58^{a} ± 0.67	

[a,b] Different superscripts in the same column belong to each marker indicating significant differences ($P < 0.05$ and $P < 0.001$). SEM - standard error of the mean; CLDN7 - claudin 7; EGFR - epidermal growth factor receptor

The results of histopathology grading and corresponding numbers of affected dogs were grade 1 (n = 13), grade 2 (n = 13), and grade 3 (n = 19). The expression level of EPCAM was not significantly associated with the histological grade *($P > 0.05$)*. The CLDN7 expression tended to increase in grade 2 compared to grade 1 but failed to reach significance *($P = 0.07$)* (7.55ΔCt ± 0.33, 6.94ΔCt ± 0.36, respectively). The EGFR expression was significantly higher in grade 2 compared to grade 1 *($P < 0.05$)* (6.49ΔCt ± 0.39 and 5.73ΔCt ± 0.26, respectively).

Discussion

Identification of CTCs is a potential marker for understanding the clinical course of canine mammary tumours (da Costa et al. 2013). Da Costa et al. (2011) specified that PCR assays were able to sensitively detect EGFR and CLDN7 in PB leukocytes. Likewise, EGFR and CLDN7 were detected in circulation by rt-PCR assay. To our knowledge, this is the first study on EPCAM expression in PB in dogs with a mammary tumour. The EPCAM and CLDN7 interact in tumour progression (Nübel et al. 2009; Wu et al. 2013). Gires et al. (2020) reported that EPCAM and its proteolytic fragments interact with EGFR for the epithelial-to-mesenchymal transition (EMT) of carcinoma cells. Similar to the previous reports (Nübel et al. 2009; Wu et al. 2013; Gires et al. 2020), EPCAM was positively correlated with EGFR and CLDN7 in this study.

The rise in EGFR expression in group MT was assumed to be due to the proliferation, growth and differentiation of the tumour cells, as reported by Koltai et al. (2018). Da Costa et al. (2013) specified that CLDN7 expression was higher in PB of dogs with non-metastatic mammary carcinomas compared to those with mammary adenomas, and CLDN7 was not expressed in PB of healthy dogs. In contrast to Jakab et al. (2008) who detected reduced expression of CLDN7 in malignant canine mammary epithelial tumor tissue, CLDN7 expression was higher in PB of dogs in group MT compared to group H. It was hypothesized that increased expression of CLDN7 in PB of dogs in group MT could be explained by the degeneration of TJ permeability due to the loss of cell-to-cell adhesion in mammary tissue which is well supported by current research (Kominsky et al. 2003; da Costa et al. 2013). Da Costa et al. (2011) investigated EPCAM in PB samples of dogs with a mammary tumour but failed to detect EPCAM in all samples. Thamm et al. (2016) indicated that mean EPCAM expression in CMT tissue was higher in tumor cells of epithelial origin than those of non-epithelial origin. To our knowledge, this was the first study to detect EPCAM in PB of dogs with mammary tumours and healthy dogs using a rt-PCR assay. However, EPCAM expression levels in PB were not sufficient to distinct the healthy dogs from the dogs with mammary tumours in this study *(P*>0.05*)*. It was suggested that non-significant results were obtained in this study due to the down-regulation of EPCAM in tumour tissue as researchers have reported for human breast cancer (Königsberg et al. 2011; Zhang et al. 2017).

Carvalho et al. (2013) reported that EGFR expression is associated with increased angiogenesis and metastasis in CMT as in human breast cancer. Also Queiroga et al. (2017) indicated that increased concentration of EGFR was determined in mammary tumour tissue of dogs with distant metastases. In line with the previous reports, increased EGFR expression in PB was detected in dogs with distant metastases. Further studies need to confirm the therapeutic target of EGFR in case of malignant CMTs. Reduced expression of CLDN7 was correlated with a higher tumour grade, metastatic disease, including loco-regional recurrences and with cellular adhesion (Sauer et al. 2005). Loss of CLDN7 may play a role in metastasis and it is considered to be related with the loss of cell adhesion (Kominsky et al. 2003). When CLDN7 expressions were evaluated in PB of the dogs with metastatic and non-metastatic mammary tumours, the non-significant results of CLDN7 expression were thought to be due to the reduced cell adhesion in tissue.

EPCAM is a transmembrane glycoprotein that regulates the cell-to-cell contact (Pavšič et al. 2014). In line with previous reports (Pavšič et al. 2014), it was suggested that EPCAM expression in PB tended to decrease in presence of ulceration on tumour skin due to the deterioration of the cell-to-cell adhesion mechanism ($P = 0.05$). Other researchers (Carvalho et al. 2013; Guimaraes et al. 2014) indicated that high EGFR immunoreactivity was associated with tumour necrosis. However, Gama et al. (2009) reported that EGFR expression in mammary tissue did not indicate significant differences

in terms of necrosis and ulceration. Although the analyzed material was different (tissue or PB), similar results were obtained (Gama et al. 2009). Kaszak et al. (2018) reported that EGFR is involved in tumour growth. Hu et al. (2021) indicated that tumour growth slowed after ulceration. It was thought to be because of EGF down-regulating its own receptor in the presence of ulceration and necrosis. Cell-to-cell adhesions in epithelial cell sheaths were maintained by claudins (Kominsky et al. 2003). However, CLDN7 expression in PB was not associated with the presence of ulceration ($P > 0.05$). It was hypothesized that non-significant results were achieved due to the deterioration of cellular adhesion from the beginning of tumour development.

Donnay et al. (1993) reported a positive correlation between oestrogen receptors and EGFR expression in the tissue of malignant CMT. Similarly, increased EGFR expression in PB was determined ($P < 0.01$) in intact dogs with mammary tumours in this study.

Queiroga et al. (2017) investigated EGFR expression in CMT tissue by ELISA assay and significantly different results were obtained for complex carcinoma and simple carcinoma compared to solid carcinoma and carcinosarcoma ($P < 0.001$). However, Guimarães et al. (2014) reported that EGFR immunoreactivity was not associated with the histological type of CMTs. Besides, the immunocytochemical expression of the TJ protein CLDN7 in smears from breast carcinomas was not significantly correlated with tumour subtypes (Soysal et al. 2013). Ring et al. (2015) hypothesized that EPCAM mRNA expression was feasible for most breast cancer subtypes. In this study, circulating mRNA expression levels of EGFR, CLDN7, and EPCAM were not significantly associated with tumour subtypes ($P > 0.05$). While EGFR expression levels showed significant differences between some tumour subtypes and healthy dogs, CLDN7 was able to distinguish only healthy dogs from dogs with mammary tumours in this study. In consideration of the reported data, it was suggested that expression levels of these tumour markers did not show an alteration in regard to the analysis techniques or epithelial tumour subtypes.

When tumour markers were evaluated according to tumour size (with reclassified staging in addition to TNM), increased EGFR expression levels were explained by the role of EGFR in the proliferation in CMT cells, as reported by Kennedy et al. (2011). Other researchers (St Arnaud et al. 1984; Bertics et al. 1985) noticed that EGFR levels were affected by many factors such as growth factors (platelet-derived growth factor, transforming growth factor) and tumour promoters (12-O-tetradecanoylphorbol 13-acetate or teleocidin). Also, EGF regulates these factors by decreasing its own receptor. As specified by the authors (St Arnaud et al. 1984), due to EGF down-regulating its receptor in tumour-growing tissue, EGFR expression levels increased in the PB of dogs with a mass less than 10 cm in diameter ($P < 0.05$). Although CLDN7 had over-expression in dogs with mammary tumours, CLDN7 expression levels were not significant ($P > 0.05$) in terms of different tumour sizes, in keeping with the reported article (Sauer et al. 2005). Tandon et al. (1990) also notified a positive correlation between the tumour mass size and rate of tumour proliferation. It has been reported that EPCAM expression was associated with worsening prognosis, and the mean tumour size of EPCAM positive breast cancer patients was more than 34 mm (Soysal et al. 2013). Also, Abd El-Maqsoud and Abd El-Rehim (2014) indicated that EpCAM over-expression was associated with tumour size ($P = 0.05$) in invasive breast cancer. Similarly, EPCAM expression was significantly ($P < 0.05$) up-regulated in increased tumour sizes (> 10 cm). It was concluded that dogs with large masses should be followed-up prognostically.

Another clinical indicator in the present study was the histological grading of tumours which had an effective role in the prognosis. Gama et al. (2009) reported that EGFR expression was not associated with tumour histological grade in canine mammary tissues. Contradictory results were obtained to their report (Gama et al. 2009) due to the difference in the analysed material (tissue or blood). Similar to Guimaraes et al. (2014),

EGFR expression was significantly associated with the histological grade of malignancy ($P < 0.05$). However, there are contradictory results with respect to EPCAM expression in terms of histological grading in human literature (Li et al. 2016). In further studies, the reflection of the EPCAM gene into the phenotype and investigation of protein levels are required to evaluate the EPCAM with regard to metastases and histological grading in dogs. The CLDN7 expression was inversely correlated with histological grading in CMT tissue (Jakab et al 2008) and in humans with breast carcinoma cells (Kominsky et al. 2003). However, CLDN7 expression in PB tended to increase in grade 2 compared to grade 1 in this study but failed to reach significance. Inconsistent results were assumed to be due to the difference in the analysed material with respect to previous reports (Kominsky et al. 2003; Jakab et al. 2008; Li et al. 2016).

In conclusion, the measurement of tumour markers in PB was found to be effective in CMTs. Besides, association between the expression of the CTC markers (EPCAM, EGFR, CLDN7) in PB and clinical indicators was proved in dogs with mammary tumours. Further studies are required to put these markers into the clinical practice by establishing procedures for early diagnosis and treatment.

Conflict of interest

The authors(s) declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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