FULL PAPER *Surgery*

Pathological Classification of Canine Mammary Tumor Based on Quantifying mRNA Levels of Hormonal Receptors, SATB1, and Snail in Tissue and Fine Needle Biopsy Samples

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ABSTRACT. Cytological diagnosis is not generally conclusive enough to identify histopathological malignancy in canine mammary tumors (CMTs). To establish cytological examination using fine needle biopsy (FNB) samples, gene expressions of hormonal receptors, human epidermal growth factor receptor 2 (HER2), and transcription regulators (Special AT-rich binding protein 1: SATB1 and Snail) were investigated in both tissue and FNB samples of CMTs. In tissue samples of malignant CMTs, especially invasive ones, low expressions of hormonal receptors and high expressions of SATB1 and Snail were detected. On discriminant analysis of tissue samples, 73.2% of CMTs were correctly classified according to histopathological examinations. In FNB samples of malignant CMTs, low expressions of hormonal receptors were detected. On discriminant analysis of FNB samples, 74.2% of CMTs were correctly classified according to histopathological examination. In conclusion, FNB gene expressions had a utility for diagnosis of CMTs malignancy in some degree. By researching more sensitive genes for malignant CMTs, the gene examination of FNB samples from CMTs will become a useful diagnostic tool that can be performed easily without anesthesia and could predict tumor malignancy and invasion prior to surgical removal.

KEY WORDS: canine, Fine Needle Biopsy, gene expression, mammary tumors.

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Canine mammary tumors (CMTs) are the most frequent neoplasms in female dogs. Based on the histopathological diagnosis, approximately half of CMTs is classified as malignant, and evaluation of tumor malignancy is clinically essential to determine the type of surgery [9, 17]. Histopathological diagnosis is the standard tool for determining tumor malignancy. However, most patients undergo surgical mammary gland removal without biopsy because general or local anesthesia is necessary to obtain tissue samples. On the other hand, fine needle biopsy (FNB) can be performed easily without anesthesia and is widely used to diagnose many types of tumors. However, cytological evaluation of CMTs is generally thought to be not conclusive enough to discriminate correctly between benign and malignant tumors [2]. Therefore, new cytological diagnostic tools that can be used for FNB samples are needed.

Recently, many proteins or gene expressions have been investigated for detecting tumor malignancy of CMTs. Lack of estrogen receptors (ER) and progesterone receptors (PR), which normal mammary glands should express, are well-known biomarkers associated with histologic tumor malignancy, lymph node involvement, and distant metastasis [11, 14, 26]. Moreover, in a study of ER and PR expression of 113 CMTs, lower PR expression was significantly associated with shorter survival times after surgical removal [11]. In recent years, human epidermal growth factor receptor 2 (HER2, c-erb-2) has been reported to have an important role in tumor aggressiveness. Its overexpression has been detected in 24–30% of human breast cancers [3, 30, 32]. Anti-HER2 antibody is widely used for HER2-overexpressing breast cancers as an antibody drug therapy. In the recent studies of HER2 expression of CMTs, overexpression of this protein was detected in 17.6–35.4% of malignant mammary tumors, and no or faint expression was seen in most benign mammary tumors [16, 20, 22, 25]. However, survival times and survival rates were better in dogs with HER2-overexpressing malignant mammary tumors than in dogs with tumors normally expressing HER2 [20].

These biomarkers, which are expected to be useful tools not only to classify CMTs as benign or malignant tumors but also to detect highly malignant, invasive tumors, were mainly evaluated using immunohistochemical (IHC) staining techniques. To identify gene biomarkers in CMTs, quantification of the mRNA levels of many genes, includ-

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ing hormonal receptors and *HER2*, have been reported [21, 23, 31, 34]. In these studies, mRNA was extracted from mammary tumor tissue samples, but few reports assessed the gene expressions of biomarkers in FNB samples.

Special AT-rich binding protein 1 (SATB1) is a matrix attachment region (MAR)-binding protein and has emerged as a key factor for gene transcription. By regulating many gene expressions through remodeling chromatin architecture, SATB1 is thought to play an essential role in T cell differentiation and activation [4, 24]. Furthermore, this nuclear protein has a cage-like distribution and tethers chromatin loops to a distinct region as a genome organizer [10]. Recently, SATB1 expression has been investigated in some tumors, including human breast cancer [12, 18, 35]. Han *et al.* reported that high SATB1 expression level was correlated to a high tumor malignancy and poor prognosis in human breast cancer [18]. By reprogramming gene expression, SATB1 has been thought to make tumor cells more aggressive. Snail, which is the zinc finger transcription factor and one of the genes regulated by SATB1, has been described as a mediator of epithelial-mesenchymal transitions (EMTs) [6, 18]. EMTs are characterized by loss of cell adhesion molecules and gain of mesenchymal markers [33]. In human breast cancer, Snail was reported as an important key mediator of tumor cell invasion and metastasis by induction of EMT [7, 29]. However, the roles of SATB1 and Snail in CMTs have not been clear.

The aim of this study was to assess the utility of biomarkers for cytological examination to predict tumor malignancy and invasiveness by comparison of mRNA levels of *ER*, *PR*, *HER2, SATB1* and *Snail* between benign and malignant CMTs and between non-invasive and invasive CMTs, and to establish a new cytological gene examination using FNB samples.

MATERIALS AND METHODS

Patients: Fifty-five dogs studied included 14 Miniature Dachshunds, 6 Shih-Tzus, 4 Beagles, 3 each of Malteses, Papillons, and Shetland Sheepdogs, 2 each of American Cocker Spaniels, Miniature Schnauzers, Welsh Corgi Penbrakes, West Highland White Terriers, and Cavalier King Charles Spaniels, 1 each of Great Pyrenees, Poodle, Chihuahua, Japanese Spitz, and Miniature Bull Terrier, and 7 mixed breeds. These dogs had not had any malignant tumors before excisional biopsy except for the malignant mammary tumors. In 15 dogs, ovariohysterectomy (OHE) had been performed prior to the removal of CMTs. One dog with a mammary tumor was male. The dogs' mean age at the time of tumor removal was 10.0 years (range, 4 to 16 years).

Samples: Fifty-six tissue samples were obtained by excisional surgery from 55 dogs with mammary tumors. The tissue samples under 0.03 grams were obtained from the marginal tumor tissues of the specimens. The mean size of the CMTs was 2.9 cm (range, 0.5 to 11 cm). FNB samples were obtained by inserting 22-G needles into the tumors through the skin in 31 CMTs prior to tissue sample collection. After these sampling, mammary tumors were examined histopathologically by a veterinary pathologist and classified according to the WHO classification [28]. Based on the histopathological examination, the CMTs were categorized into benign or malignant, and non-invasive or invasive. Invasive tumor was defined as tumor with infiltrative growth into the surrounding normal tissues or lymph and blood vessels. All tissue or cytological samples were immersed with RNA*later* (Applied Biosystems, Foster City, CA, U.S.A.) overnight at 4°C or 30 min on ice, followed by removal of the RNA*later* and storage at −80°C.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR): Total RNA was isolated from mammary tumors using an RNeasy Mini kit (Qiagen, Hilden, Germany). DNAse digestion was performed using an RNase-Free DNase kit (Qiagen). cDNA was synthesized from 1 μg of tRNA with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo dT primers (Toyobo) according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR) analysis: cDNA that had been diluted for the amplification of the target genes (*ER*, *PR*, *HER2*, *SATB1,* and *Snail*) was used for qRT-PCR analysis. A standard curve for each gene was produced using 100- and 10-fold serial dilutions of the genes as a template (10^8, 10^6, 10^4, and 10^3 copies). The reaction was performed using a Quantitect SYBR Green PCR kit (Qiagen) and an iQ5/MyiQ Single-Color (Bio-Rad Laboratories, Hercules, CA, U.S.A.) following the manufacturer's instructions, run as triplicates of each sample. The copy number of each gene expressed in CMTs was calculated from a standard curve and normalized to that of *ribosomal protein 19 (RP19)*. Each primer sequence is shown in Table 1.

Statistical analysis: The Mann-Whitney U test was used to analyze the differences in the mRNA expression levels of target genes between benign and malignant CMTs, and between non-invasive and invasive CMTs, with $P<0.05$ considered significant. The genes that had significantly different expressions among these categories were selected for discriminant analysis. Each tumor sample was classified into these categories by discriminant analysis. The relationship between tissue and FNB samples in gene expression was analyzed by Spearman's rank correlation coefficient. Data analyses were carried out with Excel Toukei 2010 (SSRI, Tokyo, Japan).

RESULTS

Histologic study: The 56 tissue samples consisted of 28 benign CMTs (2 simple adenomas, 18 complex adenomas, 8 mixed tumors) and 28 malignant CMTs (12 simple carcinomas, 13 complex carcinomas, 1 adenocarcinoma, 1 carcinosarcoma, 1 osteosarcoma); of the malignant CMTs, 16 showed tumor invasiveness to the surrounding normal tissues or lymph and blood vessels.

Gene expressions and discriminant analysis of tissue samples: The expression level of *ER* appeared significantly higher in benign (median, 0.01; average, 0.03) CMTs than in malignant (median, 0.002; average, 0.006) CMTs. The difference in *ER* expression between non-invasive (median,

Table 1. Primer nucleotide sequences

Gene	Nucleotide sequence (5' to 3')	Size (bp)	Accession No.
ERα	F: CCTTCAGTGAAGCTTCGATG	130	XM_533454
	R: AGAAGGTGGACCTGATCATG		
PR	F: CAGGAAGAGTTCCTGTGTAT	255	NM_001003074
	R: CCGGGACTGGATAAATGTAT		
HER2	F: CAGCCCTGGTCACCTACAA	120	NM_001003217
	R: CCACATCCGTAGACAGGTAG		
SATB1	F: GATTCAGCAGGAAATGAAGCG	211	XM_542770
	R: GCTCTCCTGTTCATAAATGGC		
Snail	F: GACTCCCAGACTCGCAAGG	308	XM_543048
	R: GACATGCGGGAGAAGGTTCG		
RP19	F: CCTTCCTCAAAAAGTCTGGG	95	XM_538673
	R: GTTCTCATCGTAGGGACGAAG		

F, forward; R, reverse.

0.01; average, 0.02) and invasive (median, 0.001; average, 0.004) CMTs was also significant (Fig. 1A). The tissue samples of spayed dogs expressed *ER* (median, 0.004; average, 0.009) less than those of intact dogs (median, 0.009; average, 0.020). The expression levels of *PR* were also significantly higher in benign (median, 0.05; average, 0.07) CMTs and in non-invasive (median, 0.03; average, 0.06) CMTs than in malignant (median, 0.006; average, 0.02) CMTs and invasive (median, 0.008; average, 0.02) CMTs (Fig. 1B). The tissue samples of spayed dogs expressed *PR* (median, 0.009; average, 0.02) less than those of intact dogs (median, 0.03; average, 0.06). The expression levels of *HER2* were similar in benign (median, 0.02; average, 0.04) and malignant (median, 0.02; average, 0.03) CMTs, and non-invasive (median, 0.02; average, 0.04) and invasive (median, 0.01; average, 0.03) CMTs (Fig. 1C). The expression levels of *SATB1* were similar in benign (median, 0.1; average, 0.15) and malignant (median, 0.1; average, 0.2) CMTs, but expression levels were significantly higher in invasive (median, 0.14; average, 0.33) CMTs than in non-invasive (median, 0.09; average, 0.13) CMTs (Fig. 1D). In particular, *SATB1* expression levels were high in samples from patients that had a relapse of CMTs or died within six months after tumor excision (data not shown). *Snail* was similarly expressed in benign (median, 0.05; average, 0.05) and malignant (median, 0.06; average, 0.16) CMTs, but there was a significant difference in *Snail* expression between non-invasive (median, 0.04; average, 0.07) and invasive (median, 0.07; average, 0.2) CMTs (Fig. 1E). To predict the histological malignancy of each tumor based on gene expression, *ER* and *PR*, which showed significant differences between benign and malignant CMTs, were used for the discriminant analysis. The total accuracy of classification was 73.2% (Table 2A). Four genes (*ER*, *PR*, *SATB1*, and *Snail*) that had significant differences between non-invasive and invasive CMTs were used for the discriminant analysis to predict tumor invasiveness. The total accuracy of classification was 80.0% (Table 2B).

Gene expressions and discriminant analysis of FNB samples: Quantification of gene expressions was performed in 31 FNB samples from the CMTs, including 21 benign and 10 malignant CMTs. Seven FNB samples were from invasive CMTs. *HER2* was excluded from the target genes for FNB analysis because of the tissue sample results. Significant differences in *ER* expressions that were observed in tissue samples were well preserved in FNB samples. High expressions of *ER* in benign (median, 0.06; average, 0.08) and non-invasive (median, 0.05; average, 0.08) CMTs and low expressions of *ER* in malignant (median, 0.002; average, 0.008) and invasive (median, 0.002; average, 0.005) CMTs were identified in FNB samples (Fig. 2A). *PR* expression levels of FNB samples were significantly higher in benign (median, 0.07; average, 0.15) CMTs than in malignant (median, 0.004; average, 0.008) CMTs (Fig. 2B). Moreover, a difference in *PR* expression was also detected between non-invasive (median, 0.06; average, 0.13) and invasive (median, 0.004; average, 0.007) CMTs. However, a tendency for higher expressions of *SATB1* and *Snail* in invasive tissue samples was not detected in FNB samples (Fig. 2C and 2D). On discriminant analysis of FNB samples, *ER* and *PR* were used for classification, along with both tumor malignancy and invasiveness. Using the two genes, 61.9% of benign and 100% of malignant CMTs, as well as 62.5% of non-invasive and 100% of invasive CMTs, were correctly classified (total accuracy was 74.2 and 71.0%, respectively) (Table 3A and 3B). In addition, expression levels of *ER*, *PR*, *SATB1*, and *Snail* in FNB samples were compared with each of their expression levels in the tissue samples. *ER* and *PR* had positive correlations (r_s=0.74, P<0.01 and r_s=0.83, P<0.01, respectively), but *SATB1* and *Snail* had no correlation (r_s=−0.08, P>0.05 and r_s=0.18, P>0.05, respectively).

DISCUSSION

Recently, the expressions of many genes and proteins in tissues from CMTs have been investigated, and differences in expressions between benign and malignant CMTs have been reported. In the present study, to establish a cytological gene examination that could be easily performed prior to definitive surgical therapy, tissues and FNB samples from CMTs were used for detecting mRNA levels of *ER*, *PR*, *HER2*, *SATB1*, and *Snail*.

It is widely known that protein expressions of ER and

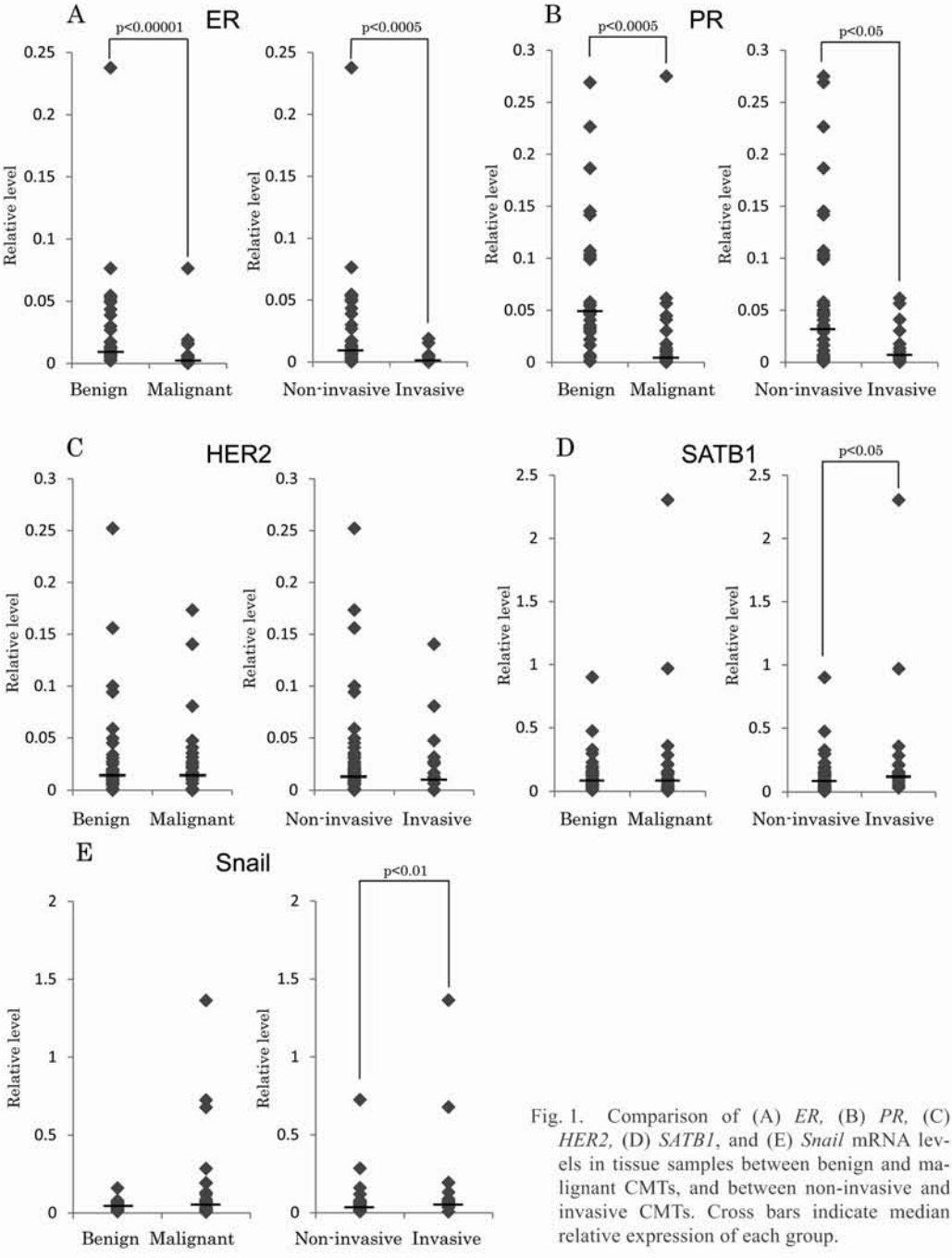

Fig. 1. Comparison of (A) *ER,* (B) *PR,* (C) *HER2,* (D) *SATB1*, and (E) *Snail* mRNA levels in tissue samples between benign and malignant CMTs, and between non-invasive and invasive CMTs. Cross bars indicate median relative expression of each group.

PR decrease in malignant CMTs. Similarly, in the present study, gene expressions of *ER* and *PR* in the tissue samples were lower in malignant CMTs than in benign CMTs, and the tissue samples from spayed dogs expressed these genes less than the samples from intact dogs. These data suggested that gene expression levels of *ER* and *PR* have a relative correlation with their protein expression levels and would be useful diagnostic tools to detect the malignancy of CMTs. In addition, *ER* and *PR* showed lower expression levels in invasive CMTs than in non-invasive CMTs. Hashimoto *et al.* also reported that protein concentrations of ER and PR decreased in proportion to progression in the clinical stage of CMTs [19]. These results indicated that *ER* and *PR* could predict CMT invasiveness. On the other hand, higher gene expressions of *ER* and *PR* in benign CMTs were detected in FNB samples, as well as in tissue samples. Thus, *ER* and *PR* might be suitable biomarkers for gene examination of CMTs using FNB samples. This would provide valuable information to help determine whether ovariohysterectomy should be done along with removal of CMTs.

Table 2. Discriminant analysis with *ER*, *PR*, *SATB1* and *Snail* expressions

A. Classification of tissue samples by the histological malignancy

Histopathological tumor type	Result of classification by discriminant analysis		Accuracy
	Benign	Malignant	
Benign (n=28)	15	13	53.60%
Malignant (n=28)	2	26	92.90%
		All samples	73.20%

B. Classification of tissue samples by the tumor invasiveness

Histopathological tumor type	Result of classification by discriminant analysis		Accuracy
	Non-invasive	Invasive	
Non-invasive (n=40)	38	2	95.00%
Invasive (n=16)	9	7	43.80%
		All samples	80.00%

Table 3. Discriminant analysis with ER and PR expressions

A. Classification of FNB samples by the histological malignancy

Histopathological tumor type	Result of classification by discriminant analysis		Accuracy
	Benign	Malignant	
Benign (n=21)	13	8	61.90%
Malignant (n=10)	0	10	100.00%
		All samples	74.20%

B. Classification of FNB samples by the tumor invasiveness

Histopathological tumor type	Result of classification by discriminant analysis		Accuracy
	Non-invasive	Invasive	
Non-invasive (n=24)	15	9	62.50%
Invasive (n=7)	0	7	100.00%
		All samples	71.00%

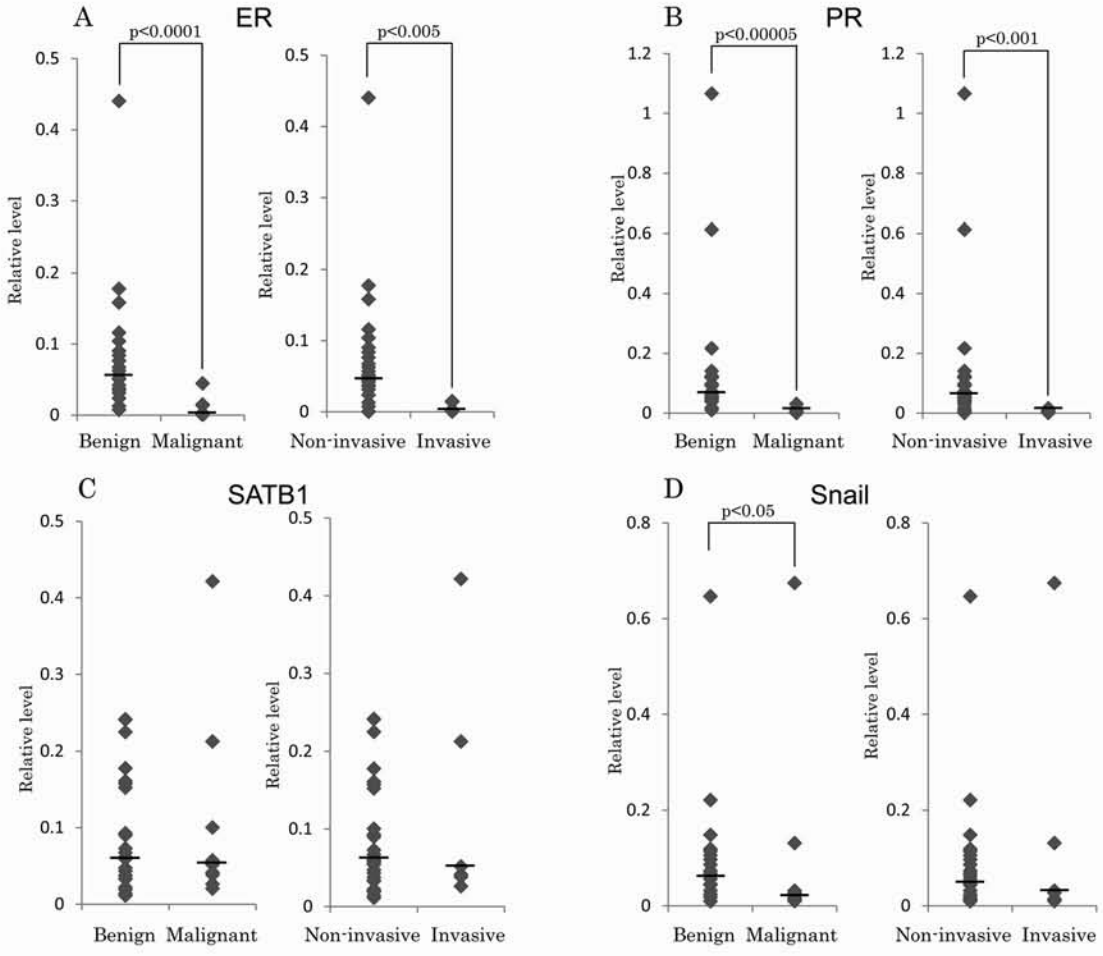

Fig. 2. Comparison of (A) *ER*, (B) *PR*, (C) *SATB1*, and (D) *Snail* mRNA levels in FNB samples between benign and malignant CMTs, and between non-invasive and invasive CMTs. Cross bars indicate median relative expression of each group.

Protein overexpression of *HER2* by malignant CMTs has been reported in several studies. However, there was no difference in *HER2* expression between benign and malignant or between non-invasive and invasive CMTs. Ahern *et al.* reported overexpression of *HER2* mRNA in 17 of 23 malignant CMTs, but in none of 5 benign CMTs [1]. A recent study reported no differences in the *HER2* mRNA levels between adenomas and carcinomas or between presence and absence of lymph node involvement of CMTs [23]. In human breast cancer, reports on the interaction between mRNA expression and HER2 protein or DNA amplification levels did not reach agreement [5, 13]. Since there was no significant difference in *HER2* expression among CMT tissue samples in the present study, *HER2* was not assessed in FNB samples.

This study investigated the mRNA expressions of *SATB1* and *Snail* in CMT tissue, and expressions of both *SATB1* and *Snail* were clearly higher in invasive CMTs than in non-invasive CMTs. One sample which expressed remarkably high *SATB1* or *Snail* might influence on the statistical analysis. However, the each sample was not excluded from the analysis because of their association with clinical features. The tissue sample expressing very high *SATB1* was obtained from the invasive CMT with involved lymph node and the skin metastasis were occurred two months after the operation. The other CMT expressing high very *Snail* was osteosarcoma with vascular invasion, and their aggressive behaviors have been generally known [27]. Further, other samples with high level of *SATB1* correlated with a poor clinical outcome. Moreover, comparison of the amino acid sequence of canine and human SATB1 showed that two important domains of canine SATB1 for DNA binding and homodimerization had 100% similarity to human SATB1 (data not shown). These results suggest that the canine SATB1 may also regulate tumor metastasis genes, such as Snail, to make tumor cells a more aggressive phenotype, as in human breast cancer. However, in the FNB samples, no differences in the expression levels of *SATB1* and *Snail* between invasive CMTs and non-invasive CMTs were detected. The cause of this result might be the possibility that small amounts of tumor cells express *SATB1* and *Snail*. Recent studies in human based on the expression of EMT markers reported that EMT occurred in a more local region at the invasive area of the tumor [8]. It might be difficult to collect *SATB1* and *Snail*-expressing tumor cells in the limited area sampled by FNB.

On discriminant analysis using gene expression levels of *ER*, *PR*, *SATB1,* and *Snail*, 73.2% of all tissue samples were correctly classified as benign or malignant CMTs, and 80.0% of all tissue samples were correctly classified as non-invasive or invasive CMTs. In FNB samples, expression levels of *ER* and *PR* had a high positive correlation to those in tissue samples. *ER* and *PR* might be suitable biomarkers for cytological gene examination. In addition, using gene expression levels of *ER* and *PR,* the accuracy of 74.2% for tumor classification according to malignancy was as the same as for tissue samples, and that for invasiveness (71.0%) was slightly lower. Aleen *et al.* reported that the accuracies of cytological examinations of FNB samples of CMTs by two cytologists were 79% and 66% [2]. It is interesting that FNB samples could be correctly classified as benign or malignant CMTs only by *ER* and *PR* levels with a similar accuracy to the report by Aleen *et al.* However, investigation of more gene expressions that can be detected even in FNB samples is needed to improve the accuracy of cytological gene examination. In this study, there were high percentages of false-positive (number of benign samples diagnosed as malignant) results in tissue and FNB samples (46 and 38%, respectively). Considering the report that concentrations of ER and PR in CMTs tended to vary with estrous cycle stage [15], it might be a difficult to classify benign or malignant CMTs with 100% accuracy only by *ER* and *PR* levels. In the future, use of other genes related to malignancy or, currently, referral for cytological examination might improve the accuracy of FNB samples. mRNA quantification of FNB samples by qRT-PCR cost much money and time, so that a simple gene examination kit should be considered.

In conclusion, the present study suggested that *ER* and *PR* are reliable biomarkers for the gene examination of FNB samples, and canine *SATB1* and *Snail* might have a role in tumor progression, as in humans. Cytological gene examination could become a useful diagnostic tool that can be performed easily without anesthesia and could predict tumor malignancy and invasiveness prior to surgical removal. To establish cytological gene examination of CMTs, more studies of gene expressions, including key biomarkers that are widely involved in the tumor mechanism, are needed.

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Supplemental Table 1. Patients' characteristics

		n
Breed	Miniature Dachshund	14
	Shih Tzu	6
	Beagle	4
	Maltese	3
	Papillon	3
	Shetland Sheepdog	3
	American Cocker Spaniel	2
	Miniature Schnauzer	2
	Welsh Corgi Penbrakes	2
	West Highland White Terrier	2
	Cavalier King Charles Spaniel	2
	Great Pyrenees	1
	Poodle	1
	Chihuahua	1
	Japanese Spitz	1
	Miniature Bull Terrier	1
	Mixed Breed	7
Sex	Female (intact)	39
	Female (spayed)	15
	Male	1

		n
Tumor size	mean ± SD	2.9 ± 2.6 cm
	median	2 cm
	range	0.5 to 11 cm
	T1 (<3 cm)	30
	T2 (3–5 cm)	15
	T3 (>5 cm)	5
	unknown	6
Tumor type	Benign	
	Simple adenoma	2
	Complex adenoma	18
	Benign mixed tumor	8
	Malignant	
	Simple carcinoma	12
	Complex carcinoma	13
	Adenocarcinoma	1
	Carcinosarcoma	1
	Osteosarcoma	1
Age	mean±SD	10 ± 2.7 years
	median	10 years
	range	4 to 16 years