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		
RPI9	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

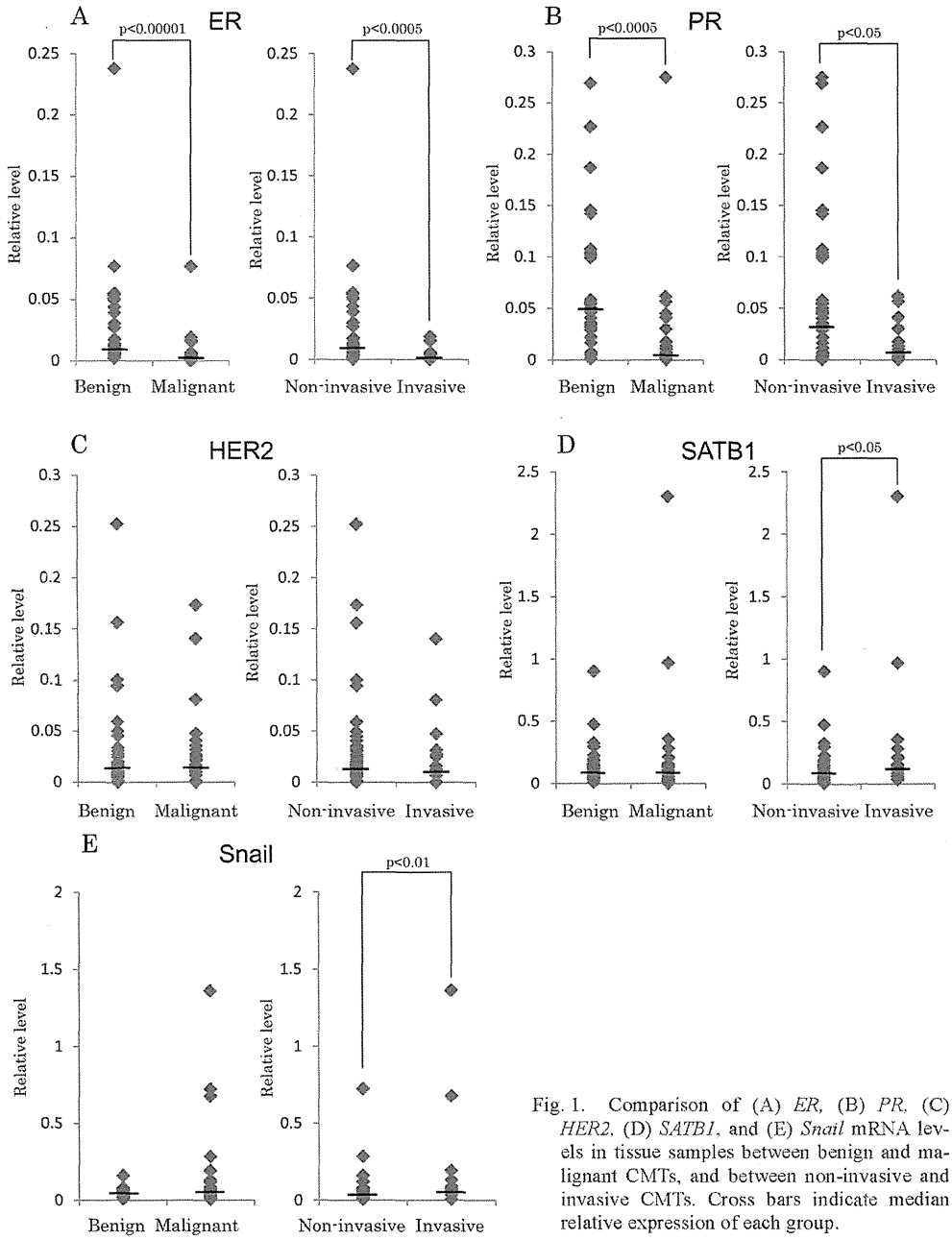

Fig. 1. Comparison of (A) *ER*, (B) *PR*, (C) *HER2*, (D) *SATB1*, and (E) *Snail* mRNA levels in tissue samples between benign and malignant CMTs, and between non-invasive and invasive CMTs. Cross bars indicate median relative expression of each group.

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%

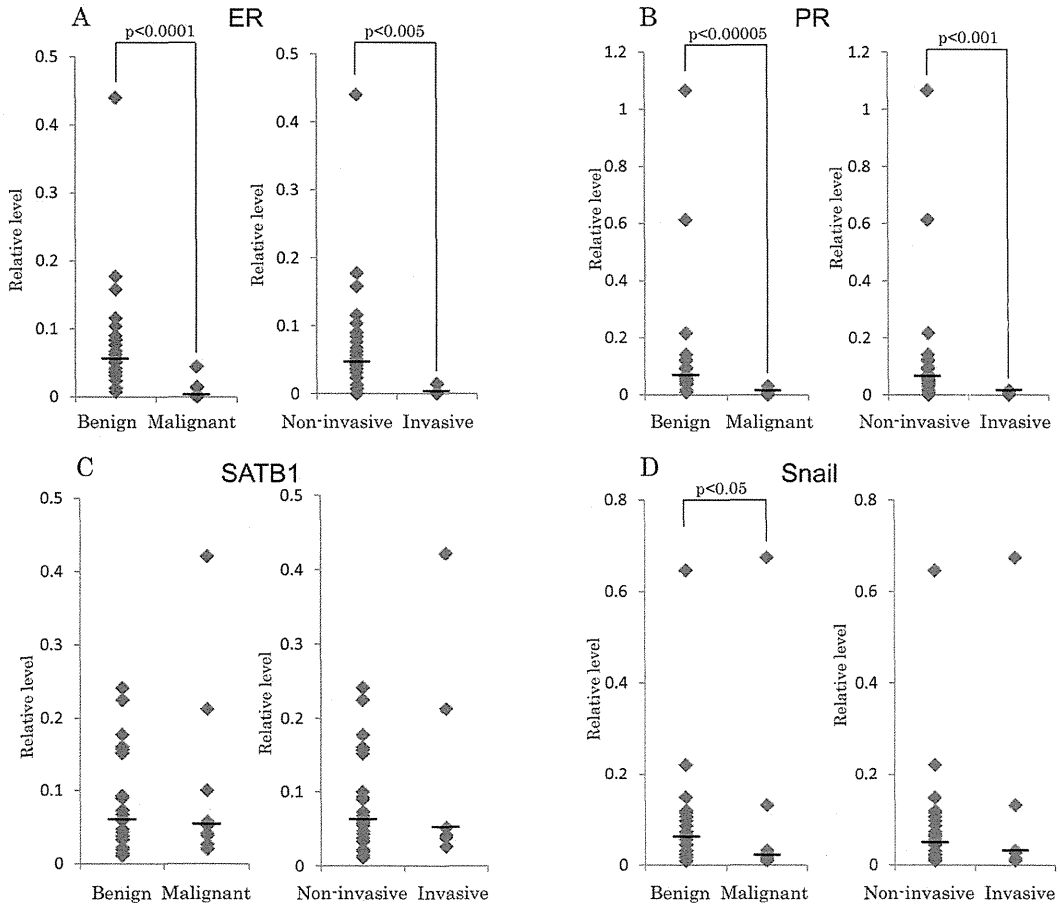

Fig. 2. Comparison of (A) *ER*, (B) *PR*, (C) *SATB1*, and (D) *Snail* mRNA levels in FNB samples between benign and malignant CMTs, and between non-invasive and invasive CMTs. Cross bars indicate median relative expression of each group.

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.

REFERENCES

1. Ahern, T. E., Bird, R. C., Bird, A. E. and Wolfe, L. G. 1996. Expression of the oncogene c-erbB-2 in canine mammary cancers and tumor-derived cell lines. *Am. J. Vet. Res.* **57**: 693–696. [Medline]
2. Allen, S. W., Prasse, K. W. and Mahaffey, E. A. 1986. Cytologic differentiation of benign from malignant canine mammary tumors. *Vet. Pathol.* **23**: 649–655. [Medline]
3. Almasri, N. M. and Al Hamad, M. 2005. Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in breast carcinoma in Jordan. *Breast Cancer Res.* **7**: R598–R604. [Medline] [CrossRef]
4. Alvarez, J. D., Yasui, D. H., Niida, H., Joh, T., Loh, D. Y. and Kohwi-Shigematsu, T. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes Dev.* **14**: 521–535. [Medline]
5. Barberis, M., Pellegrini, C., Cannone, M., Arizzi, C., Coggi, G. and Bosari, S. 2008. Quantitative PCR and HER2 testing in breast cancer: a technical and cost-effectiveness analysis. *Am. J. Clin. Pathol.* **129**: 563–570. [Medline] [CrossRef]
6. Batlle, E., Sancho, E., Francí, C., Domínguez, D., Monfar, M., Baulida, J. and García de Herreros, A. 2000. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**: 84–89. [Medline] [CrossRef]
7. Blanco, M. J., Moreno-Bueno, G., Sarrio, D., Locascio, A., Cano, A., Palacios, J. and Nieto, M. A. 2002. Correlation of Snail expression with histological grade and lymph node sta-

tus in breast carcinomas. *Oncogene* **21**: 3241–3246. [Medline] [CrossRef]
8. Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., Knuechel, R. and Kirchner, T. 2001. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 10356–10361. [Medline] [CrossRef]
9. Brodey, R. S., Goldschmidt, M. H. and Roszel, J. R. 1983. Canine mammary gland neoplasms. *J. Am. Anim. Hosp. Assoc.* **19**: 61–90.
10. Cai, S., Han, H. J. and Kohwi-Shigematsu, T. 2003. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat. Genet.* **34**: 42–51. [Medline] [CrossRef]
11. Chang, C. C., Tsai, M. H., Liao, J. W., Chan, J. P., Wong, M. L. and Chang, S. C. 2009. Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors. *J. Am. Vet. Med. Assoc.* **235**: 391–396. [Medline] [CrossRef]
12. Cheng, C., Lu, X., Wang, G., Zheng, L., Shu, X., Zhu, S., Liu, K., Wu, K. and Tong, Q. 2010. Expression of SATB1 and heparanase in gastric cancer and its relationship to clinicopathologic features. *APMIS* **118**: 855–863. [Medline] [CrossRef]
13. Cuadros, M., Talavera, P., López, F. J., García-Peréz, I., Blanco, A. and Concha, A. 2010. Real-time RT-PCR analysis for evaluating the Her2/neu status in breast cancer. *Pathobiology* 77: 38–45. [Medline] [CrossRef]
14. de Las Mulas, J. M., Millán, Y. and Dios, R. 2005. A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Vet. Pathol.* **42**: 200–212. [Medline] [CrossRef]
15. Donnay, I., Rauïs, J., Devleeschouwer, N., Wouters-Ballman, P., Leclercq, G. and Verstegen, J. 1995. Comparison of estrogen and progesterone receptor expression in normal and tumor mammary tissues from dogs. *Am. J. Vet. Res.* **56**: 1188–1194. [Medline]
16. Dutra, A. P., Granja, N. V., Schmitt, F. C. and Cassali, G. D. 2004. c-erbB-2 expression and nuclear pleomorphism in canine mammary tumors. *Braz. J. Med. Biol. Res.* **37**: 1673–1681. [Medline] [CrossRef]
17. Gilbertson, S. R., Kurzman, I. D., Zachrau, R. E., Hurvitz, A. I. and Black, M. M. 1983. Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs. *Vet. Pathol.* **20**: 127–142. [Medline]
18. Han, H. J., Russo, J., Kohwi, Y. and Kohwi-Shigematsu, T. 2008. SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. *Nature* **452**: 187–193. [Medline] [CrossRef]
19. Hashimoto, S., Yamamura, H., Sato, T., Kanayama, K. and Sakai, T. 2002. The progression of canine mammary tumors and the concentrations of estrogen and progesterone receptors in the tumor tissues. *J. Anim. Clin. Med.* **11**: 163–166.
20. Hsu, W. L., Huang, H. M., Liao, J. W., Wong, M. L. and Chang, S. C. 2009. Increased survival in dogs with malignant mammary tumours overexpressing HER-2 protein and detection of a silent single nucleotide polymorphism in the canine HER-2 gene. *Vet. J.* **180**: 116–123. [Medline] [CrossRef]
21. Kanae, Y., Endoh, D., Yokota, H., Taniyama, H. and Hayashi, M. 2006. Expression of the PTEN tumor suppressor gene in malignant mammary gland tumors of dogs. *Am. J. Vet. Res.* **67**: 127–133. [Medline] [CrossRef]
22. Kim, J. H., Im, K. S., Kim, N. H., Yhee, J. Y., Nho, W. G. and Sur, J. H. 2011. Expression of HER-2 and nuclear localization of HER-3 protein in canine mammary tumors: histopathological and immunohistochemical study. *Vet. J.* **189**: 318–322. [Medline] [CrossRef]
23. Klopfleisch, R., Klose, P. and Gruber, A. D. 2010. The combined expression pattern of BMP2, LTBP4, and DERL1 discriminates malignant from benign canine mammary tumors. *Vet. Pathol.* **47**: 446–454. [Medline] [CrossRef]
24. Kumar, P. P., Bischof, O., Purbey, P. K., Notani, D., Urlaub, H., Dejean, A. and Galande, S. 2007. Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus. *Nat. Cell Biol.* **9**: 45–56. [Medline] [CrossRef]
25. Martin de las Mulas, J., Ordás, J., Millán, Y., Fernández-Soria, V. and Ramón y Cajal, S. 2003. Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study. *Breast Cancer Res. Treat.* **80**: 363–367. [Medline]
26. Millanta, F., Calandrella, M., Bari, G., Niccolini, M., Vannozzi, I. and Poli, A. 2005. Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. *Res. Vet. Sci.* **79**: 225–232. [Medline] [CrossRef]
27. Misdorp, W., Cotchin, E., Hampe, J. F., Jabara, A. G. and von Sandersleben, J. 1971. Canine malignant mammary tumours. I. Sarcomas. *Vet. Pathol.* **8**: 99–117. [Medline]
28. Misdorp, W., Else, R. W., Hellmén, E. and Lipscomb, T. P. 1999. Histological Classification of Mammary Tumors of the Dog and Cat, 2nd Series, Vol.VII. WHO International Histological Classification of Mammary Tumors of Domestic Animals, AFIP, Washington, DC.
29. Olmeda, D., Moreno-Bueno, G., Flores, J. M., Fabra, A., Portillo, F. and Cano, A. 2007. SNAI1 is required for tumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells. *Cancer Res.* **67**: 11721–11731. [Medline] [CrossRef]
30. Perez, E. A., Roche, P. C., Jenkins, R. B., Reynolds, C. A., Halling, K. C., Ingle, J. N. and Wold, L. E. 2002. HER2 testing in patients with breast cancer: poor correlation between weak positivity by immunohistochemistry and gene amplification by fluorescence in situ hybridization. *Mayo Clin. Proc.* **77**: 148–154. [Medline]
31. Qiu, C., Lin, D. D., Wang, H. H., Qiao, C. H., Wang, J. and Zhang, T. 2008. Quantification of VEGF-C expression in canine mammary tumours. *Aust. Vet. J.* **86**: 279–282. [Medline] [CrossRef]
32. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177–182. [Medline] [CrossRef]
33. Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R. A. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**: 927–939. [Medline] [CrossRef]
34. Zavlaris, M., Angelopoulou, K., Vlemmas, I. and Papaioannou, N. 2009. Telomerase reverse transcriptase (TERT) expression in canine mammary tissues: a specific marker for malignancy? *Anticancer Res.* **29**: 319–325. [Medline]
35. Zhao, X. D., Ji, W. Y., Zhang, W., He, L. X., Yang, J., Liang, H. J. and Wang, L. L. 2010. Overexpression of SATB1 in laryngeal squamous cell carcinoma. *ORL J. Otorhinolaryngol. Relat. Spec.* **72**: 1–5. [Medline] [CrossRef]

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

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Original Article

Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence

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ABSTRACT — The human steroid and xenobiotic receptor (SXR), (also known as pregnane X receptor PXR, and NR1I2) is a low affinity sensor that responds to a variety of endobiotic, nutritional and xenobiotic ligands. SXR activates transcription of Cytochrome P450, family 3, subfamily A (CYP3A) and other important metabolic enzymes to up-regulate catabolic pathways mediating xenobiotic elimination. One key feature that demarcates SXR from other nuclear receptors is that the human and rodent orthologues exhibit different ligand preference for a subset of toxicologically important chemicals. This difference leads to a profound problem for rodent studies to predict toxicity in humans. The objective of this study is to generate a new humanized mouse line, which responds systemically to human-specific ligands in order to better predict systemic toxicity in humans. For this purpose, the ligand binding domain (LBD) of the human SXR was homologously knocked-in to the murine gene replacing the endogenous LBD. The LBD-humanized chimeric gene was expressed in all ten organs examined, including liver, small intestine, stomach, kidney and lung in a pattern similar to the endogenous gene expressed in the wild-type (WT) mouse. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the human-selective ligand, rifampicin induced Cyp3a11 and Carboxylesterase 6 (Ces6) mRNA expression in liver and intestine, whereas the murine-selective ligand, pregnenolone-16-carbonitrile did not. This new humanized mouse line should provide a useful tool for assessing whole body toxicity, whether acute, chronic or developmental, induced by human selective ligands themselves and subsequently generated metabolites that can trigger further toxic responses mediated secondarily by other receptors distributed body-wide.

Key words: Steroid and xenobiotic receptor, Pregnane X receptor, Humanized mouse, Ligand binding domain, Knock-in mouse

INTRODUCTION

Most orally administered xenobiotics are metabolized first by the intestine and then by the liver after portal transport. The expression levels of enzymes involved in xenobiotic metabolism are regulated at the transcriptional level by key xenobiotic sensors including the steroid and xenobiotic receptor (SXR), also known as the pregnane X receptor (PXR), pregnane activated receptor (PAR) and NR1I2 (Bertilsson *et al.*, 1998; Lehmann *et al.*, 1998; Blumberg *et al.*, 1998). SXR is important in the field of toxicology for at least two reasons. Firstly, this receptor system induces the expression of CYP3A and CYP2B enzymes, the major metabolizers of pharmaceu-

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ticals and xenobiotics. Therefore, SXR is a key mediator of drug- and chemical-induced toxicity as well as drug-drug and drug-nutrient interactions (Zhou *et al.*, 2004). Secondly, the orthologous rodent and human receptors exhibit differential sensitivity for a subset of chemical ligands important in the field of toxicology. For example, rifampicin (RIF) is a specific and selective activator of human SXR, whereas pregnenolone 16a-carbonitrile (PCN) is selective for the rodent orthologue.

Rodent-human differences in CYP3A and CYP2B-mediated responses to xenobiotics can be a profound problem in toxicologic studies where rodents are used to predict the toxicity of a compound in humans (Ma *et al.*, 2007). Therefore, development of a murine model that reconstructs the SXR-mediated systemic response of humans is of a great significance in toxicology.

Human and rodent SXRs share ~95% amino acid sequence identity in the DNA-binding domain (DBD) but only about 77% identity in the LBD. Tirona *et al.* (2004) analyzed the ligand selectivity of a human-rat chimeric protein and showed that the species differences are primarily defined by sequence differences in the LBD. Watkins and colleagues showed that the key residues responsible for the majority of the ligand selectivity were Leu 308 (human) and Phe305 (rat and mouse). Crystallographic analysis located these amino acids within or neighboring the flexible loop that forms a part of the pore to the ligand-binding cavity. Swapping the rodent and human-specific residues was shown to modulate the activation by the human-selective activator RIF *in vitro* (Watkins *et al.*, 2001). According to those findings, a simple replacement of the mouse LBD with the human sequence should be sufficient to "humanize" the ligand binding properties as well as activation of the downstream target genes.

Three kinds of humanized mice have already been generated. One is the SXR-null/Alb-SXR mouse (Alb-SXR mouse) made by crossing the SXR knockout mice with a transgenic mouse line that expresses human SXR in liver under the control of the albumin promoter (Xie *et al.*, 2000). Gonzalez and colleagues generated a transgenic mouse expressing a human BAC containing the entire hSXR gene in a SXR null background, thus controlled under human SXR promoter (SXR BAC mouse) (Ma *et al.*, 2007). Another mouse is the human SXR genome knock-in mice (hSXR genome mouse) (Scheer *et al.*, 2008). The human SXR genomic region from exon 2 to exon 9 was knocked-in to mouse SXR exon 2. This mouse expresses the human full length SXR mRNA under the control of mouse SXR promoter regulation. Although useful for toxicology studies, these mice have disadvantages in that the human SXR is expressed only in the liver (Alb-SXR mouse), hSXR mRNA is not expressed in all of the tissues where SXR is known to be expressed (SXR BAC mouse), and there might be potential differences in the binding affinities of hSXR DNA-binding domain (DBD) to *cis*-acting elements in mouse SXR target genes (hSXR genome mouse).

As noted above, it is known that the critical differences between human and rodent ligand-selectivity reside in the LBD. Therefore, when our project to generate a humanized SXR mouse was initiated, we reasoned that altering the LBD would be sufficient to generate a humanized ligand selectivity. We decided to retain the mouse DBD to avoid any potential differences between the binding affinities of the chimeric receptor for cis-acting elements in the mouse genome. To maintain the tissue-specific expression pattern of the endogenous gene, we inserted the human cDNA encoding the region carboxyl-terminal to the DBD into the mouse gene. This retains all of the 5' and 3' regulatory elements in the mouse gene, as well as introns 1 and 2, which contain important elements for regulating SXR expression (Jung *et al.*, 2006).

Here we report a new line of mouse (hSXRki mouse) in which a cDNA encoding the human LBD is homologously recombined into the mouse gene after exon 3. The tissue distribution of the resulting chimeric mouse DBD-human LBD mRNA is comparable to that of the WT mouse. The hSXRki mouse showed a fully humanized response to the human-selective activator RIF in that the Cyp3a11 mRNA was induced in liver and mucosa of small intestine in response to RIF, but not the rodent-selective compound PCN. This new mouse line should provide a useful tool for assessing the whole body toxicity induced by a human selective SXR ligand itself and its subsequently generated metabolite(s) that can trigger further toxic responses through other pathways body-wide.

MATERIALS AND METHODS

Generation of hSXRki knock-in mice

A DNA fragment of mouse SXR intron 2 to exon 3 was PCR amplified using mouse BAC DNA (BAC clone No. RP23-351P21) as a template. Primers used were BAC39486FW and mSXR462RV (for sequences of the primers see Table1). This fragment was connected to the LBD of human SXR cDNA from amino acid 105 through the carboxyl terminus amplified by the PCR primers: hSXR904FW and hSXR1887RVEcoRI (template; human SXR cDNA). The 3'UTR of bovine growth hormone (BGH) was added to 3' to the terminal codon. This concatenated fragment was introduced to a vector, which

Table 1. List of primer pairs

Purpose	Primer name	Sequence (5' to 3')
Targeting vector construction	BAC39486FW	CCATGGGTACCACGAATAACAA
	mSXR462RV	CATGCCACTCTCCAGGCA
	hSXR904FW	AAGAAGGAGATGATCATGTCCG
	hSXR1887RVEcoRI	CCGAATTCTCATCATCAGCTACCTGTGATACCGAACA
Genotyping	NeoAL2	GGGGATGCGGTGGGCTCTATGGCTT
	SXR RC RV5	TGAGAGTGCACAAGTTCAAGCT
	WTInt5	AGTGATGGGAACCACTCCTG
	WTEx6RV	TGGTCCTCAATAGGCAGGTC
	mhSXRE4	GTGAACGGACAGGGACTCAG
	mhSXRSARV	CTCTCCTGGCTCATCCTCAC
Percellome quantitative RT-PCR	Cyp3a11 FW	CAGCTTGGTGCTCCTCTACC
	Cyp3a11 RV	TCAAACAACCCCCATGTTTT
	Ces6 FW	GGAGCCTGAGTTCAGGACAGAC
	Ces6 RV	ACCCTCACTGTTGGGGTTC
	mouse SXR FW	AATCATGAAAGACAGGGTTC
	mouse SXR RV	AAGAGCACAGATCTTTCCG
	human SXR FW	ATCACCCGGAAGACACGAC
	human SXR RV	AAGAGCACAGATCTTTCCG
	mouse-human SXR FW	CCCATCAACGTAGAGGAGGA

has the neomycin resistance gene with loxP sequence at both ends, removable with Cre recombinase (Saga *et al.*, 1999). A 7kb KpnI fragment containing intron 2 was used as a long arm and 1.3kb PstI-EcoRI fragment containing from exon 8 to intron 8 was used as a short arm for homologous recombination (Fig. 1). The resulting targeting vector was linearized with SacII and introduced by electroporation to TT2 ES cell line (Yagi *et al.*, 1993) and neomycin resistant clones were selected, PCR genotyped, and confirmed by the Southern blotting. For generation of chimeric mice, these ES clones were aggregated with ICR 8-cell embryos and transferred to pseudopregnant female recipients. The chimeric mice born were bred with ICR females. Germ line transmission of the targeted allele was confirmed by PCR. A mouse was crossed with a CAG-Cre transgenic mouse (Sakai and Miyazaki, 1997) to evict the neomycin resistance gene, and back crossed to C57BL/6 CrSlc (SLC, Inc., Shizuoka, Japan) at least 6 generations and used for the analysis.

PCR Genotyping (See Table 1 for primer sequences)

Primers for identification of homologously recombined ES clones were NeoAL2 and SXR RC RV5. DNA purified from the tail of each mouse was used for PCR genotying. Primers for WT detection were WTInt5 and WTEx6RV amplifying a product of 755 bp. Primers for confirmation of removal of the neomycin resistance gene were mhSXRE4 and mhSXRSARV amplifying a product of 1,223 bp.

Southern blot analysis

To confirm homologous recombination, DNA from ES cell cultures was purified and digested with BamHI and XhoI, then electrophoresed and analyzed by Southern hybridization (Saga *et al.*, 1997). Mouse SXR exon 9 region which remains after homologous recombination was used for the probe. The restriction fragments from the WT allele and targeted allele are 2,305 bp and 1,925 bp, respectively.

Chemicals

RIF (molecular weight 822.95) and PCN (molecular weight 341.49) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corn oil was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Quantitative RT-PCR (Percellome PCR) (See Table 1 for primer sequences)

The method for Percellome quantitative RT-PCR was described previously (Kanno *et al.*, 2006). Briefly, tissue pieces stored in RNAlater (Ambion, Austin, TX, USA) were homogenized and lysed in RLT buffer (Qiagen GmbH., Germany) and 10 μl aliquots were used

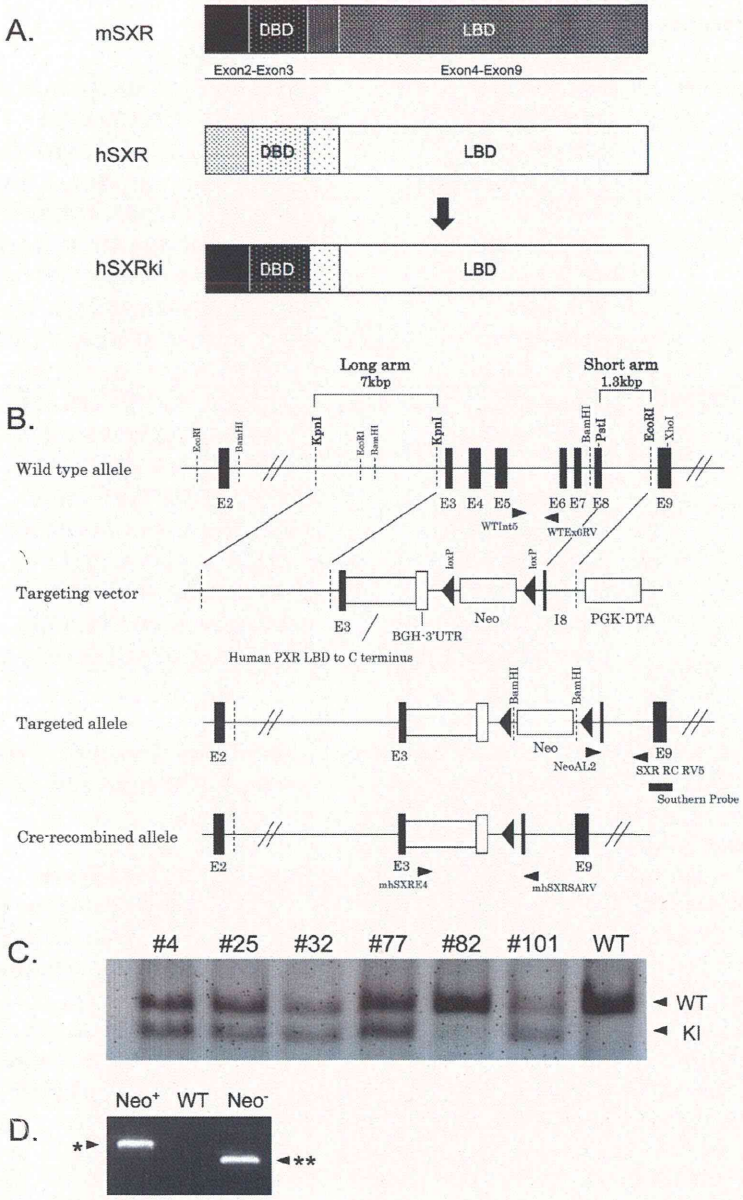

Fig. 1. Targeting strategy used to generate the hSXRki mouse. A) Diagram of hSXRki chimeric protein. Hinge region and ligand binding domain (LBD) of human SXR are knocked-in to mouse SXR, resulting in chimeric protein having murine N-ternial domain and DNA binding domain (DBD). B) Targeting strategy used to generate the hSXRki mouse. The chimeric mouse DBD and human LBD fragment, followed by the BGH 3' UTR were knocked-in to the mouse SXR gene. The genomic region spanning from exon 3 to exon 8 was substituted by the inserted fragment with the remainder of the gene remaining intact. C) Confirmation of homologous recombination by southern blot analysis. Six ES clones positive for recombination by PCR genotyping were further analyzed by southern blot (clones #4 ~ #101). Lower bands (1925 bp) indicate successful homologous recombination; upper bands (2305 bp) correspond to WT allele. Clones #4, #25, #32, #77 and #101 were confirmed as homologous recombinants; clones #4 and #25 were used for the generation of chimeric mice. D) Confirmation of Cre-mediated removal of the neomycin resistance gene. Mouse tail genome DNA was PCR amplified with the primer set, mhSXRE4 and mhSXRSARV. *: 2,858 bp (for the mice having the neomycin resistance gene), **: 1,223 bp (for the mice without the neomycin resistance gene).