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Expression Patterns of the *erbB* Subfamily mRNA in Canine Benign and Malignant Mammary Tumors

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ABSTRACT. *ErbB* subfamily genes, known as proto-oncogenes, encode receptor tyrosine kinases, and are expressed in relation to tumorigenesis of the mammary gland in humans. In this study, we examined the expression of *erbB* subfamily mRNAs in two canine normal mammary glands and 12 mammary tumor samples by reverse transcriptase-coupled polymerase chain reaction (RT-PCR). Each primer set was designed from the nucleotide sequence of the region conserved in *erbB* subfamily cDNA among other species. No *erbB* subfamily mRNAs were expressed in the normal mammary gland. In contrast, all of the subfamily mRNAs were expressed in a benign mammary tumor, and more than one type of the subfamily mRNA were observed in 11 malignant mammary tumors. The length of RT-PCR products were 380 bp for *erbB1*, 500 bp for *erbB2*, 644 bp for *erbB3*, and 416 bp for *erbB4*. These sequences were highly homologous to the cDNA sequences of other species. Therefore, these results suggest that the expression of *erbB* subfamily mRNAs in canine mammary tumors plays an important role in tumorigenesis of the mammary gland.

KEY WORDS: canine, *erbB* subfamily, mammary tumor, RT-PCR, tumor marker.

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EGF receptor subfamily genes encode 4 structurally related receptor type-tyrosine kinases, *c-erbB*, *c-erbB2*, *c-erbB3* and *c-erbB4*. While the members of this subfamily play roles in the proliferation and differentiation of normal epithelium, over expression of the ErbB proteins has been reported in various human tumor cells [4]. It has been suggested that the aberrant activation of their kinase activities contributes to tumorigenesis or progression [8]. It is known that ligand-binding stimulates intra- and intermolecular phosphorylation of *erbB* receptor molecules [20]. For example, the phosphorylation of *erbB2* products is stimulated by binding EGF with its receptor, *erbB* [10, 24]. The amplification of the *erbB2* gene and/or overexpression of the gene product are frequently observed in a number of human breast tumors of poor prognosis [18, 22]. Thus, the *erbB* proteins seem to have a significant contribution to the incidence and progression of human breast tumors.

While the expression of these genes in relation to tumorigenesis of breast tumors has been investigated extensively in human tumors, only a limited number of papers are available concerning the status of these genes in animal tumors [1, 21, 25]. Recently, mammary tumors as well as other tumors have become clinically important deseases in veterinary medicine of companion animals. Therefore, the study of the state of the expression of cancer related genes in various tumor tissues of companion animals has become a necessity. The reverse transcriptase-coupled polymerase chain reaction (RT-PCR) is a useful method for examining the expression of a specific gene, because the primers can be synthesized using the nucleotide sequences conserved among mammalian species [15, 16]. In this study, we describe the analysis of the expression patterns of *erbB* subfamily mRNA in canine mammary tumor samples.

MATERIALS AND METHODS

Normal and tumor samples: For a normal control, a piece of mammary gland tissue was excised under general anesthesia from a clinically normal female beagle. Other samples were obtained from a tumor resected surgically at the Veterinary Teaching Hospital of Osaka Prefecture University. For the histological examination, a part of the tumor samples were fixed in 10% formalin and embedded in paraffin. Thin sections were, then, prepared and stained with haematoxylin-eosin. The histopathological diagnoses of these tumors are listed in Table 1.

Total RNA purification from normal and tumor tissues: All the tumor tissues were separated from the connective tissue and cut into small pieces in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline [PBS(-); 137 mM NaCl, 2.7 mM

Table 1. The histopathological diagnoses of canine mammary tumors

Sample No.	Type of tumor
1	adenocarcinoma
2	benign mixed tumor
3	malignant mixed tumor
4	malignant mixed tumor
5	malignant mixed tumor
6	malignant mixed tumor
7	malignant mixed tumor
8	adenocarcinoma
9	malignant mixed tumor
10	adenocarcinoma
11	malignant mixed tumor
12	malignant mixed tumor

Table 2. Primer sets used in RT-PCR for the *erbB* subfamily and α-tubulin

Primer	Target mRNA	Sequence	Length
B1-fw	erbB1	5'-TACAGCTTTGGTGCCACCTG-3'	20mer
B1-rv		5'-GGCCAAGCCTGAATCAGCAA-3'	20mer
B2-fw	erbB2	5'-TGGCTGCAAGAAGATCTTTG-3'	20mer
B2-rv		5'-TGCAGTTGACACACTGGGTG-3'	20mer
B3-fw	erbB3	5'-GCAGAGGGCAAAGTATGTGA-3'	20mer
B3-rv		5'-AGCTCTGTCTCTTTGAAGAT-3'	20mer
B4-fw	erbB4	5'-AGGAGTGAAATTGGACACAG-3'	20mer
B4-rv		5'-AATGCTTGAAGGTCTCCATT-3'	20mer
Tub-fw	α-tubulin	5'-TCCATCCTCACCACCCACAC-3'	20mer
Tub-rv		5'-CGCTTGGTCTTGATGGTGGC-3'	20mer

KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4] containing gentamycin sulfate at 0.8 mg/mL. The samples were, then, washed in PBS(-) to remove necrotic tissues. Total RNA from each specimen was prepared by the acid-guanidium thiocyanate-phenol-chloroform method [5], and stored at –80°C as described previously [15].

Amplification of canine erbB subfamily cDNA from total RNAs of normal and tumor tissues: Canine *erbB* subfamily cDNA fragments were amplified by RT-PCR using total RNAs obtained from a canine normal mammary gland and tumor tissues as described previously with slight modifications [15]. Ten micrograms of total RNA were incubated with 200 units of Superscript II (Gibco BRL) and 500 *n*g of poly dT oligonucleotide (12–18 mer, Pharmacia) at 37°C for 1 hr. The synthesized single-strand cDNA was amplified with 2.5 units of Taq polymerase (Toyobo) and *erbB* subfamily primers (Table 2) for 25 cycles (94°C for 0.5 min; 55°C for 1 min; and 72°C for 2 min) followed by incubation at 72°C for 5 min for complete elongation using a thermal cycler (Perkin-Elmer GENEAMP PCR system 2400). The sequences of the RT-PCR primers were selected from the homologous region of the sequences reported for other mammals and chicken *erbB* subfamily cDNA using the Entrez search system. An α-tubulin primer set was used as the internal standard for the amount of mRNA included in the reaction mixture [15]. RT-PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

DNA sequencing of the RT-PCR products: Each RT-PCR product amplified using primer sets for the *erbB* family was purified from agarose gel by GFX™ PCR DNA and a Gel Band Purification Kit (Amersham Pharmacia Biotech) and subcloned into a plasmid vector pCR2.1 by a TA cloning kit (Invitrogen). The nucleotide sequence of each clone was determined by the dideoxytermination method [23] using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit and a Genetic analyzer A310 (Perkin Elmer Applied Biosystems). The obtained sequence data were analyzed for homology with those reported for other species by gene analysis software.

RESULTS

Expression of erbB subfamily mRNAs in canine normal mammary gland: Expression of four *erbB* subfamily mRNAs in normal canine mammary gland was examined by the RT-PCR method. The primer set to α-tubulin mRNA was used as a positive control. The expected size of each RT-PCR product from previously reported human data is 380 bp for erbB1, 500 bp for erbB2, 644 bp for erbB3 and 416 bp for erbB4. The amplified bands with these expected sizes were not observed in the two samples obtained from canine normal mammary glands, while the RT-PCR product obtained with the α-tubulin primer set was clearly detected (data not shown).

Expression of erbB subfamily mRNA in canine mammary tumors: We examined the expression of four *erbB* subfamily mRNAs in 12 canine mammay tumor samples by RT-PCR. The types of the tumor samples are listed in Table 1. Although no PCR products obtained with the primer sets for the *erbB* subfamily were detected in two normal mammary glands, at least one type of *erbB* subfamily mRNA was expressed in all of the tumor samples (Fig. 1). The amplified products were purified from agarose gel and their nucleotide sequences were determined (Fig. 2). As summarized in Table 3, each RT-PCR product was highly homologous to other species, showing that these products were amplified from canine *erbB* subfamily cDNAs.

Although the amounts of *erbB1* mRNAs relative to that of α-tubulin mRNA expressed in 12 canine mammary tumor samples were variable, the mRNA was expressed in all the tumor samples including a benign mixed tumor (Fig. 3). The expression of *erbB2*, *erbB3* and *erbB4* was observed in 10, 6 and 9 samples, respectively. Interestingly, all of the *erbB* subfamily mRNAs were strongly expressed in the benign mixed tumor sample. No apparent correlation was observed between the types of malignant mammary tumor and the pattern of expression of *erbB* subfamily mRNAs.

DISCUSSION

It has been reported that *erbB* subfamily proto-oncogenes play important roles in the development of human and

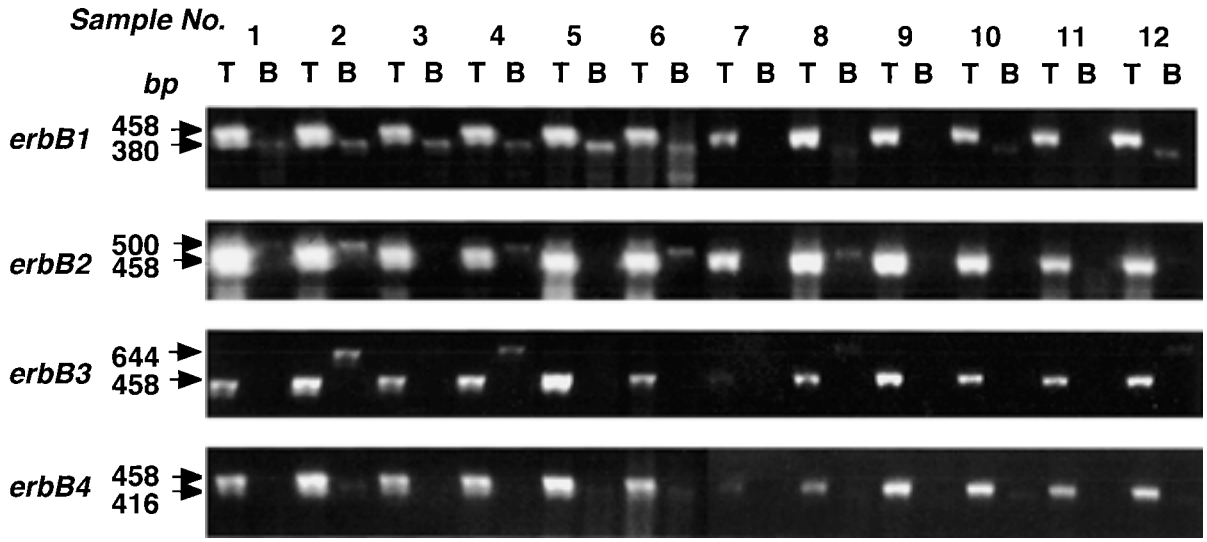

Fig. 1. Expression of *erbB* subfamily mRNAs in canine mammary tumor samples. The RT-PCR products were examined by 1% agarose gel electrophoresis. The sample numbers correspond to those in Table I. B: *erbB* subfamily, T: α-tubulin.

mouse mammary glands, although these genes are expressed in different parts of the tissue [11, 18]. Recent reports have shown that *erbB1*, *erbB2* and *erbB4* are expressed in human breast tissue in relation to ductal growth, lobuloalveoli formation, and secretion of milk protein, respectively [7, 18, 26]. On the other hand, although *erbB3* is also expressed in human or mouse mammary glands, the exact role of the protein is still not clear in detail. Products of the *erbB* subfamily genes are known to form homo- or heterodimers with other molecules of the subfamily and undertake signal transduction to intracellular cascades by their tyrosine kinase acitivities [28].

In human mammary tumors, overexpression and amplification of the *erbB2* gene has frequently been observed [17], which suggests that *erbB2* relates to tumorigenesis of mammary gland. Recently, it has been reported that *erbB1*, *erbB2* and *erbB4* form heterodimers with *erbB2* in human mammary tumors [3, 12]. The formation of heterodimers has been suggested to play an important role in signal amplification [2, 9, 13]. The enhanced expression of *erbB3* mRNA is observed in human mammary tumor, though the significance is still under investigation [27]. Despite the high incidence of canine mammary tumors, only a limited amount of information is available on the status of tumor related genes, including the *erbB* subfamily genes.

In this study, it was shown that more than one of the *erbB* subfamily mRNAs were expressed in canine mammary tumors, while they were not detected in a normal mammary gland. Though we could not see a direct correlation between the combination of expressed genes and the histological type of tumors, our results suggest that the expression of *erbB* subfamily mRNAs plays a significant role in the tumorigenesis of the mammary gland. To understand the mechanism, more information should be accumulated about the status of these genes in normal mammary glands during hyperplasia and pregnancy, since it is known that estrogen regulates the expression of the *erbB* subfamily [26] which are functional under the physiological conditions described above.

As described above, it is known that the *erbB2* gene is frequently overexpressed or amplified in human mammary tumors. Co-expression of *erbB* and *erbB2* genes in mouse fibroblasts enhances the effect of EGF on the transformed phenotype [14]. A relatively high incidence (10/12) of the expression of both genes may suggest an important contribution to the tumorigenesis in canine mammary tumors.

We previously reported that *c-kit* proto-oncogene is frequently expressed in canine mammary tumors as well as in other types of canine tumors [15]. It is known that *c-kit* protein, like *erbB* proteins, belongs to the superfamily of receptor tyrosine kinase [6] and relates to cell proliferation and differentiation [17]. Thus, it is very interesting to note that a variety of receptor tyrosine kinases are expressed in canine mammary tumors. Further study on the cross-talk among these receptors is needed to understand the significance of the expression of the tumor related genes including the *erbB* subfamily and *c-kit*.

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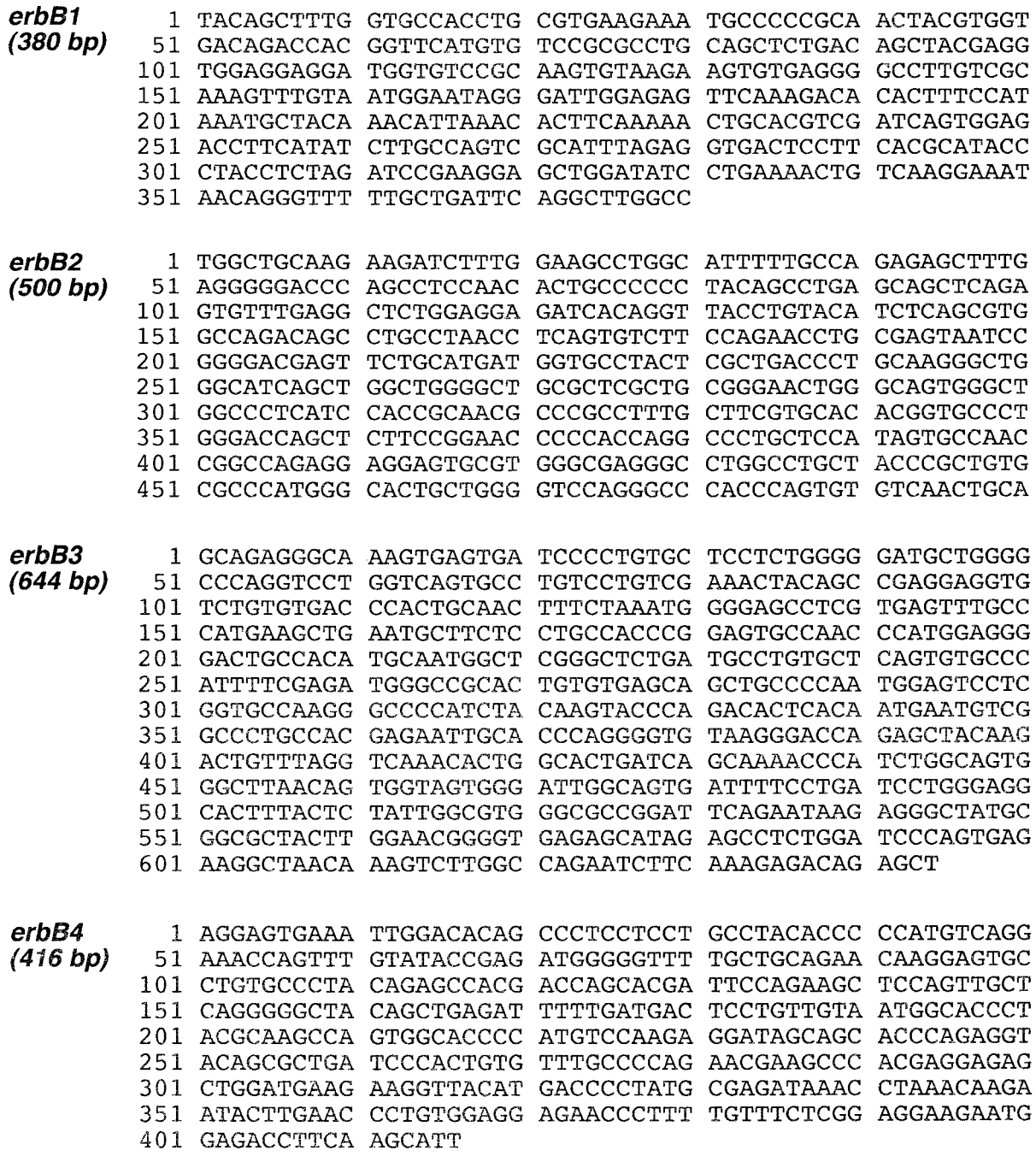

Fig. 2. cDNA sequences of the RT-PCR products amplified by the primer sets for *erbB* subfamily cDNAs.

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Table 3. Comparison of RT-PCR products of the canine *erbB* subfamily to other species

Species	Homology (%)			
	erbB1 (380 bp/126 aa)*	erbB2 (500 bp/166 aa)*	erbB3 (644 bp/214 aa)*	erbB4 (416 bp/138 aa)*
Human	85.5%/95.2%	91.8%/91.0%	92.1%/92.5%	93.3%/98.6%
Mouse	84.2%/90.5%	–	–	–
Rat	82.6%/90.5%	84.1%/86.8%	84.5%/85.5%	86.1%/92.0%
Hamster	–	85.0%/86.1%	–	–
Chicken	74.5%/77.8%	–	–	–

* Each cDNA length and numbers of putative amino acids were obtained from nucleotide sequence of each RT-PCR product amplified using *erbB* subfamily primer sets. Unknown sequences of *erbB* subfamily cDNA in several species are indicated by "–".

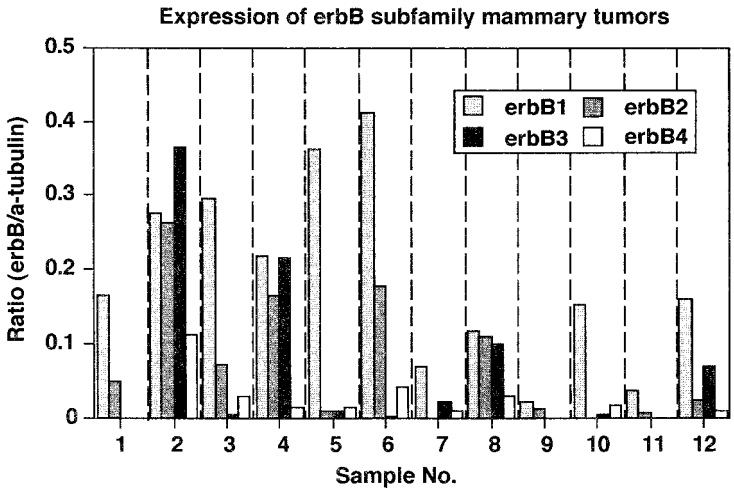

Fig. 3. Relative amounts of the RT-PCR of *erbB* subfamily mRNAs in canine mammary tumor samples. The numbers of the samples correspond to those in Table 1. Each amount was calculated from the ratio of fluorescent intensity of each product band on agarose gel to that of α-tubulin.

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