

Partial sequence and activation of canine erb B2 oncogene in the mammary tumor

Noboru ITOH²⁾, Hiroyuki TANIYAMA³⁾, Masaharu MORIYOSHI⁴⁾, Osamu FUJITA⁵⁾,
Tutomu KOBAYASHI¹⁾, Masayoshi YAMADA¹⁾ and Akira YUASA¹⁾

(June 1997)

Introduction

Mammary tumors are the most common tumor found in dogs³⁾. Some investigators have proposed that mammary tumors in dogs can be considered as an animal model for human breast cancer⁷⁾.

Of the several tumor markers now being studied, the HER-2/neu oncogene, also known as neu or c-erb-B2, has been shown to be of primary importance. Several investigators have found amplification of the erb B2 gene in various tumor collected from human^{4,8,9,12,13)}.

Investigation of the role of erb B2 in canine mammary tumors is necessary for oncogenesis of dogs as experimental models for human breast cancer. In this study, we partially sequenced the canine erb B2 oncogene, and examined its tissue-specific expression by Northern blot analysis.

Materials and Methods

Dogs and tissue — Tumor tissue was collected during surgery from 3 dogs. The three tumors were identified as multiple mixed tumors (benign), mammary adenocarcinoma (malignant), and malignant myoepithelioma (malignant) by histological examination. Histologically

identified normal mammary glands collected from a dog which possessed multiple mixed tumors (benign) were used as our normal control. The tissue samples were immediately put on ice, and cut into sections for histologic and oncogenic analyses. Those tissue specimens taken for histologic diagnosis were fixed in neutral formalin solution, and processed for light microscopy. Those intended for oncogenic analysis were frozen and stored at -70°C until use.

Preparation of canine cDNA from mammary gland tissue — Frozen tissue specimens (approximately 0.1g) from the canine mammary glands were put into RNA solTM B (Biotex Lab Inc., U.S.A.), and homogenized by Physcotron (Niti-On Medical Supply Co., Ltd., Japan). The samples were purified using chloroform, isopropanol, and 75% ethanol. Precipitates were dried and dissolved in Diethylpyrocarbonate (DEPC)-treated H₂O.

From this RNA (3 μg in 13 μl DEPC-treated H₂O), cDNA was synthesized using a randomhexamer (Life Technologies, Inc., U.S.A.), a 10X synthesis buffer, 10 mM deoxyribonucleoside 5'-triphosphates mixture (dNTP), 0.1 M dithiothreitol (DTT) and a Super Script IITM (Life Technologies, Inc., U.S.A.). The resulting single first-

-
- 1) 酪農学園大学獣医学科, 獣医生化学教室 小林 努・山田雅良・湯浅 亮
Department of Veterinary Medicine, Veterinary Biochemistry, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan.
 - 2) 酪農学園大学家畜病院 伊東 登
Veterinary Medical Teaching Hospital, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan.
 - 3) 酪農学園大学獣医学科, 獣医病理学教室 谷山弘行
Department of Veterinary Medicine, Veterinary Pathology, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan.
 - 4) 酪農学園大学獣医学科, 獣医臨床繁殖学教室 森好政晴
Department of Veterinary Medicine, Veterinary Obstetrics, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan.
 - 5) 白石動物病院 藤田 修
Shiroishi Veterinary Hospital

strand cDNA was used as a template for Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction — Degenerate oligonucleotide 5' primer, AACTGCACCCACTC-CTGTGTGG, and degenerate oligonucleotide 3' primer, GATCCAAGCACCTTCACCTTCC, were designed by determining the regions of strongest homology existing between human and rat erb B2^{1,11}. The regions corresponding to the primer sequences were located at nucleotides 2079-2058 and 2356-2335 of the human erb B2 (Fig. 1). These primers were synthesized using the Expedite™ Nucleic Acid Synthesis System (Perceptive Biosystem, U.S.A.)

To amplify erb B2, 2 μ l of 0.25 μ g/ μ l of single-stranded DNA were placed in a 0.5 ml tube containing 5 μ l of a 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH8.3], 30 mM MgCl₂, and 0.1 % [w/v] gelatin), 1 μ l of a dNTP mixture containing 10 mM each of dATP, dCTP, dGTP and dTTP, and 2.5 μ l of each primer (50 mg/ml). This mixture was overlaid with 30 μ l of mineral oil (Sigma Chemical Co., U.S.A.) and the reaction mixture was placed in a Program Control System PC-800 (Astec, Japan). PCR was initiated with the following parameters: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2

min (extension) for 38 cycles. The amplified DNA was analyzed by electrophoresing the reaction mixture through a 2% agarose gel containing 0.5 μ g/ml of ethidium bromide, and visualized with a UV light.

Cloning of erb B2 PCR fragment — Approximately 2 μ l of the erb B2-specific PCR reaction product was mixed with 50 ng of pMOS Blue vector (Amersham International plc, England), and 30 μ l of reaction solution A and 5.5 μ l of reaction solution B from Takara DNA Ligation System (Takara Shuzo Co., Ltd., Japan) were added. This mixed solution was then incubated for 12 hr at 16°C. Transformed cells, JM109 (Takara Shuzo Co., Ltd., Japan) were placed on L-broth agar plates containing 20 μ g/ml of ampicillin, and coated with 25 μ l of X-gal (40 mg/ml) and 100 mM of isopropyl thio- β -galactoside (IPTG) for 1hr before plating the bacteria. After a overnight incubation at 37°C, individual white colonies were collected and used to inoculate 5 ml of L-broth containing 20 μ g/ml of ampicillin. After 12 hr, 5 μ l of the culture were used as a substrate in a PCR reaction to determine which cultures contained the proper recombinant plasmid DNA.

DNA sequencing — The reaction mixture

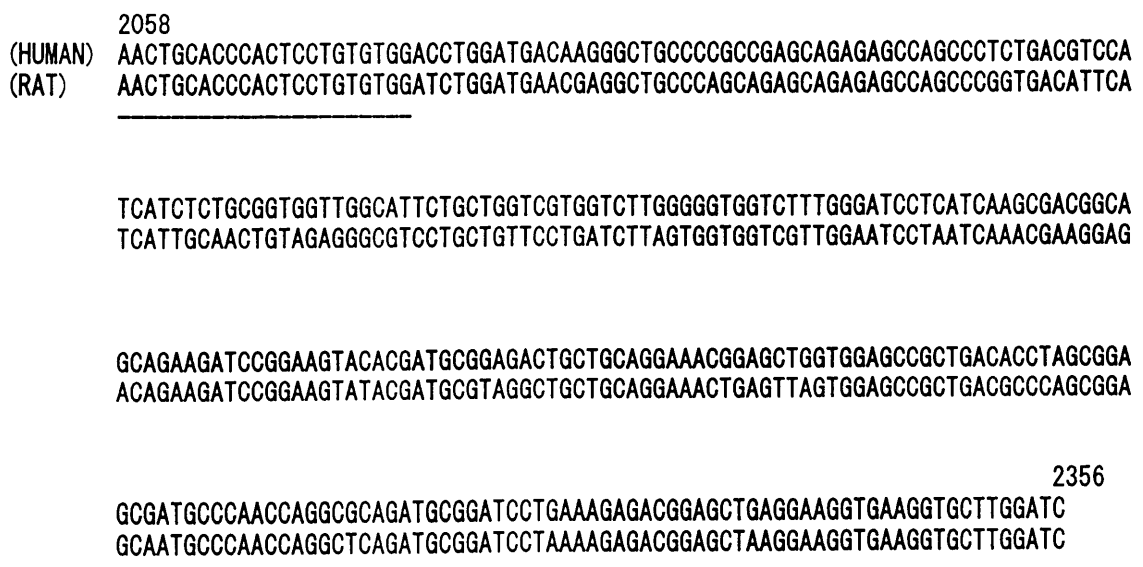


Fig. 1 Comparison of erb B2 sequences. Numbering above the nucleotide acids were reported by Yamamoto et al [11]. Dashed lines mark the homologous sequence between humans and mice; these oligonucleotide sequences were used as 3' and 5' primers in the PCR reaction for detecting canine erb B2 gene.

containing plasmid DNA was purified by alkaline method²⁾, and DNA sequencing was carried out using the Cycle Sequencing Method (Takara Shuzo Co., Ltd., Japan). Sequencing was carried out using the cDNA sample taken from the tissue of the multiple mixed tumor.

Northern blot analysis— Total RNA was isolated from 0.2 g of each specimen. Tissue samples were collected from the tumors described above. The RNA samples obtained were dissolved and stored at -30°C in DEPC-treated H_2O . Total RNA was denatured with formamide, subjected to electrophoresis on a 1% agarose gel containing 18% formaldehyde, and then transferred to a nylon membrane. The PCR product described previously was used as the probe for erb B2. The resulting product was labeled with a DIG RNA Labeling Kit (Boehringer Mannheim GmbH, Germany). Hybridization was completed at 65°C for 16 h in a solution produced according to the protocol of the DIG RNA Labeling Kit. After hybridization, the filters were washed twice with a 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.5% SDS at 65°C for 40 min. The filters were finally washed with 0.2X SSC containing 0.5% SDS at 65°C for 40 min, and then dried and autoradiographed at room temperature for 30 min.

Results

About 300 base pair fragments were obtained from both tumor tissue and normal tissue by reverse transcriptase-PCR using the erb B2-specific degenerate primers (AACTGCACCCACTC-CTGTGTGG and GATCCAAGCACCTTCAC-CTTC)(Fig. 2). There was no difference in expression order between normal tissue and tumor tissue.

Each fragment from the tumor tissue was cloned into a PCR vector and sequenced (Fig. 3). Because the sequence matched that of human erb B2 and rat neu cDNA, it was found that the cloned DNA indeed represents a cDNA derived from the canine erb B2 mRNA. The nucleotide sequence was found to exhibit 89.0% and 82.7% homology with human and rat gene, respectively.

The amino acid sequence corresponding to

the nucleotide sequence between 2082 and 2334, which includes the transmembrane region of the erb B2 protein, is shown in Fig. 4. Squares with asterisks indicate transmembrane regions. Amino acid sequences in the cytoplasmic domain were strongly homologous among the three species, although sequences in the transmembrane region and extracellular domain revealed a number of differences.

Northern blot analysis was performed to examine the expression of the gene for erb B2 in both normal and tumor tissue. Figure 5 shows that erb B2 was expressed in both normal and tumor tissue at level 28S. The expression order was high in lanes 2 and 3 tumor tissue, but low in lane 1 normal mammary tissue. The expression order was particularly high in lane 3 tumor tissue.

Discussion

Erb B2 oncogene has been extensively investigated in human breast cancer^{9,13)}. Investigation



Fig. 2 Detection by reverse transcriptase-PCR of mRNA encoding canine erb B2. RNA from normal and cancer tissue was isolated, converted to single-strand cDNA, and amplified by PCR using erb B2-specific oligonucleotide primers. Positive signals of expected size, about 300bp, were detected in cDNA prepared from normal control tissue (lane 3) and cancer tissue (lane 2: multiple mixed tumor). Lane 1 contains a 1 Kb DNA Ladder used as the molecular weight marker.

2058

AACTGCACCCACTCCTGTGTGGACCTGGACGAGAAGGGCTGTCCCGCCGAGCAGAGCCAGCCCTGTGACATCCATC

LeuAspGluLysGlyCysProAlaGluGlnArgAlaSerProValThrSerIle
637

ATTGCCGCTGTGGAGGGCATTCTGCTGGCTGTGGTCTGTTGGGCTGGTCCTCGGCATCCTGATCAAGCGAAGGGCGCAG

IleAlaAlaValGluGlyIleLeuLeuAlaValValValGlyLeuValLeuGlyIleLeuIleLysArgArgArgGln

AAGATCCGGAAGTACACTATGCGGAGGCTGCTGCAGGAAACCGAGCTGGTGGAGCCGCTGACGCCTAGTGGAGCGATG

LysIleArgLysTyrThrMetArgArgLeuLeuGlnGluThrGluLeuValGlnProLeuThrProSerGlyAlaMet

2356

CCCAACCAGGCTCAGATGCGGATCCTGAAAGAGACAGAGCTGAGGAAGGTGAAGGTGCTTGGATC

ProAsnGlnAlaGlnMetArgIleLeuLysGluThrGluLeu
720

Fig. 3 Sequence of canine erb B2. Numbering refers to the amino acid sequence above and the DNA sequence below. Dashed lines mark the homologous sequence between humans and mice; these oligonucleotide sequences were used as 3' and 5' primers in the PCR reaction for detecting canine erb B2 gene.

(Extracellular domain)
637
(Dog) LDEKGCPAEQRASPVTSIIAAVEGILLAVVGLVGLIKRRRQK
(Human) --D-----L---S---V---V---L---V---F-----Q--
(Rat) --ER-----F---T---V---FLILVV-V-----

IRKYTMRRLLQETELVEPLTPSGAMPNQAMRILKETEL

(Cytoplasmic domain) 720

Fig. 4 Comparison of erb B2 amino acid sequence. Numbering above the line refers to the amino acids below. Dashes indicate identity with the canine sequence, and asterisks indicate the transmembrane region reported by Yamamoto et al [11].

of erb B2 in canine mammary tumors is useful for comparative oncogenesis, and dogs may be used as experimental models for human breast cancer. However, only a few reports have been published concerning oncogenes in spontaneously arising tumors in domestic animals^{5,6}.

The nucleotide sequence obtained from

canine cDNA in this study is considered to be the canine erb B2 fragment, because the nucleotide sequence was found to exhibit 89.0% and 82.7% homology with human and rat erb B2 genes, respectively. Cloning of the canine erb B2 gene, using PCR oligonucleotides based on human and murine sequences, suggests that this technique could be valuable in cloning canine oncogenes where the sequence data of other animals, such as humans or rats, is available.

Many differences were seen in the transmembrane region among humans, rats and dogs, although differences in the amino acids between the three species were mainly restricted to hydrophobic amino acids. Differences in the sequence of amino acids among the three species were small in the cytoplasmic and extracellular domains.

Yamamoto et al.¹¹ reported that threonine residue at position 686 corresponded to the phosphorylation site in the EGF (epidermal growth factor) receptor. As threonine was seen in all three species, this amino acid may play an impor-

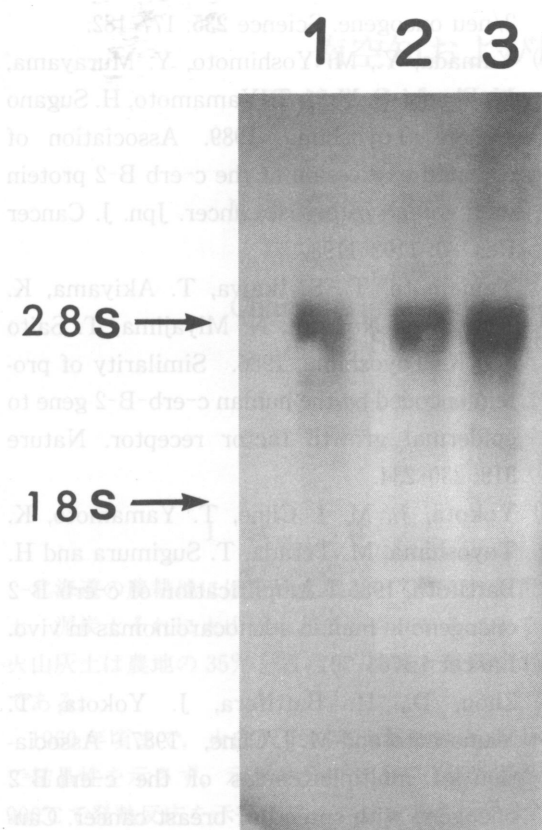


Fig. 5 Northern blot analysis of canine erb B2. Each lane was loaded with 5 μ g of total RNA prepared from normal control tissue (lane 1) and various mammary tumor tissues (lane 2: mammary adenocarcinoma, and lane 3: malignant myoepithelioma).

tant role in signal transmission in all three species.

Since Slamon et al.⁹⁾ first reported that the c-erb B2 was amplified in human breast cancer, the relationship between c-erb B2 and human cancer, including breast cancer, has been intensively investigated. Amplification of erb B2 has been identified, not only in breast cancer, but also in cancer of the stomach, carcinoma of the colon, and cerebral tumor^{4,12,13)}. This amplification of erb B2 was not identified in all of the cases: for example, amplification was only seen in about 20% of the breast cancers. In non-amplified expressed cases, expression of erb B2 in the stage of transcription or translation was detected using Western blotting¹⁰⁾. Elucidation of the role of c-erb B2 in cancer tissues may be useful for decisions related to prognosis and method of treat-

ment.

As there have been few reports about erb B2 in canine tumors, detection of such expressions in the translation stage of canine erb B2 may be useful for comparative pathology. In the present study, relatively high expressions were seen in the tumor tissues. The expression in malignant myoepithelioma (malignant) was most significant. Further study of this correlation between malignancy of tumor and expression of erb B2 would be of great interest.

In the present study, canine erb B2 gene was partially sequenced, and an over-expression of the erb B2 gene in mammary gland tumors was suggested. However, further studies are needed, using a wider variety of tumor tissue to clarify the correlation between erb B2 expression and tumor malignancy.

Summary

Canine oncogene, erb B2 was shown to be partially sequenced and its activation was detected in mammary tumor. The cDNA was obtained by reverse transcriptase-polymerase chain reaction (PCR), as follows: the cDNA was synthesized from total RNA prepared from canine mammary gland tissue, and PCR was carried out using 5' primer and 3' primer designed according to the homologous region between human and rat erb B2 gene. About 300 base pair fragments were obtained. The nucleotide sequence of these fragments was found to exhibit 89.0% and 82.7% homology with that of the corresponding regions of human and rat erb B2, respectively. Northern blot analysis demonstrated that this erb B2 mRNA was expressed in both normal and tumor tissue. The expression levels of erb B2 mRNA in several specimens of canine mammary tumor tissue were higher than those in normal mammary tissue.

Acknowledgements

We wish to thank Associate Professor Hiroshi Yokota and Associate Professor Hiroyuki Okada of Rakuno Gakuen University, for their valuable help. This work was partially supported by a Grants-in-Aid to Cooperative Research

from Rakuno Gakuen University, 1995.

References

- 1) Bargmann, C. I., M-C. Hung and R. A. Weinberg, 1986. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* **319**: 226-230.
- 2) Birnboim, H. C. and J. Doly, 1979. A rapid alkaline extraction procedure for recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513-1523.
- 3) Brodey, R. S., M. H. Goldschmidt and J. R. Roszel, 1983. Canine mammary gland neoplasm. *J. Am. Anim. Hosp. Assoc.* **19**: 61-90.
- 4) Masuda, H., H. Battifora, J. Yokota, S. Meltzer and M. J. Cline, 1987. Specificity of proto-oncogene amplification in human malignant disease. *Mol. Biol. Med.* **4**: 213-227.
- 5) Miyoshi, N., S. Tateyama, K. Ogawa, D. Nosaka, T. Ohashi and B. Sunyasootcharee, 1991. Proto-oncogenes of genomic DNA in clinically normal animals of various species. *Am. J. Vet. Res.* **52**: 940-944.
- 6) Miyoshi, N., S. Tateyama, K. Ogawa, R. Yamaguchi, H. Kuroda, N. Yasuda and T. Shimizu, 1991. Abnormal structure of the canine oncogene, related to the human c-yes-1 oncogene, in canine mammary tumor tissue. *Am. J. Vet. Res.* **52**: 2046-2049.
- 7) Pierrepoint, C. G., 1985. Possible benefits to veterinary medicine of considering the dog as a model for human cancer. *J. Small Anim. Pract.* **26**: 43-47.
- 8) Semba, K., N. Kamata, K. Toyoshima and T. Yamamoto, 1985. A v-erb B-related protooncogene, c-erb B-2, is distinct from the c-erb-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **82**: 6497-6501.
- 9) Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich and L. McGuire, 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177-182.
- 10) Yamada, Y., M. Yoshimoto, Y. Murayama, M. Ebuchi, S. Mori, T. Yamamoto, H. Sugano and K. Toyoshima, 1989. Association of elevated expression of the c-erb B-2 protein with spread of breast cancer. *Jpn. J. Cancer Res.* **80**: 1192-1198.
- 11) Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito and K. Toyoshima, 1986. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**: 230-234.
- 12) Yokota, J., M. J. Cline, T. Yamamoto, K. Toyoshima, M. Terada, T. Sugimura and H. Battifora, 1986. Amplification of c-erb B-2 oncogene in human adenocarcinomas in vivo. *Lancet* **1**: 765-767.
- 13) Zhou, D., H. Battifora, J. Yokota, T. Yamamoto and M. J. Cline, 1987. Association of multiple copies of the c-erb B-2 oncogene with spread of breast cancer. *Cancer Res.* **47**: 6123-6125.

犬の癌遺伝子 erb B2 の部分シーケンスと乳腺腫瘍組織における発現

要 約

イヌの癌遺伝子 erb B2 を部分シーケンスし、イヌ乳腺腫瘍においてその発現を認めた。以下のよう、PCR 法によりイヌ erb B2 遺伝子の一部の cDNA が得られた。すなわち、既に報告されているヒトとラットの erb B2 遺伝子配列の共通配列をもとに 5' および 3' プライマーを作成し、イヌ乳腺腫瘍組織から作成した total RNA から cDNA を作成した。その結果、約 300 塩基対の遺伝子が得られた。クローニング後、シーケンスしたところ、ヒトおよびラットの当該部分との相同性は、それぞれ 89.0% および 82.7% であった。イヌ正常乳腺組織および腫瘍組織から抽出した RNA を用いて、ノーザンハイブリダイゼーションを行った。その結果、一部の乳腺腫瘍組織において、正常乳腺組織に比べて、より多い erb B2 の発現がみられた。