Expression of the PTEN tumor suppressor gene in malignant mammary gland tumors of dogs

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Objective—To determine whether changes in expression level of the phosphatase and tensin homolog deleted on the chromosome 10 (PTEN) gene are associated with malignant transformation in mammary gland tumors in dogs.

Sample Population—Specimens of 5 benign and 8 malignant mammary gland tumors and 2 unaffected mammary glands from dogs.

Procedure—The open reading frame (ORF) sequence of PTEN gene in each specimen was analyzed via a direct-sequencing method; expression levels of PTEN gene were quantified via a competitive reverse transcription (RT)-PCR method.

Results—Compared with findings in clinically normal mammary gland tumors, amounts of PTEN mRNA were increased 2- to 4-fold in 4 of the 5 benign mammary gland tumor samples. In contrast, PTEN expression was remarkably low in 4 of the 8 malignant tumor samples (approx 12% to 37% of the level in unaffected mammary gland specimens). Gene amplification via the RT-PCR method with total RNA prepared from malignant tumor samples as a template yielded 3 bands that were smaller than the full-length ORF product of PTEN gene; in 2 of those 3 RT-PCR products, exons 6 and 7 or exons 3 to 8 were absent. No mutation was detected in the full-length ORF product of PTEN gene.

Conclusions and Clinical Relevance—Results suggested that a decreased level of PTEN gene expression (compared with unaffected mammary gland tissue) is associated with malignancy in canine mammary tumors. Analysis of PTEN gene expression level in dogs with mammary gland tumors may provide useful prognostic information. (Am J Vet Res 2006;67:127-133)

Mammary gland tumors are the most common neoplasms in female dogs. In dogs, mammary gland tumors are age-dependent neoplasms that arise from different types of tissues (epithelial or glandular tissues and mesenchymal or connective tissues) in the mammary gland. The most common types are tumors that develop from glandular tissues, which include adenomas, carcinomas, and adenocarcinomas. Approximately 50% of all mammary gland tumors are malignant and have the potential to metastasize. It has been reported that the tumor suppressor gene PTEN (also known as mutated in multiple advanced cancer 1) is frequently mutated or deleted in neoplasms, such as glioblastoma, melanoma, prostate cancer, endometrial cancer, and breast cancer in humans. In addition, it has been reported that the PTEN gene is mutated or deleted in melanoma and osteosarcoma cell lines and tumor samples obtained from dogs. The PTEN protein consists of a catalytic N-terminal phosphatase domain that acts on both protein and lipid substrates and a C-terminal C2 domain that interacts in a Ca2+-independent manner with phospholipid substrates. The major physiological substrate of the PTEN protein is phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P3); the action of PTEN on PtdIns[3,4,5]P3 antagonizes the signaling activity of phosphatidylinositol-3 kinase, thereby blocking the activation of the protein kinase B pathway. The phosphatase activity of PtdIns[3,4,5]P3 suppresses cell proliferation, cell survival, and tumor-directed angiogenesis. The protein phosphatase activity of the PTEN protein is associated with downregulation of focal adhesion kinase phosphorylation, which leads to inactivation of the Ras/mitogen-activated protein kinase pathway, the PTEN protein activity negatively affects cell proliferation, cell migration, and tumor-cell invasion. Therefore, it has been thought that a loss of PTEN activity may confer increased survivability, proliferative potential, and invasive capacity on tumor cells and may promote progression toward a more malignant phenotype. The PTEN protein suppresses cellular growth via downregulation of phosphatidylinositol-3 kinase, resulting in G1 arrest and cell death in human breast cancer cell lines. Furthermore, in human breast cancer tissues, a low expression level of PTEN mRNA is commonly detected and it is thought that a decrease in the level of PTEN gene expression is associated with a larger tumor size and higher histologic malignancy grade. Although it has been shown that changes in expression or mutation of various genes, such as p53 and estrogen receptor, are associated with mammary gland tumorigenesis and malignant transformation in dogs, the relationships between changes in the level of expression or mutation of the PTEN gene and tumorigenesis or malignancy grade of canine mammary gland

ORF Open reading frame
RT Reverse transcription

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tumors remain unclear. The purpose of the study reported here was to determine whether changes in expression level of the PTEN gene are associated with malignant transformation in mammary gland tumors in dogs.

**Materials and Methods**

**Tissues**—Mammary gland tumor tissues were obtained from 13 client-owned dogs (age range, 6 to 18 years) during surgical treatment at the Veterinary Teaching Hospital of Rakuno Gakuen University. Of the 13 dogs, 8 had malignant mammary gland tumors and 5 had benign mammary gland tumors (Table 1). Small specimens of unaffected mammary gland tissues and kidney tissues were obtained via biopsy procedures during anesthesia from 2 clinically normal dogs that were bred at the breeding facility of the School of Veterinary Medicine, Rakuno Gakuen University. The procedures used for handling of the dogs and transferring samples for use in the present study were approved by the Animal Research Committee of the School of Veterinary Medicine, Rakuno Gakuen University. Tissue specimens were fixed in neutral-buffered 10% formalin and routinely processed. Sections were stained with H&E, and histologic diagnoses were established by use of the general World Health Organization classification of mammary tumors.14-33

**Total RNA preparation**—Small pieces of tissues from 8 malignant and 5 benign mammary gland tumors and from 2 nonneoplastic mammary glands were rapidly frozen in liquid nitrogen and stored at −80°C until processed. Total RNA was isolated by use of an acid guanine thiocyanate-phenol chloroform method (with slight modification) involving RNA isolation solution.7 The RNA concentrations were determined via UV spectrophotometry.6

**Direct-sequencing and subcloning**—The first-strand cDNA was synthesized from 2.5 µg of total RNA prepared from each of the nonneoplastic mammary gland samples and mammary gland tumor samples by use of a reverse transcriptase.1 The sequence of the ORF that translates as an amino acid sequence with 1,212 bp of the canine PTEN gene was available (GenBank accession No. U924331262). A fragment, including the full-length ORF was amplified via a PCR technique involving a forward primer (PTEN-F, 5'-AGCCACCG-CTGGTGTTTTATCCCTCTTGAT-3'). The PCR procedure involved a cycle sequencing kit with an autosequencer according to the manufacturer's protocol and analyzed by use of sequence analysis software. Alternatively, the PCR products were cloned into a plasmid vector by use of a ligation kit and DH5-alpha competent cells. A plasmid clone DNA was prepared by use of a plasmid preparation kit and cleaved by NdeI at a position beyond the 3' end of the 208-bp fragment. By use of an in vitro transcription kit, competitor RNA for the PTEN gene was synthesized from the cleaved plasmid and treated with RNase-free DNase I for 15 minutes. The concentrations of PTEN competitor RNA were determined via UV spectrophotometry.9 As an internal control, β-actin mRNA was used. For quantitative determination of β-actin mRNA, a 459-bp fragment was first amplified by use of forward (CBF1, 5'-CCATCTCTGCTGGTGACCTG-3') and reverse (CBR1, 5'-ATCTTCTCATCTTGTGCTGGGCCAG-3') primers based on the canine β-actin sequence (GenBank accession No. AF021873) from cDNA of a nonneoplastic mammary gland sample (Figure 1). A plasmid vector expressing the 410-bp competitor RNA of β-actin mRNA with an internal 49-bp deletion was constructed with PCR products from the 459-bp fragment as a template and with CBF2 (5'-CCATCTCCT-GGTCTGACCCGCTACGAGCCTCCACACTG-3') and CBR2 (5'-AGCAATGCGCCAGGTATATGG-3') primers, as described previously.

**Quantitative determination of PTEN gene expression by use of a competitive RT-PCR method**—For quantitative determination of PTEN mRNA, a competitive RT-PCR procedure was performed essentially according to the method described by Celi et al.3 First, cDNA was synthesized from 2.5 µg of total RNA isolated from nonneoplastic mammary gland and mammary gland tumor samples by use of 50 pmol of primer CPR2 (5'-CTGGGTGTCAGAGTCAGTGGTG-3') for PTEN or primer CPR1 (5'-AGCAATGCGCCAGGTATATGG-3') for β-actin and reverse transcriptase in the presence of 6,300 pg of competitor RNA or 500 pg of competitor RNA for PTEN or primer CPR2 (5'-AGCAATGCGCCAGGTATATGG-3') for β-actin and reverse transcriptase in the presence of 6,300 pg of competitor RNA or 500 pg of competitor RNA for PTEN or primer CPR1 and CBF1 and CBR2 primers for the PTEN gene and by use of CBF1 and CBR2 primers for the β-actin gene in a 30-µL reaction mixture containing 10 mM Tris-Cl (pH, 8.3 at 25°C), 50 mM KCl, gelatin (0.001% [wt/vol]), 300 ng of cDNA, 0.01 units of Taq polymerase, 0.2 mM dNTP, 15 mM MgCl2, and 0.5 µM each of primer by use of a thermal cycler2 under the following conditions: initial denaturation at 94°C for 1 minute, 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes. The PCR products underwent electrophoresis in 1% (wt/vol) agarose gel buffered with Tris-acetate-EDTA solution; the gels were stained with ethidium bromide and then examined by use of a UV transilluminator. The PCR products were purified by use of a PCR product purification kit. Sequences of the PCR products were determined by use of a terminator sequencing method involving a cycle sequencing kit with an autosequencer according to the manufacturer's protocol and analyzed by use of sequence analysis software. Alternatively, the PCR products were cloned into a plasmid vector by use of a ligation kit and DH5-alpha competent cells.

**Competitor RNA preparation**—For the competitive RT-PCR procedure, expression vectors for competitor RNA were constructed essentially according to the method described by Celi et al.3 First, 249-bp fragments of the canine PTEN gene were amplified via a PCR method in which cDNA from a nonneoplastic mammary gland sample was used as a template; the procedure was the same as that described previously except that polymerization time was 30 seconds and different forward and reverse primers were used (CPF1, 5'-GTAAGACTGTACCTCAA-3' and CPR1, 5'-AGCATTGTGGGATTT-3', respectively) (Figure 1). Subsequently, 208-bp fragments with internal 41-bp deletion were synthesized by use of a deletion PCR procedure involving a modified forward primer (CPF2, 5'-GTAAGACCTGTACCTCACA-3') and CPR1. The PCR procedure for amplification of the 208-bp fragment was performed as described except that polymerization time was 30 seconds and 300 ng of the 249-bp PCR product was used as a template. The 208-bp fragment with internal deletion was purified by use of a PCR product purification kit and cloned into a plasmid vector in which competitor RNA was directed by T7 RNA polymerase promoter.7 The plasmid clone DNA was prepared by use of a plasmid preparation kit and cleaved by NdeI at a position beyond the 3'-end of the 208-bp fragment. By use of an in vitro transcription kit, competitor RNA for the PTEN gene was synthesized from the cleaved plasmid and treated with RNase-free DNase I for 15 minutes. The concentrations of PTEN competitor RNA were determined via UV spectrophotometry.9 As an internal control, β-actin mRNA was used. For quantitative determination of β-actin mRNA, a 459-bp fragment was first amplified by use of forward (CBF1, 5'-CCATCTCTGCTGGTGACCTG-3') and reverse (CBR1, 5'-ATCTTCTCATCTTGTGCTGGGCCAG-3') primers based on the canine β-actin sequence (GenBank accession No. AF021873) from cDNA of a nonneoplastic mammary gland sample (Figure 1). A plasmid vector expressing the 410-bp competitor RNA of β-actin mRNA with an internal 49-bp deletion was constructed with PCR products from the 459-bp fragment as a template and with CBF2 (5'-CCATCTCCT-GGTCTGACCCGCTACGAGCCTCCACACTG-3') and CBR2 (5'-AGCAATGCGCCAGGTATATGG-3') primers, as described previously.

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was amplified via the RT-PCR method with total RNA prepared from malignant tumor samples, benign tumor samples, nonneoplastic mammary gland tissue, and clinically normal kidney tissues as templates and primers Ex2 (5'-AGACTTGAGGGCCTATACAG-3') and Ex9 (5'-TAA-CATCTGCGTGACAGA-3'). Primers Ex2 and Ex9 were designed for amplification from exons 2 to 9 of PTEN gene mRNA. The PCR procedures were the same as described for amplification of the full-length ORF of PTEN cDNA.

**Results**

Expression levels of the PTEN gene in samples of mammary gland tumors from 13 dogs were assessed via a competitive RT-PCR method. The PTEN mRNA and PTEN competitor RNA were amplified to 199- and 158-bp fragments, respectively, and β-actin mRNA and β-actin competitor RNA were amplified to 409- and 360-bp fragments, respectively (Figure 1). Electrophoretic patterns of the competitive RT-PCR products for the PTEN gene were evaluated (Figure 2). By use of β-actin mRNA as an internal control, relative amounts of PTEN mRNA were normalized in such a way that the expression level of PTEN mRNA in nonneoplastic mammary gland tissue was 1.0. Compared with findings in the nonneoplastic mammary glands, relative amounts of PTEN mRNA were increased by 2- to 4-fold in 4 of the 5 benign mammary gland tumors; in contrast, PTEN expression level was remarkably decreased in 4 of the 8 malignant mammary gland tumors (Table 1). In the malignant tumor samples in which a reduced expression level was detected, relative amounts of PTEN mRNA were approximately 12% to 37% of those in nonneoplastic mammary gland samples.

In the present study, the region of junction sequence between phosphatase and C2 domain was amplified via a competitive RT-PCR method (Figure 1). Therefore, PTEN mRNA was not amplified if deletion had occurred in this region. To determine whether deletion mutation of the PTEN gene had occurred in malignant tumor samples in which decreased amounts of PTEN mRNA were detected, PTEN cDNA was amplified via the RT-PCR method with total RNA prepared from malignant tumor samples as a template and PTEN-F and PTEN-R primers (the use of which results in amplification of the full-length ORF of a canine PTEN gene). Three bands (994, 350, and approx 600 bp) smaller than the full-length (1,212-bp) ORF were detected among the amplified products. The small PCR prod-
Products were cloned (Figure 3). Sequence determination of 2 clones revealed that clone 2 lacked exons 6 and 7 and clone 3 lacked exons 3 through 8. However, no mutation was detected in the full-length ORF product of the PTEN gene. Clones 2 and 3 were deleted coding sequences of the functional domain of the PTEN gene. When cDNA from exons 2 to 9 of the canine PTEN gene was amplified via the RT-PCR method with total RNA prepared from malignant tumor samples as a template and primers Ex2 and Ex9, 3 bands smaller than typical length (987 bp) were detected in the amplified products in 7 of the 8 malignant tumor samples (Figure 4). Analyses of benign tumor samples, nonneoplastic mammary gland tissue, and normal kidney tissue revealed PCR products that were of typical length and 125-bp long. Results of sequence analyses of the PCR products indicated that the 769- and 125-bp bands corresponded to clones 2 and 3, respectively (data not shown). A 500-bp band had a sequence that was unrelated to the PTEN gene (data not shown).

**Discussion**

The tumor suppressor protein PTEN, which has both lipid phosphatase and protein phosphatase activities, downregulates cell proliferation and interactions with the extracellular matrix and maintains normal sensitivity of cells to induction of apoptosis.16-21 It has been reported that the PTEN gene is mutated in several organ tumors, including breast cancer in humans5,7-11,37,38 and melanoma12 and osteosarcoma in dogs.13 Of the neoplasms that may develop in female dogs, mammary gland tumors are the most common and frequently malignant and may metastasize.2,3,19 However, the potential role of the PTEN protein in canine mammary tumor development has not been investigated. In the present study, the relative amounts of PTEN mRNA were increased in most of the benign mammary gland tumor samples, compared with amounts in nonneoplastic mammary gland samples; in contrast, the relative amounts of PTEN mRNA were markedly decreased in 4 of the 8 malignant tumor samples. Therefore, a decrease in the level of PTEN gene expression may be associated with malignancy in canine mammary gland

Table 1—Relative PTEN gene expression level in samples of neoplastic and nonneoplastic canine mammary tissue by breed and age of dog, classification of tissue sample, and tumor type.

<table>
<thead>
<tr>
<th>Classification of tissue sample</th>
<th>Breed of dog</th>
<th>Age (y)</th>
<th>Type of mammary gland tumor</th>
<th>Expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaffected (normal)</td>
<td>Beagle</td>
<td>NK</td>
<td>NA</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>NK</td>
<td>NA</td>
<td>1.0</td>
</tr>
<tr>
<td>Malignant tumor</td>
<td>Mixed</td>
<td>13</td>
<td>Adenocarcinoma (papillary), lymph node metastasis</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>12</td>
<td>Myoepithelioma</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Shih Tzu</td>
<td>10</td>
<td>Adenocarcinoma</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Siberian Husky</td>
<td>7</td>
<td>Squamous cell carcinoma</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>10</td>
<td>Adenocarcinoma</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>German Shepherd Dog</td>
<td>9</td>
<td>Adenocarcinoma</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>NK</td>
<td>Adenocarcinoma</td>
<td>1.0</td>
</tr>
<tr>
<td>Benign tumor</td>
<td>Shih Tzu</td>
<td>10</td>
<td>Mixed tumor</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>6</td>
<td>Mixed tumor, papilloma</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>NK</td>
<td>Mixed tumor</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Maltese</td>
<td>18</td>
<td>Mixed tumor</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Maltese</td>
<td>18</td>
<td>Mixed tumor</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Each expression level was calculated from calibration curves derived as a result of competitive RT-PCR, and each expression ratio was normalized by use of the PTEN gene expression level in nonneoplastic mammary gland samples (ratio, 1.0). Each expression ratio represented the mean of the results of 2 separate experiments.

NK = Not known. NA = Not applicable.
tumors. Of the 6 samples of mammary gland adenocarcinoma that were analyzed in our study, 3 had decreased amounts of PTEN mRNA (compared with the findings in nonneoplastic samples); thus, histologic types of tumors may not be directly related to a change in PTEN gene expression level. In human mammary glands, a high concentration of PTEN protein is associated with ductal hyperplasia (with or without atypia), compared with the amount in normal mammary gland epithelial cells.10 Although the reason for increased amounts of PTEN mRNA detected in benign mammary gland tumors of the dogs in our study remains unclear, the expression level of PTEN may increase compensationally to antagonize cell proliferation in tissues with hyperplasia and benign tumors because the PTEN protein downregulates cell proliferation. The decreased amount of PTEN mRNA identified in samples of malignant mammary gland tumors may result in a reduction of the downregulation by PTEN on migration and invasion of tumor cells and tumor cell growth.

In the present study involving dogs, 2 amplified products smaller than the full-length (1,212-bp) ORF were derived from total RNA samples obtained from malignant mammary gland tumors. Sequence determination of the 2 small products revealed that 1 lacked exons 6 and 7 and the other lacked exons 3 through 8. Although truncated mRNA of PTEN caused by an alternative splicing mechanism has been identified in human cells,39 the lack of 1 exon or contiguous multiple exons is called exon skipping.40 Exon skipping is associated with genetic disorders in humans such as Ehlers-Danlos syndrome types I and IV.41,42 In our study, the smallest fragment (clone 3) was detected in all RNA samples derived from malignant and benign mammary gland tumors and nonneoplastic mammary glands. Furthermore, a small mRNA sequence affected by exon skipping was detected in the RNA sample prepared from kidneys. Because clone 3 had lost the entire functional domain of PTEN, the role of the small sequences of mRNA remains unknown. A fragment lacking exons 6 and 7 (clone 2) was detected in RNA samples from 4 of 5 malignant mammary gland tumors but not in samples from benign mammary gland tumors, nonneoplastic mammary glands, and kidneys. Therefore, the presence of the clone 2 product may be associated with malignant transformation in mammary gland tumors of dogs. The C-terminal region of the phosphatase domain and all of the functional C-terminal domain containing a C2 domain and a PSD-95/Dlg/ZO-1 homology domain-binding motif, which are essential for regulating PTEN protein stability and enzymatic activity,43,44 were deleted in clone 2. If the transcript affected by exon skipping is translated, the truncated form of PTEN may negatively affect the function of normal nontruncated PTEN products. The mechanism by which products of PTEN affected by exon skipping occur remains unknown. Ueda et al45 reported mutations in introns 2 and 7 of the PTEN gene in human breast cancer tissues; therefore, a mutation in introns of the PTEN gene may cause exon skipping.

The competitive RT-PCR technique is a quantitative method in which known amounts of copies of a synthetic, mutated, or deleted internal standard (called the competitor) are introduced with the sample into the PCR reaction mixture. This is a highly sensitive method for analysis of gene expression level.36,46 In the present study, the competitor was designed against the junction region between phosphatase and the C2 domain in exons 6 and 7. Occasionally, exons 6 and 7 were missing in clones 2 and 3 as a result of exon skipping. Although products affected by exon skipping may have resulted in reduced amounts of PTEN mRNA in malignant mammary gland tumors of dogs, compared with amounts in nonneoplastic tissue samples, exon-skipped transcripts comprised only a small percentage of the total PTEN gene transcripts. Therefore, exon skipping is not likely to contribute notably to changes in total PTEN mRNA amounts. Recently, it has been reported...
that the low expression level of the PTEN gene in invasive breast cancer tissues is caused by hypermethylation on the promoter region of the PTEN gene and that the hypermethylation is related to tumor size and grade in naturally occurring breast cancers in humans. Presently, it is not known whether the promoter sequence of the PTEN gene is hypermethylated in malignant mammary gland tumors of dogs. On the basis of our data, we suggest that a decrease in the expression level of the PTEN gene may provide useful prognostic information.

References

36. Celi FS, Zenilman ME, Shuldiner AR. A rapid and versatile


