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Expression Patterns of the *slit* Subfamily mRNA in Canine Malignant Mammary Tumors

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ABSTRACT. Slit, a secreted protein, functions as a chemorepellent factor in axon guidance and neuronal migration and as an inhibitor in leukocyte chemotaxis. In humans, *slit2* protein attracts endothelial cells and promotes tube formation in the tumor angiogenic mechanism. In this study, we cloned a part of the canine *slit* subfamily and examined the expression of *slit* subfamily mRNAs in 3 normal canine mammary glands and 11 mammary tumor samples by RT-PCR. The cloned part of the *slit* gene sequences showed high similarity to those of the human, mouse, and rat. The mRNAs were expressed at low levels in the normal mammary gland. The expression levels of *slit1* mRNA were low in both the normal and tumor tissues. In contrast, the expression of *slit2* mRNA increased in most of the malignant mammary tumors, and an increase in *slit3* mRNA expression was observed in 2 of the malignant mixed tumors. These results suggest that the expression of *slit2* plays an important role in tumor angiogenesis in canine mammary gland tumors and that *slit2* can be a putative marker for malignancy diagnosis of these tumors.

KEY WORDS: canine mammary gland tumor, malignancy, robo, slit, tumor marker.

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Mammary tumors are one of the most common neoplasms in female dogs and women. Canine mammary tumors may account for half of all tumors in bitches, and approximately 40–50% of them are considered malignant [2, 14]. In recent years, advancement of a veterinary medicine has made the research of cancer-related genes necessary for malignancy diagnosis.

The slit family comprises large extracellular matrix secreted and membrane-associated glycoproteins. Three vertebrate orthologs, *slit1*, *2*, and *3*, have been isolated. Each displays overlapping, but distinct, patterns of expression in the vertebrate nervous system [16, 17]. Human Slits are candidate ligands for the repulsive guidance receptors of the *robo* gene family. Slit-Robo interactions mediate the repulsive cues on axons and growth cones during neural development [1]. It has also been reported that slits inhibit leukocyte chemotaxis induced by chemotactic factors [18].

In human prostate tumors, one of the most common malignancies, there is a trend towards increased *slit1* expression compared with normal prostate tissue [9]. Increased *slit2* and/or *slit3* expression is also observed in several other prostate tumors, especially hormone-refractory prostate tumors. Wang *et al.* reported *slit2* expression in a large number of solid tumors and *robo1* expression in vascular endothelial cells [20]. Slit2 protein attracts endothelial cells and promotes tube formation through the slit-robo signaling cascade. These findings indicate that the angiogenic function of slit-robo signaling is a novel target as well as a molecular tool for cancer therapy. The expression level of *slit2* mRNA may become a useful marker for detection of cancer malignancy. However, it has been reported that *slit2* promoter is epigenetically inactivated in human colorectal cancer [5] and glioma [4].

In this study, we cloned canine slit genes and analyzed the expression patterns of the *slit* subfamily mRNA in canine mammary tumors.

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MATERIALS AND METHODS

cDNA cloning of canine slits: Total RNA was extracted from the brain of a 3-year-old male beagle with Sepasol I super (Nacalai tesque, Kyoto, Japan). The total RNA (5 μg) was denatured at 70°C for 10 min, cooled immediately, and reverse transcribed using 100 units of ReverTra Ace (Toyobo Bio, Osaka , Japan), 5 pmol of oligo-dT primer, and 1 mM dNTPs in a total volume of 20 μl at 42°C for 60 min. After heating at 95°C for 5 min, polymerase chain reaction (PCR) amplification was performed with 2.5 units of Blend *Taq*™ DNA polymerase (Toyobo Bio), 20 nmol of dNTPs, and 20 pmol of primers. PCR was conducted for 25 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. The primers were chosen with the assistance of Primer 3.0 (Web software provided by Steve Rozen and the Whitehead Institute/MIT Center for Genome Research) based on the sequence similarity among human, mouse, and rat slits. We performed BLAST searches against dbEST, htgs, and nr (the nonredundant set of the GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequence chosen as primers and to confirm the absence of DNA polymorphisms. The primer sets used

Table 1. The histopathological diagnoses of canine mammary tumors

Case No.	Type of tumor
1	Complex adenoma
2	Complex adenoma
3	Complex carcinoma
4	Complex carcinoma
5	Complex carcinoma
6	Complex carcinoma
7	Complex carcinoma
8	Complex carcinoma
9	Simple carcinoma (Anaplastic)
10	Simple carcinoma (Tubulopapillary)
11	Simple carcinoma (Solid)
12	Simple carcinoma (Solid)
13	Simple carcinoma (Solid)

are listed in Table 2. They were tested by PCR to ensure that they yielded a single band on agarose gel, and the PCR products were purified and sequenced to confirm primer specificity.

Canine mammary tissues: Sixteen samples of mammary tissue, including three normal, 2 benign, and 11 malignant tumor tissues, were used for this study. The diagnosis of each tumor was based on the World Health Organization (WHO) classification (11). The diagnoses are summarized in Table 1. All procedures were conducted in accordance with Osaka Prefecture University's Policy on the Use of Animals.

Real-time PCR: Total RNA was extracted from surgically harvested tissues and reverse-transcribed as described above. The primers used for real-time PCR were chosen from the cloned canine *slit* sequences with the assistance of Primer 3.0. All PCR reactions were performed by using an iCycler iQ Detection System (Bio-Rad, CA) and SYBR® Green Real-Time PCR Master Mix (Toyobo Bio). The thermal cycling conditions were comprised of an initial denaturation step at 95°C for 10 min and 90 cycles at 95°C for 30 sec and 62 to 68°C (optimized for each primer set) for 1 min. Quantitative values were obtained from the threshold cycle (Ct) number at which the increase in signal associated with exponential growth of the PCR products starts to be detected using iCycler iQ Optical System Software Ver.3.0A, Bio-Rad. We also quantified β-actin transcripts as an endogenous mRNA controls. The final results were expressed as N-fold differences in target gene expression relative to the β-actin gene (10).

RESULTS

Characterization of canine slit cDNA (slit1, slit2, and slit3): Partial cDNA sequences were obtained from the canine brain tissue (Fig. 1). The amplified sizes were approximately 650 bp, 820 bp, and 600 bp for *slit1, slit2*, and *slit3*, respectively, which was as expected from the sequence data for other species. Each band was excised from agarose gel, and its nucleotide sequences were determined as shown in Fig. 2 and submitted to the DDBJ nucleotide database under accession No. AB194047, AB194048, and AB194049. The nucleotide and deduced amino acid sequences had homology to those of other species, including the human, mouse, and rat (Table 3).

Expression of slit genes in normal mammary tissue and tumors: Expression of the three *slit* mRNAs in normal canine mammary glands and tumor samples was examined by RT-PCR with the primer sets used for cloning. The types of tumor samples are listed in Table 1. It was difficult to detect an obvious band for *slit1*, although both *slit2* and *slit3* expression was detectable on agarose gel (data not shown). Since more apparent bands for *slit2* and *slit3* were observed, we evaluated the expression of the genes by real-time PCR. The experiments revealed that the *slit2* and *slit3* mRNAs were strongly expressed in some malignant tumors (Fig. 3). The expression of *slit1* mRNA was too low to evaluate, both

Table 2. Primer sets in cloning and real-time PCR for the canine slit subfamily and β-actin

Gene	Sequence	Length
Cloning		
slit1	5'-TGGACCGGGACACTTACC-3'	18 mer
	5'-TCGAGGGGCACGCATTTC-3'	18 mer
slit2	5'-GACTGTGAAAATAACTCTACGTGTGTC-3'	27 mer
	5'-CATAGAGTGGAGAGTCAAAATTCAGAG-3'	27 mer
slit3	5'GAAGCTCATCACTGTGAATTTCGT-3'	24 mer
	5'-ACTCACACACTACGCTGTCCTTCT-3'	24 mer
Real-time PCR		
slit1	5'-TGCTATCTACAGTGCTGAAACAATCA-3'	26 mer
	5'-AGTGTGTAGTGCTTTCCAAAGTTGTC-3'	26 mer
slit2	5'-ACTGCCAGGATCACAAGTGTAAAA-3'	24 mer
	5'-GTTCTGACAGTCGAAGTTGTCACAG-3'	25 mer
slit3	5'-GGCATCCTACTCTACAAAGGAGACAA-3'	26 mer
	5'-GTTTAGCATCACCAGCTCCACACTAT-3'	26 mer
β-actin	5'-GCCATCTCTTGCTCGAAGTC-3'	20 mer
	5'-TACAGCTTCACCACCACAGC-3'	20 mer

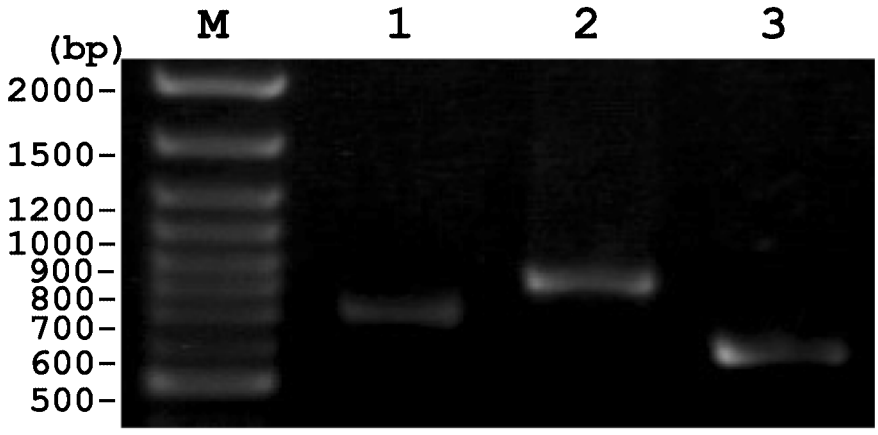

Fig. 1. RT-PCR of the slit subfamily from the canine brain. The RT-PCR products were examined using 1% agarose gel electrophoresis. Each part of the slit cDNAs was amplified with the primer sets in Materials and Methods. Lane 1 is for *slit1*, lane 2 is for *slit2*, and lane 3 is for *slit3*.

slit1 **(673 bp)**

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TGGACCGGGA CACTTACCTG CAGTTCACTG ACCTGCAGAA CTGGCCTCGG GCCAGCATCA
CTCTTCAGGT CTCCACAGCA GAGGACAATG GGATCCTCCT CTACAATGGG GATAATGACC
ACATTGCAGT TGAGCTGTAC CAGGGCCATG TCCGTGTTAG CTACGACCCA GGCAGCTACC
CCAGCTCTGC TATCTACAGT GCTGAAACAA TCAACGATGG GCAGTTCCAC ACAGTTGAGC
TGGTGGCCTT TGACCAGATG GTGAACCTCT CCATTGATGG TGGCAGCCCC ATGACCATGG
ACAACTTTGG AAAGCACTAC ACACTCAACA GTGAGGCCCC CCTCTATGTG GGAGGGATGC
CCGTGGATGT GAACTCAGCT GCCTTCCGCC TGTGGCAGAT CCTCAATGGC ACCAGCTTCC
ACGGTTGCAT CCGGAATCTA TACATCAACA ACGAACTGCA GGACTTCACC AAGACACAGA
TGAAGCCGGG CGTGGTGCCC GGCTGCGAGC CCTGCCGAAA ACTCTACTGT CTACATGGCA
TTTGCCAGCC CAACGCCACC CCAGGGCCCG TGTGCCACTG CGAGGCTGGC TGGGGGGGCC
TGCACTGTGA CCAGCCAGTG GACGGCCCCT GCCATGGCCA CAAGTGTGTC CATGGGAAAT
GCGTGCCCCT CGA
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slit2 **(826 bp)**

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GACTGTGAAA ATAACTCTAC GTGTGTCGAT GGAATTAATA ACTACACATG CCTTTGTCCG
CCTGAGTACA CAGGCGAGTT GTGTGAGGAG AAGCTGGACT TCTGCGCTCA GGACCTGAAC
CCCTGCCAGC ACGACTCCAA GTGCATCCTG ATGCCCAAAG GATTCAAATG CGACTGCACG
CCGGGGTACG TGGGCGAGCA CTGCGACATC GACTTCGACG ACTGCCAGGA TCACAAGTGT
AAAAACGGAG CGCACTGCAC GGACGCGGTG AACGGCTACA CGTGCACCTG CCCCGAAGGC
TACAGCGGCT TGTTCTGTGA ATTCTCCCCG CCCATGGTCC TCCCACGCAC CAGCCCCTGT
GACAACTTCG ACTGTCAGAA CGGGGCGCAG TGCATCGTCA GGGCGGGCGA GCCAATCTGC
CAGTGTCTGC CCGGCTACCA GGGGGACAAG TGTGAGAAGT TGGTCAGCGT GAACTTCGTG
AACAAAGAGT CGTATCTTCA AATTCCTTCA GCCAAGGTCC GGCCCCAGAC GAACATCACC
CTGCAGATTG CCACCGACGA AGACAGCGGG ATCCTCCTGT ACAAGGGCGA CAAGGACCAC
ATTGCCGTGG AGCTGTATCG GGGACGGGTG CGCGCCAGCT ACGACACCGG CTCGCACCCC
GCTTCTGCCA TTTACAGCGT GGAGACGATC AATGATGGAA ACTTTCACAT TGTGGAACTA
CTTGCCCTGG ATCAGAGCCT GTCCCTCTCC ATGGATGGAG GGAGCCCCAA AATCATCACC
AACTTGTCAA AGCAGTCCAC TCTGAATTTT GACTCTCCAC TCTATG
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slit3 **(599 bp)**

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GAAGCTCATC ACCGTCAACT TCGTGGGCAA AGACTCCTAC GTGGAACTAG CCTCCGCCAA
GGTCCGGCCC CAGGCCAACA TCTCCCTGCA GGTGGCCACT GACAAGGACA ACGGCATCCT
ACTCTACAAA GGAGACAATG ACCCCTTGGC ACTGGAGCTG TACCAGGGCC ACGTGAGGCT
CATCTATGAC AGTCTGAGCT CCCCACCAAC CACGGTGTAC AGTGTGGAGA CGGTGAATGA
TGGGCAGTTT CATAGTGTGG AGCTGGTGAT GCTAAACCAG ACCCTGAACC TGGTAGTGGA
TAAAGGAGCC CCCAAGAGCC TGGGAAAGCT CCAGAAGCAG CCAGCAGTAG GCATCAACAG
CCCCCTATAC CTCGGAGGCA TCCCCACCTC TACCGGCCTC TCGGCCCTGC GCCAGGGCAC
AGACCGGCCA CTGGGGGGCT TCCACGGCTG CATCCACGAA GTGCGCATCA ACAACGAGCT
GCAAGACTTC AAGGCCCTCC CACCACAGTC CCTGGGAGTT TCACCAGGCT GCAAGTCCTG
TACGGTGTGC AAGCATGGCC TGTGCCGCTC TGTGGAGAAG GACAGCGTGG TGTGTGAGT
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Fig. 2. Nucleotide sequences of the canine *slits*. These nucleotide sequences were determined in part for *slit1*, *slit2*, and *slit3* cDNA obtained by RT-PCR from the canine brain.

Table 3. Comparison of RT-PCR products of the canine *slit* family to other species

Species	Homology (%)		
	slit1 (673 bp/223 aa)*	*slit2* (826 bp/275 aa)*	*slit3* (599 bp/199 aa)*
Human	91.9/97.3	86.4/96.7	93.0/98.5
Mouse	94.7/99.1	84.1/95.2	90.5/98.0
Rat	99.6/99.1	84.5/95.6	89.5/98.5

* Each cDNA length and number of putative amino acids was obtained from nucleotide sequence of each RT-PCR product amplified using the primer set in Table 2.

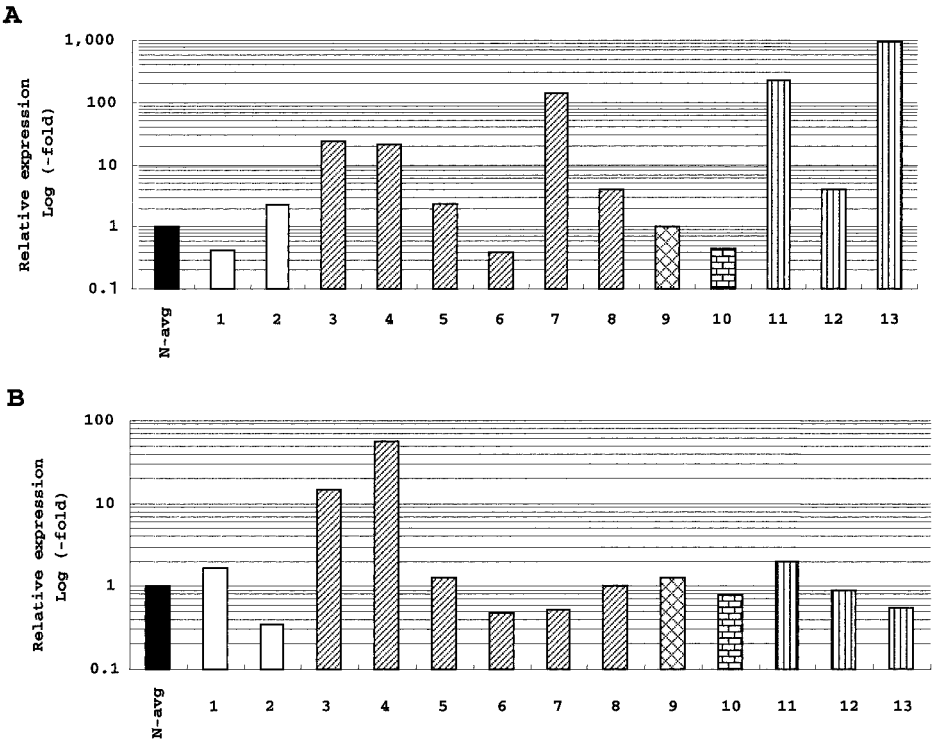

Fig. 3. Expression of *slits* in mammary tumors. Relative expression levels of slit mRNAs in 3 normal canine mammary tissue samples and 14 mammary tumor samples by real-time PCR method (Slit2-A, Slit3-B). The expression levels are shown as the ratio between the target gene and reference gene (*β-actin*) in order to correct for variations in starting amount of RNA. The ratios for each type of analysis were normalized such that the mean ratio in the normal mammary tissue samples is 1. The sample numbers correspond to those in Table 1.

in the normal and tumor tissues, using the real-time PCR system (data not shown). In the *slit2* analyses, 5 of 6 cases of complex carcinoma showed increased expression (Fig. 3A). In regard to *slit3*, only two cases of complex carcinoma showed more than a 10-fold increase in expression (Fig. 3B), and the expression levels of the rest were not significantly different from those of the normal tissues.

DISCUSSION

Slit is known as a chemorepellent guidance cue in the development of Drosophila [12]. Many investigations in neurobiology and immunology have established the primary function of slit signaling as guidance of neuronal migration and leukocyte trafficking by acting as a chemorepellent cue [19]. Slit may act as a signal molecule in the movement of epithelial sheaths [15] and for guidance of muscle precursor cell migration in Drosophila [8]. Recently, it has been reported that *slit2* acts in tumor angiogenesis as an attractant molecule for endothelial cells in migration toward tumor masses and formation of new blood vessels [20]. Therefore, it has been thought that the expression of *slit2* can be a useful marker for evaluation of tumor malignancy in humans. On the other hand, it has also been reported that *slit2* expres-

sion is suppressed by the epigenic system in glioma, colorectal, lung, and breast cancers (3, 4, 5). In this study, our aim was to estimate the expression of *slit* mRNAs in canine mammary gland tumors.

We cloned and determined partial sequences of canine *slit1*, *slit2*, and *slit3* (Fig. 2). The mRNA and deduced amino acid sequences of the canine *slits* were highly conserved among humans, mice, and rats (Table 3).

Analyses of the expression pattern of the canine *slit* genes revealed that most of the malignant tumors showed higher *slit2* expression, while most of the malignant tumor tissues showed a similar level of expression for *slit1* (data not shown) and *slit3* compared to the normal and benign tissues (Fig. 3). The microenvironment in tumors is known to induce vascular endothelial cells to form new blood vessels to support expansion of the tumor mass [6]. New blood vessels arise from preexisting capillaries or postcapillary venules in tumors, and/or by recruiting endothelial precursor cells from the bone-marrow stem cell pool [13]. In spite of the extensive study of angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and cell adhesion molecules such as integrins and cadherins [7], the tumor-endothelial cell communication signals for directing movement are still not well-defined. A relative high incidence of the expression of *slit2* gene indicates that the gene may not be influenced by epigenic inactivation and may play an important contribution in canine mammary tumors. It is possible that *slit2* in malignant mammary tumors may act to attract vascular endothelial cells to form new blood vessels to the tumor mass. Further study of anginogenic signaling in canine mammary gland tumors is needed to understand the significance of the expression of tumor-related genes including slits.

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