

## Matrix Metalloproteinase Inhibitor RECK Expression in Canine Tumors

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**ABSTRACT.** Matrix metalloproteinases (MMPs) selectively degrade the extracellular matrix, and they have been reported to play an important role in tumor invasion, metastasis and angiogenesis. These enzymes are closely related to tumor malignancy and patient survival time. Recently, reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene was identified as an endogenous membrane-anchored MMP inhibitor. The down-regulation of RECK has been implicated in tumor progression. In this study, the expression levels of the *RECK* messenger ribonucleic acid (mRNA) in various spontaneously developed canine tumors were investigated by using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), and the correlation between *RECK* and clinicopathological factors, as well as *MMP-9* expression were analyzed. The median age of 36 dogs investigated in this study was 9 years old (range, 1–15 years old). Quantitative RT-PCR could detect low levels of expression of *RECK* mRNA in the tumor samples. The expression levels of *RECK* mRNA in some tumor tissue samples were significantly lower than those in normal tissue samples. No significant associations of *RECK* with clinicopathological factors were observed. Using the Mann-Whitney *U* test, the expression level of the *MMP-9* mRNA was observed to be significantly correlated to *RECK* expression ( $p < 0.05$ ).

**KEY WORDS:** canine, matrix metalloproteinase, proteinase inhibitor, RECK, tumor malignancy.

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Metastasis and invasion of tumor cells are correlated with tumor malignancy. Matrix metalloproteinase (MMP)-2 (72 kilodalton, Gelatinase A) and MMP-9 (92 kilodalton, Gelatinase B) have been reported to play an important role in the pathology of cancer, particularly in invasion, metastasis and angiogenesis. Increased concentrations of gelatinases are associated with poor prognosis of a number of human malignant tumors [5, 15, 16].

Matrix metalloproteinases are a family of more than 20 zinc-dependent neutral endopeptidases [3]. All MMPs are expressed as proenzymes and are subsequently activated to become proteolytic enzymes [28]. MMPs selectively degrade the extracellular matrix, and gelatinases degrade the type-IV collagen, which is the main component of the basement membrane. The activity of MMPs is closely regulated by endogenous nonspecific (2-macroglobulin) and specific inhibitors (tissue inhibitors of matrix metalloproteinases [TIMP]-1 to -4) [9, 13, 31].

A new endogenous specific MMP inhibitor gene, termed as the reversion-inducing cysteine-rich-protein with Kazal motifs (*RECK*) gene, was recently isolated by cDNA expression cloning. This gene induces flat morphology when expressed in the v-Ki-ras-transformed NIH/3T3 cell line [32]. *RECK* negatively regulates MMP-2, MMP-9, and membrane-type matrix metalloproteinase (MT1-MMP [MMP-14]) [25, 32]. *RECK* has been reported to be an endogenous MMP inhibitor and a membrane-anchored glycoprotein (approximately 110 kDa) with multiple epidermal growth factor-like repeats and serine protease inhibitor-like domains; however, it has no structural homology with TIMPs. The *RECK* gene was detected in various human tis-

sues, but it could not be detected in tumor derived cell lines and oncogene-transformed cells [32]. The down-regulation of *RECK* has been experimentally implicated in tumor progression and angiogenesis. In addition, patients with hepatocellular carcinoma, breast carcinoma and pancreatic carcinoma showed that high *RECK* expression was significantly correlated with long survival time [10, 21, 31].

Spontaneously developing canine tumors are considered to be useful models of human cancer because the survival time in dogs is shorter than that in humans, and the biological behavior of these tumors is very similar to that in humans [6, 18, 23]. In addition, it was reported that in spontaneously developed canine tumors, MMP-2 and MMP-9 expressions might be involved in tumor malignancy, similar to human patients [17, 18]. In our previous study, we confirmed that the cDNA sequence of canine *RECK* was considerably similar to that of mice and humans [33]. Thus, it will be expected that *RECK* may play an important role in canine tumor malignancy, as it does in humans.

The objective of this study is to investigate the expression of *RECK* in various spontaneously developing canine tumors. For this reason, *RECK* mRNA expression level in different canine tumor specimens was quantified by real-time fluorescence RT-PCR and correlated with clinicopathological factors and *MMP-9* mRNA expression.

### MATERIALS AND METHODS

*Animals and tissue samples:* Tissue specimens were obtained from 36 dogs with spontaneously developed canine tumors. The samples were obtained after the dogs

underwent biopsy or resection of their primary tumors. Normal tissue sections that were apparently distant from primary tumor or tissues obtained from clinically normal dogs were also analyzed. The canine tumors were classified in accordance with the World Health Organization's (WHO) classification system [26]. Immediately after resection, the tissue samples were frozen in liquid nitrogen or stored overnight at 4°C in RNA preservation solution (RNAlater; Ambion, Austin, TX, U.S.A.), followed by storage at -80°C until RNA extraction.

**RNA extraction and reverse transcription:** Total RNA was extracted from the tissue samples by the guanidine isothiocyanate method [4]. The total RNA (2 µg) was then denatured at 70°C for 10 min, cooled immediately, and added to a solution containing 200 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.), 50 nmol dithiothreitol, 10 pmol poly (dT) primer and 20 nmol dNTPs in a total volume of 20 µl. After the poly (dT) primer was annealed at 20°C for 10 min, cDNA synthesis was performed at 37°C for 1 hr.

**Quantitative RT-PCR analysis of canine RECK gene:** All the PCR reactions were performed using a detection kit (LightCycler-FirstStart DNA Master SYBR Green kit, Roche Molecular Biochemicals, Mannheim, Germany) [37]. The primers were synthesized to amplify a specific segment of the cDNA sequence of canine *RECK* and *MMP-9*. In order to ensure the fidelity of mRNA extraction and reverse transcription, and to normalize the samples, all the samples were subjected to PCR amplification with oligonucleotide primers that were specific to the constitutively expressed gene *Ribosomal protein S26 (RPS26)*. The primer sequences for the each gene were as follows: *RECK* forward primer, 5'-TCC CTG AAG ACC ACA CAG CT-3'; reverse primer, 5'-CAA CCA ACT TCC CCT GCA GA-3'; *MMP-9* forward primer, 5'-CGC TTC TGG AGT TTC GAC GT-3'; reverse primer, 5'-GTC CTG GCA GAA GTA GGC TT-3' and *RPS26* forward primer, 5'-GCC ATT AAG AAA TTC GTC ATT CG-3'; reverse primer, 5'-CGA GAT CGA TTC CTG ACT AC-3'.

The PCR reaction mixture containing the following reaction components was prepared to the indicated end-concentration: 13.4 µl water, 2.4 µl MgCl<sub>2</sub> (4 mM), 0.1 µl forward primer (100 µM), 0.1 µl reverse primer (100 µM) and 2.0 µl LightCycler Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals). As a PCR template, 18 µl of master-mix and 2 µl of cDNA as a PCR template were added to glass capillaries.

The cycling conditions were as follows; initial denaturation at 95°C for 30 sec, followed by 35 cycles (*RECK*, *MMP-9*) or 30 cycles (*RPS26*) at 95°C for 0 sec, 60°C for 5 sec, and 72°C for 10 sec. In order to improve the SYBR Green I quantification, the temperature of the fluorescence measurement point was set at 84°C (*RECK*), 85°C (*MMP-9*), and 83°C (*RPS26*), respectively. The expression level of each mRNA in the tissue samples was determined relative to the standard curve by using the LightCycler computer software (Roche Molecular Biochemicals). The identity of each

PCR product was confirmed by electrophoresis.

The expression of the *RECK* mRNA in five canine tumor cell lines (mast cell tumor [12], mammary gland tumor, transitional cell carcinoma, osteosarcoma [14], and high metastatic osteosarcoma [1]) was also analyzed by quantitative RT-PCR method.

**Gelatin zymography:** Using a previously reported method, gelatin zymography was performed to measure the production and activity of gelatinases obtained from tumor cell lines in the conditioned media [2, 36]. Briefly, 20 µl of supernatant of each conditioned medium was mixed with an equal volume of 2x SDS-PAGE sample buffer without boiling. The samples were fractionated using electrophoresis in a 10% polyacrylamide gel containing gelatin (0.5 mg/ml). In order to remove the SDS, the gels were soaked in 2.5% Triton X-100 containing 10 mM Tris (pH8.0) for 30 min at room temperature, and incubated in a digestion buffer (50 mM Tris [pH 8.0], 0.5 mM CaCl<sub>2</sub> and 1 µM ZnCl<sub>2</sub>) at 37°C for 30 hr, to enable the proteinase digestion of the substrate. The gels were stained with 0.25% Coomassie brilliant blue R-250 and destained with 10% isopropanol and 5% acetic acid. The gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

**Statistical analysis:** The differences between the *RECK* mRNA expression level in normal and concurrent tumor tissue specimens were assessed by the nonparametric Mann-Whitney *U* test. For statistical purposes, the undetectable *RECK* mRNA expression level was set at 0. The correlation between the *RECK* mRNA expression and clinicopathological parameters were tested with the nonparametric Mann-Whitney *U* test or Kruskal-Wallis test. The *RECK* mRNA expression level in different tumors was tested with the nonparametric Kruskal-Wallis test. The Pearson's correlation coefficient test was used to evaluate the association between the *RECK* mRNA expression and the *MMP-9* mRNA expression. The statistical analyses were performed using the Stat View 5.0 software (SAS Institute, Cary, NC, U.S.A.).

## RESULTS

**Animals:** Tissue samples were obtained from 36 dogs, including 20 uncastrated males, 4 castrated males, 11 unsplayed females, and 1 splayed female. The median age of the dogs was 9 years (range, 1–15 years) (Table 1). The dogs were of the following breeds: mixed breed dogs (n=12), Golden Retriever (n=6), Siberian Husky (n=3), Beagle (n=2), Pomeranian (n=2), Shetland Sheep Dog (n=2), and one each of the Bernese Mountain Dog, Cavalier King Charles Spaniel, Labrador Retriever, Maltase, Miniature Dachshund, Miniature Schnauzer, Samoyed, Shih Tzu, and Welsh Corgi.

**RECK mRNA expression in tumor and normal tissue samples:** The expression levels of the *RECK* mRNA in tumor and normal tissues were compared (Fig. 1). In osteosarcoma and hemangiosarcoma tissues, there was no signifi-

Table 1. Clinical data of tumor bearing dogs and *RECK/RPS26* value in various canine tumor tissues

Case	Tumor Type	Age	Sex	Metastasis		Tumor Location	Tumor Stage	<i>RECK/RPS26</i>
				Lymph Node	Distant			
1	HPC	9	f	-	-	skin	T2N0M0	5.6234
2	HPC	8	f	-	-	skin	T2N0M0	2.3143
3	HPC	11	m	-	-	skin	T2N0M0	0.5785
4	HPC	10	f	-	-	skin	T3N0M0	3.9679
5	HPC	15	m	-	-	skin	T3N0M0	0.2143
6	HPC	8	f	-	-	skin	T3N0M0	0.4413
7	HPC	8	m	-	-	skin	T3N0M0	4.0142
8	HPC	13	f	-	-	skin	T3N2aM0	12.1291
9	HPC	9	f	-	-	skin	T4N0M0	0.1296
10	HPC	7	c	-	-	skin	T4N1aM0	0.4298
11	HS	5	m	+	-	skin	T3N1bM0	2.7561
12	HS	6	c	-	-	spleen	-	0.6765
13	HS	4	m	-	+	spleen	-	1.4248
14	HS	9	m	+	-	spleen	-	1.8044
15	HS	10	s	-	+	spleen	-	11.3482
16	OS	1	m	+	-	bone	T1M0	0.5127
17	OS	9	m	-	-	bone	T2M0	0.3306
18	OS	3	m	-	-	bone	T2M0	0.0194
19	OS	8	f	-	+	bone	T2M1	6.2455
20	OS	9	m	-	+	bone	T2M1	26.3755
21	SCC	2	f	+	-	oral	StageIII	3.3494
22	SCC	9	m	-	-	oral	StageIII	2.7255
23	SCC	8	f	-	-	skin	T2N1bM0	9.7039
24	SCC	8	m	+	-	nasal	T2N1bM0	0.0903
25	MCT	5	m	-	-	skin	StageIb	0.0000
26	MCT	12	m	-	-	skin	StageIIa	0.0000
27	MCT	13	f	-	-	skin	StageIIIb	0.0000
28	MCT	13	c	-	+	skin	StageIV	0.0000
29	MM	13	m	+	-	oral	StageIII	15.8476
30	MM	12	f	+	+	oral	StageIV	0.0519
31	PAGA	13	m	-	-	skin	T2N0M0	0.0695
32	PAGC	10	m	-	-	skin	T3N0M0	0.0584
33	AGC	9	m	+	-	skin	T4N2aM0	0.6446
34	FS	9	m	+	-	oral	StageIII	0.4569
35	HCC	15	m	-	-	liver	T3N0M0	0.9676
36	TCC	10	c	+	-	bladder	T2N1M0	0.0081

HPC; Hemangiopericytoma, HS; Hemangiosarcoma, OS; Osteosarcoma, SCC; Squamous cell carcinoma, MCT; Mast Cell Tumor, MM; Malignant Melanoma, PAGA; Perianal Gland Adenoma, PAGC; Perianal Gland Adenocarcinoma, AGC; Apocrine Gland Carcinoma, FS; Fibrosarcoma, HCC; Hepatocellular Carcinoma, TCC; Transitional Cell Carcinoma. m; intact male, c; castrated male, f; intact female, s; spayed female.

cant correlation between normal and tumor tissues. In hemangiopericytoma and skin tumor, the *RECK* mRNA expression in the tumor tissues was significantly lower than that in normal tissues ( $p < 0.05$ ). In hepatocellular carcinoma, the *RECK* mRNA expression was low in the tumor tissues when compared with that in normal liver tissues.

*RECK* mRNA expression in different tumor types: The results of histopathological examination of the 36 tumor tissues and *RECK/RPS26* are listed in Table 2. The nonparametric Kruskal-Wallis test revealed no statistical difference among the various tumors ( $p = 0.084$ ). Among these tumors, *RECK* mRNA expression could not be detected only in all of the mast cell tumors (MCT,  $n = 4$ ).

The *RECK* mRNA expression in tumor cell lines was also examined. Among all the tumor cell lines, the cell line of osteosarcoma showed significantly high *RECK* expression ( $p < 0.05$ ).

*Correlation of RECK mRNA expression with clinicopathological features and MMP-9 mRNA expression in the dogs:* The Mann-Whitney *U* test and the Kruskal-Wallis test revealed that there was no significant difference in the clinicopathological variables, including age, sex, existence of lymph node metastasis, and distant metastasis (Table 3). Distant metastasis was confirmed in the lung (osteosarcoma, melanoma), heart, and liver (hemangiosarcoma). *MMP-9* mRNA expression level was significantly correlated to the *RECK* mRNA expression ( $p = 0.022$ ) (Table 3). However, on analysis by the Pearson's correlation coefficient test, no correlation between them was observed, and the value of correlation coefficient was  $-0.037$  ( $p = 0.842$ ) (Fig. 2).

*Gelatin zymography:* Variable levels of pro-MMP-2 and pro-MMP-9 were detected in the conditioned medium. MMP-9 was significantly activated in the transitional cell carcinoma cell line. The secretion of gelatinase in osteosar-

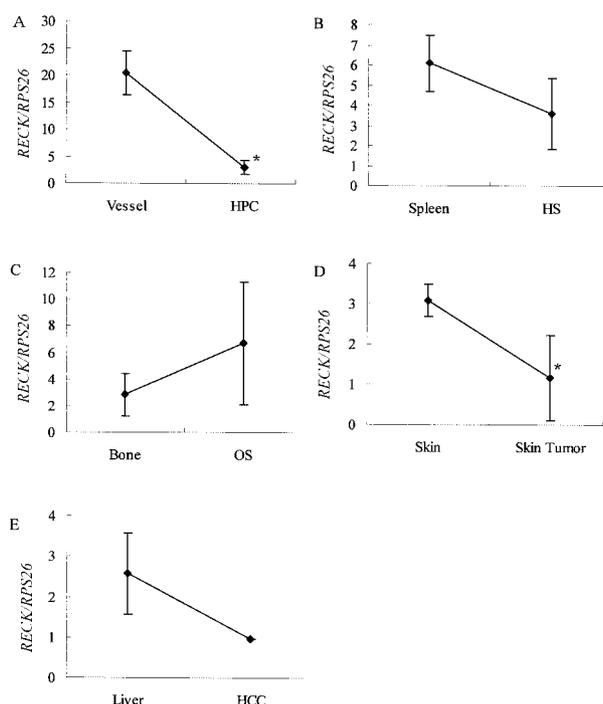


Fig. 1. Levels of the *RECK/RPS26* expression in canine tumor tissues and original normal tissues. A: Hemangiopericytoma (HPC), B: Hemangiosarcoma (HS), C: Osteosarcoma (OS), D: Skin tumor, E: Hepatocellular Carcinoma (HCC). In hemangiopericytoma and skin tumor, the *RECK* expression in the tumor tissues were significantly lower than that in normal tissues by Mann-Whitney *U* test (\* $p < 0.05$ ). (*RECK*: reversion-inducing-cysteine-rich protein with Kazal motifs, *RPS26*: ribosomal protein S26) Bar: mean value  $\pm$  standard error.

Table 2. *RECK/RPS26* value in various canine tumors and tumor cell lines

Tumor Type	Number of cases	<i>RECK/RPS26</i> Mean $\pm$ S.E.
Clinical Case		
Hemangiopericytoma	10	2.9800 $\pm$ 1.1910
Hemangiosarcoma	5	3.6020 $\pm$ 1.9653
Osteosarcoma	5	6.6967 $\pm$ 5.0538
Mast Cell Tumor	4	Undetectable
Squamous cell carcinoma	4	3.9673 $\pm$ 2.0385
Malignant Melanoma	2	7.9498
Apocrine Gland Adenocarcinoma	1	0.0695
Fibrosarcoma	1	0.4569
Hepatocellular Carcinoma	1	0.9676
Perianal Gland Adenocarcinoma	1	0.0584
Transitional Cell Carcinoma	1	0.0081
Perianal Gland Adenoma	1	0.0695
Tumor Cell Lines		
Mast Cell Tumor	3	0.0603 $\pm$ 0.0247
Mammary Gland Tumor	3	0.2761 $\pm$ 0.1568
Transitional Cell Carcinoma	3	0.1115 $\pm$ 0.0383
Osteosarcoma	3	1.1762 $\pm$ 0.0820
High Metastatic Osteosarcoma	3	0.4297 $\pm$ 0.1119

S.E.: Standard Error

coma was low as compared to that in other tumor cell lines (Fig. 3).

## DISCUSSION

In the present study, expression of endogenous MMP inhibitor RECK was investigated in various spontaneously developing canine tumors. The quantification of *RECK* mRNA is reported to be an effective prognostic marker in human hepatocyte carcinoma [10], breast carcinoma [30], and pancreatic carcinoma [21]. In most of the previous studies, the expression level of the *RECK* mRNA was quantified by Northern blotting [10, 21, 32]. In these studies, the expression level was observed to be considerably low in normal human tissues and tumor cell lines. Therefore, the purification of poly A (+) mRNA from the total RNA was necessary in order to detect the low expression of the *RECK* mRNA by Northern blotting, and a large amount of samples was required for purification. The quantitative RT-PCR used in this study is an easy and useful method to measure the mRNA of specific genes, and it is suitable for treating a large number of samples. Moreover, it can be performed with a small amount of tissue sample.

A majority of tumor tissues showed low levels of *RECK* mRNA expression as compared to normal tissues. However, the expression of *RECK* mRNA in some tumor tissues was detected to be higher than that in normal tissues. The higher expression in the tumor tissues is inconsistent with the fact that the expression of the *RECK* gene is suppressed in rodent and human tumor-derived cell lines [32]. In this study, *RECK* mRNA expression was also suppressed in a majority of the canine tumor cell lines. One explanation for this suppression is that the selection of RECK-negative populations occurs due to the culture passaging, which may be result in suppression of *RECK* mRNA. The other explanation is that specific stimulus may affect the *RECK* expression *in vivo* or *in vitro*. For example, stromal cells affect *RECK* expression in tumor cells either directly or by secreting growth factors.

Among all the analyzed sample, those obtained from a normal vessel showed the highest *RECK* mRNA expression. This may be due to the fact that RECK was proven to show high expression in perivascular smooth muscle cells [25]. The tumor vessels have no perivascular smooth muscle cells [7]; therefore, *RECK* mRNA expression in tumor tissues was generally regarded to be low. Considering this fact, there was a possibility that preeminent *RECK* mRNA expression in tumor tissues were caused by the contamination of perivascular smooth muscle cells.

A slight significant difference was observed in the *RECK* mRNA expression in various tumor types. However, *RECK* expression in osteosarcoma was considered to be higher than that in other tumors. The *RECK* mRNA expression in the osteosarcoma cell line was significantly higher than that in other tumor cell lines, and it inclined to be higher in the spontaneously developed osteosarcoma. The *RECK* expression levels could differ depending on the type of tumor. A

Table 3. Comparison between clinicopathological features and *RECK/RPS26* value

Factor	Category	Cases	(%)	<i>RECK/RPS26</i> Median	<i>p</i>
Age <sup>a)</sup>	<5 years old	6	(16.7)	0.97	N.S. (0.656)
	≥ 5 years old	30	(83.3)	0.61	
Sex <sup>b)</sup>	intact male	20	(55.6)	0.55	N.S. (0.130)
	castrated male	4	(11.1)	0.22	
	intact female	11	(30.6)	3.35	
	spayed female	1	(2.8)	11.35	
Regional lymph node <sup>a)</sup>	metastasis	10	(27.8)	0.64	N.S. (0.784)
	normal	26	(72.2)	0.58	
Distant metastasis <sup>a)</sup>	metastasis	6	(16.7)	8.80	N.S. (0.384)
	normal	30	(83.3)	0.48	
<i>MMP-9/RPS26</i> <sup>a)</sup>	< 20	19	(52.8)	0.13	0.022
	≥ 20	17	(47.2)	1.80	

*MMP-9*: Matrix metalloproteinase-9, N.S.: Not significant.  
a) *p* for Mann-Whitney *U* test, b) *p* for Kruskal-Wallis test.

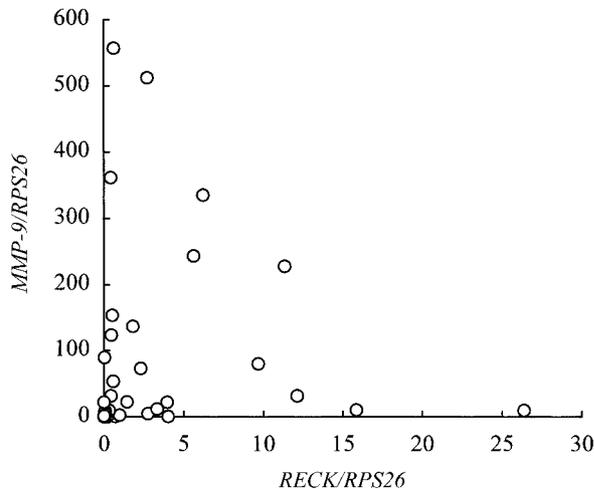


Fig. 2. The correlation between *RECK* and *MMP-9* expression in messenger ribonucleic acid level. The *RECK* and the *MMP-9* expression levels were evaluated by Pearson's correlation coefficient test. No significant correlation was observed ( $p=0.842$ ).

large number of tumor tissue samples needs to be analyzed in order to evaluate the possible differences of *RECK* mRNA expression in various tumors.

The *RECK* mRNA could not be detected in all the MCT samples using the quantitative RT-PCR analysis. Canine MCT is known to be a highly infiltrative tumor, and very wide margin is needed for complete resection [34]. Highly invasive tumors were expected to show low *RECK* mRNA expression and high *MMP-9* mRNA expression. Thus, MCT may have a MMPs regulation mechanism that is different from the *RECK* pathway. This result indicates that the *RECK* mRNA expression level is not effective as a prognostic marker in canine MCT.

In the present study, no MCTs showed a high *MMP-9* mRNA expression. On the other hand, gelatin zymography

showed the secretion of pro-*MMP-9* from MCT cells. This result may be related to the fact that *RECK* has been shown to inhibit pro-*MMP-9* secretion and activation but not the production [32].

The Pearson's correlation coefficient test revealed that the *RECK* mRNA expression in hepatocellular carcinoma was significantly and positively correlated with *MMP-9* expression. Moreover, the expression levels of *RECK* and *MMP-9* were similar at mRNA level and at protein level [21]. The correlation between these two factors was also investigated in canine tumors. The Mann-Whitney *U* test revealed that the levels of *MMP-9* and *RECK* mRNA expression correlated significantly and positively; however, no correlation was observed by the Pearson's correlation coefficient test. The *RECK* transfection in cultured cell had not been proven to affect *MMP-9* mRNA expression, and was reported to suppress pro-*MMP-9* secretion and activation [32]. In the present study, a positive correlation of *MMP-9* and *RECK* expression may be determined based on the *MMP-9* expression in the inflammatory cell. Another possibility is that these two genes are regulated by a common mechanism. The expression of the *RECK* gene was observed to be regulated by Sp1 transcriptional factors, in a manner same as that for *MMP-9* [8, 27, 29].

TIMPs are also endogenous MMP inhibitors and their higher expression results in the poor prognosis of human breast carcinoma, colon carcinoma, and stomach carcinoma [19, 22]. The difference between *RECK* and TIMPs is in their localization; *RECK* is membrane anchored, whereas TIMPs are secreted. Thus far, the tumor promoting mechanism of TIMPs is relatively unclear. The function of TIMPs is to inhibit MMPs [20]; however, they also act as growth factors of tumors [11, 24, 35]. In comparison with TIMPs, *RECK* has been reported to be an effective prognosis marker in human patients and a similar condition [10, 21, 31] was expected in dogs. Further study is required to determine the usefulness of *RECK* as a prognostic marker in canine spontaneously developed tumors.

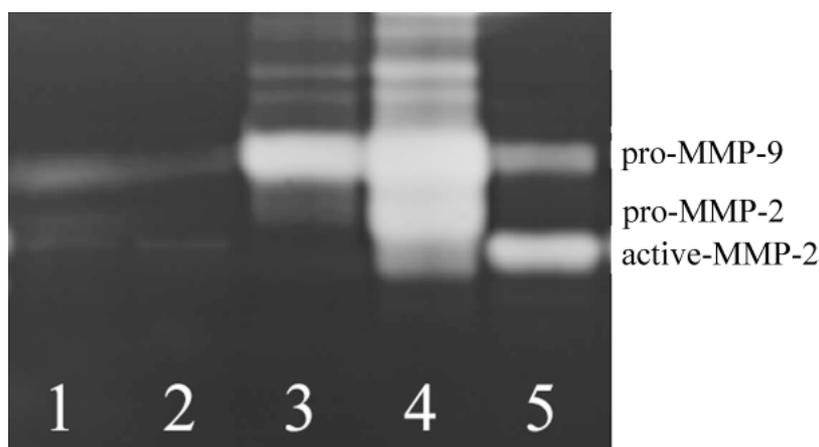


Fig. 3. The gelatin zymography in tumor cell lines. lane 1; high metastatic osteosarcoma, lane 2; osteosarcoma, lane 3; mast cell tumor, lane 4; transitional cell carcinoma, lane 5; mammary gland tumor.

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