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NOTE

Surgery

mRNA expression of tumor-associated genes in canine grade I meningiomas

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ABSTRACT. This study was undertaken to establish a method for measuring mRNA expression by using real-time RT-PCR in the diagnosis of canine meningiomas. When performing real-time RT-PCR, it is essential to include appropriate control tissues and to select appropriate housekeeping genes as an internal standard. Based on the results of our study, *RPS18* constitutes a suitable internal standard for the comparison of mRNA expression between normal meninges and meningiomas. The results showed increased mRNA expression of *VEGFA* and *EGFR*; however, mRNA expression of *KDR* was reduced. Measuring mRNA expression by using real-time RT-PCR with appropriate control tissues and internal standards can provide useful information to understanding the pathogenesis of canine meningiomas, which corresponds with immunohistochemical findings.

KEY WORDS: canine meningioma, housekeeping genes, real-time RT-PCR, tumor-associated genes

Meningiomas are the most common intracranial tumors in dogs [16]. Based on the histopathological features, meningiomas are categorized by the World Health Organization (WHO) into two major groups: benign slow-growing tumors (meningothelial, fibrous, transitional, angiomatous, papillary, granular, and myxoid) and faster-growing anaplastic tumors [9]. Meningiomas are also divided into three grades: grade I, benign; grade II, atypical with intermediate histological features; and grade III, malignant [7].

Overexpression of growth factor associated genes, such as those for vascular endothelial growth factor (*VEGF*), VEGF receptor (*VEGFR*), epidermal growth factor receptor (*EGFR*), and platelet-derived growth factor receptor (*PDGFR*), has been reported in canine meningiomas [3]. Increased *VEGF* expression has been reported in canine meningiomas and is associated with a higher proliferative index and poorer prognosis [4, 10, 13].

Immunohistochemical examination is required to demonstrate the existence of tumor-associated factors in human and canine brain tumors [2, 8, 12]. Although some institutions perform biopsies of brain tumors in dogs, it is more difficult to examine small biopsy samples. Examination of mRNA expression could provide information corresponding to the immunohistochemical findings. Real-time RT-PCR requires a small amount of a specimen, a short time to perform, and minimal skills; its advantage is that it can be used to measure mRNA expression, particularly in brain tumors. It is very important during the performance of real-time RT-PCR to have appropriate control tissues and to select appropriate housekeeping genes as an internal standard in order to measure mRNA expression accurately.

In this study, the mRNA expression in canine samples was examined. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*), TATA-binding protein (*TBP*), and ribosomal protein S18 (*RPS18*) served as housekeeping genes. *VEGFA*, kinase insert domain-containing receptor (*KDR*) (also called *VEGFR-2*), *EGFR*, and *PDGFRA* were the target genes. The aim of this study was to understand the pathogenesis of canine meningioma by measuring accurate mRNA expression of tumor-associated genes compared with appropriate control tissue and internal standard.

Lesional tissue samples were obtained from 14 clinical cases of grade I meningioma (meningothelial meningioma, 1; fibrous meningioma, 7; transitional meningioma, 3; and psammomatous meningioma, 3) that presented to the Nihon University Animal Medical Center between 2011 and 2014. The dogs were of various breeds, sex, and age, and all of them were aged over 8 years. Control tissue samples (7 samples of normal meninges and 7 samples of normal cerebral white matter) were obtained from 5 euthanized beagle dogs. All experiments were approved by the Nihon University Animal Care and Use Committee and conducted in accordance with their guidelines. All samples were stored at −80°C immediately after the surgical resection or autopsy until total RNA extraction was performed. Total RNAs were extracted from the samples using TRIzol reagent (Life Technologies Co., Carlsbad, CA, U.S.A.) according to the manufacturer's instruction. First-strand cDNA synthesis was performed using 500 *n*g of total RNA with a PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). Real-time RT-PCRs were performed using 2 *μl* of

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Table 1. Primer sequences

Gene	Primer sequence	Length (bp)
GAPDH	Forward: TGTGTCCGTCGTGGATCTGA	150
	Reverse: TTGATGTTGAAGTCGCAGGAG	
ACTB	Forward: CGCAAGTACTCTGTGTGGATTGG′	118
	Reverse: ATTTGCGGTGGACGATGGA	
TBP	Forward: ATGGTGTGTACGGGAGCCAAG	184
	Reverse: ACTGTTGGTGGGTCAGCACAAG	
RPS18	Forward: ATAGCCTTTGCCATCACAGCAATTA	86
	Reverse: TTGGTGAGATCGATGTCTGCTTTC	
VEGFA	Forward: TCAGGACACTGCTGTACTTTGAGG	133
	Reverse: GGCTTGTCAGGAGCAAGTGAA	
KDR	Forward: CTTGGACAGCATCACCAGTAGTCAG	131
	Reverse: TGAGATGCTCCAAGGTCAGGAA	
EGFR	Forward: GAAAGCTTGACCAAGCAAGCAC	89
	Reverse: ACGGGACAGTACGTTAAGATGAACA	
PDGFRA	Forward: GTCACATTCATCCAAACCGTCAA	136
	Reverse: ACCTGCCAGGAGCACGTACA	

the first-strand cDNA in 25 μl of the total reaction volume and SYBR Premix Ex Taq™ II (Takara Bio Inc.). PCRs were conducted using the Thermal Cycler Dice Real Time System II TP900 (Takara Bio Inc.). The PCR reactions consisted of 1 cycle of denaturing at 95°C for 5 sec and annealing and extension at 60°C for 30 sec. Results were analyzed using the second derivative method and the comparative cycle threshold method using version 5.11B of the software (Takara Bio Inc.) of the instrument. The primers for the housekeeping genes and for the target genes are shown in Table 1. To determine the appropriate internal standard in this study, quantitative real-time RT-PCR for housekeeping genes was performed using cDNAs made from equivalent amounts of total RNA extracted from normal cerebral white matters, normal meninges, and meningiomas. Subsequently, the cycle threshold (Ct) values for all tissues were analyzed using Kruskal-Wallis one-way analysis of variance. Furthermore, the Ct values of housekeeping genes in normal meninges and menigiomas were examined using BestKeeper (https://www.gene-quantification.de/bestkeeper.html) and NormFinder (https://moma.dk/normfinder-software) softwares for determination of stable housekeeping genes [1, 11]. The housekeeping gene with the lower standard deviation of Ct values and with the values of Pearson correlation coefficient close to 1.0 in BestKeeper, or with 0.5 or less of the stability values in NormFinder could be suitable as internal standards. Relative mRNA expressions of the target genes were calculated using the comparative (ΔΔCt) method based on the values for the appropriate housekeeping genes and the mean values for normal meninges. The relative mRNA expressions were compared between the meningiomas and the normal meninges by using the Mann-Whitney test.

The amplicons of the target genes and housekeeping genes, except for *GAPDH*, showed a single peak and a single band in the dissociation curve and in the agarose gel electrophoresis. *GAPDH* was excluded for inappropriate amplification. The Ct values for *ACTB*, *RPS18*, and *TBP* in the normal cerebral white matter, normal meninges, and meningiomas are shown in Fig. 1. The Ct values for *ACTB* differed significantly between normal cerebral white matter and normal meninges and between normal meninges and meningiomas. In BestKeeper, the standard deviation of Ct values in *ACTB*, *RPS18*, and *TBP* were 1.79, 1.58, and 1.35, respectively, and the values of Pearson correlation coefficient in *ACTB*, *RPS18*, and *TBP* were 0.96, 0.97, and 0.87, respectively. In NormFinder, the stability values in *ACTB*, *RPS18*, and *TBP* were 0.036, 0.015, and 0.049, respectively. It was determined that *RPS18* was the most stable housekeeping gene, though *ACTB* and *TBP* were also confirmed to be usable by NormFinder.

The relative mRNA expression of target genes (*VEGFA*, *KDR*, *EGFR*, and *PDGFRA*) is shown in Fig. 2. The relative mRNA expression of target genes was based on the *RPS18* Ct values obtained from the appropriately selected internal standard, and the values were normalized from the mean relative expression in normal meninges. *VEGFA* and *EGFR* mRNA expression was significantly higher in meningiomas. There was no significant difference in *PDGFRA* mRNA expression in meningiomas. *KDR* mRNA expression in meningiomas was significantly lower than that in normal meninges.

The mRNA expression of housekeeping genes was also different among tissues obtained from the normal cerebral white matter, normal meninges, and meningiomas. Therefore, it was very important to choose the appropriate control tissue and internal standard for comparative evaluation of mRNA expression. In this study, normal meninges as control tissue and *RPS18* as an internal standard gene were chosen for the evaluation of mRNA expression.

The results showed that mRNA expression of *VEGFA* and *EGFR* was increased in grade I meningioma as speculated from previous reports [3, 5, 14]. However, the mRNA expression of *KDR* was decreased significantly; this was in contrast to findings in human meningiomas [6]. It has been reported that the expression of *VEGFR-1* mRNA in canine meningioma varies [5]. It has been suggested that VEGFR-1 may inhibit tumor growth via regulation of VEGF bioavailability through ligand binding to alternatively spliced soluble receptors, and downregulation of VEGFR-1 may provide a biological advantage for tumor growth [15]. It is possible that the expression of VEGF receptors, including the KDR, varies significantly as a response to significant increases in the levels of VEGF in some brain tumors.

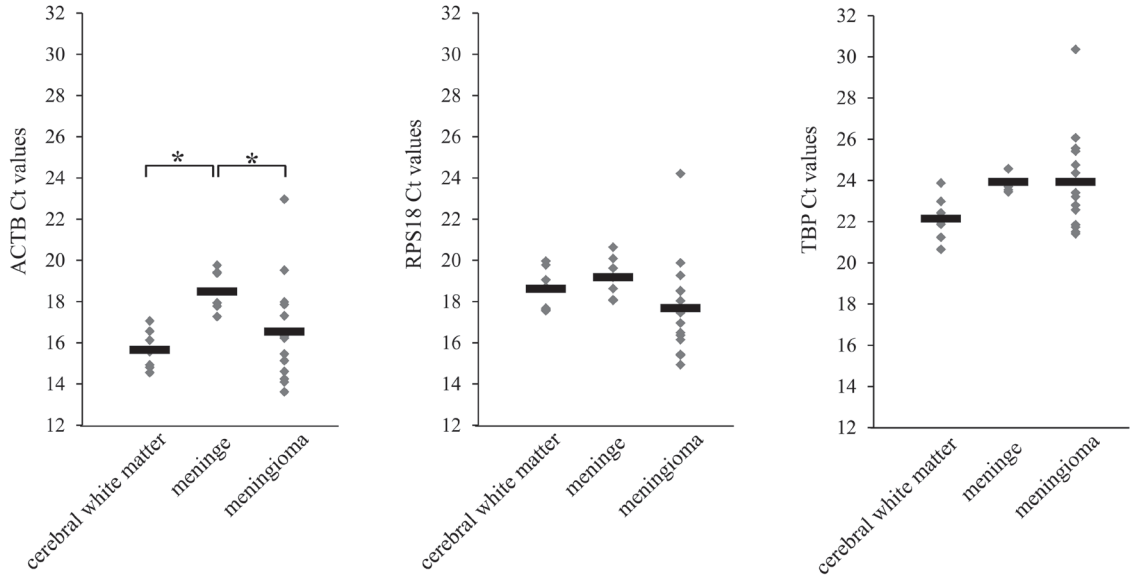

Fig. 1. Ct values for housekeeping genes in normal brain tissues and meningiomas. Plots show Ct values in samples of normal cerebral white matter, meninges, and meningiomas; the bars indicate the mean Ct values. **P*<0.05.

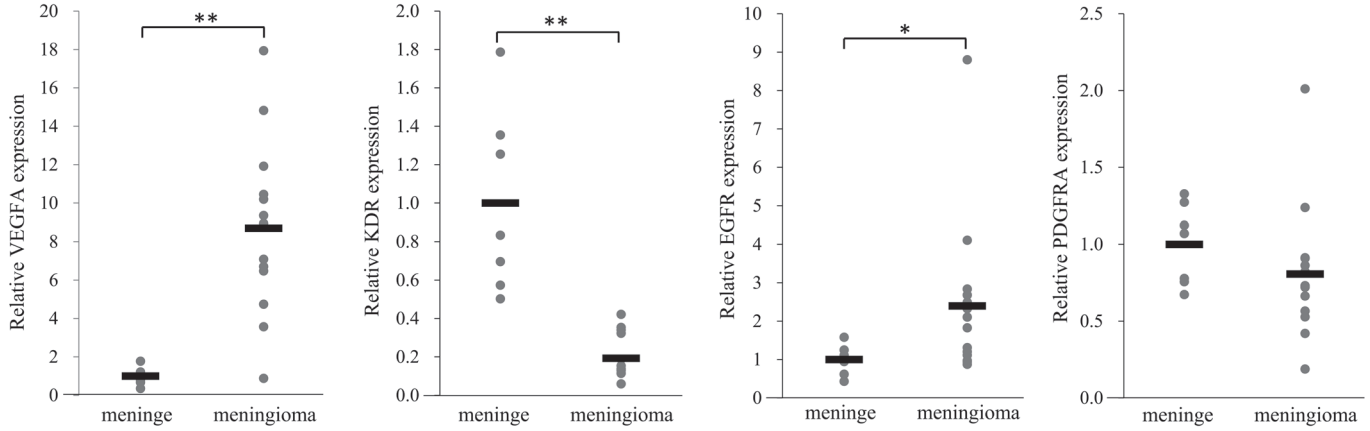

Fig. 2. mRNA expression of *VEGFA*, *KDR*, *EGFR*, and *PDGFRA*. Plots show individual values of relative mRNA expression in normal meninges and in meningiomas; the bars indicate the mean values of relative mRNA expression. **P*<0.05, ***P*<0.01.

Based on the results of comparative examination of well-used housekeeping genes, *RPS18* was proven to be a suitable internal standard for comparison of mRNA expression between normal meninges and meningiomas in dogs. The comparative mRNA expression in this study was measured more accurately than it is in conventional methods because normal meninges and *RPS18* were chosen as control tissue and internal standard, respectively. Measuring mRNA expression with real-time RT-PCR using appropriate control tissue and internal standard can provide useful information to understanding the pathogenesis of canine meningiomas, and this information corresponds with the immunohistochemical findings. In addition, the techniques require a smaller amount of tissues and a shorter time to perform than immunohistochemical examination.

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