J Vet Sci. 2025 Mar;26(2):e22
https://doi.org/10.4142/jvs.24165
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Research Report

Molecular and Cellular Biology

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A novel thrombospondin-1 variant as a potential diagnostic biomarker and therapeutic target in canine mammary tumor and osteosarcoma cells

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OPEN ACCESS

Received: Jun 6, 2024
Revised: Dec 3, 2024
Accepted: Jan 5, 2025
Published online: Feb 25, 2025

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ABSTRACT

Importance: Thrombospondin-1 (TSP1) is a vital glycoprotein that plays a key role in critical biological functions, including cell migration, invasion, angiogenesis, and proliferation. Understanding the roles of its variants, particularly TSP1 variant (TSP1V), is critical for cancer biology and therapy.
Objective: This study examined the expression of the transcriptional variant TSP1V, focusing on canine TSP1 sequences.
Methods: The expression of canine TSP1 sequences was analyzed using a reverse transcription polymerase chain reaction (RT-PCR) to identify the *TSP1* wild-type (*dTSP1W*) and novel canine *TSP1V* transcripts (*dTSP1V*). The effects of damnacanthal and genistein, anticancer compounds, on the viability of canine mammary and osteosarcoma cell lines were assessed by modulating the expression ratios of *TSP1V* to *TSP1W* at the transcriptional level. RT-PCR analysis compared the relative *dTSP1V* and *dTSP1W* concentrations in normal and tumor canine mammary tissues.
Results: Two *dTSP1V* were identified. Treatment with damnacanthal and genistein decreased cell proliferation in canine mammary and osteosarcoma cell lines, associated with changes in the *TSP1V* to *TSP1W* expression ratio. RT-PCR analysis revealed increased *dTSP1V* expression in normal tissues, while *dTSP1W* expression was elevated in tumor tissues.
Conclusions and Relevance: TSP1V may be a potential diagnostic biomarker and therapeutic target for mammary tumors and osteosarcoma in dogs. The differential expression of *dTSP1V* and *dTSP1W* in normal versus tumor tissues underscores the importance of TSPIV in cancer biology, expanding the understanding of its role beyond human thyroid cancer and laying the groundwork for future research in other cancers and species.

Keywords: Biomarker; canine mammary tumor; osteosarcoma; thrombospondin-1; damnacanthal

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Conflict of Interest

The authors declare no conflicts of interest.

Funding

This work was supported by the Research Institute for Veterinary Science and BK21 PLUS Program for Creative Veterinary Science Research Center, Seoul National University, and by a National Research Foundation of Korea (NRF) grant funded by the Korean government (2018R1A2B200292314) and by the clinical research grant (NCC2510420-1) provided by the National Cancer Center of Korea.

INTRODUCTION

Thrombospondin-1 (TSP1) is a crucial glycoprotein intricately facilitating several cellular processes, including angiogenesis, cell proliferation, migration, and invasion. TSP1 expression helps regulate malignant cell behavior, influencing carcinogenesis, tumor progression, and metastasis [1]. In human breast cancer and osteosarcoma, elevated TSP1 levels have been correlated with a poor prognosis because of its ability to promote metastasis by increasing cell invasion [2-4]. Therefore, targeting TSP1 expression in breast cancer and osteosarcoma could have significant therapeutic benefits.

Canine mammary tumors (CMTs) are one of the most prevalent tumors in female dogs, while osteosarcoma primarily affects large and giant dog breeds, often presenting aggressive behavior. Both cancer types have a high potential for malignancy. Malignant mammary tumors can metastasize, similar to canine osteosarcoma (COS), which often spreads to the lungs and other bones. A promising strategy for preventing and treating these cancers involves inducing TSP1 variant transcripts through alternative splicing. Recently, novel TSP1 transcripts with anti-TSP1 activity were identified in human thyroid cancer [5]. In addition, several anticancer compounds induced a transcriptional variant of TSP1 (*TSP1V*), in thyroid cancer. Thus, TSP1V is a potential diagnostic biomarker and a promising therapeutic target in cancer.

CMT is the most commonly diagnosed neoplasm among dog malignancies in female dogs [6]. Specifically, CMT often manifests as a malignant entity with significant morbidity and mortality. An evaluation of the malignancy grades depends on an amalgamation of parameters, encompassing the tumor type, the presence of conspicuous nuclear and cellular pleomorphism, mitotic indices, necrotic foci, peritumoral and lymphatic invasions, along regional lymph node metastases [7]. Therefore, the early detection and accurate diagnosis of mammary tumors are crucial, underscoring the need for novel diagnostic biomarkers specifically designed for CMT. Considering the similarities in clinical behavior between CMT and human breast cancer [8], an examination of the role and significance of *TSP1V* in the context of CMT is warranted.

The bioactive compound damnacanthal, a derivative of anthraquinone sourced from *Morinda citrifolia* (noni) roots, has attracted attention for its anticancer prowess across a spectrum of tumor types, including breast, colon, liver, and lung cancers. Its anticancer mechanisms encompass cell cycle arrest and apoptosis induction [9-11]. The orchestration of damnacanthal-triggered apoptosis entails the protracted activation of p21, culminating in the transcriptional upregulation of *p53* and the *Bax* gene [10]. In particular, damnacanthal impedes the nuclear factor-κB/receptor-interacting protein-2/caspase-1 signaling pathway by suppressing p56lck tyrosine kinase. These findings have implications beyond cancer treatment because they suggest the potential utility of damnacanthal in managing mast cell-mediated allergic disorders [12]. In addition, damnacanthal enhances the expression of the pro-form of NAG-1 in colorectal cancer, augmenting its anticancer efficacy [9]. Nevertheless, further research is needed to elucidate the complex molecular mechanisms underlying the anticancer potential of damnacanthal. This study investigated the expression profiles of TSP1V and TSP1W in canine tumors, which coincided with an examination of the effects of damnacanthal on TSP1 expression within the realm of these malignancies. These findings have the potential to open new avenues for diagnostic and therapeutic strategies related to TSP1V in breast cancer and other types of cancer.

METHODS

Ethics approval

The study was approved by the Institutional Animal Care and Use Committee of the Seoul National University (SNU) (approval No. SNU-230504-1; May 8, 2023).

Cell culture and reagents

The CF41.Mg and D17 cells were obtained from the American Type Culture Collection (ATCC, USA), whereas the canine progenitor epidermal keratinocyte (CPEK) cells were purchased from CELLnTEC. These cells underwent testing by the ATCC for post-freeze viability, growth properties, morphology, mycoplasma contamination, species determination (cytochrome c oxidase I assay and short tandem repeat analysis), and sterility. The cell lines were revived immediately upon receipt, aliquoted, and frozen in liquid nitrogen. The CF41.Mg, CPEK, and D17 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) with 1% penicillin/streptomycin (Gibco Life Technologies, USA). All cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. *Trans*-chalcone, genistein, sulindac sulfide, and piroxicam were supplied by Sigma-Aldrich (USA), while tolfenamic acid was purchased from Cayman Chemical Company (USA). Damnacanthal was acquired as described elsewhere [13].

Tissue samples

The canine mammary tissues used in this study were provided for research purposes by the Korea Animal Medical Center (Korea) in the form of a pair of tumor tissues and adjacent normal tissues (**Table 1**). All owners received an explanation of the study in oral and written form and provided written informed consent for participation before the study commenced.

Cell proliferation assay

The cells were seeded in 96-well plates (5,000 cells/well) and incubated overnight with 100 μL of complete medium. In the following days, the cells were treated with trans-chalcone (20 μM), damnacanthal (50 μM), genistein (100 μM), tolfenamic acid (100 μM), and piroxicam (100 μM) with complete medium for 24 h. A cell proliferation assay was performed according to the manufacturer's instructions using the CellTiter 96 AQueous One Solution (Promega, USA). After 24 h, the medium was exchanged with 20 μL of One Solution reagent in 100 μL of complete medium and incubated for 1 h at 37°C. The cell viability was evaluated by measuring the absorbance at 490 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific). Each experiment was performed in quintuplicate.

Table 1. Demographics of the patients

Breed	Age	Weight (kg)	Ovariohysterectomy	Diagnosis	Histological stage (S)	Histological grade (G)
Cocker Spaniel	13	13.6	Yes	Mammary carcinoma, intraductal papillary	S3	G2
Shih Tzu	11	6.6	No	Mammary carcinoma (comedocarcinoma subtype)	S3	G2
Poodle	17	3.95	No	Mammary gland adenoma	S1	G1

https://doi.org/10.4142/jvs.24165

Colony formation assay

Two thousand D-17 (ATCC) and CF41.Mg (ATCC) cells were seeded into six-well plates and treated with either 0.1% dimethyl sulfoxide (DMSO), damnacanthal (25 μM), or genistein (50 μM) for 12 days. The treatment-containing cell culture medium was refreshed every two to three days. The cultures were terminated once visible colonies formed. The cells were fixed with 4% paraformaldehyde (PC2031-050-00; Biosesang, Korea) for 15 min and stained with a 0.2% crystal violet solution (V5262; Sigma-Aldrich) for 10 min at room temperature. Colony counting was performed using ImageJ software (National Institutes of Health, USA). Each treatment condition was tested in quadruplicate.

Western blot analysis

The cells were cultured in 100 mm dishes until reaching 70% confluence and harvested using radioimmunoprecipitation assay buffer supplemented with proteinase and phosphatase inhibitors. Protein separation was performed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes (GVS Filter Technology, USA), which were then blocked with 0.05% Tris Buffered Saline with Tween 20 (TBS-T) buffer containing 5% non-fat milk at room temperature (RT) for 1 h, followed by overnight incubation with an appropriate primary antibody diluted in 0.05% TBS-T buffer containing 5% non-fat milk at 4°C. Anti-thrombospondin 1 (TSP1; #37879) was purchased from Cell Signaling Technology (USA), and anti-β-actin (sc-47778) was obtained from Santa Cruz Technology (USA). The membranes were washed three times with 0.05% TBS-T buffer for 10 min and incubated with secondary antibodies diluted in 0.05% TBS-T buffer containing 5% non-fat milk at RT for 2 h. After washing, protein expression was detected using enhanced chemiluminescence Western blotting detection reagent (Thermo Fisher Scientific) and analyzed with an Alliance Q9 Advanced chemiluminescence analyzer (UVITEC Cambridge, UK), according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR)

The cells were cultured in a six-well plate until they reached 70% confluence and treated with DMSO, *trans*-chalcone (10 μM), damnacanthal (12.5, 25, and 50 μM), genistein (25, 50, and 100 μM), tolfenamic acid (50 μM), or piroxicam (50 μM) in serum-free medium for 4, 8, or 24 h at 37°C under humid conditions with 5% CO_2. The total RNA was isolated using TRIzol reagent (Ambion, USA), and reverse transcription was performed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to the respective manufacturers' instructions. GoTaq Green Master Mix (Promega) with the indicated primers detailed in **Table 2** was used for PCR. The thermal cycling conditions for RT-PCR were performed under the following conditions: an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, appropriate annealing temperatures for each primer for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated by electrophoresis on 1.5% agarose gel and photographed by Alliance Q9 Advanced analyzer (UVITEC Cambridge). All samples were analyzed in triplicate and quantified using ImageJ software.

Statistical analysis

Parametric tests, including unpaired Student's *t*-tests and one-way analysis of variance, were used for statistical analysis. A Mann–Whitney U test was utilized for the non-parametric test. Data processing and statistical analyses were performed using Microsoft Office Excel (Microsoft, USA) and GraphPad Prism, version 9.5.1 (GraphPad Software Inc., USA). All experimental data are presented as the mean ± standard error of the mean for parametric data; *p* values were considered significant.

https://doi.org/10.4142/jvs.24165

Table 2. Primer used in this study

	Forward primer (5′→3′)	Reverse primer (5′→3′)
Exon 2-5	GTTCCTGTTGCGTGTGTGCG	CGGATGCTGTCCTGAAGTGTG
Exon 5-7	TCCGCACTAACTACATCGGC	AGGACACCTTCTTGCAGATGG
Exon 7-9	CATCATGCCCTGCTCCAACG	GGGGGCTAGGAGAATTGCAGA
Exon 9-11	GGAAACCATGTGAAGGCGAA	AGTACACTTGACCCCAGCAA
Exon 9	TGGTCGTCTTGTTCCGTGAC	CAGGCATCTTTCTTGCAGGC
Exon 10	CTGGTCACTGTGGGACATCTG	
Exon 11-13	TTGCTGGGGTCAAGTGTACT	TGCAGTCATGTGTCCCATCT
Exon 13-15	TGGGACACATGACTGCAACA	TTGTCCGTGTCAGCCTGATC
Exon 15-17	GACAACTGCCCCTACAACCA	ATTGGGCACAAGTCTGCAGT
Exon 17-19	GACTCAGACCGCATTGGAGA	CGGAAAGGCCAGAGTATCCC
Exon 19-22	AGTTCAATGCTGTGGACTTTAGTG	ACCATGGTAACTGTTCATGCTCT
Intron 9 (1)		CCCGTTTATGACCCACCCAA
Intron 9 (2)		CTCCTCGGACACACGAAGAG
dNAG-1	AGGAGCTTCGGAAACGCTAC	CCTACACGACTGAGCTGACG
dGAPDH	GTGTCCCCACCCCCAATGTA	ACCCGGTTGCTGTAGCCAAA

RESULTS

Identification of TSP1V in canine cells

A previous study reported that *TSP1V*, which is the transcription variant of *TSP1*, has anticancer activity in thyroid cancer. Therefore, this study examined the expression pattern of the canine *TSP1* variant (*dTSP1V*) in canine cell lines by modulating TSP1 expression [5]. First, this study compared the exon sequences in humans and other species (mouse, canine, and feline) using the Multiple Sequence Alignment of Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and found high similarity in exon sequences between humans and the other species (mouse, 88.51%; canine, 90.34%; feline, 91.55%) (**Fig. 1A**). Moreover, the protein sequences of these species also showed high similarity: mouse, 94.7%; canine, 97.18%; feline, 97.44% (**Supplementary Fig. 1**). This high similarity suggests that dogs may have a comparable alternative splicing mechanism for producing dTSP1V. RT-PCR was performed to examine intron retention in TSP1 in canine cell lines (CPEK, D17 [COS], and CF41.Mg [CMT]). Ten pairs of primers were designed to cover different regions of dTSP1, and RT-PCR was conducted using canine-specific primers (**Table 2**). Some parts of *dTSP1* showed extra bands that were expected to be generated by intron retention (**Fig. 1B**). Sequencing of these extra bands confirmed intron retention. The transcriptional variants were found by designing a reverse primer from intron 9 and conducting RT-PCR using exon 7, exon 5, and exon 2 forward primers and an intron 9 reverse primer. The RT-PCR result suggested two forms of *dTSP1* variants produced by intron retention in canines (**Fig. 1C and D**). The minor form of *TSP1V* (*TSP1Vm*) was barely expressed in the PCR product from exon 2 to intron 9. Therefore, this study focused on *dTSP1V* (major) in the subsequent experiments in this study. The data suggested that *TSP1V*, produced by intron retention, is generated in humans and canine cells.

Damnacanthal and genistein inhibit the proliferation of canine breast cancer and osteosarcoma cells

An MTS assay was conducted to examine the anti-proliferative activity of several phytochemicals and nonsteroidal anti-inflammatory drugs (NSAIDs) on CF41.Mg and D17 cells (**Fig. 2**). The cells were treated with 20 μM *trans*-chalcone, 50 μM damnacanthal, 100 μM genistein, 100 μM tolfenamic acid, and 100 μM piroxicam in complete medium (**Fig. 2**). Additional experiments were conducted to evaluate the effects of these compounds at half doses in a serum-free medium (**Supplementary Fig. 2**). The results showed that four of the five tested compounds inhibited the proliferation of CF41.Mg cells, while two compounds

A

TSP1	Human exon2	Human exon3	Human exon4	Human exon5	Human exon6	Human exon7	Human exon8	Human exon9	Human exon10	Human exon11	Human exon12	Human exon13	Human exon14	Human exon15	Human exon16	Human exon17	Human exon18	Human exon19	Human exon20	Human exon21	Human exon22	Avg.
Mouse	75.79	83.04	97.37	89.50	84.55	91.49	88.51	93.22	89.08	91.41	89.54	89.95	88.89	91.25	90.76	89.79	90.35	91.91	91.84	90.00	70.56	88.51
Canine	85.42	89.64	92.11	89.50	86.18	92.55	91.38	92.09	91.95	87.50	90.85	90.87	91.67	92.50	93.28	94.47	91.67	92.65	93.88	95.00	71.90	90.34
Feline	88.54	90.18	93.42	90.50	93.50	93.62	90.23	90.96	94.25	89.06	90.85	91.78	94.44	89.38	92.44	94.04	92.11	93.75	94.90	95.00	79.65	91.55
Avg.	83.25	87.62	94.30	89.83	88.08	92.55	90.04	92.09	91.76	89.32	90.41	90.87	91.67	91.04	92.16	92.77	91.38	92.77	93.54	93.33	74.04	90.13

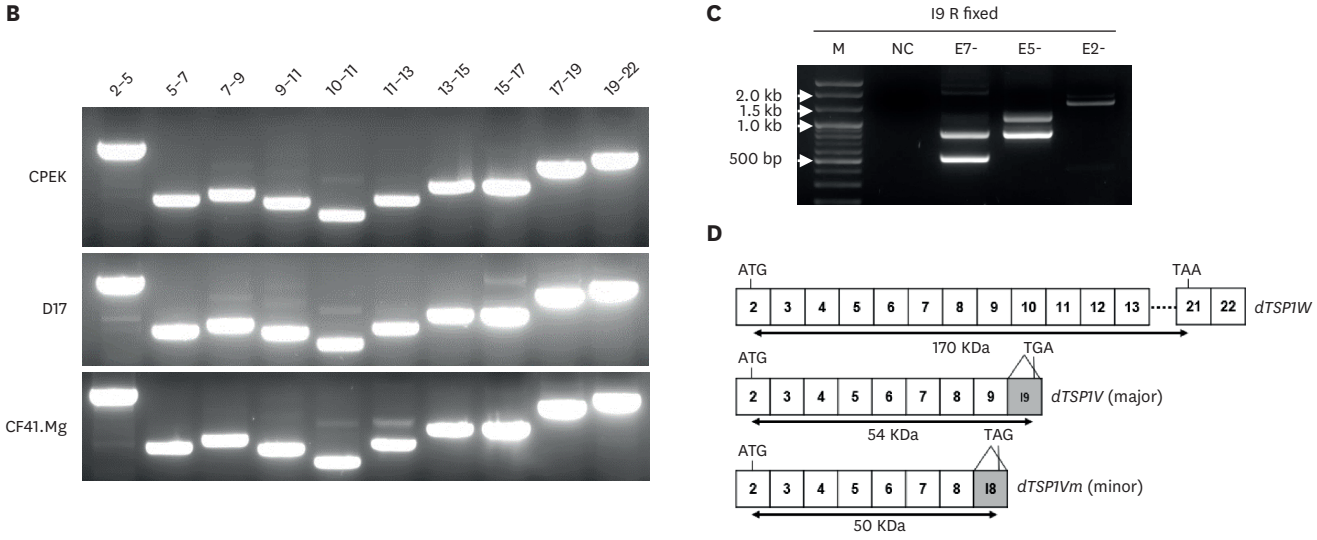

Fig. 1. Identification of the transcriptional variants of canine *TSP1* (*dTSP1*) in canine cell lines. (A) Comparison of the exon sequences between humans and other species (mouse, canine, and feline). (B) RT-PCR was performed using RNA from canine cell lines (CPEK, D17, and CF41.Mg) as templates, with primers designed to cover 10 *dTSP1* parts. (C) RT-PCR using RNA from CPEK cells, using a fixed intron 9 reverse primer and forward primers for exons 7, 5, and 2. The upper and lower band represent *dTSP1V* (minor) and *dTSP1V* (major) (100 bp DNA Ladder; SolGent, Korea), respectively. (D) Schematic diagram of the *dTSP1W, dTSP1V* (major), and *dTSP1V* (minor) sequences.
TSP1, thrombospondin-1; RT-PCR, reverse transcription polymerase chain reaction; CPEK, canine progenitor epidermal keratinocyte; Avg, average.

inhibited the proliferation of D17 cells (**Fig. 2A and E**). Damnacanthal and genistein, which had inhibitory effects on both cell lines, were selected for confirmation using dose- and time-dependent treatments. As expected, these compounds showed a time- and dose-dependent inhibition of cell proliferation in both cell lines (**Fig. 2B, C, F, and G**). The colony formation assay validated this result. As shown in **Fig. 2D and H**, damnacanthal and genistein significantly inhibited colony formation, supporting their anti-proliferative effects on these cells.

TSP1V expression is upregulated by damnacanthal in CMT and COS cells

This study measured the expression of wild-type dog TSP1 (dTSP1W) and its variant dTSP1V in CMT and COS cells at the RNA and protein levels. RNA analysis showed that *dTSP1W* was expressed in all tested canine cell lines (CPEK, CF41.Mg, and D17), whereas *dTSP1V* was rarely expressed (**Fig. 3A**). dTSP1W (180 KDa) and dTSP1V (54 KDa) were detected at the protein level (**Fig. 3B**). Subsequently, RNA was extracted from the cells treated with various compounds, and conventional RT-PCR was performed. Damnacanthal significantly upregulated *dTSP1V* in CF41.Mg and D17 cells while simultaneously downregulating *dTSP1W* in the canine cell lines (**Fig. 3C and F**). These observations showed a dose and duration dependence (**Fig. 3D, E, G, and H**).

https://doi.org/10.4142/jvs.24165

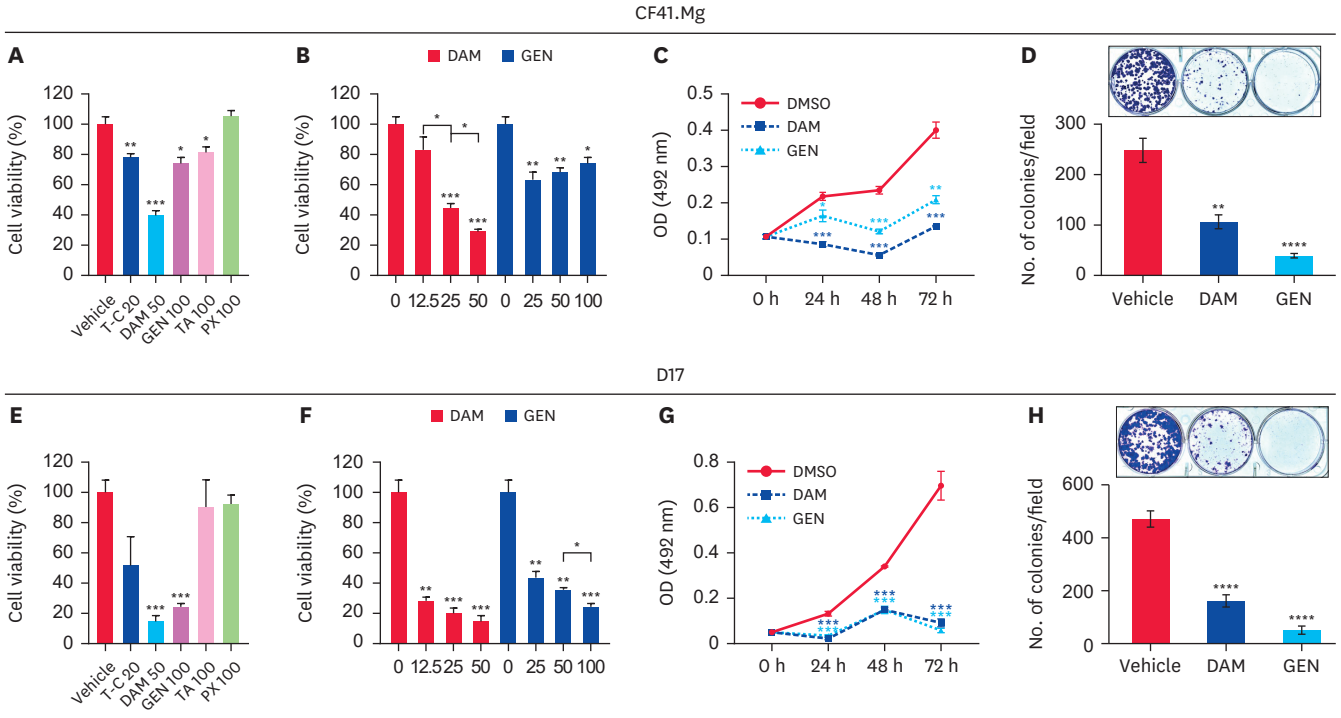

Fig. 2. Effects of anticancer compounds on cell proliferation in CF41.Mg (CMT) and D17 (COS) cell lines. (A, E) MTS assay results showing the impact of phytochemicals and nonsteroidal anti-inflammatory drugs (T-C, 20 μM; DAM, 50 μM; GEN, 100 μM; TA, 100 μM; and PX, 100 μM) on CF41.Mg and D17 cell lines in complete medium. (B, F) MTS assay results showing cell viability in CF41.Mg and D17 cell lines treated with varying concentrations of DAM (12.5, 25, and 50 μM) and GEN (25, 50, and 100 μM) in a complete medium. (C, G) MTS assay results showing cell viability in CF41.Mg and D17 cell lines treated with DAM and GEN for different durations (24, 48, and 72 h) in a complete medium. (D, H) Colony formation assay results for CF41.Mg and D17 cell lines treated with 25 μM DAM and 50 μM GEN. Representative images are displayed, and the quantified colony formation data are provided below.
CMT, canine mammary tumor; COS, canine osteosarcoma; T-C, *trans*-chalcone; DAM, damnacanthal; GEN, genistein; TA, tolfenamic acid; PX, piroxicam; OD, optical density; DMSO, dimethyl sulfoxide.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

TSP1 may serve as a biomarker of canine breast cancer

Finally, this study examined tissue samples from dogs with mammary tumors to evaluate the potential of TSP1 as a biomarker for canine mammary cancer. RNA was extracted from mammary tumor tissues and adjacent normal tissues, followed by RT-PCR analysis. The results indicated that *dTSP1V* expression was more abundant in the adjacent normal tissues, whereas the *dTSP1W* expression levels were elevated in tumor tissues. Nonsteroidal anti-inflammatory drug-activated gene (NAG-1) was reported to be linked to high tumor grades, estrogen receptor (ER)-negativity, and human epidermal growth factor receptor 2 (HER2) overexpression in patients with breast cancer [14]. *dNAG-1* was also expressed more strongly in mammary tumors than in normal tissues (**Fig. 4A and B**). Furthermore, the ratio of *dTSP1V* to *dTSP1W* was significantly different between normal and tumor tissues (**Fig. 4C**). These results suggest that the *dTSP1V* to *dTSP1W* expression ratio could be a diagnostic marker for breast cancer in dogs.

DISCUSSION

Previous research has presented contrasting evidence regarding the involvement of TSP1 in breast cancer progression, with some studies suggesting its potential as a therapeutic target. For example, PKHB1, a thrombospondin-1 peptide mimic, was reported to exhibit antitumor

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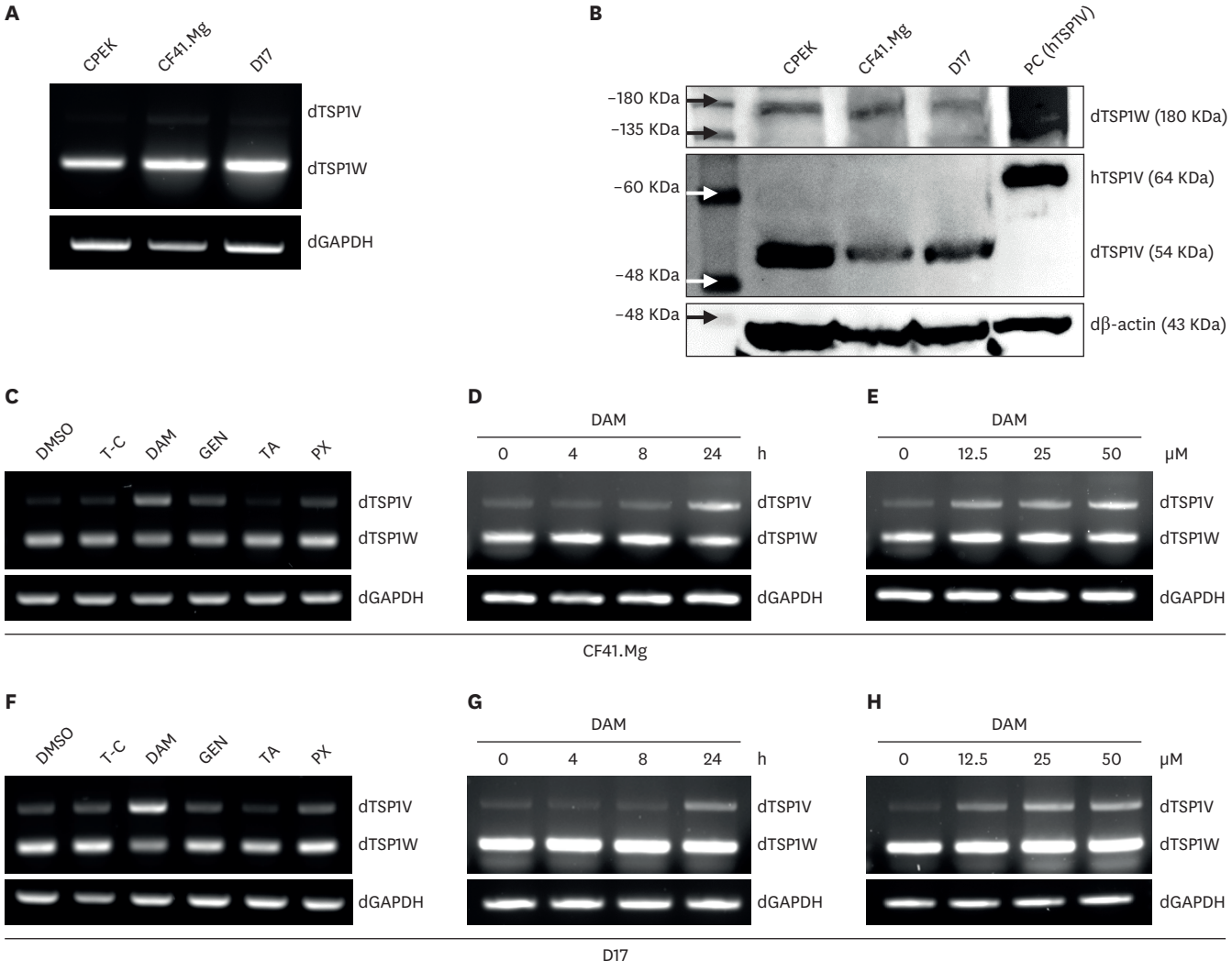

Fig. 3. Effect of compound treatment on intron-retained *dTSP1V* expression in CMT and COS. (A) RT-PCR was performed using exon 9 forward, exon 9 reverse, and intron 9 reverse primers to detect *dTSP1* expression in RNA samples from canine cell lines (CPEK, CF41.Mg, and D17). (B) Protein samples from canine cell lines (CPEK, CF41.Mg, and D17) were subjected to Western blot analysis with an anti-TSP1 antibody for detection. The PC refers to cells overexpressing the human TSP1V expression vector in BCPAP cells. (C, F) Canine cell lines, CF41.Mg and D17, were treated with various phytochemicals and NSAIDs (T-C, 10 μM; DAM, 25 μM; GEN, 50 μM; TA, 50 μM; and PX, 50 μM) in serum-free medium. RNA was extracted from the treated cells and analyzed by RT-PCR, as described in (A). (D, G) RNA was extracted from CF41.Mg and D17 cell lines following treatment with DAM for different durations (4, 8, and 24 h) in serum-free medium, and subsequently analyzed using RT-PCR. (E, H) RNA from CF41.Mg and D17 cell lines were extracted after treatment with varying concentrations of DAM (12.5, 25, and 50 μM) in a serum-free medium, and subsequently analyzed using RT-PCR. The PCR results shown in C to H were performed in triplicate and analyzed individually using Image J.
CMT, canine mammary tumor; COS, canine osteosarcoma; RT-PCR, reverse transcription polymerase chain reaction; TSP1, thrombospondin-1; PC, positive control; NSAID, nonsteroidal anti-inflammatory drug; T-C, *trans*-chalcone; DAM, damnacanthal; GEN, genistein; TA, tolfenamic acid; PX, piroxicam; CPEK, canine progenitor epidermal keratinocyte.

properties and promote immune system activation via immunogenic cell death induction in breast cancer cells [15]. In addition, exosomal TSP1 has been implicated in promoting breast cancer invasion by activating focal adhesion kinase and matrix metalloproteinases-9, with strong correlations observed between the extracellular matrix stiffness, exosomal TSP1 concentration, and metastasis-related mortality [16]. These multifaceted roles of TSP1 in breast cancer underscore the need for further research to understand its potential as a therapeutic target in breast cancer. The varying effects of TSP1 in breast cancer may be due to differences in the levels of TSP1V expression under different experimental conditions.

https://doi.org/10.4142/jvs.24165

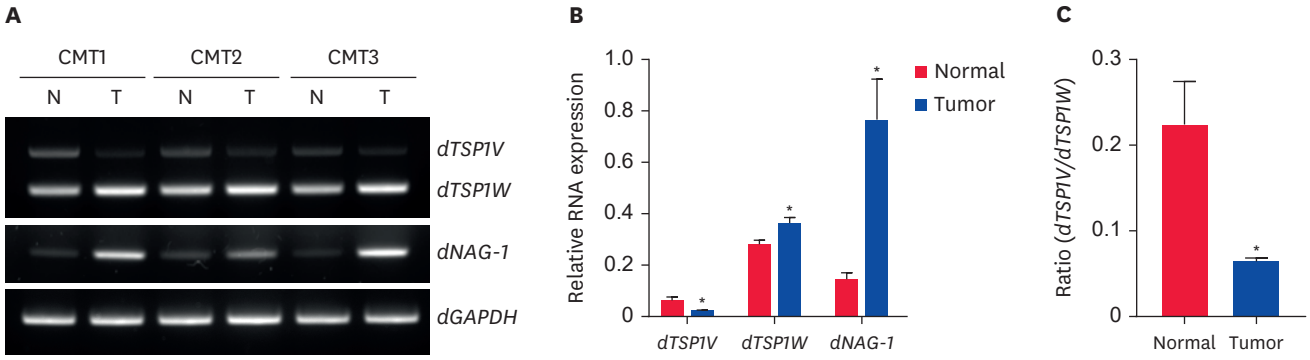

Fig. 4. *dTSP1* expression in tumor and adjacent normal tissues from CMT patients. (A) RT-PCR was conducted using primers for *dTSP1* and *dNAG-1* with RNA extracted from tumor tissues and adjacent normal tissues of CMT patients. (B) Expression levels of *dTSP1W*, *dTSP1V*, and *dNAG-1* in the tumor and adjacent normal tissues of CMT patients, normalized to *dGAPDH*. (C) *dTSP1V* to *dTSP1W* ratio in tumor versus adjacent normal tissues from CMT patients.
CMT, canine mammary tumor; RT-PCR, reverse transcription polymerase chain reaction; N, normal; T, tumor.
$^{*}p < 0.05$ (n = 3).

In a previous study, *TSP1V,* produced by intron retention during alternative splicing, inhibited tumorigenesis in thyroid cancer by suppressing *TSP1W* [5]. The current study showed that TSP1V is expressed in human thyroid cancer, canine breast cancer, and osteosarcomas (**Fig. 2**), suggesting that alternative splicing mechanisms are conserved across species.

Considering the limited research on the molecular and cellular biology of CMTs and osteosarcoma, chemical candidates were selected based on studies of human breast cancer and osteosarcoma. In addition, certain chemicals, such as tolfenamic acid and piroxicam, have been reported to inhibit cell survival in canine cancers [17-19]. These compounds, which have demonstrated anticancer activity in human cancers, might have similar effects in canine cancers, and the experiments were designed accordingly. Damnacanthal and genistein had promising anti-proliferative effects in CMT and COS (**Fig. 3**). Damnacanthal is an anthraquinone isolated from the root of *Morinda citrifolia* L. (noni), and genistein is an isoflavonoid present in high quantities in soybeans, both of which have many pharmacological properties including anticancer activity [20,21]. The mechanisms of the anticancer activity of genistein have many pathways, including suppression of tyrosine kinases, regulation of Hedgehog-Gli1 signaling, modulation of epigenetic activities, seizing of cell cycle and Akt and MEK signaling pathways [21-24]. This study suggested a novel mechanism of genistein and damnacanthal in the modulation of *TSP1W* and *TSP1V* expression providing a novel mechanism for its anticancer activity (**Figs. 2** and **3**).

The *TSP1V* to *TSP1W* expression ratio could be used to differentiate benign thyroid nodules from differentiated thyroid carcinoma in follicular neoplasms of thyroid cancer [5]. In CMT, the expression ratio in mammary tumors significantly differed from the adjacent normal tissues (**Fig. 4**), suggesting that the *dTSP1V* to *dTSP1W* expression ratio may serve as a diagnostic biomarker for CMT. Furthermore, previous studies suggested that NAG-1 (also known as GDF15) could serve as a valuable diagnostic and prognostic biomarker for various cancers, including colorectal, thyroid, lung, and pancreatic cancer [25-28]. In particular, breast cancer cells with acquired radioresistance exhibited increased NAG-1 expression and enhanced epithelial-to-mesenchymal transition features, such as migration and invasion [29]. The results showed increased *dNAG-1* expression in tumor tissues compared to the adjacent normal tissues, suggesting that *dNAG-1* could serve as a biomarker for canine mammary tumorigenesis. Further research will be needed to validate the diagnostic and prognostic role of dNAG-1 in CMTs.

https://doi.org/10.4142/jvs.24165

This study shed light on the involvement of *TSP1W* and *TSP1V* in CMT and COS, highlighting the potential use of the *dTSP1V*/*dTSP1W* expression ratio as a diagnostic biomarker for CMT. Furthermore, these findings suggest that damnacanthal and genistein could be promising therapeutic agents for CMTs. On the other hand, further investigations are needed to uncover the underlying mechanisms and determine the clinical significance of *TSP1W* and *TSP1V* as potential diagnostic and therapeutic targets.

ACKNOWLEDGMENTS

The authors are particularly grateful for the valuable critiques provided by Ms. Pattawika Lertpatipanpong at the Seoul National University College of Veterinary Medicine.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Protein sequence comparison between human and other species (mouse, canine, and feline).

Supplementary Fig. 2

Anticancer compounds affect cell proliferation in CF41.Mg (CMT) and D17 (COS) cell lines. (A and D) MTS data of phytochemicals and nonsteroidal anti-inflammatory drugs (T-C, 10 μM; DAM, 25 μM; GEN, 50 μM; TA, 50 μM; and PX, 50 μM) treatment in CF41.Mg and D17 cell lines with serum-free medium. (B and E) Cell viability measured by MTS assay in CF41.Mg and D17 cell lines treated with different doses of damnacanthal (12.5, 25, and 50 μM) and genistein (25, 50, and 100 μM) in a serum-free medium. (C and F) Cell viability measured by MTS assay in CF41.Mg and D17 cell lines treated with different times (24, 48, and 72 h) of damnacanthal and genistein in a serum-free medium.

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