

Expression Profiling of Receptor-Activator of Nuclear Factor-Kappa B Ligand in Soft Tissue Tumors

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Bone and soft tissue tumors are derived from mesenchymal cells, and they are hard to treat. Receptor-activator of nuclear factor-kappa B ligand (RANKL) is an essential cytokine for osteoclast differentiation and activation and is expressed on the surface of osteoblasts or stromal cells. In this study, to explore the potential of denosumab treatment for soft tissue tumors, we analyzed the expression profiles of RANKL mRNA in 425 tumor specimens of 33 histological types by real-time RT-PCR. Denosumab is a monoclonal antibody that prevents the binding of RANKL to receptor-activator of nuclear factor-kappa B (RANK). For comparison, the relative expression levels of RANK and osteoprotegerin (OPG) mRNAs were also measured. OPG functions as a soluble decoy receptor for RANKL. Higher expression levels of RANKL mRNA were detected in calcifying aponeurotic fibroma, fibrosarcoma, calcifying epithelioma, myositis ossificans, heterotopic calcification, giant cell tumor of the tendon sheath (GCTTS), and pigmented villonodular synovitis (PVNS), compared with the levels of other tumor types. Moreover, the expression levels of RANK mRNA were highest in GCTTS, followed by myositis ossificans and PVNS, whereas the expression levels of OPG mRNA were greatly varied among these histological types. We then analyzed RANKL protein expression by immunohistochemistry in 57 tumor specimens with higher expression levels of RANKL mRNA. RANKL-positive cells were detected in GCTTS, PVNS, myositis ossificans, heterotopic calcification, and calcifying aponeurotic fibroma. In conclusion, RANKL is expressed in subsets of soft tissue tumors with calcification, and denosumab is a potential therapeutic option for soft tissue tumors expressing RANKL.

Keywords: calcification; immunohistochemistry; mRNA; receptor-activator of nuclear factor-kappa B ligand; soft tissue tumor

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Introduction

Bone and soft tissue tumors are derived from mesenchymal cells. It is often hard to treat soft tissue tumors. Receptor-activator of nuclear factor-kappa B ligand (RANKL) is an essential cytokine for osteoclast differentiation and activation, which causes bone resorption. RANKL is expressed on the surface of osteoblasts or stromal cells, and it binds the receptor, receptor-activator of nuclear factor-kappa B (RANK). RANKL induces osteoclast progenitors expressing RANK to transform into osteoclasts (Yasuda et al. 1998). Osteoprotegerin (OPG), which is a member of

the tumor necrosis factor receptor superfamily, functions as a soluble decoy receptor for RANKL and is an effective inhibitor of osteoclast maturation and activation (Tsuda et al. 1997; Huang et al. 2000). RANKL is expressed not only in bone cells (including osteoblasts, osteocytes, and hypertrophic chondrocytes), but also in specific cell types of a wide variety of tissues, including the spleen, heart, lung, brain, thymus, T and B lymphocytes, and mammary epithelial cells (Kartsogiannis et al. 1999; Rintas et al. 2014). Moreover, recent studies have reported that RANKL appears to be involved in many cellular processes, including vascular calcification and immunity, although not all its

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functions are clear (Davenport et al. 2016; Lin et al. 2016). RANKL promotes vascular calcification by inducing bone morphogenetic protein-2 (BMP-2) release from human aortic endothelial cells (Davenport et al. 2016). Denosumab is a fully human monoclonal antibody against RANKL that specifically inhibits osteoclast differentiation and bone resorption by preventing RANKL-mediated formation and activation of osteoclasts (Thomas and Skubitz 2009; Lewiecki 2010; Branstetter et al. 2015). Denosumab inhibits bone destruction in patients with multiple myeloma-associated osteolytic bone disease, bone metastases from solid cancers, and giant cell tumors of the bone (Fizazi et al. 2011; Henry et al. 2011). We previously investigated RANKL expression in various primary bone tumors to identify malignancies that may respond to denosumab (Yamagishi et al. 2016). Bone and soft tissue tumors are derived from mesenchymal tissues such as the bone, muscle, nerve and fat tissue, and therefore may express RANKL. However, malignant bone and soft tissue tumors are rare; thus, the information about RANKL expression in soft tissue tumors is limited. In this study, we analyzed the expression levels of RANKL, RANK and OPG mRNAs, as well as RANKL protein expression, in various soft tissue tumors to identify potential lesions that may respond to denosumab by using real-time reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), respectively.

Materials and Methods

Specimens

We obtained 425 clinical specimens from patients with soft tissue tumors and tumor-like lesions who were treated at our institute between 2007 and 2014. The specimens were preserved at -80°C . There were 238 male and 187 female patients. The patients' diseases encompassed 33 histological types, including 17 benign tumor types, 3 intermediate tumor types, and 13 malignant tumor types (Table 1). We also included 18 samples of giant cell tumor of bone (GCTB), as a positive control for RANKL expression (Yamagishi et al. 2016). The diagnoses were independently determined histopathologically by two experienced pathologists according to the World Health Organization classification (Fletcher et al. 2013). We obtained written informed consent from all patients. The study design was approved by the Ethics Committee of the School of Medicine, Niigata University.

Quantitative real-time RT-PCR

Total RNA was extracted from fresh-frozen specimens. First-strand cDNA was synthesized from total RNA using the PrimeScript RT Reagent Kit (TaKaRa Bio, Shiga, Japan). RT-PCR was performed using SYBR Premix EX Taq II (Tli RNaseH Plus; TaKaRa), and the Thermal Cycler Dice Real Time System TP800 (TaKaRa). The primers used were: human RANKL (forward, 5'-GCCTTTCAAGGAGCTGTGCAA-3'; reverse, 5'-ATCTAACCATGAGCCATCCACAT-3'), RANK (forward, 5'-GCCATCATCTTTGGCGTTT-3'; reverse, 5'-CAAAGTTTGGCGTGTGTACTG-3'), OPG (forward, 5'-CAATTTGCCTGGCACCAAAG-3'; reverse, 5'-AGGTGAGGTAGCATGTCCAATGT-3'), and glyceraldehyde-3-phosphate dehy-

drogenase (GAPDH) (forward, 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse, 5'-TGGTGAAGACGCCAGTGG-3'). The relative expression level of each mRNA was adjusted as a ratio to expression in RPMI 8226, a human multiple myeloma cell line that expresses RANKL. We selected RPMI 8226 as the calibrator because RANKL expression has been confirmed in multiple myeloma and denosumab is approved for multiple myeloma (Kawano et al. 2012). We could not use a normal tissue as there is no appropriate normal tissue that constitutively expresses stable RANKL mRNA.

IHC

We also evaluated RANKL expression by IHC. Of the 425 patient samples, 57 specimens were selected for evaluation of RANKL protein expression. These tumors have calcification or osteoclast-like giant cells, which were highly suspected to express RANKL protein. Formalin-fixed, paraffin-embedded tissue sections ($4\ \mu\text{m}$) were deparaffinized and hydrated, and heat-induced epitope retrieval was performed inside an autoclave at 121°C for 20 minutes in Histofine antigen retrieval buffer, pH 9 (Nichirei Bioscience, Tokyo, Japan). We used 3% hydrogen peroxide to inactivate the endogenous peroxidase activity. The slides were incubated overnight at 4°C with rabbit anti-human RANKL antibody (ab9957; Abcam, Cambridge, UK) (Scotto di Carlo et al. 2018). The primary antibody was visualized by using the Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei Bioscience) and 3,3'-diaminobenzidine (Simple Stain DAB, Nichirei Bioscience).

Results

RANKL expression

The median relative expression levels of RANKL mRNA were 1,323 (range, 542-4,528) in calcifying aponeurotic fibroma, 651 (range, 5.91-1,296) in fibrosarcoma, 561 (range, 19.9-606) in myositis ossificans, 279 (range, 76.7-482) in desmoplastic fibroblastoma, 99.7 (range, 40.7-412) in heterotopic calcification, 160 (range, 102-173) in nodular fasciitis, 93.8 (range, 78.3-94.6) in granular cell tumor, 51.6 (range, 0.83-1944) in calcifying epithelioma, 42.6 (range, 16.3-184) in PVNS, and 40.0 (range, 13.7-641) in giant cell tumor of the tendon sheath (GCTTS) (Fig. 1A, B, Table 2). These results indicate that tumors with calcification tend to show higher expression levels of RANKL mRNA. However, the median RANKL expression level was ≤ 10 in most of soft tissue tumors; thus, the relative expression levels of RANKL mRNA were lower in soft tissue tumors than the median level detected in GCTB, a positive control for detecting RANKL mRNA (Table 2).

RANK expression

The median relative expression levels of RANK mRNA were 268 (range, 20.6-1,093) in GCTTS, 165 (range, 38.0-185) in nodular fasciitis, 154 (range, 40.1-274) in myositis ossificans, 130 (range, 41.1-1,105) in PVNS, 83.1 (range, 64.8-210) in heterotopic calcification, and 91.7 (range, 13.9-146) in angiosarcoma (Fig. 2A, B). The median RANK expression levels were ≤ 30 in most of tumor histological types; thus, the relative expression levels of RANK mRNA were lower than the median level detected

Table 1. List of histological types and numbers of clinical specimens.

Histology	Cases (n = 425)	Sex	
		M (n = 238)	F (n = 187)
Benign			
Lipoma	117	67	50
Schwannoma	58	34	24
Hemangioma	16	6	10
Neurofibroma	14	3	11
PVNS	10	2	8
GCTTS	9	4	5
Angiomyoma	8	3	5
Fibroma	7	6	1
Calcifying epithelioma	5	1	4
Planter fibromatosis	4	4	0
Calcifying aponeurotic fibroma	3	3	0
Granular cell tumor	3	0	3
Leiomyoma	3	1	2
Nodular fasciitis	3	1	2
Myositis ossificans	3	2	1
Heterotopic calcification	3	1	2
Desmoplastic fibroblastoma	2	2	0
Intermediate or malignant tumor			
WDL	43	25	18
UPS	31	16	15
Myxoid liposarcoma	12	5	7
Desmoid	8	4	4
DFSP	8	5	3
Epithelioid sarcoma	6	5	1
Synovial sarcoma	6	6	0
Leiomyosarcoma	4	3	1
MPNST	4	3	1
Angiosarcoma	3	2	1
Myxofibrosarcoma	3	3	0
Extraskeletal myxoid chondrosarcoma	3	3	0
Rhabdomyosarcoma	2	1	1
Fibrosarcoma	2	1	1
Dedifferentiated liposarcoma	2	1	1
ASPS	2	1	1
GCTB (positive control)	18	14	4

ASPS, alveolar soft part sarcoma; DFSP, dermatofibrosarcoma protuberans; GCTB, giant cell tumor of bone; GCTTS, giant cell tumor of the tendon sheath; MPNST, malignant peripheral nerve sheath tumor; PVNS, pigmented villonodular synovitis; UPS, undifferentiated pleomorphic sarcoma; WDL, well-differentiated liposarcoma.

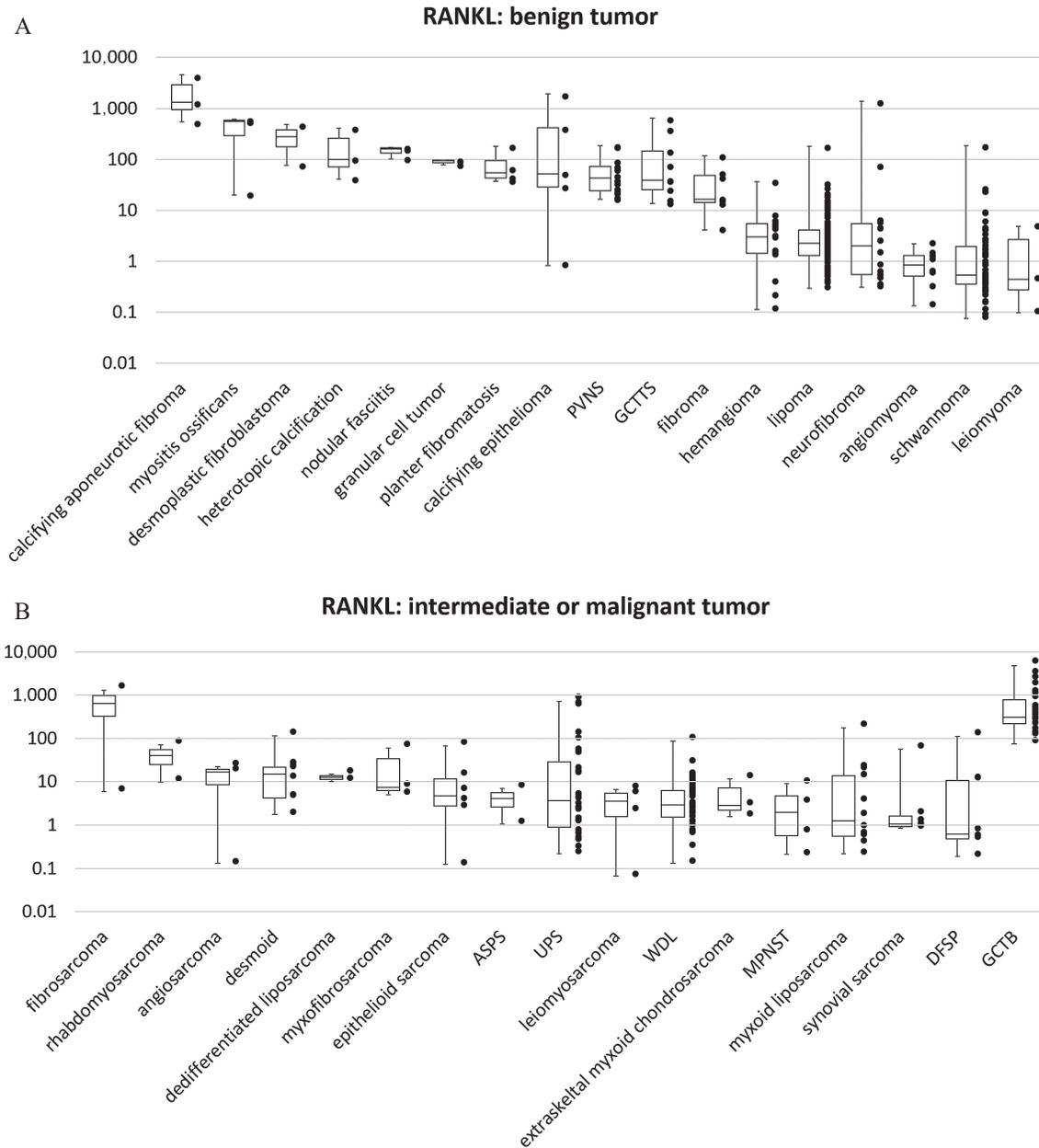


Fig. 1. Dot plot for relative RANKL mRNA levels in soft tissue tumors.

A. Benign tumor. B. Intermediate or malignant tumor. The relative expression levels of RANKL mRNA were measured by real-time PCR.

RANKL, receptor-activator of nuclear kappa B-ligand; PCR, polymerase chain reaction; PVNS, pigmented villonodular synovitis; GCTTS, giant cell tumor of the tendon sheath; ASPS, alveolar soft part sarcoma; MFH, malignant fibrous histiocytoma; WDL, well-differentiated liposarcoma; MPNST, malignant peripheral nerve sheath tumor; DFSP, dermatofibrosarcoma protuberans; GCTB, giant cell tumor of bone.

in GCTB (Table 2). Moreover, unlike the expression profiles of RANKL mRNA, the relative expression levels of RANK mRNA were not greatly varied, irrespective of the histological types.

OPG expression

The median relative expression levels of OPG mRNA were 29,476 (range, 2,262-55,246) in extraskelletal myxoid chondrosarcoma, 21,165 (range, 9,977-26,144) in calcify-

ing aponeurotic fibroma, 1,626 (range, 4.54-18,122) in epithelioid sarcoma, 1,462 (range, 179-2,138) in myositis ossificans, 1,389 (range, 62.2-9,088) in angiosarcoma, 622 (range, 198-854) in heterotopic calcification, 248 (range, 93.1-2,452) in PVNS, and 86.4 (range, 32.9-1,350) in GCTTS (Fig. 3A, B). Overall, the relative expression levels of OPG mRNA were higher in most of soft tissue tumors than the median level detected in GCTB (Table 2). In particular, the expression levels of OPG mRNA were highest

Table 2. The median expression levels of RANKL, RANK and OPG mRNAs as assessed by quantitative real-time polymerase chain reaction.

Histology	Median		
	RANKL	RANK	OPG
Benign			
Calcifying aponeurotic fibroma	1,323	37.9	21,165
Myositis ossificans	562	154	1462
Desmoplastic fibroblastoma	279	82.4	422
Heterotopic calcification	100	83	621
Nodular fasciitis	160	165	1,159
Granular cell tumor	94.8	4.89	54.2
Planter fibromatosis	54.3	19.5	214
Calcifying epithelioma	51.6	50.9	124
PVNS	42.6	158	264
GCTTS	39.0	268	86.4
Fibroma	16.6	4.92	469
Hemangioma	3.03	7.48	579
Lipoma	2.26	24.5	1,349
Neurofibroma	1.98	28.6	1,077
Angiomyoma	0.84	18.6	389
Schwannoma	0.53	31.5	715
Leiomyoma	0.45	6.32	387
Intermediate or malignant tumor			
Fibrosarcoma	651	2.53	47.3
Rhabdomyosarcoma	40.4	12.4	1,090
Angiosarcoma	16.8	91.7	1,389
Desmoid	15.1	4.20	506
Dedifferentiated liposarcoma	12.7	24.1	433
Myxofibrosarcoma	7.50	24.1	279
Epithelioid sarcoma	4.77	12.8	1,626
ASPS	4.09	1.80	204
UPS	3.65	20.5	194
Leiomyosarcoma	3.60	5.74	16.9
WDL	2.93	26.0	744
Extraskeletal myxoid chondrosarcoma	2.88	7.86	29,476
MPNST	1.99	8.47	44.2
Myxoid liposarcoma	1.25	20.7	139
Synovial sarcoma	1.06	2.55	66.5
DFSP	0.63	17.0	154
GCTB (positive control)	307	212	225

ASPS, alveolar soft part sarcoma; DFSP, dermatofibrosarcoma protuberans; GCTB, giant cell tumor of bone; GCTTS, giant cell tumor of the tendon sheath; MPNST, malignant peripheral nerve sheath tumor; OPG, osteoprotegerin; PVNS, pigmented villonodular synovitis; RANK, receptor-activator of nuclear kappa B; UPS, undifferentiated pleomorphic sarcoma; WDL, well-differentiated liposarcoma.

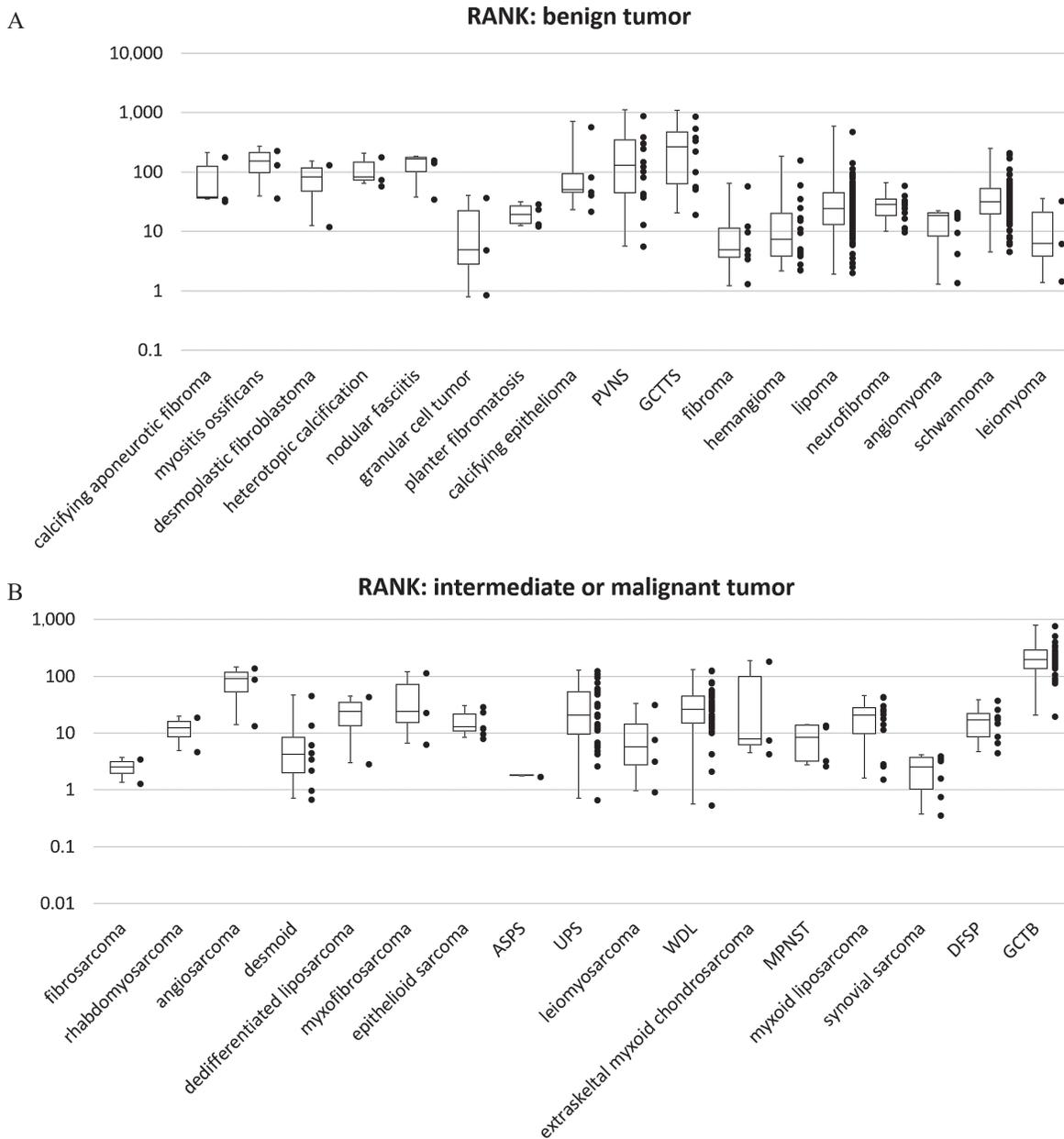


Fig. 2. Dot plot for relative RANK mRNA expression levels in soft tissue tumors.

A. Benign tumor. B. Intermediate or malignant tumor. The relative expression levels of RANK mRNA were measured by real-time PCR.

RANK, receptor-activator of nuclear kappa B; PCR, polymerase chain reaction; PVNS, pigmented villonodular synovitis; GCTTS, giant cell tumor of the tendon sheath; ASPS, alveolar soft part sarcoma; MFH, malignant fibrous histiocytoma; WDL, well-differentiated liposarcoma; MPNST, malignant peripheral nerve sheath tumor; DFSP, dermatofibrosarcoma protuberans; GCTB, giant cell tumor of bone.

in calcifying aponeurotic fibroma and extraskeletal myxoid chondrosarcoma.

IHC

We performed IHC for RANKL in 57 cases with high RANKL mRNA expression (Table 3). RANKL protein was detected in 29 cases, which comprised 5 cases of GCTTS, 3 of PVNS, 2 of myositis ossificans, 1 of heterotopic calcification, 2 of calcifying aponeurotic fibroma, 5 of undifferentiated pleomorphic sarcoma, and 11 of other histological

types. However, the staining was not as strong as what we observed in the GCTB samples (Fig. 4A, B). RANKL-positive cells were scattered, even in calcifying aponeurotic fibroma, which expressed the highest RANKL mRNA levels among the soft tissue tumors (Fig. 4C, D). RANKL expression was also observed in heterotopic calcification (Fig. 4E, F). Similarly, a small number of RANKL-positive cells were observed in GCTTS and PVNS, in which osteoclast-like giant cells were also observed (Fig. 4G, H). The staining pattern of RANKL was membranous and/or cyto-

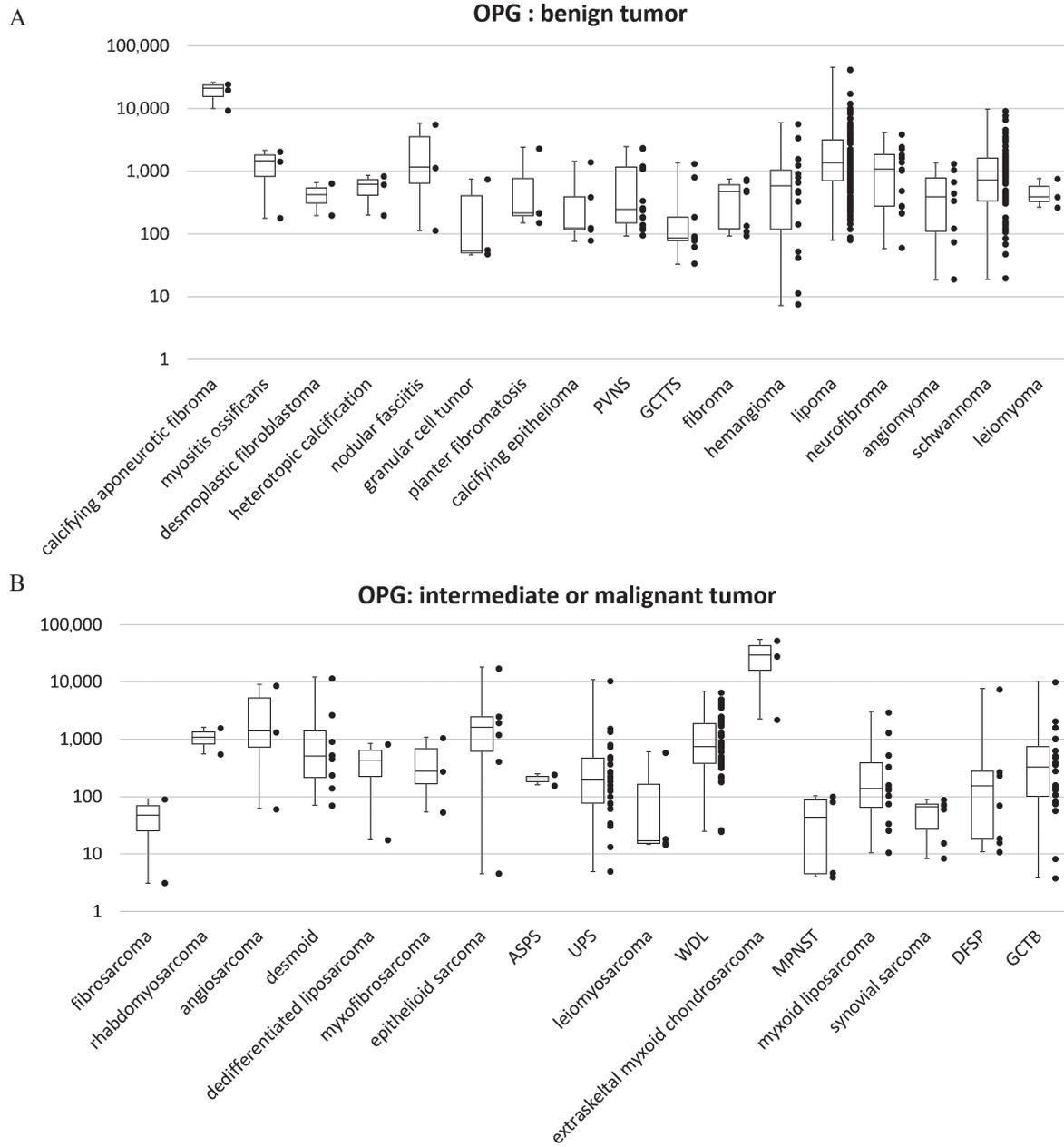


Fig. 3. Dot plot for relative OPG mRNA expression levels in soft tissue tumors. A. Benign tumor. B. Intermediate or malignant tumor. The relative expression levels of OPG mRNA were measured by real-time PCR.

OPG, osteoprotegerin; PCR, polymerase chain reaction PVNS, pigmented villonodular synovitis; GCTTS, giant cell tumor of the tendon sheath; ASPS, alveolar soft part sarcoma; MFH, malignant fibrous histiocytoma; WDL, well-differentiated liposarcoma; MPNST, malignant peripheral nerve sheath tumor; DFSP, dermatofibrosarcoma protuberans; GCTB, giant cell tumor of bone.

plasmic with minimal background, as previously reported (Taylor et al. 2017). In the present study, RANKL staining of GCTB and calciifying aponeurotic fibroma showed a membranous pattern; however, it has a cytoplasmic pattern in GCTTS and heterotopic calcification. The staining intensity was not strong in GCTTS and PVNS, but RANKL-positive cases were found in 5 of 6 and 3 of 4 cases, respectively. Moreover, RANKL-positive cells were sparsely distributed in UPS, but calcification or osteoclast-like giant

cells were not detected (data not shown).

Discussion

RANKL is an essential mediator of osteoclast formation, function, and survival; it promotes the recruitment of osteoclast-like giant cells, which are responsible for bone resorption (Branstetter et al. 2015). RANKL is considered a therapeutic target in certain bone tumors such as GCTB, metastatic bone tumors, and multiple myeloma.

Table 3. List of histological types that were examined by immunohistochemistry.

Histology	Cases (n = 57)	Median of RANKL expression	Cases with presence of RANKL-positive cells
Calcifying aponeurotic fibroma	2	2,926	2
Fibrosarcoma	1	1,297	1
Myositis ossificans	3	562	0
Desmoplastic fibroblastoma	1	482	0
Heterotopic calcification	3	99.7	1
Nodular fasciitis	3	160	2
Granular cell tumor	3	93.8	1
Calcifying epithelioma	2	235	0
PVNS	4	20.7	3
GCTTS	6	38.4	5
Desmoid	7	11.5	3
Dedifferentiated liposarcoma	2	12.7	1
Myxofibrosarcoma	1	60	0
UPS	9	83.8	5
WDL	2	55.8	1
MPNST	1	8.99	0
Neurofibroma	2	734	1
Myxoid liposarcoma	1	175	1
DFSP	1	111	1
Schwannoma	3	26.8	1

DFSP, dermatofibrosarcoma protuberans; GCTTS, giant cell tumor of the tendon sheath; MPNST, malignant peripheral nerve sheath tumor; PVNS, pigmented villonodular synovitis; UPS, undifferentiated pleomorphic sarcoma; WDL, well-differentiated liposarcoma.

Recently, RANKL expression has also been reported in various other giant cell-rich tumors that cause bone resorption. A case of an aneurysmal bone cyst (ABC) with high RANKL expression displayed a therapeutic response to anti-RANKL therapy (Pelle et al. 2014). Moreover, histological findings indicated the presence of osteoclast-like giant cells in ABC (Fletcher et al. 2013). Additionally, Boyce et al. (2012) reported the presence of remarkable RANKL expression in a fibrous dysplasia. When considering soft tissue tumors, Tajima et al. (2015) observed osteoclast-like giant cells in breast leiomyosarcoma. Furthermore, Terasaki et al. (2015) reported the occurrence of osteoclast-like giant cell in uterine leiomyosarcomas, as well as high RANKL mRNA expression. In the present study, RANKL expression was observed in soft tissue tumors of various histological types; its expression was high in some histological tumors, such as calcifying aponeurotic fibroma, calcifying epithelioma, heterotopic calcification, PVNS, GCTTS, and fibroblastic tumors. RANKL was also observed in other histological types, although its expression level was lower. However, RANKL expression

was case-dependent and not significantly different among tumor subtypes. Our previous study showed high RANKL expression in bone tumors that contained osteoclast-like giant cells, such as GCTB, and in chondrosarcoma with abundant calcification (Yamagishi et al. 2016). In this study, similar results were observed in soft tissue tumors such as calcifying aponeurotic fibroma, calcifying epithelioma, heterotopic calcification, GCTTS, and PVNS. However, the expression levels of RANKL mRNA were lower in most of soft tissue tumors than those in GCTB (see Table 2).

In contrast, a number of recent studies have linked RANKL to calcification in vessels or in other organs, such as the kidney, lung, and heart. Davenport et al. (2016) reported that RANKL promotes vascular calcification by inducing osteoblastic behavior in vascular smooth muscle cells. They showed that RANKL induces vascular endothelial cells to release BMP-2, which in turn acts on adjacent vascular smooth muscle cells in a paracrine fashion to induce osteoblastic activity, dependent on their expression of runt-related transcription factor-2 (Runx2) and alkaline

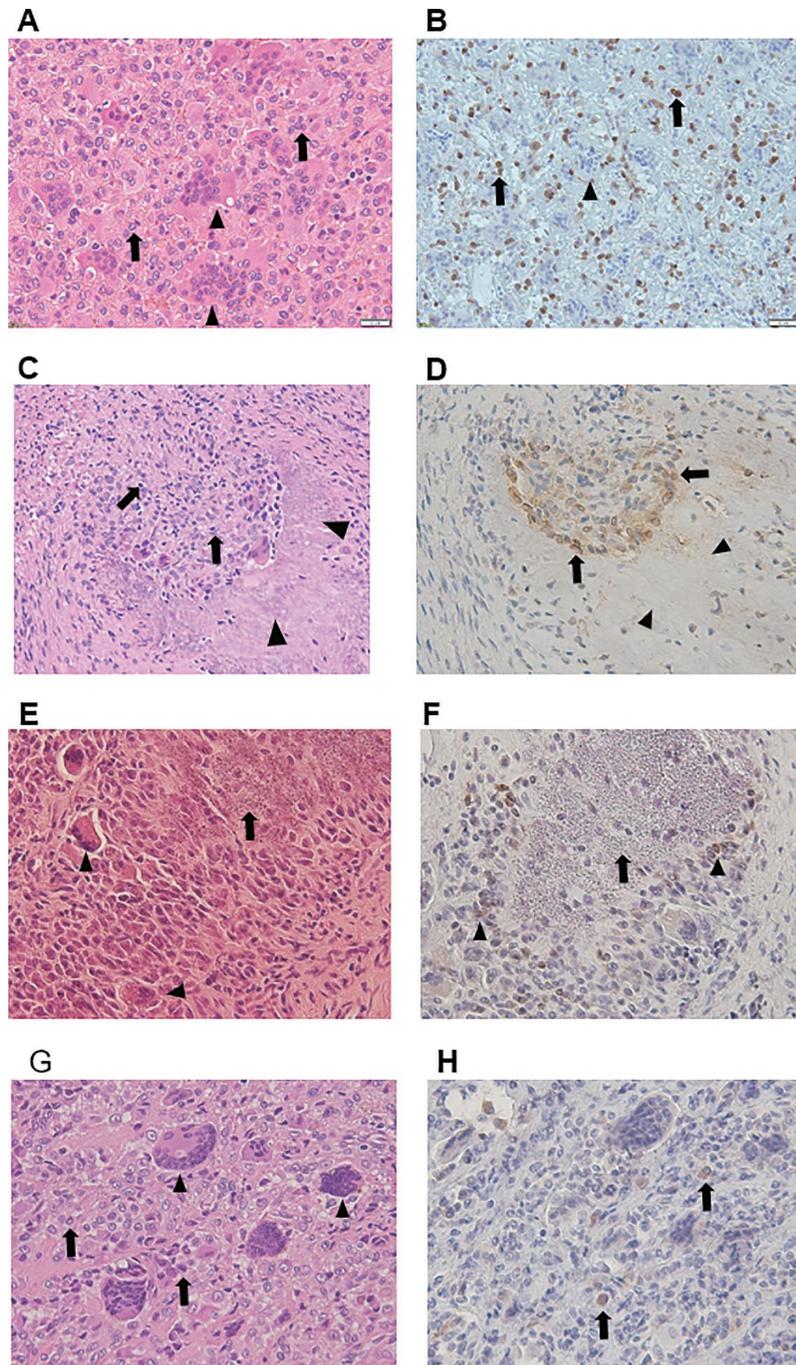


Fig. 4. Immunohistochemical analysis of RANKL expression in soft tissue tumors.

Serial tissue sections were used in the immunohistochemical analysis of RANKL expression in soft tissue tumors.

A, Hematoxylin-eosin staining of giant cell tumor of bone (GCTB). Tumor stromal cells (arrows) and osteoclast-like giant cells (arrowheads) were observed. The value of RANKL mRNA expression is 732. B, Immunohistochemistry for RANKL in GCTB. RANKL-positive stromal cells (arrows) surrounding osteoclast-like giant cells (arrowhead) were detected. RANKL was detected in the cytosol. C, Hematoxylin-eosin staining of calcifying aponeurotic fibroma. Tumor stromal cells (arrows) and calcification (arrowheads) were observed. The value of RANKL mRNA expression is 1,323. D, Immunohistochemistry for RANKL in calcifying aponeurotic fibroma. RANKL-positive stromal cells (arrows) were highly observed around calcification areas (arrowheads). Osteoclast-like giant cells were attracted by RANKL. RANKL was detected in the cytosol. E, Hematoxylin-eosin staining of heterotopic calcification. Stromal cell calcification (arrow) and osteoclast-like giant cells (arrowheads) were observed. The value of RANKL mRNA expression is 411. F, Immunohistochemistry for RANKL in heterotopic calcification. RANKL-positive stromal cells (arrowheads) were observed around calcification area (arrow). RANKL was detected in cytosol. G, Hematoxylin-eosin stain of GCTTS. Tumor stromal cells (arrows) and osteoclast-like giant cells (arrowheads) were observed. The value of RANKL mRNA expression is 145. H, Immunohistochemistry for RANKL in GCTTS. RANKL-positive stromal cells (arrows) were observed in a scattered manner. RANKL was detected in the cytosol.

phosphatase (Davenport et al. 2016). Furthermore, Lin et al. (2016) reported that Runx2 induces smooth muscle cells to differentiate into osteoblast-like cells, and also prompts the maturation of chondrocytes from smooth muscle cells. Rinotas et al. (2014) observed heterotopic calcification, including the vessels, kidney, lung, heart, and tongue in transgenic mice with systemic RANKL expression; this can lead to soft tissue calcification. Heterotopic calcification may often become intractable after total hip arthroplasty or in patients with connective tissue diseases (Feinblatt et al. 2005; Gutierrez et al. 2012). It may also become unmanageable when severe symptoms of widespread ossification in myositis ossificans arise (Cohen et al. 1993; Yamagishi et al. 2016). In the present study, RANKL was detected in some soft tissue tumors that exhibited calcification; this is consistent in part with the aforementioned studies that show association of RANKL with vascular calcification. We hypothesize that calcification in soft tissue tumors or tumor-like lesions, such as calcifying aponeurotic fibroma, calcifying epithelioma, myositis ossificans, and heterotopic calcification, may be related to RANKL overexpression.

RANKL expression in fibroblastic tumors such as fibrosarcoma, desmoplastic fibroblastoma, and planter fibromatosis was also high, but the number of specimens of these tumor types was small, and additional cases are required to investigate the significance of RANKL expression in such tumors.

We performed IHC for RANKL protein in cases that expressed high levels of RANKL mRNA. RANKL-positive cells were observed in some samples; however, they were scattered even in tumors with high RANKL mRNA expression. A few RANKL-positive cells were observed in two calcifying aponeurotic fibroma cases, two myositis ossificans cases, and one heterotopic calcification case, including those in which RANKL mRNA expression was high. However, the number of RANKL-positive cells was not as high as in bone tumors, even though they were observed around calcified areas, suggesting that RANKL protein may be also involved in the regulation of bone growth and turnover.

A small number of RANKL-positive cells were observed in GCTTS and PVNS specimens in which osteoclast-like giant cells were also observed. Although RANKL mRNA and protein expression were case-dependent and not significantly different among tumor subtypes, it was apparent that osteoclast-like giant cells and areas of calcification were more common in soft tissue tumors positive for RANKL protein expression.

Our data also showed that soft tissue tumors that had high RANKL mRNA expression do not necessarily have high protein expression; rather, high RANKL mRNA levels may only be an indicator of positivity for RANKL protein expression. It is possible that RANKL staining was poor because we usually prepared decalcified tumor specimens with calcification and perform paraffin fixation, and decalcification can reduce antigenicity. We think that the differ-

ences are due not only to the decreased antigenicity caused by decalcification but also other variables in the staining process. Additional studies are required.

Unlike the expression profiles of RANKL mRNA in soft tissue tumors, the relative expression levels of RANK mRNA were not greatly varied, although RANK mRNA levels were highest in GCTTS, followed by PVNS, both of which contain osteoclast-like giant cells. Because RANKL induces RANK-expressing osteoclast progenitors to transform into osteoclasts, RANK may be expressed in soft tissue tumors with osteoclast-like giant cells. By contrast, the relative expression levels of OPG mRNA were greatly varied, depending on the tumor types (Table 2).

In conclusion, most of soft tissue tumors express RANKL, RANK, and OPG mRNAs, although the expression levels of each mRNA were greatly varied, depending on histological types. Moreover, RANKL protein-positive cells are present in subsets of soft tissue tumors, including calcifying aponeurotic fibroma, GCTTS and PVNS. We suggest that RANKL may be involved in soft tissue calcification. Taken together, we propose that denosumab is a potential therapeutic option for soft tissue tumors expressing RANKL.

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Conflict of Interest

The authors declare no conflict of interest.

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