

High-level expression of podoplanin in benign and malignant soft tissue tumors: Immunohistochemical and quantitative real-time RT-PCR analysis

YONGJUN XU¹, AKIRA OGOSE¹, HIROYUKI KAWASHIMA¹, TETSUO HOTTA¹,
TAKASHI ARIIZUMI¹, GUIDONG LI¹, HAJIME UMEZU² and NAOTO ENDO¹

¹Division of Orthopedic Surgery, Niigata University Graduate School of Medical and Dental Sciences, 757-1, Asahimachi-dori, Niigata City, Niigata Prefecture 951-8510; ²Division of Pathology, Niigata University Medical and Dental Hospital, 1-754, Asahimachi-dori, Chuo-ku, Niigata City, Niigata Prefecture 951-8520, Japan

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Abstract. Podoplanin is a 38 kDa mucin-type transmembrane glycoprotein that was first identified in rat glomerular epithelial cells (podocytes). It is expressed in normal lymphatic endothelium, but is absent from vascular endothelial cells. D2-40 is a commercially available mouse monoclonal antibody which binds to an epitope on human podoplanin. D2-40 immunoreactivity is therefore highly sensitive and specific for lymphatic endothelium. Recent investigations have shown widespread applications of immunohistochemical staining with D2-40 in evaluating podoplanin expression as an immunohistochemical marker for diagnosis and prognosis in various tumors. To determine whether the podoplanin (D2-40) antibody may be useful for the diagnosis of soft tissue tumors, 125 cases, including 4 kinds of benign tumors, 15 kinds of malignant tumors and 3 kinds of tumor-like lesions were immunostained using the D2-40 antibody. Total RNA was extracted from frozen tumor tissue obtained from 41 corresponding soft tissue tumor patients and 12 kinds of soft tissue tumor cell lines. Quantitative real-time PCR reactions were performed. Immunohistochemical and quantitative real-time RT-PCR analyses demonstrated the expression of the podoplanin protein and mRNA in the majority of benign and malignant soft tissue tumors and tumor-like lesions examined, with the exception of alveolar soft part sarcoma, embryonal and alveolar rhabdomyosarcoma, extraskeletal Ewing's sarcoma/peripheral primitive neuro-ectodermal tumor and lipoma, which were completely negative for podoplanin. Since it is widely and highly expressed in nearly

all kinds of soft tissue tumors, especially in spindle cell sarcoma, myxoid type soft tissue tumors and soft tissue tumors of the nervous system, podoplanin is considered to have little value in the differential diagnosis of soft tissue tumors.

Introduction

Podoplanin, also called the E11 antigen, is a 38 kDa mucin-type transmembrane glycoprotein that was first reported in lymphatic endothelial cells, epithelial cells of the choroid plexus, alveolar type I cells, osteoblasts, and peritoneal mesothelial cells by Wetterwald *et al* in 1996 (1). This protein was subsequently identified as a 43 kDa protein on the surface of rat glomerular epithelial cells (podocytes), and because it was found to be involved in the flattening of foot processes in puromycin-induced nephrosis, it was named podoplanin (2). Recent investigations have demonstrated that podoplanin is regulated by the lymphatic-specific homeobox gene Prox 1, a master gene that controls the development of lymphatic progenitors from embryonic veins (3).

Podoplanin is homologous to the hT1 α -2 (also known as Aggrus) gene which encodes the type I alveolar cell-specific antigen and to the oncofetal antigen M2A, which is recognized by the commercially available D2-40 antibody. Podoplanin promotes platelet aggregation, and possesses a platelet aggregation-stimulating (PLAG) domain. Thr52 is important for the activity of this domain (10). Tumor-induced platelet aggregation occurred early in the development of metastatic lesions, so it may be involved in cancer cell migration, invasion, metastasis, and malignant progression (4-12). Human podoplanin is expressed by several normal tissue cells, including: mesothelial cells (13,14), osteocytes and osteoblasts (1,13,15,16), stromal reticular cells, follicular dendritic cells of lymphoid tissue, ependymal cells of the central nervous system (CNS), and ovarian and testicular cells of both germ cell and sex-cord stromal origin, basal epithelial keratinocytes of the skin, cervix, and esophagus, and myoepithelial cells of the breast (1,5,6,14,17-22). Podoplanin is a useful marker of Kaposi's sarcoma (22-24),

Correspondence to: Dr Akira Ogose, Division of Orthopedic Surgery, Niigata University Graduate School of Medical and Dental Sciences, 757-1, Asahimachi-dori, Niigata City, Niigata Prefecture 951-8510, Japan
E-mail: aogose@med.niigata-u.ac.jp

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and other vascular lesions, such as epithelioid hemangioendotheliomas of the head, neck and liver (25,26), and hemangioblastomas of the CNS (27). Membranous and cytoplasmic immunoreactivity for podoplanin was detected in 86-100% of epithelioid mesotheliomas, respectively, whereas podoplanin is invariably negative in lung adenocarcinomas (14).

Strong podoplanin immunoreactivity was detected in primary and metastatic seminoma and testicular germ-cell tumors of young adults derived from intratubular germ cell neoplasia and carcinoma *in situ* (6,28,29). Podoplanin expression is strongly expressed in pilocytic astrocytomas, glioblastomas, and primary central nervous system germ cell tumors (30-32). It is also strongly expressed in epidermal squamous cell carcinoma (33), oral leukoplakia (34-36), and skin adnexal carcinomas (37), whereas podoplanin is negative in metastatic adenocarcinomas and histiocytic neoplasms (38). The expression of podoplanin in a wide range of benign and malignant soft tissue tumors is not previously well characterized. In this study, we analyzed the expression of podoplanin in benign and malignant soft tissue tumors and tumor-like lesions at both protein and mRNA level by immunohistochemical and quantitative real-time RT-PCR analysis.

Materials and methods

Tissue samples. Tumor specimens were obtained during surgery from 20 patients with undifferentiated high grade pleomorphic sarcomas [malignant fibrous histiocytomas (MFH), 20 pleomorphic types], 10 patients with myxofibrosarcoma, 31 patients with liposarcomas (8 well differentiated, 19 myxoid, and 4 dedifferentiated type 4 cases), 4 patients with biphasic synovial sarcomas, 7 patients with rhabdomyosarcomas (3 pleomorphic, 1 embryonal, and 3 alveolar type cases), 9 patients with malignant peripheral nerve sheath tumors, 3 patients with leiomyosarcomas, 3 patients with fibrosarcomas (2 adult cases, 1 sclerosing epithelioid case), 1 patient with extraskeletal chondrosarcoma, 3 patients with alveolar soft part sarcomas, 4 patients with epithelioid sarcomas, 3 patients with extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumors (PNET), 4 patients with dermatofibrosarcoma protuberans, 6 patients with lipomas, 4 patients with schwannomas, 3 patients with fibromas, 3 patients with myxomas, 1 patient with pigmented villonodular synovitis (PVNS), 3 patients with ganglion tumors, and 3 patients with chronic expanding hematomas. Informed consent had been obtained previously from patients or their guardians. The tumor specimens were routinely fixed in 10% buffered formalin for 18-20 h at room temperature and paraffin-embedded. Sections (4 μ m) were cut and attached to APS coated glass slides. Hematoxylin and eosin was used for routine staining. The histology of these tissue samples was confirmed by experienced pathologists.

Cell lines and culture conditions. A human fibrosarcoma cell line (HT1080; Health Science Research Resources Bank), two human malignant fibrous histiocytoma cell lines (NMFH-1 and NMFH-2; Niigata University), two human synovial sarcoma cell lines (HS-SY-II; Dr H. Sonobe, Department of Pathology, Kochi Medical School, Kochi, Japan, and SYO-1;

Dr T. Ozaki, Okayama University), a human alveolar soft part sarcoma cell line (ASPS-KY; Dr S. Yanoma, Kanagawa Cancer Center, Yokohama, Japan), a human myxoid type liposarcoma cell line (402-92; Dr P. Åman, Department of Clinical Genetics, University Hospital, Lund, Sweden), a human malignant peripheral nerve sheath tumor cell line (NMS2; Niigata University), 3 human epithelioid sarcoma cell lines (SFT8606; Dr H. Iwasaki, Fukuoka University School of Medicine, Fukuoka, Japan, NEPS; from a patient with epithelioid sarcoma, and FU-EPS-1; Dr H. Iwasaki), and a human Ewing's/primitive neuroectodermal tumor (PNET) cell line (SKNMC; American Type Culture Collection, Bethesda, MD, USA) were incubated at 37°C in an atmosphere containing 5% CO₂ in culture media supplemented with 10% FBS, 0.6% Kanamycin Sulfate (Gibco, Invitrogen), and 1% Antibiotic-Antimycotic (Gibco, Invitrogen).

The NMFH-1, NMFH-2, HS-SY-II, ASPS-KY, 402-92, SFT8606, NEPS, FU-EPS-1, and NMS2 cells were cultured in RPMI-1640 culture medium (Gibco, Invitrogen), and the HT1080 and SKNMC cells were cultured in MEM- α medium (Gibco, Invitrogen); SYO-1 cells was cultured in medium DMEM (Gibco, Invitrogen). The medium was replaced twice a week. Culture cells were harvested for evaluation by quantitative real-time RT-PCR.

Quantitative real-time PCR. Total RNA was extracted from the frozen tumor tissue that has been obtained from 41 various soft tissue tumor patients, consisting of 3 cases of pleomorphic malignant fibrous histiocytomas, 4 myxoid malignant fibrous histiocytomas, 5 myxoid liposarcomas, 4 well differentiated liposarcomas, 1 case of dedifferentiated liposarcoma, 3 cases of malignant peripheral nerve sheath tumors, 2 synovial sarcomas, 2 dermatofibrosarcomas, 2 alveolar soft part sarcomas, 1 case each of rhabdomyosarcoma, leiomyosarcoma, fibrosarcoma, extraskeletal chondrosarcoma, epithelioid sarcoma, extraskeletal Ewing's sarcoma, 3 schwannomas, 4 lipomas, 1 fibroma and 1 case of pigmented villonodular synovitis, as well as soft tissue tumor-derived cell lines using Isogen (Nippongene, Toyama, Japan).

RNA was purified by standard techniques. The initial cDNA strand was synthesized using the PrimeScript[®] RT reagent kit (Takara, Shiga, Japan) according to the manufacturer's protocol. In brief, 0.5 μ g of total RNA was reverse-transcribed at 37°C for 15 min in 10 μ l of 1X Prime Script buffer containing PrimeScript RT enzyme mix I, Random 6 mers (100 μ M) and Oligo dTPrimers (50 μ M), and RT enzyme was deactivated at 85°C for 5 sec. We performed PCR using the primers for podoplanin sequence. Total RNA from a normal human adult kidney (BioChain Institute, Hayward, USA) was used for calibration of the soft tissue tumors and their cell lines. Real-time PCR was performed using the Thermal Cycler Dice[®] Real Time system (Takara) with SYBR[®] Premix Ex Taq[®]/MTM II (Takara) following the manufacturer's instructions. Briefly, real-time PCR reactions were performed using the following program: 10 sec at 94°C, 40 cycles of 5 sec at 95°C and 30 sec at 60°C, and finally 15 sec at 95°C, 30 sec at 60°C and 15 sec at 95°C for dissociation. The results for each sample were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The data were analyzed using the software program provided by Takara. The results were

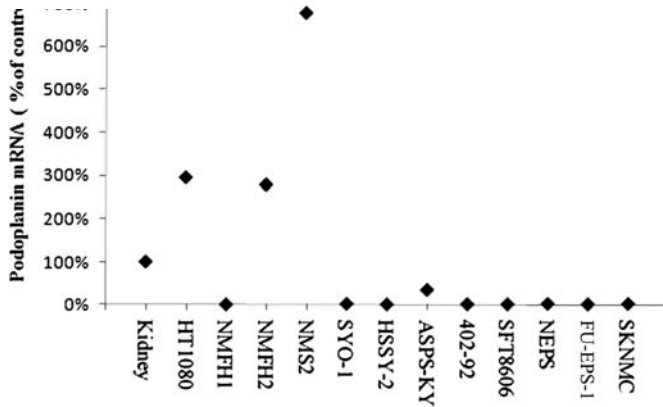


Figure 1. The expression of podoplanin mRNA in 12 kinds of soft tissue tumor cell lines, including a fibrosarcoma cell line (HT1080), 2 kinds of malignant fibrous histiocytomas (MFH) cell lines (NMFH1 and NMFH2), a malignant peripheral nerve sheath tumor cell line (NMS2), 2 kinds of synovial sarcoma cell lines (HS-SY-II and SYO-1), a alveolar soft part sarcoma cell line (ASPS-KY), a myxoid liposarcoma cell line (402-92), 3 kinds of epithelioid sarcoma cell lines (SFT8606, NEPS and FU-EPS-1), a Ewing's sarcoma/primitive neuroectodermal tumor (PNET) cell line (SKNMC). The normal human kidney was used for calibration. The results for each sample were normalized by the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal standard. Podoplanin mRNA was expressed at higher levels in HT1080, NMFH2 and NMS2 than in control kidney specimen, whereas podoplanin was expressed at lower levels in other kinds of cell lines than in kidney.

expressed as normalized ratios. The Primers for podoplanin and GAPDH (Table III) were designed and validated using a Perfect Real-time Primer Support System (Takara).

Immunohistochemical analysis. Immunohistochemical analysis of podoplanin in soft tissue tumors was performed. Tissue sections (4 μ m) from paraffin-embedded soft tissue tumor specimens were mounted on APS coated glass slides. In brief, the tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Slides were steamed for antigen retrieval with 10 mM citrate buffer at pH 6.0 (Dako Cytomation, Carpinteria, CA) at 85-100°C for 40 min. Part of the samples were treated with 0.2% trypsin solution at 37°C for 20 min for antigen retrieval to avoid damage and exfoliation of the samples by the hot steam.

After cooling for 15 min, the slides were immersed in methanol containing 0.3% hydrogen peroxide for 20 min at room temperature to block the endogenous peroxidase activity, and then were incubated with phosphate buffered saline containing 10% goat serum (Dako, Denmark) for 20 min at room temperature to reduce nonspecific reactions. The slides were then incubated with the monoclonal mouse antibody (D2-40, Dako) against human podoplanin at a 1:100 dilution at 4°C overnight, followed by reacting them with a universal immunoperoxidase polymer, anti-mouse and anti-rabbit (Histofine Simple Stain MAX PO (MULTI); Nichirei, Tokyo, Japan) antibodies for 30 min at room temperature. The peroxidase activity was detected with 3'-diaminobenzidine tetrahydrochloride (Nichirei). The slides were counterstained with hematoxylin (Vector Laboratories, Inc. Burlingame, CA).

For histological scoring, representative areas of each tissue section were selected and evaluated independently by 2 investigators, who were blinded to the clinical information pertaining to the subjects. The immunostaining was graded on a sliding scale of 0 to 3+ according to the percentage of reactive cells as follows: 0, no staining, 1+, 0-10% cells stained with weak intensity, 2+, 10-40% cells stained with variable intensity, 3+, >40% of cells stained with strong intensity.

Results

The expression of podoplanin mRNA in soft tissue tumor cell lines. To quantify the expression of podoplanin mRNA in human soft tissue tumor cell lines, we performed quantitative real-time PCR analyses of 12 soft tissue tumor cell lines. The relative podoplanin mRNA expression levels of each tumor are shown in Fig. 1. Podoplanin transcript levels in the HT1080, NMFH-2 and NMS2 tumor cell lines were significantly higher than in the control kidney specimen. In contrast, the podoplanin transcript levels in the other tumor cell lines were lower than in the kidney.

The expression of podoplanin mRNA in soft tissue tumors. To quantify the expression of podoplanin mRNA in human soft tissue tumors, we performed quantitative real-time PCR analyses of soft tissue tumors from 42 patients. The relative podoplanin mRNA expression levels of each tumor are shown in Figs. 2 and 3. Podoplanin transcript levels in a wide range of benign and malignant soft tissue tumors, as well as tumor-like lesions were significantly higher than in the control kidney specimen. Only in primitive neuroectodermal tumor, alveolar soft part sarcomas, lipomas, some liposarcomas (well differentiated type), was podoplanin mRNA expressed at a lower level.

Immunohistochemical analysis of podoplanin in malignant soft tissue tumors. High-level podoplanin expression was observed in various types of malignant soft tissue tumors when the anti-podoplanin antibody (D2-40) was used, especially in spindle cell sarcoma, myxoid type and the nervous system soft tissue tumors, whereas round cell sarcoma exhibited no reactivity for D2-40 and was completely negative for podoplanin. The immunohistochemical results are summarized in Table I and Fig. 4. Podoplanin immunoreactivity was detected in 17 of 20 (85%) tumors. In four of the cases of undifferentiated high grade pleomorphic sarcomas (pleomorphic malignant fibrous histiocytomas); the staining was graded as 3+, 2+ in six cases, and as 1+ in the other seven of these.

Pleomorphic malignant fibrous histiocytomas showed membranous immunoreactivity for D2-40. In 3 cases, there was no detectable podoplanin expression. Immunoreactivity to D2-40 was observed in all of the samples of myxofibrosarcoma (10/10, 100%). In 5 of these cases, the staining was graded as 3+, in 2 as 2+, and in 3 as 1+. The reaction with the D2-40 antibody was characterized by a strong, thick membranous staining pattern. Among liposarcomas, the well differentiated types showed weak immunoreactivity to D2-40, and only 1 of 8 (12.5%) was considered positive. The

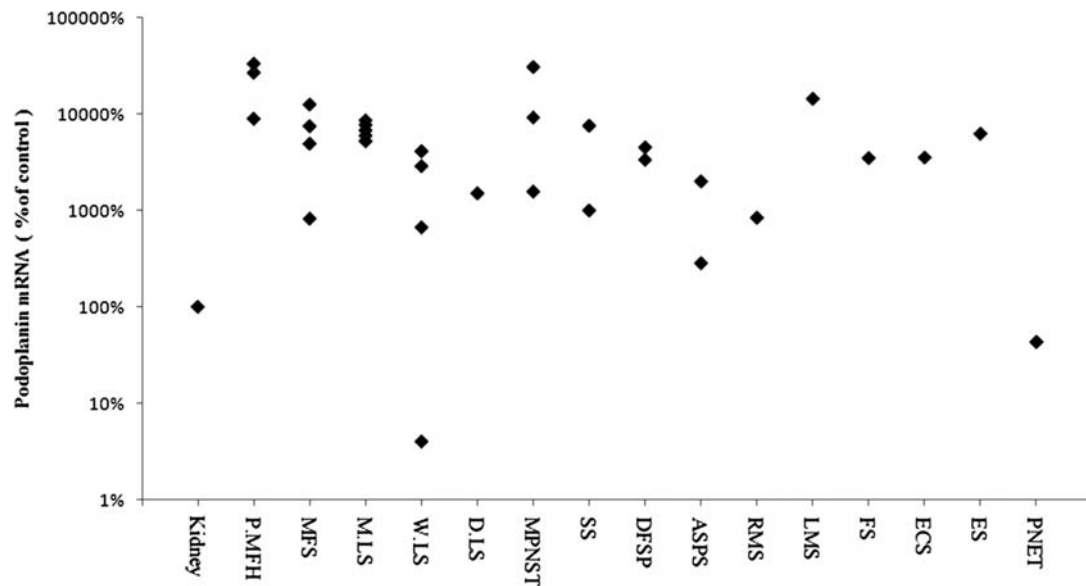


Figure 2. The expression of podoplanin mRNA in 15 kinds of malignant soft tissue tumors. The normal human kidney was used for calibration. The results for each sample were normalized by the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal standard. The expression of podoplanin mRNA in 3 undifferentiated high grade pleomorphic sarcomas (pleomorphic malignant fibrous histiocytomas) and 4 myxofibrosarcomas. Podoplanin mRNA was expressed at higher levels in all 3 pleomorphic MFH and 4 myxofibrosarcomas than in kidney. P.MFH, pleomorphic malignant fibrous histiocytoma; MFS, myxofibrosarcoma. The expression of podoplanin mRNA in 5 myxoid liposarcomas, 4 well differentiated liposarcomas and in 1 case of dedifferentiated liposarcoma. Podoplanin mRNA was expressed at higher levels in almost all the cases than in kidney except for W.LS4, which expressed podoplanin at lower level than in kidney. M.LS, myxoid liposarcoma; W.LS, well differentiated liposarcoma; D.LS, dedifferentiated liposarcoma. The expression of podoplanin mRNA in 3 malignant peripheral nerve sheath tumors, 2 biphasic synovial sarcomas, 2 dermatofibrosarcoma protuberans and 2 cases alveolar soft part sarcomas. Podoplanin mRNA was expressed at higher levels in all the cases than in kidney. MPNST, malignant peripheral nerve sheath tumors; SS, biphasic synovial sarcoma; DFSP, dermatofibrosarcoma protuberans; ASPS, alveolar soft part sarcoma. The expression of podoplanin mRNA in 1 rhabdomyosarcoma, 1 leiomyosarcoma, 1 fibrosarcoma, 1 extraskeletal chondrosarcoma, 1 epithelioid sarcoma and 1 case of extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumor. Podoplanin mRNA was expressed at higher levels in almost all the cases than in kidney except for PNET, which expressed podoplanin at lower level than in kidney. RMS, Rhabdomyosarcoma; LMS, leiomyosarcoma; FS, fibrosarcoma; ECS, extraskeletal chondrosarcoma; ES, epithelioid sarcoma; PNET, extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumor.

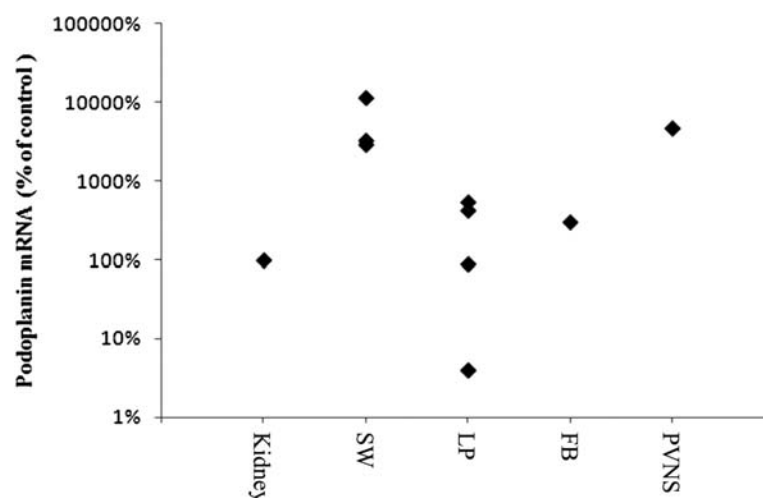


Figure 3. The expression of podoplanin mRNA in 3 types of benign soft tissue tumors and a tumor-like lesion. The normal human kidney was used for calibration. The results for each sample were normalized by the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal standard. Podoplanin mRNA was expressed at higher levels in 3 schwannomas, 2 lipomas, 1 fibroma and 1 pigmented villonodular synovitis than in kidney, whereas podoplanin was expressed at lower levels in 2 lipomas (LP1 and LP4) than in kidney. SW, schwannoma; LP, lipoma; FB, fibroma; PVNS, pigmented villonodular synovitis.

staining was graded as 1+ and the reaction was characterized by a dot-like membranous staining pattern.

In contrast, myxoid types showed strong immunoreactivity to D2-40, and 18 of 19 (94.7%) tumors exhibited

positive reactivity. The staining was graded in 11 cases as 3+, in 5 as 2+, in 2 as 1+. The reaction with the D2-40 antibody was characterized by a strong, thick membranous staining pattern. Podoplanin immunoreactivity was detected



Tumor type	No. of cases	Grade of reactivity ^a				No. (%) of positive cases	Average grade
		0	1+	2+	3+		
Malignant fibrous histiocytoma							
Undifferentiated high grade pleomorphic sarcoma (Pleomorphic MFH)	20	3	7	6	4	17 (85)	1.55
Myxofibrosarcoma	10	0	3	2	5	10 (100)	2.20
Liposarcoma							
Well differentiated	8	7	1	0	0	1 (12.5)	0.125
Myxoid	19	1	2	5	11	18 (94.7)	2.37
Dedifferentiated	4	0	1	3	0	4 (100)	1.75
Synovial sarcoma							
Biphasic	4	0	1	0	3	4 (100)	2.50
Rhabdomyosarcoma							
Pleomorphic	3	0	1	2	0	3 (100)	1.67
Embryonal	1	1	0	0	0	0 (0)	0.00
Alveolar	3	3	0	0	0	0 (0)	0.00
Malignant peripheral nerve sheath tumor	9	0	4	4	1	9 (100)	1.67
Leiomyosarcoma	3	0	1	0	2	3 (100)	2.33
Fibrosarcoma	3	0	1	0	2	3 (100)	2.33
Extraskeletal chondrosarcoma	1	0	0	1	0	1 (100)	2.00
Alveolar soft part sarcoma	3	3	0	0	0	0 (0)	0.00
Epithelioid sarcoma	4	3	1	0	0	1 (25)	0.25
Extraskeletal Ewing's sarcoma/ peripheral primitive neuroectodermal tumor	4	2	0	1	1	0 (0)	0.00
Dermatofibrosarcoma protuberans	4	2	0	1	1	2 (50)	1.25

^a0, no staining; 1+, 0-10% tumor cell reactive; 2+, 10-40% tumor cell reactive; 3+, >40% tumor cell reactive.

in 4 of 4 (100%) of the dedifferentiated type tumors. In 3 of these cases, the staining was graded as 2+, and in 1 as 1+. The reaction was characterized by a strong, thick staining pattern in dedifferentiated areas. Podoplanin immunoreactivity was detected in 4 of 4 (100%) biphasic synovial sarcomas; the staining was graded in 3 cases as 3+, and in 1 as 1+. The reaction showed a dot-like, diffuse membranous staining pattern. Three of three pleomorphic rhabdomyosarcomas (100%) showed positive reactivity for the D2-40 antibody. The staining was graded 2+ in 2 cases, and in 1 case as 1+. The reaction was characterized by a diffuse, dot-like membranous staining pattern, whereas three of the alveolar and one embryonal rhabdomyosarcoma were completely negative for D2-40.

Podoplanin immunoreactivity was detected in 9 of 9 (100%) malignant peripheral nerve sheath tumors. The staining was graded in 1 case as 3+, in 4 as 2+, and in 4 as 1+. The reaction showed a diffuse, dot-like membranous staining pattern. Three of three leiomyosarcomas (100%) showed positive reactivity for D2-40. The staining was graded

in 2 cases as 3+, and in 1 as 1+. The reaction was characterized by a strong, diffuse, ring-like staining pattern. Among the fibrosarcomas, 2 of 2 adult types and 1 of 1 sclerosing epithelioid tumor showed positive reactivity for D2-40. The staining of adult types was graded in 1 case as 3+, and in 1 as 1+, and the sclerosing epithelioid type was graded in 1 case as 3+. The reaction of sclerosing epithelioid type was characterized by a strong, diffuse cytoplasmic staining pattern. Podoplanin immunoreactivity was detected in 1 case of extraskeletal chondrosarcoma. The staining was graded as 2+ and showed a discontinuous membranous staining pattern.

One of four epithelioid sarcomas (25%) exhibited positive reactivity for D2-40. The staining was graded in one cases as 1+, and as 0 in the other three of the tumors. The reaction was characterized by a dot- and ring-like staining pattern for D2-40. Two of the four dermatofibrosarcoma protuberans (50%) showed positive reactivity for D2-40, and the staining was graded in 1 case as 3+, and in the other case as 2+. The reaction was characterized by a strong, diffuse and ring-like pattern for D2-40. None of the alveolar soft part sarcomas,

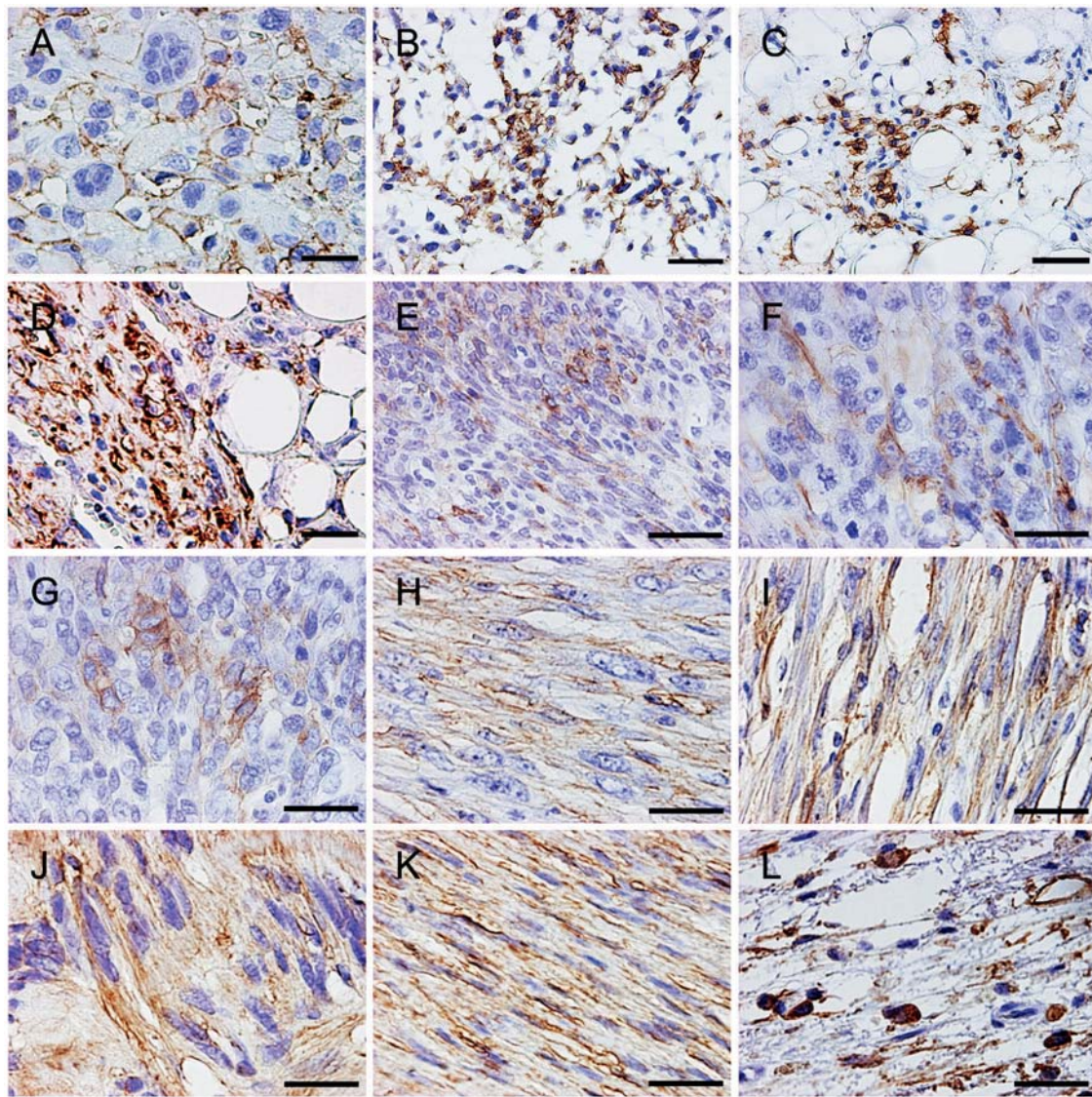


Figure 4. Strong podoplanin immunoreactivity in malignant and benign soft tissue tumors. Podoplanin expression was determined by the anti-podoplanin antibody D2-40. (A) Pleomorphic malignant fibrous histiocytomas showed membranous immunoreactivity for D2-40. (B) Myxofibrosarcomas showed strong, thick membranous staining pattern. (C) Myxoid liposarcomas showed strong, thick membranous staining pattern. (D) Dedifferentiated liposarcoma showed strong, thick staining pattern, especially in dedifferentiated areas. (E) Biphasic synovial sarcomas showed dot-like, diffuse membranous staining pattern. (F) Pleomorphic rhabdomyosarcomas showed diffuse, dot-like membranous staining pattern. (G) Malignant peripheral nerve sheath tumors showed diffuse, dot-like membranous staining pattern. (H) Leiomyosarcomas showed strong, diffuse, ring-like staining pattern. (I) Fibrosarcoma showed a strong, diffuse cytoplasmic staining pattern. (J) Schwannoma were a strong, diffuse, ring-like cytoplasmic staining pattern. (K) Fibroma showed a strong, diffuse, ring-like staining pattern. (L) Myxoma showed a strong, diffuse, ring-like cytoplasmic staining pattern for D2-40. Bar, (A-D) 100 μ m; (E-L) 50 μ m.

embryonal and alveolar rhabdomyosarcomas, nor the extra-skeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumors (PNET) exhibited reactivity for D2-40, and all were completely negative for podoplanin.

Immunohistochemical analysis of podoplanin in benign soft tissue tumors and tumor-like lesions. The immunohistochemical results are summarized in Table II and Fig. 5. Two of three cases of myxoma showed positive reactivity for D2-40, and staining was graded in these 2 cases as 3+. The reaction showed a strong, diffuse, ring-like cytoplasmic staining pattern for D2-40. Four of four (100%) schwannomas exhibited positive reactivity for D2-40. The staining was graded in 3 cases as 3+, and in 1 case as 2+. The reaction was characterized by a strong, diffuse, ring-like cytoplasmic

staining pattern. Two of three cases (66.7%) of fibromas showed positive reactivity for D2-40. The staining was graded in 1 case as 3+, and in 1 case as 1+, and showed a strong, diffuse, ring-like staining pattern. The lipoma exhibited no reactivity for D2-40, indicating that it was completely negative for podoplanin. Among the tumor-like lesions, 1 case of pigmented villonodular synovitis (PVNS) showed positive reactivity, and the staining was graded as 3+. The reaction exhibited discontinuous, and a diffuse membranous staining pattern was observed. The ganglion tumors and chronic expand expanding hematoma exhibited positive reactivity for D2-40. The staining of the ganglion tumors was graded as 3+ in one case, and as 2+ in the other two, and similar staining patterns were present in chronic expand expanding hematoma.



Tumor and tumor-like lesion type	No. of cases	Grade of reactivity ^a				No. (%) of positive cases	Average grade
		0	1+	2+	3+		
Lipoma	6	6	0	0	0	0 (0)	0.00
Schwannoma	4	0	0	1	3	4 (100)	2.75
Fibroma	3	1	1	0	1	2 (66.7)	1.33
Myxoma	3	1	0	0	2	2 (66.7)	2.00
Pigmented villonodular synovitis	1	0	0	0	1	1 (100)	3.00
Ganglion	3	0	0	2	1	3 (100)	2.33
Chronic expanding hematoma	3	0	0	2	1	3 (100)	2.33

^a0, no staining; 1+, 0-10% tumor cell reactive; 2+, 10-40% tumor cell reactive; 3+, >40% tumor cell reactive.

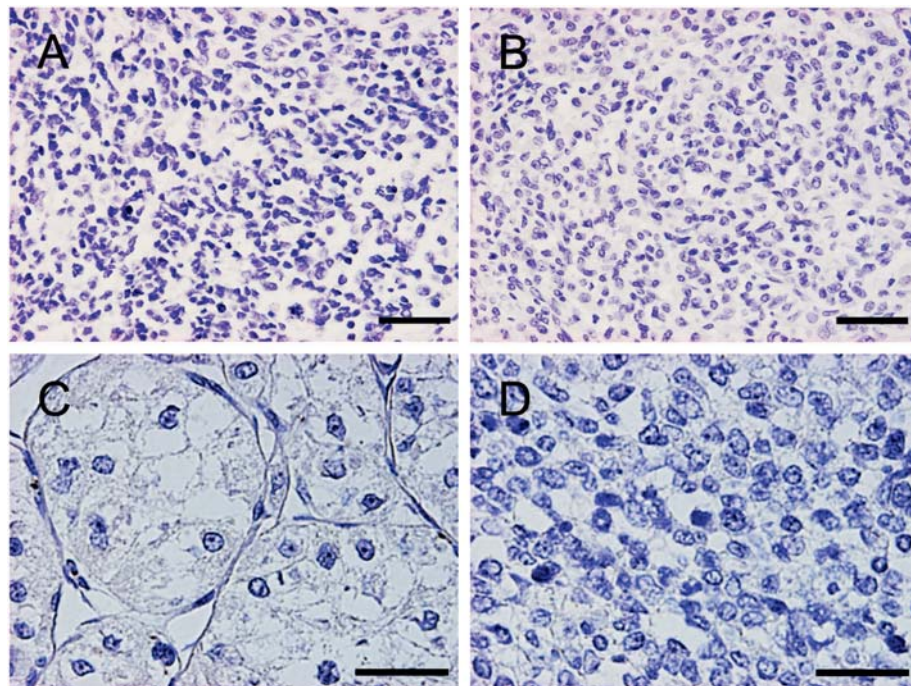


Figure 5. No podoplanin immunoreactivity in soft tissue sarcomas. Podoplanin expression was determined by the anti-podoplanin antibody D2-40. (A) Alveolar rhabdomyosarcomas, (B) embryonal rhabdomyosarcoma, (C) alveolar soft part sarcomas, (D) extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumor (PNET). None of (A-C), nor (D) exhibited reactivity for D2-40, and all were completely negative for podoplanin. Bar, (A and B) 100 μ m; (C and D) 50 μ m.

Discussion

Jokinen *et al* (39) reported that 16 cases of 21 (76%) schwannomas showed strong and diffuse cytoplasmic positive reactivity for D2-40, and 3 of 4 cases (75%) of epithelioid malignant peripheral nerve sheath tumors showed a strong, diffuse membranous staining pattern for D2-40, which were similar to our immunohistochemical staining results in the same tumor types. Ordóñez *et al* (14) reported that 4 of 6 biphasic synovial sarcoma (66.7%) showed positive reactivity for podoplanin in the epithelioid component of the tumor, which was also similar to our immunohistochemical staining results in the same type of tumors. Although podoplanin expression has been detected in various human

tumors, the expression of podoplanin in the majority of benign and malignant soft tissue tumors has not been well examined, except for schwannoma, epithelioid malignant peripheral nerve sheath tumors, and biphasic synovial sarcoma.

In the present study, immunohistochemical and quantitative real-time RT-PCR analyse demonstrated that there is expression of podoplanin at the protein and mRNA levels in the majority of benign and malignant soft tissue tumors and tumor-like lesions. Furthermore, it seemed that podoplanin immunoreactivity showed a very high correlation with cell morphology in soft tissue sarcoma. Spindle cell soft tissue sarcoma exhibited relatively strong immunoreactivity for D2-40 and was positive for podoplanin. We found that in 10 (100%) of 10 myxofibrosarcomas, in 18 (94.7%) of 19

Table III. Primers used for quantitative real-time polymerase chain reaction.

Name	Sequence	Primer ID	GenBank accession no.
Podoplanin	5'-TGA CTCCAGGAACCAGCGAAG-3'	HA071355-F	NM_006474
	5'-GCCAATGCCTGTTACACTGTTGA-3'	HA071335-R	
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	HA067812-F	NM_002046
	5'-TGGTGAAGACGCCAGTGGA-3'	HA067812-R	

GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

myxoid liposarcomas, in 4 (100%) of 4 dedifferentiated liposarcomas, in 4 (100%) of 4 biphasic synovial sarcomas, in 3 (100%) of 3 leiomyosarcomas, in 3 (100%) of 3 pleomorphic rhabdomyosarcomas, in 9 (100%) of 9 malignant peripheral nerve sheath tumors, and in 3 (100%) of 3 fibrosarcomas, there was widespread immunoreactivity for podoplanin with variable levels of intensity, frequency and staining patterns in most of the malignant soft tissue tumor types. In contrast, there was only limited and weak staining in 1 (12.5%) of 8 well differentiated liposarcomas and in 2 (50%) of 4 dermatofibrosarcoma protuberans. No immunoreactivity was seen in any of the 3 alveolar soft part sarcomas, the 3 extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumors, the 1 embryonal rhabdomyosarcoma or any of the 3 alveolar rhabdomyosarcomas, which demonstrated that round cell soft tissue sarcomas exhibited no immunoreactivity for podoplanin. Similar staining patterns were seen in benign soft tissue tumors and tumor-like lesions.

There was positive reactivity to the D2-40 antibody in 2 (66.7%) of 3 myxomas, in 4 (100%) of 4 schwannoma, in 2 (66.7%) of 3 fibroma. Strong, diffuse expression of podoplanin protein has been also found in all tumor-like lesions, including the 1 pigmented villonodular synovitis, 3 ganglion tumors and the 3 chronic expanding hematomas. It has been reported that podoplanin induces an actin cytoskeleton rearrangement by increasing the epithelial-mesenchymal transition (EMT) based on the binding activity with plasma membrane protein ERM (ezrin, radixin and moesin) to activate RhoA. This series of events in podoplanin-positive cancer cells may facilitate EMT, which plays a role in the plasma membrane extension and actin cytoskeleton rearrangement (40-42).

In this study, quantitative real-time RT-PCR analyses confirmed that almost all of the tumor tissue that was examined contained podoplanin mRNA transcripts at a level higher than that in human kidney, except for 1 case of well differentiated liposarcoma, 1 case of extraskeletal Ewing's sarcoma/peripheral primitive neuroectodermal tumor, and 2 lipomas. The results are in accord with the ubiquitous expression of the podoplanin protein in the immunohistochemical staining results of the corresponding tumors. Our findings demonstrated that the majority of benign and malignant soft tissue tumors and tumor-like lesions express podoplanin mRNA at a relatively high level. Therefore, it seems that podoplanin expression cannot be used to distinguish benign and malignant soft tissue tumors.

Although most of the malignant soft tissue tumors were confirmed to have strong expression levels of both podoplanin

mRNA and protein, the majority of their corresponding soft tissue cell lines had lower expression of podoplanin mRNA. The expression of podoplanin in the HT1080 human fibrosarcoma cell line was reported at a low level unless the cells were stimulated with transforming growth factor- β , thus suggesting that podoplanin mRNA is translated to podoplanin protein by complex biological regulation (43,44).

High-level expression of podoplanin at the mRNA transcriptional level was detected in almost all of the different types of soft tissue sarcoma cells. Podoplanin mRNA might thus be detected in rare viable soft tissue tumors in the peripheral blood circulation. The use of reverse transcriptase-polymerase chain reaction (RT-PCR) for determination of the podoplanin mRNA level, and the real-time quantitative amount of podoplanin mRNA in the peripheral blood may reflect the size and dissemination of the tumor, and the level of the relative quantitative podoplanin mRNA may be reflect responses to treatment and prognosis of tumors, such as the likelihood of late recurrence, long-term survival, and can represent a rapid and precise method for determination of the response to chemotherapy or radiotherapy. Thus, the podoplanin mRNA RT-PCR assay of viable soft tissue tumor cells in the peripheral blood of cancer patients might be of some value as for follow-up and treatment monitoring of soft tissue sarcomas.

In conclusion, podoplanin is widely expressed at a high level in various benign and malignant soft tissue tumors, especially in spindle cell sarcoma, myxoid type and soft tissue tumors of the nervous system, whereas round cell sarcoma exhibited no reactivity for the D2-40 antibody and were completely negative for podoplanin. We did not find any valuable tissue-specificity for the differential diagnosis of soft tissue tumors. Thus, our results suggest that podoplanin has little value in the diagnosis of soft tissue tumors. However, evaluation of the podoplanin mRNA of circulating soft tissue tumor cells might be of some value for follow-up and monitoring the treatment response of soft tissue sarcomas.

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