BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Identification of Coiled-Coil Domain—Containing Protein 180 and Leucine-Rich Repeat—Containing Protein 4 as Potential Immunohistochemical Markers for Liposarcoma Based on Proteomic Analysis Using Formalin-Fixed, Paraffin-Embedded Tissue

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Recent technical improvements in both mass spectrometry and protein extraction have made it possible to use formalin-fixed, paraffin-embedded (FFPE) tissues for proteome analysis. In this study, comparable proteome analysis of FFPE tissues revealed multiple candidate marker molecules for differentiating atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL) from lipoma. A total of 181 unique proteins were identified for ALT/WDL. Of the identified proteins, coiled-coil domain—containing protein 180 (CCDC180) and leucine-rich repeat—containing protein 4 (LRRC4) were studied as candidate markers of ALT/WDL. CCDC180 and LRRC4 immunohistochemistry clearly stained tumor cells of ALT/WDL and dedifferentiated liposarcoma and could differentiate them from lipoma with high accuracy. Cell biological methods were used to further examine the expression of the candidate marker molecules in liposarcoma cells. In liposarcoma cells, knockdown of CCDC180 and LRRC4 inhibited cell proliferation. CCDC180 inhibited cell migration, invasion, and apoptosis resistance in WDL cells. Adipogenic differentiation suppressed the expression of CCDC180 and LRRC4 in WDL cells. These results indicated that LRRC4 and CCDC180 are novel immunohistochemical markers for differentiating ALT/WDLs. Their expression was associated with adipocyte differentiation and contributed to malignant potentials of WDL cells. Proteome analysis using a standard stock of FFPE tissues can reveal novel biomarkers for various diseases, which contributes to the progress of molecular pathology. (*Am J Pathol* 2019, 189: 1015–1028; <https://doi.org/10.1016/j.ajpath.2019.01.013>)

Proteomics is the large-scale analysis of a proteome, a data set of total proteins in a biological system including their amount, cellular structure, and function. Every alteration of proteins directly reflects some biological or clinical events and can become an important factor of pathologic abnormality. Recently, mass spectrometry (MS)—based proteomics has been used to discover biomarkers for a variety of diseases as well as to elucidate molecular mechanisms.^{1–5} In comparison with mRNA-based analytical methods, MS-based proteomics

has the advantage of enabling discovery of immunohistochemical markers because immunohistochemistry is based on the detection of proteins.⁶

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Formalin-fixed, paraffin-embedded (FFPE) tissues are archived in a hospital to be generally prepared for histopathological examination with hematoxylin and eosin staining and immunohistochemistry. FFPE tissues can be stored for a long period, resulting from rigid cross-linking of molecular components by formaldehyde. Long-term preserved FFPE tissues are the best source for retrospective studies because they are stocked along with detailed information about patients, including clinicopathological features, blood test results, outcome, response to therapy, and adverse events.^{7,8} On the other hand, the preservative advantage has inversely prevented FFPE tissues from being used for some biochemical analyses other than immunohistochemistry because of the difficulty in extraction of a sample with high quality. However, recent technical improvements in mass spectrometry and protein extraction have made it possible to use FFPE tissues for proteome analysis,^{9–11} resulting in identification of novel biomarkers for pancreatic cancer,^{12,13} rheumatoid arthritis,¹⁴ and colorectal cancer.¹⁵

Lipomatous tumors represent the largest single group of mesenchymal tumors, including benign-type lipoma and malignant liposarcoma.¹⁶ Among the liposarcomas, atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL) represents the largest subgroup and is similar to benign lipoma in histologic appearance. In clinical pathology, it is important to differentiate ALT/WDL from lipoma because ALT/WDL can proceed on to dedifferentiation, a critical risk factor for distant metastasis. Genomic PCR and fluorescence *in situ* hybridization, for detecting amplification of chromosome 12q13-q15 in ALT/WDL, are helpful for differential diagnosis, although those analyses are not appropriate for clinical practice everywhere. Overexpressions of E3-ubiquitin–protein ligase Mdm2 (MDM2), cyclin-dependent kinase 4 (CDK4), and high-mobility group protein, which result from 12q13-q15 amplification, are also used as ALT/WDL markers in

immunohistochemistry.^{16–22} However, immunohistochemistry of the molecules has limited applicability to practical use because of the small number of immunopositive cells in specimens. Therefore, useful immunohistochemical markers are required to distinguish ALT/WDL from lipoma.

In this study, comparable proteome analysis of FFPE tissues revealed multiple candidate marker molecules for differentiating ALT/WDL from lipoma. It was also confirmed that identified molecules, coiled-coil domain–containing protein 180 (CCDC180) and leucine-rich repeat–containing protein 4 (LRR4), were useful in immunohistochemistry. In addition, it was demonstrated that they contributed to malignant roles of WDL cells and that they were up-regulated in association with dedifferentiation.

Materials and Methods

Materials

Dithiothreitol, iodoacetamide, ammonium bicarbonate, acetonitrile, ethanol, guanidine hydrochloride (guanidine-HCl), hydrochloric acid, formic acid, dexamethasone, and cisplatin were purchased from Wako (Osaka, Japan). Heptane, methanol, acetic acid, and chloroform were purchased from Kanto Chemical (Tokyo, Japan). 3-Isobutyl-1-methylxanthine, oleic acid, trifluoroacetic acid (TFA), and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). The Qproteome FFPE tissue kit was purchased from Qiagen (Hilden, Germany). Antibodies used in this study are listed in Table 1.

Patient Samples

The present research protocol was approved by the Sapporo Medical University Ethics Committee (Sapporo, Japan), and informed consent was obtained from all participating

Table 1 List of Antibodies Used in This Study

Antibody	Clonality	Species	Supplier	Dilution	Application
Anti-C9orf174 (CCDC180)	Polyclonal	Rabbit	Abcam (Cambridge, UK)	1:1000	WB
Anti-netrin G2 ligand (LRR4)	Monoclonal	Mouse	Abcam	1:1000	WB
Anti-FABP4	Polyclonal	Rabbit	Abcam	1:1000	WB
Anti-actin	Polyclonal	Rabbit	Sigma-Aldrich	1:3000	WB
Anti-CCDC180	Polyclonal	Rabbit	Atlas Antibodies (Stockholm, Sweden)	1:50	IHC
Anti-LRR4	Polyclonal	Rabbit	Atlas Antibodies	1:200	IHC
Anti-chymase	Monoclonal	Mouse	Thermo Fisher Scientific (Waltham, MA)	1:4000	IHC
Anti-MDM2	Monoclonal	Mouse	Calbiochem (La Jolla, CA)	1:50	IHC
Anti-CDK4	Monoclonal	Mouse	Invitrogen (Carlsbad, CA)	1:50	IHC
Anti-Ki-67	Polyclonal	Rabbit	Cell Signaling Technology, Inc. (Beverly, MA)	1:100	IHC
Anti-cleaved caspase-3	Polyclonal	Rabbit	Cell Signaling Technology, Inc.	1:100	IHC
Anti-cleaved PARP	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:200	IHC

CDK4, cyclin-dependent kinase 4; FABP4, fatty acid–binding protein 4; IHC, immunohistochemistry; MDM2, E3-ubiquitin–protein ligase Mdm2; PARP, poly (ADP-ribose) polymerase; WB, Western blot analysis.

patients. The tissues used in this study were from 47 patients who underwent surgical resection for ALT/WDL, dedifferentiated liposarcoma (DDL), or lipoma at Sapporo Medical University Hospital (Sapporo, Japan) during the period from 2006 to 2017. FFPE tissue blocks from 22 cases of ALT/WDL, 18 cases of DDL, and 25 cases of lipoma were obtained from the pathology file of Sapporo Medical University Hospital. Clinicopathological features of the patients are summarized in [Supplemental Table S1](#). After detailed histopathological analysis, 4 of 22 ALT/WDL cases and 4 of 25 lipoma cases were selected for downstream peptide sample preparation.

Peptide Sample Preparation

Peptide sample preparation was performed, as described before, with some modifications.^{15,23–25} FFPE tissue blocks were divided into sections on a microtome to eight serial sections (10 to 15 μm thick and approximately 100 mm^2 in size). Each of the FFPE tissue sections was deparaffinized by 0.5 mL heptane in a 2.0-mL microtube at room temperature for 90 minutes. After adding 25 μL of methanol, the tissue was collected by centrifugation. The supernatant was removed, and the pellet was left to dry in air at room temperature. Subsequent protein extraction was performed by using a Qproteome FFPE tissue kit or the guanidine-HCl

method ([Figure 1](#)). In cases in which the Qproteome FFPE tissue kit was used, the air-dried tissue pellet was resuspended in 100 μL of extraction buffer, briefly sonicated, and incubated at 100°C for 20 minutes. It was then incubated in the tube at 80°C for 2 hours with agitation and centrifugation ($14,000 \times g$ for 15 minutes at 4°C). After cooling, the supernatant was collected in a new microtube. To purify the protein sample, 400 μL of methanol, 100 μL of chloroform, and 300 μL of water were added to each tube in turn, followed by vortex mixing for 10 seconds and centrifugation. After removal of the upper layer, the protein was formed as a white gel-like pellet on the bottom of the tube when centrifuged after adding 300 μL of methanol. After washing the pellet with ethanol and centrifugation, the pellet was dissolved in 20 μL of β -ME Sample Treatment (Cosmo Bio, Tokyo, Japan). The protein samples were applied to 4% to 20% gradient SDS polyacrylamide gel (Cosmo Bio) and electrophoresed to stack the protein into the gel until the dye front reached to 5 cm below the bottom of the sample-loaded well. The protein-stacked gels were excised, and the gels were further divided into approximately 1-mm square cubes. After reduction and alkylation, in-gel digestion was performed with trypsin overnight at 37°C, as described by Shevchenko et al.²⁶ In cases in which the guanidine-HCl method was used to extract proteins from the pellet, the dried material was resuspended in 250 μL of 6 mol/L guanidine-HCl/50 mmol/L ammonium

Figure 1 Comparable proteome analysis of atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL) and lipoma formalin-fixed, paraffin-embedded (FFPE) tissues. **A:** Experimental workflow. 1) FFPE tissue specimens were obtained, 2) eight serial sections (10 to 15 μm thick and approximately 100 mm^2 in size) from FFPE tissues were deparaffinized by heptane in a microtube, 3) subsequent protein extraction was performed by using a Qproteome FFPE tissue kit or guanidine-HCl method, 4) disulfide bonds were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide, and the sample was digested overnight with trypsin, 5) peptides were purified, and 6) matrix-assisted laser desorption/ionization—time-of-flight/time-of-flight (MALDI-TOF/TOF) analysis was performed. **B:** Venn map of proteins that were identified by mass spectrometry—based global proteomic analysis from ALT/WDL and lipoma of FFPE tissues (Unused ProtScore >0.15).

Table 2 List of siRNAs Used in This Study

Name of siRNA	Gene	Sequence of siRNA
siCCDC#1	<i>CCDC180</i>	5'-GCCAAUACUUCUUUGUGCGUGAAAU-3'
siCCDC#2	<i>CCDC180</i>	5'-GGUUGCUGACGAAUCUGCAAGUGAA-3'
siLRRC#1	<i>LRRC4</i>	5'-CUGGUUACCAGCCGGCAUA-3'
siLRRC#2	<i>LRRC4</i>	5'-GUAUAUCUCUGAGGGAGCU-3'

bicarbonate/20 mmol/L dithiothreitol (pH 8.5), briefly sonicated, and incubated at 100°C for 20 minutes and then incubated in the tube at 80°C for 2 hours with agitation. After cooling, the recovered proteins were quantified with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). Iodoacetamide was added to a final concentration of 40 mmol/L, and the sample was incubated at room temperature in the dark for 1 hour. The alkylation reaction was quenched by adding 3 µL of 2 mol/L dithiothreitol. For tryptic digestion, the sample was diluted (1:6) with 50 mmol/L ammonium bicarbonate (pH 8.1) to reduce the concentration of guanidine-HCl buffer (final concentration of 1 mol/L) and digested with trypsin overnight. After digestion, the mixture was acidified with TFA (final concentration of 0.1%). To remove interfering substances before mass spectrometry, the mixture was desalted with MonoSpin C18 (GL Sciences, Tokyo, Japan), according to the manufacturer’s instructions. Finally, peptides were eluted with 0.1% formic acid in 60% acetonitrile and stored at –20°C until analysis.

Mass Spectrometry

Peptides were subsequently separated on a HiQsilC18W-3 column (100 µm inner diameter × 100; KYA Technology, Tokyo, Japan), according to a previous report.²⁵ Elution solvent A and solvent B were 0.1% TFA and 0.1% TFA, respectively, in 70% acetonitrile. The gradient was 5% to 50% for solvent B over 50 minutes at a flow rate of 300 nL/minute. Separated peptides were spotted onto 384-well AB OptiTOF MALDI Plate Inserts (AB Sciex, Foster City, CA). The 150-nL peptide fractions were overlaid with 700 nL aCHCA (Sigma, Tokyo, Japan) in 80 mg/mL ammonium citrate, 70% acetonitrile, and 0.1% TFA. A 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex), with 4000 Series Explorer version 3.5 software (AB Sciex), was used for mass spectrometric analyses. The mass accuracy was calibrated by using tryptic bovine serum albumin standard (KYA Technology) and a six-peptide mixture (AB Sciex). MS/MS acquisitions of MS spectra with signal to noise ratio >100 were performed using air as the collision gas with a collision energy of 1 kV. Peptide tolerance was 100 ppm, and MS/MS tolerance was 1 Da. Data were processed by Protein Pilot version 3.0 (AB Sciex, Framingham, MA) using the Paragon search algorithm.²⁷ MS/MS data were searched against the human International Protein Index database version 3.63 (European Bioinformatics Institute, Cambridgeshire, UK). The Unused ProtScore reflects the total amount of unique peptide evidence related to a given protein.

Immunohistochemistry of Surgical Specimens

Specimens of 22 cases of ALT/WDL, 18 cases of DDL, and 25 cases of lipoma, obtained by surgical resections during the period from 2006 to 2017, were retrieved from the pathology file of Sapporo Medical University Hospital. The hematoxylin and eosin slides of all cases were reviewed to select representative sections. New sections from paraffin blocks were examined by the labeled polymer method. Immunohistochemical labeling was visualized using Dako REAL EnVision Detection System (Agilent Technologies, Inc., Santa Clara, CA), as per the manufacturer’s protocol.^{28–30} Antigen of CCDC180, LRRC4, chymase, MDM2, and CDK4 retrieval was performed by immersing sections in 10 mmol/L tris/1 mmol/L EDTA buffer (pH 9.0) or 10 mmol/L citrate buffer (pH 6.0) and boiling them in a microwave (95°C, 30 minutes), followed by overnight incubation at 4°C with antibodies to the following antigens: CCDC180, LRRC4, chymase, MDM2, and CDK4. The intensity of staining was assessed as strong (3+), moderate (2+), weak (1+), or negative (0). All slides were independently evaluated by two pathologists (A.T. and M.M.). Discordant cases were discussed, and a consensus was reached.³¹

Cell Line Culture and Treatment

The human liposarcoma cell lines 93T449 and SW872 were purchased from ATCC (Manassas, VA). 93T449 cells (derived from well-differentiated liposarcoma) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). SW872 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum. The medium for the cell lines contained 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were plated on 60-mm culture dishes (Corning Glass Works, Corning, NY) and incubated in a humidified 5% CO₂ incubator at 37°C. 93T449 cells were treated with 40 mmol/L cisplatin for 24 hours.

Induction of Adipocyte Differentiation of 93T449 Cells

One-day post-confluent 93T449 cells were treated with an adipogenic cocktail containing 0.5 mmol/L 3-isobutyl-1-methylxanthine and 0.25 µmol/L dexamethasone. After 4 days, the medium was replaced with fresh Dulbecco’s modified Eagle’s medium supplemented with 8% fetal bovine serum containing 100 µmol/L oleic acid, and the medium was

replaced every 2 to 3 days for 21 days. Cells were harvested for Western blot analysis or examined by using Oil Red O staining (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Cells in 60-mm culture dishes were washed with phosphate-buffered saline, fixed with 10% formalin neutral buffer solution for 5 minutes, incubated with 0.3% (w/v) Oil Red O in 60% (v/v) isopropanol for 30 minutes at room temperature, and washed with distilled water. Fat droplets in the adipogenesis-induced cells were evaluated with a phase-contrast microscope.³²

Gene Silencing Using siRNA

siRNA targeting the mRNA sequences of human *CCDC180* or *LRRC4* and siRNA universal negative control were purchased from Sigma-Aldrich. Transfection of siRNA was performed using RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's instructions. siRNAs used in this study are listed in Table 2.

Immunohistochemistry of Cell Block

Cell blocks were prepared by using the sodium alginate method.²⁸ Briefly, cells were collected in a 2.0-mL tube and washed with phosphate-buffered saline. Supernatant was discarded after centrifugation, and cells were fixed in 10% formalin solution for 12 to 24 hours at 4°C. Fixative was discarded after centrifugation, and aggregated cells were gently suspended in 1% sodium alginate solution (Wako Pure Chemical Industries, Ltd). Then, 1 mol/L CaCl_2 was added to form gel, and the fixed cell-containing gel was embedded in paraffin. Thin sections (4 μm thick) were cut from paraffin-embedded cell blocks and stained with hematoxylin and eosin. Deparaffinized sections were immersed in 10 mmol/L sodium citrate buffer (pH 6.0) and autoclaved for antigen retrieval. Primary antibodies were anti-Ki-67 or cleaved caspase-3. Immunohistochemical labeling was visualized using the EnVision system, as per the manufacturer's protocol.^{28,29}

RNA Isolation

RNA isolation and RT-PCR were performed, as described previously.³³ Primers used for RT-PCR of *CCDC180* and *LRRC4* are listed in Table 3.

Western Blot Analysis

Western blot analysis was performed, as described previously.³⁴ Immunoreactive bands were detected by the Clarity

Western electrochemiluminescence substrate system (Bio-Rad Laboratories, Hercules, CA) using AE-9300H EzCapture MG (ATTO, Tokyo, Japan). Densitometry was performed on immunoblot images using ImageJ software version 1.45s (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>).³⁵

Plate Colony-Forming Assay

Cells were seeded in 6-well plates at a density of 2500 cells per well. After incubation for 10 to 14 days, cells were washed with phosphate-buffered saline twice, fixed with methanol for 15 minutes, and stained with 0.04% crystal violet for 15 minutes at room temperature. The colony is defined to consist of at least 50 cells. Visible colonies were counted. Each experiment was independently repeated six times.³⁶

Cell Proliferation Assay

A WST-8 assay (Dojindo Laboratories, Kumamoto, Japan) was performed, as described previously.³⁷ Cells were seeded in 96-well plates, and its viability was assessed at 24 hours after incubation by using the Cell Counting Kit-8 (Dojindo Laboratories), according to the manufacturer's instructions. Absorbance at a wavelength of 450 nm was measured by using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories).

Cell Migration and Invasion Assay

A cell migration and invasion assay was performed, as described previously.³⁷ The migration assay was performed using Transwell (Corning Glass Works; 8- μm pore polycarbonate membrane insert) in 24-well dishes. Biocoat Matrigel (Corning Glass Works; pore size, 8- μm) was used to evaluate cell invasion potential. Migrated or invaded cells on the lower side of the filters were examined and counted under a microscope (Olympus, Tokyo, Japan).

Statistical Analysis

The measured values are presented as means $\pm$ SD. The statistical significance of differences was evaluated using the unpaired two-tailed *t*-test, the *U*-test, the Fisher exact test, and the Kruskal-Wallis test. Statistical significance was accepted when $P < 0.05$. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR

Table 3 List of Primers Used for RT-PCR

Gene	Forward primer	Reverse primer	Product size, bp
<i>CCDC180</i>	5'-AAGAAGGCTGTACCCAGTGC-3'	5'-TCTCCCAGAGGAGGGTCAAG-3'	192
<i>LRRC4</i>	5'-TTCCTTCCGAGATGGAAAGAGG-3'	5'-GCTGCACACAGAATCCACAC-3'	211
<i>GAPDH</i>	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'	452

is a modified version of R commander version 1.6-3,³⁸ designed to add statistical functions frequently used in biostatistics.

Results

Comparable Proteome Analysis of ALT/WDL and Lipoma FFPE Tissues

To discover useful molecular markers for differentiating ALT/WDL from lipoma, comparable proteome analysis of FFPE tissues was performed from patients with ALT/WDL and from patients with lipoma. First, protein samples were extracted from FFPE tissues of ALT/WDL (four cases) and lipoma (four cases) in two different ways (Figure 1A) to increase the number of proteins identified at the subsequent analytical step.³⁹ The protein samples were enzymatically processed into peptide fragments and then the peptide samples were examined by a matrix-assisted laser desorption/ionization—time-of-flight/time-of-flight analyzer to obtain mass spectra of the fragmented peptides. The obtained mass data of the peptides were separately analyzed by using ProteinPilot software version 3.0 (AB Sciex)²⁷ to identify which proteins existed in each of the 16 samples. By using a Qproteome extraction kit, a total of 605 proteins were identified, and 381 and 307 proteins were identified for the four ALT/WDL cases and four lipoma cases, respectively. In Unused ProtScore of >0.15, an indicator of experimental reliability in mass spectrometry, a total of 308 proteins were identified, and 207 and 160 proteins were identified for the four ALT/WDL cases and four lipoma cases, respectively. By using a guanidine-HCl extraction method, a total of 300 proteins were identified, and 171 and 182 proteins were

identified for the four ALT/WDL cases and four lipoma cases, respectively. In Unused ProtScore of >0.15, a total of 164 proteins were identified, and 100 and 106 proteins were identified for the four ALT/WDL cases and four lipoma cases, respectively. The identified protein data sets were finally integrated into two groups, ALT/WDL and lipoma, after eliminating duplicate proteins for the two methods.

A total of 408 proteins, including 181 proteins identified only for ALT/WDL (unique proteins for ALT/WDL), 142 proteins identified for lipoma (unique proteins for lipoma), and 85 proteins identified for both of them, were identified in Unused ProtScore of >0.15 (Figure 1B). Lipid droplet-associated proteins, including perilipin-1 and a lipid metabolism-associated protein, fatty acid-binding protein 4,^{40–43} were identified for both ALT/WDL and lipoma, indicating the validity of the current protocol. Total proteins identified in the comparable proteome analysis are listed in Supplemental Table S2.

In gene ontology (GO) classification by a web-based gene set analysis tool kit, WebGestalt version 2013 (<http://webgestalt.org>),⁴⁴ the identified proteins were distributed throughout the cell both in ALT/WDL and lipoma with similar tendencies, indicating that there are no characteristics to distinguish between them in the context of biological process, molecular function, and cellular component (Supplemental Figure S1).

Immunohistochemistry of Candidate Marker Molecules in Surgical Specimens of Liposarcoma and Lipoma

Of the 181 unique proteins for ALT/WDL, 15 molecules were identified in multiple cases and emerged as candidate

Table 4 List of Unique Proteins for ALT/WDL

UniProt accession	Protein names	Gene names	Chromosomal location	Unused ProtScore*	Total ProtScore†	%Cov
P23946	Chymase‡	CMA1	14q12	15.74	15.74	48.58
Q6NZY1	Tryptase beta 2 protein	TPSB2	16p13.3	14.59	14.59	28.37
Q9BYX7	Putative β-actin-like protein 3	POTEKP	2q21.1	7.70	7.70	13.60
Q6GMX4	IGL protein	IGL	22q11.22	6.00	6.00	16.95
P52926	HMGI-C§	HMGA2	12q14.3	4.00	4.00	42.20
P02763	α-1-Acid glycoprotein 1	ORM1	9q32	2.07	2.07	11.94
Q9P1Z9	CCDC180‡	CCDC180	9q22.33	2.00	2.00	2.02
Q9Y355	Apolipoprotein A1	APOA1	11q23.3	2.00	2.00	16.42
O95197	Reticulon-3	RTN3	11q13	1.15	1.15	0.89
Q5TEC6	Histone H3	HIST2H3PS2	1q21.1	0.89	6.73	36.76
Q8WZ42	Titin	TTN	2q31.2	0.61	0.61	0.50
Q9HBW1	LRRC4‡	LRRC4	7q32.1	0.28	0.28	3.22
Q15149	Plectin	PLEC	8q24.3	0.25	0.25	1.46
Q9BX59	Tapasin-related protein	TAPBP1	12p13.31	0.24	0.24	1.92
Q96QW8	DJ576K7.1	FRAP1	1q36.22	0.19	0.19	2.91

*Unused ProtScore indicates the amount of total, unique peptide evidence related to a given protein.
†Total ProtScore indicates the sum of all peptide evidence related to a given protein.
‡Selected proteins as candidate marker molecules.
§A known ALT/WDL marker.
%Cov, percentage of matching amino acids from identified peptides; ALT, atypical lipomatous tumor; HMGI-C, high-mobility group protein; WDL, well-differentiated liposarcoma.

Figure 2 Immunohistochemistry of CCDC180 and LRRC4 in surgical specimens of atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL), dedifferentiated liposarcoma (DDL), and lipoma. **A:** Immunohistochemistry of CCDC180 and LRRC4. CCDC180 and LRRC4 are observed in the cytoplasm of tumor cells of ALT/WDL and DDL, whereas both of them are almost absent in lipoma. **Insets:** High-magnification images. **B:** Immunoreactive intensities of CCDC180 and LRRC4 are significantly higher in ALT/WDL and DDL than in lipoma, similar to the known biomarkers E3-ubiquitin–protein ligase Mdm2 (MDM2) and cyclin-dependent kinase 4 (CDK4). Immunoreactive intensities of CCDC180 and MDM2 are significantly higher in DDL than in ALT/WDL. * $P < 0.05$, ** $P < 0.01$ (U-test). Scale bars = 50 μm (A). Insets are approximately 50 μm in width. H&E, hematoxylin and eosin.

marker molecules for ALT/WDL (Table 4). A known ALT/WDL marker, HMIG-C, was included in the list (Table 4). To cut the list of candidate molecules, Prognoscan, a database for meta-analysis of the prognostic value of genes (<http://www.prognoscan.org>),⁴⁵ and The Cancer Genome

Atlas (<https://cancergenome.nih.gov>) were searched. Of the 15 candidates, three molecules, CCDC180, LRRC4, and chymase, were selected on the basis of biological interest. CCDC180 was associated with poor prognosis of lung adenocarcinoma,⁴⁶ LRRC4 expression was increased in

several cancers,⁴⁷ and chymase played some role in tumor angiogenesis.⁴⁸

Immunohistochemistry of CCDC180, LRRC4, and chymase was performed in FFPE surgical specimens from patients with ALT/WDL (22 cases) and lipoma (25 cases). In addition to ALT/WDL, 18 cases of DDL were also examined. For comparison, immunohistochemistry of known ALT/WDL markers, MDM2 and CDK4, was also performed. Immunoreactivity of CCDC180 and LRRC4 was strongly observed in the cytoplasm of tumor cells of ALT/WDL and DDL, whereas immunoreactivity of both of the molecules was almost absent or weak in lipoma (Figure 2A). The intensities of staining with anti-CCDC180, anti-LRRC4, anti-MDM2, and anti-CDK4 antibodies were scored as strong (3+), moderate (2+), weak (1+), or negative (0) and are summarized in Figure 2B and Supplemental Tables S3, S4, and S5. Immunoreactivity of CCDC180 and LRRC4 was significantly high in DDL, followed by ALT/WDL and lipoma, in that order (Figure 2B). In differentiation between lipoma and ALT/WDL, a cutoff value of 2+ produced high accuracy: sensitivity and specificity were 86.4% and 72.0%, respectively, for CCDC180; they were 63.6% and 96.0%, respectively, for LRRC4; they were 63.6% and 96.0%, respectively, for MDM2; and they were 81.8% and 88.0%, respectively, for CDK4. Considering the combination of positivity of the four molecules, CCDC180, LRRC4, MDM2, and CDK4, the highest accuracy was obtained when any of the three markers was positive (Table 5). These results indicate that a panel of multiple markers improved the accuracy compared with that of a single marker or a panel of MDM2 and CDK4. Neither the expression of CCDC180 nor that of LRRC4 was correlated with age, sex, location, or tumor size (Supplemental Table S6). The number of chymase-immunopositive cells was slightly larger in ALT/WDL than in lipoma, although antichymase antibody stained not tumor cells but mast cell–like blood cells (data not shown). These results indicated that, among the candidate molecules, CCDC180 and LRRC4 are useful markers to distinguish ALT/WDL from lipoma.

Candidate Marker Molecules Contribute to Malignant Potentials and Apoptosis Resistance in Liposarcoma Cells

Next, cell biological methods were used to further examine the expression of CCDC180 and LRRC4 in ATL/WDL. In cultured human liposarcoma cell lines SW872 (derived from unclassified liposarcoma) and 93T449 (derived from WDL), protein expression of CCDC180 and LRRC4 was confirmed by Western blot analysis (Figure 3A).

To determine whether CCDC180 expression and LRRC4 expression have a role in malignant potentials, CCDC180 and LRRC4 expression was knocked down independently and their effect was evaluated. CCDC180-specific siRNAs were used, and they effectively reduced CCDC180 expression in protein levels in 93T449 cells (Figure 3A). In a WST-8 cell proliferation assay, the percentage of proliferative cells in *CCDC180* knockdown (KD) cells was significantly lower than that in control cells ($P < 0.01$) (Figure 3B). In a colony-forming assay, the number of colonies in *CCDC180* KD cells was significantly smaller than that in control cells ($P < 0.05$) (Figure 3D). In Ki-67 labeling analysis, the percentage of Ki-67–positive cells was significantly lower in *CCDC180* KD cells than in control cells ($P < 0.01$) (Figure 3F). KD of LRRC4 showed an effect similar to that of CCDC180 in the WST-8 assay, colony-forming assay, and Ki-67 labeling analysis ($P < 0.01$) (Figure 3, C, E, and G). These results indicate that the expression levels of CCDC180 and LRRC4 modulate the proliferation of liposarcoma cells.

In a migration assay and an invasion assay, the percentages of migrating and invasive cells were significantly lower in *CCDC180* KD cells than in control cells ($P < 0.05$ and $P < 0.01$, respectively) (Figure 4, A and B), indicating that CCDC180 expression contributes to cell migration and invasion. Knockdown of *LRRC4* had different effects from those of knockdown of *CCDC180* in those assays: the number of migrating cells was not affected by *LRRC4* KD (Supplemental Figure S2A), and

Table 5 Sensitivity and Specificity of a Single Marker or Multiple Markers in Immunohistochemistry

Marker	Lipoma (n = 25)	ALT/WDL (n = 22)	Sensitivity, %	Specificity, %	PPV, %	NPV, %
MDM2 ⁺	1	14	63.6	96.0	93.3	75.0
CDK4 ⁺	3	18	81.8	88.0	85.7	84.6
CCDC180 ⁺	7	19	86.4	72.0	73.1	85.7
LRRC4 ⁺	1	14	63.6	96.0	93.3	75.0
MDM2 ⁺ /CDK4 ⁺	0	12	54.5	100.0	100.0	71.4
CCDC180 ⁺ /LRRC4 ⁺	1	13	59.1	96.0	92.9	72.7
Any two positive	4	19	86.4	84.0	82.6	87.5
Any three positive	0	15	68.2	100.0	100.0	78.1
All positive	0	9	40.9	100.0	100.0	65.8

ALT, atypical lipomatous tumor; CDK4, cyclin-dependent kinase 4; MDM2, E3-ubiquitin–protein ligase Mdm2; NPV, negative predictive value, PPV, positive predictive value; WDL, well-differentiated liposarcoma.

Figure 3 Knockdown (KD) of *CCDC180* or *LRRC4* inhibits proliferation of liposarcoma cells. **A**: Expression of *CCDC180* or *LRRC4* was knocked down by transfection of gene-specific siRNAs. Western blot analysis (β -actin used as a loading control). **B–G**: *CCDC180* or *LRRC4* KD significantly inhibits proliferation of 93T449 cells (derived from well-differentiated liposarcoma) in multiple assays, including a WST-8 assay (**B** and **C**), a colony formation assay (**D** and **E**), and immunostaining with anti-Ki-67 (proliferation marker) antibody (**F** and **G**). Data are expressed as means $\pm$ SD (**B–G**). $n = 5$ (**B**, **C**, **F**, and **G**); $n = 6$ (**D** and **E**). $*P < 0.05$, $**P < 0.01$ (t -test). Scale bars = 10 μ m (**F** and **G**). Original magnification, $\times 400$ (**F** and **G**). ABS, absorbance; SC, scrambled siRNA; siCCDC, siRNA for *CCDC180*; siLRRC, siRNA for *LRRC4*.

Figure 4 Knockdown (KD) of *CCDC180* inhibits migration, invasion, and apoptosis resistance of liposarcoma cells. **A:** Transwell migration assay. *CCDC180* KD significantly inhibits migration of 93T449 cells. **B:** Matrigel invasion assay. *CCDC180* KD significantly inhibits invasion of 93T449 cells. **C:** *CCDC180* KD significantly increases the number of cleaved caspase-3–positive apoptotic cells induced by treatment with cisplatin (40 μ mol/L, 24 hours). Data are expressed as means $\pm$ SD (**A–C**). $n = 3$ (**A–C**). * $P < 0.05$, ** $P < 0.01$ (t -test). Scale bars = 10 μ m (**C**). Original magnification: $\times 40$ (**A** and **B**); $\times 400$ (**C**). SC, scrambled siRNA; siCCDC, siRNA for *CCDC180*.

the number of invasive cells was larger in *LRRC4* KD cells than in control cells ($P < 0.01$) (Supplemental Figure S2B).

Next, the relationship between apoptosis resistance and *CCDC180* and *LRRC4* expression was examined. The number of cleaved caspase-3–positive cells, an indicator of apoptotic cells, was increased by knockdown of *CCDC180*, indicating that *CCDC180* plays an anti-apoptotic role in liposarcoma cells (Figure 4C). The antiapoptotic effect of *CCDC180* became more obvious by treatment with cisplatin, an apoptosis-inducing agent, with the number of cleaved caspase-3–positive cells being significantly higher in *CCDC180* KD cells than in control cells ($P < 0.05$) (Figure 4C). The numbers of cleaved poly (ADP-ribose) polymerase–positive cells and apoptotic bodies, downstream apoptotic indicators of cleaved caspase-3, were also significantly increased by knockdown of *CCDC180* under an apoptosis-inducing condition (Supplemental Figure S3). However, knockdown of *LRRC4* did not significantly alter the number of cleaved

caspase-3–positive cells, even after treatment with cisplatin (Supplemental Figure S2C).

Adipogenic Differentiation Suppresses Expression of *CCDC180* and *LRRC4* in Liposarcoma Cells

To elucidate the molecular mechanisms responsible for expression of *CCDC180* and *LRRC4*, 93T449 cells were chemically induced to undergo adipogenic differentiation because alteration of gene expression is frequently associated with dedifferentiation. An adipogenesis-inducing cocktail (3-isobutyl-1-methylxanthine, dexamethasone, and oleic acid) increased the number of Oil Red O–positive cells, an indicator of adipocyte differentiation, and protein expression of fatty acid–binding protein 4, indicating that the cocktail effectively induced differentiation of the liposarcoma cells into adipocyte-like cells (Figure 5A). As expected, mRNA and protein level expression of *CCDC180* and *LRRC4* was decreased after adipogenic differentiation by the cocktail (Figure 5, B and C). These results suggested

Figure 5 Adipogenic differentiation suppresses expression of CCDC180 and LRRC4 in liposarcoma cells. **A:** Adipogenic differentiation is induced by treatment with an adipogenesis-inducing cocktail (3-isobutyl-1-methylxanthine, dexamethasone, and oleic acid). Oil Red O staining shows lipid accumulation in 93T449 cells. **Top panels:** Low-magnification images of whole cell culture plates. **Bottom panels:** High-magnification images of **top panels**. **Insets:** Magnified view of the Oil Red O–positive cells. **B and C:** CCDC180 and LRRC4 are decreased after adipogenic differentiation. **B and C:** RT-PCR (**B**) and Western blot analysis (**C**). Scale bars = 100 μ m (**A**). **Insets** are approximately 100 μ m in width. FABP4, fatty acid–binding protein 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

that overexpression of CCDC180 and LRRC4 is induced during dedifferentiation of adipocytes.

Discussion

Multiple candidate marker molecules were discovered for differentiating ALT/WDL from lipoma by using comparable proteome analysis of FFPE tissues. Uncommon molecules, LRRC4 and CCDC180, were identified as useful immunohistochemical markers for ALT/WDL. Furthermore, it was demonstrated that they contributed to malignant properties of liposarcoma cells and were up-regulated in association with dedifferentiation.

In this study, protein samples were prepared from FFPE tissues by using two different methods because complementary use of different methods has been suggested to increase proteome coverage and depth.³⁹ A total of 605

proteins were identified by using a Qproteome extraction kit, 300 proteins were identified by using a guanidine-HCl extraction method, and 809 proteins were identified in total, indicating that use of the two methods provided more candidates. In Unused ProtScore of >0.15 , 308 proteins were identified by using a Qproteome extraction kit, 164 proteins were identified by using a guanidine-HCl extraction method, and 408 proteins were identified in total, indicating that the use of the two methods provided more candidates. The identified proteins included lipid metabolism–associated protein, fatty acid–binding protein 4, and lipid droplet–associated protein, perilipin-1, indicating that ALT/WDL and lipoma were derived from adipose tissues.^{40–43} Subsequent selection of candidate marker molecules for ALT/WDL was based on the presence or absence of a protein.

Amplification of chromosome 12q is a well-known marker for ALT/WDL, which is shown in 80% of cases. Amplification of 12q13–12q15 leads to formation of a typical supernumerary ring and/or giant marker chromosomes and to gene amplification of *MDM2*, *CDK4*, and *HMG2*, the coding proteins of which were increased in ALT/WDL and detected by immunohistochemistry.^{16,17,19,21,22,49,50} One of the known marker molecules, high-mobility group protein (encoded by *HMG2*), was included in the list of the consequent 15 candidates after a list of 181 unique proteins was narrowed down (**Table 4**). These results indicated that our analytical strategy, consisting of protein extraction from FFPE tissues, sample processing, mass spectrometry, and filtering the candidate molecules, is an appropriate strategy. In this study, other markers, *MDM2* and *CDK4*, were not identified, possibly because of technical difficulties, such as low ionization efficiency of their fragmented peptides. Another possible explanation is that the expression level of *MDM2* and *CDK4* proteins may be insufficient to be detected by the matrix-assisted laser desorption/ionization–time-of-flight/time-of-flight analyzer because the number of *MDM2*-expressing cells was small in immunohistochemistry of ALT/WDL.¹⁹

A proteomics approach provided an opportunity for us to focus on three uncommon molecules, CCDC180, LRRC4, and chymase. Subsequent validation by immunohistochemistry in surgical specimens confirmed that anti-CCDC180 and anti-LRRC4 antibodies clearly stained tumor cells of ALT/WDL and DDL, with a higher immunopositive rate in DDL than in ALT/WDL. Both of the antibodies can differentiate them from lipoma with high accuracy comparable to that of *CDK4* and *MDM2* (**Figure 2**). Neither the expression of CCDC180 nor that of LRRC4 was associated with any clinicopathological features in this study (**Supplemental Table S6**), possibly because of the insufficient sample size. Larger sample size may detect association between some clinicopathological features and expression of CCDC180 and LRRC4 in a future study.

Beyond the immunohistochemical utility, cell biological examination revealed that knockdown of CCDC180 or LRRC4 decreased malignant potentials of WDL cells,

indicating that high expression levels of the molecules play important roles in WDL cells. Little is known about *CCDC180*. The *CCDC180* gene is located on chromosome 9q22 and was originally identified as a Paget disease-sensitive gene, although its role has not been elucidated.⁵¹ This is the first study to demonstrate tumor-associated roles of *CCDC180*, such as cell proliferation, invasion, migration, and resistance to cisplatin-induced apoptosis (Figures 3 and 4). *LRRC4* is also an uncommon molecule. The *LRRC4* gene is located on chromosome 7q31-32.⁵² *LRRC4* is mainly expressed in brain tissue and plays tumor-suppressing roles in glioma and glioblastoma.^{53–57} In contrast, *LRRC4* contributes to the proliferation of liposarcoma cells (Figure 3, C, E, and G). The difference in the roles of *LRRC4* in liposarcoma and glioblastoma cells may depend on the presence of *LRRC4* in their parental nonneoplastic tissues: *LRRC4* was expressed in the brain but not in nonneoplastic adipose tissue (data not shown).

More important, the expression of *CCDC180* and *LRRC4* was reduced by induction of adipocyte differentiation in WDL cells, suggesting that their high expression in ALT/WDL is not an instance of chance but reflects dedifferentiation during tumor development. Furthermore, the immunohistochemical analysis showed that immunoreactive intensities of the two molecules were higher in DDL than in ALT/WDL, especially for *CCDC180*, with a significant difference, suggesting that the expression level of the two molecules is associated with adipocyte differentiation. *CCDC180* and *LRRC4* may contribute to malignant potentials in cooperation with other molecules that are induced in association with adipocyte dedifferentiation, such as CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor.^{58–61} NF- κ B signaling may also play some roles in malignant potentials of liposarcoma cells because NF- κ B signaling generally works in opposition to peroxisome proliferator-activated receptor activation in various cancers^{62,63} and was reported to be regulated by *LRRC4*.⁶⁴ These findings provide a new insight into an understanding of the roles and regulation of *CCDC180* and *LRRC4* in malignancies. For a better understanding of the role of the molecules, the signaling pathways responsible for malignant properties should be analyzed in a future study.

In conclusion, we identified *LRRC4* and *CCDC180* as novel immunohistochemical markers for differentiating ALT/WDL and showed they contributed to malignant potentials of WDL cells. These results suggested that the molecules are potential therapeutic targets of liposarcoma. Proteome analysis using a standard stock of FFPE tissues can reveal novel biomarkers for various diseases, which contributes to the progress of molecular pathology.

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Supplemental Data

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