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Original Article

**Increased ectodomain shedding of lung-epithelial cell adhesion molecule
1 as a cause of increased alveolar cell apoptosis in emphysema**

Table of Contents

Supplementary methods (pages 2-11)

Figure legends for supplementary figures S1-S8 (pages 12-15)

Supplementary methods

Antibodies and reagents

A rabbit anti-CADM1 polyclonal antibody directed against the C-terminal 15-amino acid peptide was generated in our laboratory and described previously.¹ CADM1 α CTF, β CTF and ICD were recognizable by this antibody (Supplementary figure S3). Other primary antibodies used were against E-cadherin (clone 36; BD Bioscience, San Jose, CA, USA), FLAG (M2; Sigma-Aldrich, St. Louis, MO, USA), CoxIV (3E11; Cell Signalling Technology, Danvers, MA, USA), G3PDH (Merck Millipore, Billerica, MA, USA), and mitochondria (clone 113-1 recognizing a ~60-kDa mitochondrial protein; Merck Millipore). Peroxidase- and fluorophore-conjugated secondary antibodies were obtained from Amersham (Buckinghamshire, England) and Jackson ImmunoResearch (West Grove, PA, USA), respectively. PMA and trypsin were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

NCI-H441 human lung epithelial cells were purchased from the American Type Culture Collection (Rockville, MD, USA) in 2010 (Lot No. 58294188), and all experimentation using this cell line proceeded within 6 months after resuscitation. NCI-H441 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), antibiotics containing 100 units/mL penicillin and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 5 mM HEPES buffer (Sigma-Aldrich) at 37°C in 5% CO₂/95% air. To induce CADM1 ectodomain shedding, NCI-H441 cells were incubated in RPMI-1640 medium containing 200 nM PMA, 0.0125% w/v trypsin (a concentration low enough to prevent

cell detachment), or a mixture of both, for 20 min. Lung cancer cell lines A549 and NCI-H596 were described previously.²

Human samples

Human lung tissues were obtained from patients who were diagnosed with lung cancer or tumorous masses and underwent pulmonary lobectomy or segmentectomy at Hiroshima University Hospital (Hiroshima, Japan) between 2008 and 2012. All patients with a smoking habit were obliged to quit smoking more than 1 month before the date of surgery. Immediately after the operation, non-cancerous portions (approximately 2 cm³) of the surgical specimens were cut into two; one was fixed with 10% buffered formalin to prepare hematoxylin and eosin (H&E)-stained tissue sections, and the other was frozen to prepare lung tissue lysates. When an H&E-stained specimen was diagnosed as being emphysematous consistently by two pathologists, the patient was included in the present study as an “emphysematous lung” case. In contrast, when two pathologists consistently diagnosed that an H&E-stained specimen had little pathologic changes, the patient was included as a “normal lung” case. Frozen lung tissues from either case group were broken into pieces, and were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Sigma-Aldrich). Insoluble components containing the nuclei were removed by centrifugation, and the supernatant was used as a lung tissue lysate. All patients provided written informed consent to participate in this study, and our institutional review board approved the experimental protocol (approval number, Eki-350). Normal (n = 3) and emphysematous (n = 3) lungs were also obtained from autopsied patients who did not have lung cancer and died in Kinki University Hospital (Osaka, Japan). The

tissue lysates were subjected to Western blot analyses. The Ethics Committee of Kinki University approved the experimental protocol (25-088).

Western blot analysis

Western blot analyses were conducted, and immunoreactive band intensities were quantified using NIH ImageJ software, as described previously.³

Plasmid constructs

To express α CTF exogenously on the cell membrane, the polymerase chain reaction (PCR)-based overlap extension method was applied to a plasmid vector expressing the full-length human CADM1 (pCX4bsr-CADM1)⁴ using two primer sets: A, forward, 5'-AGTCTGAGGGCAGGTGCCCGACAT-3' (annealing to the 5' untranslated region immediately upstream of the signal peptide start codon), and reverse, 5'-*cgagccttcttcacctgc*CAGATTCTGCCCATCACCTGTG-3' (the underlined lower-case and upper-case portions correspond to the N-terminal codon of α CTF and the C-terminal codon of the signal peptide sequence, respectively); and B, forward, 5'-CTGGCAGGTGAAGAAGGCTCG-3' (complementary to the italicised portion of set A reverse primer), and reverse, 5'-CAGTTGGACACCTCATTGGAAC-3' (annealing to the 3' untranslated region of CADM1). The final PCR product encoded a large-deletion form of CADM1, in which the signal peptide (amino acids 1–44) was ligated upstream of α CTF (amino acids 363–442) with a three amino acid (45–47) insertion (numbered according to human CADM1, <http://www.uniprot.org/uniprot/Q9BY67>). This PCR product was inserted into pTA2 TA-cloning vector (Toyobo, Osaka, Japan). After amplification, the insert was excised by *Eco*RI digestion, and ligated to pCX4bsr vector

via the *EcoRI* site (pCX4bsr-SP- α CTF). Directional ligation was confirmed by sequencing.

When the α CTF intracytoplasmic domain amino acid sequence was analysed with a computer-assisted algorithm WoLF PSORT (<http://wolfpsort.seq.cbrc.jp/>), the domain was predicted to localise to mitochondria. According to this algorithm, we designed a mutant form of α CTF with an intracytoplasmic domain that was predicted to localise not to mitochondria but to the cytosol (supplementary figure S3). To obtain this mutant, in the pCX4bsr-SP- α CTF construct, a portion of the α CTF cytoplasmic region, 5'-gcc gat gac gca gca gac gca gac aca gct ata atc aat gca gaa gga gga cag-3' encoding ADDAADADTAIINAEGGQ (amino acids 414–431, 18 amino acids long), was mutated to 5'- ggc ggt ggc gga gga ggc gga ggc gca ggt gta atc aat gca gaa gga-3' encoding GGGGGGGGAGGINAEG (16-amino acid-long) using multiple steps of site-directed mutagenesis (pCX4bsr-SP- α CTFmut). These two vector constructs were used as templates in PCR together with a primer set: forward, 5'-CCCAAGCTTgcaGGTGAAGAAGGCTCGATCAGG-3' (containing a *HindIII* site, underlined, immediately upstream of the N-terminal codon of α CTF, lower-case); and reverse, 5'-GACAAACGCACACCGGCCTTATTCC-3' (annealing to the vector sequence downstream of the multiple cloning site). Two PCR products were digested by *EcoRI* and *HindIII* digestion and then were inserted into the p3xFLAG-CMV-9 vector (Sigma-Aldrich) via *HindIII* and *EcoRI* sites (p3xFLAG- α CTF and p3xFLAG- α CTFmut). The absence of mutation was verified by sequencing.

Transfection

Cells were grown to 60–70% confluence, and were transfected with the indicated plasmid vectors using the Lipofectamine LTX and Plus reagents (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence and mitochondrial labelling

Cells were grown in coverslip-like-bottomed culture dishes of a 35-mm diameter (μ -Dish; ibidi, Munich, Germany), and were either treated with a mixture of PMA (200 nM) and trypsin (0.0125% w/v) or transfected as described above, or left untreated. After 2 days of transfection or 20 min of treatment, cells were incubated in RPMI-1640 medium containing 10% FBS and 200 nM Mitotracker (Molecular Probes, Eugene, OR, USA) for 30 min, and washed with PBS. Then cells were fixed with ice-cold methanol for 10 min, blocked with 2% bovine serum albumin for 30 min, and incubated with the anti-CADM1 antibody overnight at 4°C. Cells were washed three times, and incubated with an Alexa Fluor 488-conjugated anti-rabbit IgG antibody for 2 h at 4°C for visualisation. Stained images were captured using a confocal laser microscope (LSM510 Meta; Carl Zeiss, Oberkochen, Germany). Intensity correlation between red and green fluorescence was quantified using the Colocalization Analysis plugin of ImageJ software, and the overlap coefficient was calculated for each image. The mean and standard deviation (SD) of the overlap coefficient were calculated from more than 10 images per each type of cells.

Double immunofluorescence of lung sections were performed as described previously.⁵ Briefly, paraffin-embedded lung sections were deparaffinised, rehydrated, antigen retrieved by microwave heating in 10 mM citrate buffer (pH 6.0), blocked with 2% bovine serum albumin, and incubated with a mixture of antibodies against CADM1 and

mitochondria (clone 113-1) overnight at 4°C. Sections were incubated with an Alexa Fluor 488-conjugated anti-rabbit IgG and an Alexa Fluor 568-conjugated anti-mouse IgG antibody for 2 h, followed by nuclear counterstaining with DAPI. Triple stained images were captured using a confocal laser microscope (AZ-C2 plus; Nikon, Tokyo, Japan), and merged on the differential interference contrast image. Alveolar epithelial cells were identifiable by CADM1 membranous immunostaining (green). CADM1 immunostaining was occasionally detected in the cytoplasm, and appreciably colocalised with mitochondrial immunostaining (red). The number (%) of cells with this colocalisation signal (yellow) was counted in 100 alveolar epithelial cells for each case, and the mean and SD were calculated from the results of four cases for each of normal and emphysematous lungs.

Cell fractionation

Cells (2.1×10^7) grown in culture dishes were treated with trypsin (0.25% v/w), suspended in PBS, and collected as two (1×10^6 and 2×10^7) cell pellets by centrifugation. The small cell pellet was lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Sigma-Aldrich). Insoluble components containing the nuclei were removed by centrifugation, and the supernatant was used as a whole cytoplasmic lysate. The large cell pellet was separated into cytosolic and mitochondrial fractions using a Mitochondria Isolation Kit for Mammalian Cells (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Mitochondrial membrane potential

JC-1 dye (Molecular Probes, Life Technologies) was used to measure mitochondrial inner membrane potential ($\Delta\Psi_m$) in living cells. When $\Delta\Psi_m$ is high, JC-1 accumulates in the mitochondria, forming aggregates that fluoresce red. When $\Delta\Psi_m$ is low, JC-1 exists in the cytoplasm as monomers that fluoresce green. The ratio of red-to-green fluorescence is proportional to $\Delta\Psi_m$. Cells were grown to 60–70% confluence in coverslip-like-bottomed culture dishes of a 35-mm diameter (μ -Dish; ibidi) and were transfected or treated as indicated. At the indicated time points, cells were incubated in RPMI1640 containing 10% FBS and 1.0 $\mu\text{g/mL}$ JC-1 at 37°C for 20 min, rinsed twice with PBS, and incubated in RPMI1640 containing 10% FBS for 10 min. Then, the cell culture was transferred into a temperature-controlled (37°C) chamber unit (CZL-3; Carl Zeiss) that was supplied with 5% CO₂ and placed on an automated stage of an LSM510 Meta confocal microscope (Carl Zeiss). About 10–20 cells were observed per field of view using a 63 \times objective lens, and total intensities of red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence per field were measured with a morphometric analysis tool attached to the microscope computer. Approximately 20 fields of view were analysed for each experimental group, and the mean and SD of the red/green fluorescence intensity ratio were calculated. Experiments were repeated independently three times, with essentially similar results.

TUNEL assay

TUNEL assays were conducted on cultured cells and formalin-fixed, paraffin-embedded lung sections using the In Situ Cell Death Detection Kit (Roche Applied Science, Upper Bavarie, Germany) according to the manufacturer's instructions. Briefly, cells were grown to 60–70% confluence in μ -Dishes (ibidi) and transfected with indicated vectors

or left untransfected. After 48 h, cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate (pH7.4), and then were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and FITC-labelled dUTP for 1 h at 37°C, followed by nuclear counterstaining with DAPI. E-cadherin immunofluorescence was performed for lung sections as described previously⁶ prior to the assay. Briefly, thin sections were prepared from paraffin-embedded tissues, deparaffinised, rehydrated, autoclaved in 10 mM citrate buffer (pH 6.0) for 20 min at 121°C, blocked with 2% bovine serum albumin, and incubated with the anti-E-cadherin antibody for 2 h, followed by an incubation with an Alexa Fluor 568-conjugated anti-mouse IgG antibody for 2 h. Then, sections were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and FITC-labelled dUTP, followed by nuclear counterstaining with DAPI. Double-stained cultured cells and triple-stained lung sections were observed through a fluorescence microscope (Axio Observer D1; Carl Zeiss). When a cell had TUNEL signals within the DAPI nuclear stain, the cell was deemed TUNEL-positive. Alveolar epithelial cells in lung sections were identified by membranous staining for E-cadherin, and the number of TUNEL-positive cells was counted in 500 alveolar epithelial cells from three or four sections for each case. Data are expressed as mean \pm SD of the proportion of TUNEL-positive cells for each experimental group.

Reverse transcription-PCR

The methods of RNA extraction, reverse transcription, and PCR to detect CADM1 isoforms were essentially identical to those described previously,¹ except that an

oligonucleotide 5'-AAAATAGCGCCCCAGAATGATGAGC-3' was used as a reverse primer in PCR to amplify human CADM1 mRNA.

Statistical analysis

Statistical differences among experimental groups were analysed using the Mann–Whitney *U*-test for the quantified Western blot data and proportions of TUNEL-positive cells and alveolar epithelial cells with CADM1-mitochondria colocalisation, and Student's *t*-test for JC-1 green/red fluorescence intensity ratios. In multiple testing, all pairwise comparisons were Bonferroni corrected, with the allowance being set < 0.05 . The data subjected to *t*-tests were confirmed to meet the normality assumption by the F-test. Correlations were analysed using the Spearman's rank test. A *P* value ≤ 0.05 was considered significant.

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epithelium and its down-regulation in pulmonary adenocarcinoma other than bronchioloalveolar carcinoma. *Lab Invest* 2003;83:1175-83.

Figure legends

Supplementary figure S1

Histological diagnosis of “normal” and “emphysematous” lungs.

Formalin-fixed, paraffin-embedded lungs were cut into sections, and stained with hematoxylin and eosin (H&E). Representative histological pictures of “normal” and “emphysematous” lungs are shown in the upper and lower panels, respectively. Bar = 100 μm .

Supplementary figure S2

Graphs with band intensity ratios of E-cadherin/ β -actin and CADM1 full-length/ β -actin on the X- and Y-axes, respectively. These two ratios were well approximated as linear in both normal (left) and emphysematous (right) lungs. Correlation and statistical significance were analysed by the Spearman’s rank test, and R^2 and P -values are shown. White circles, normal lung non-smoker; triangles, normal lung smoker; black circles, emphysematous lung smoker.

Supplementary figure S3

Schematic presentation of the structure of α CTF and its mutant form α CTFmut.

Mutations in α CTFmut, amino acid substitution and deletion (–), are shown with single-letter codes. Conserved amino acid residues are shaded. Amino acid residues positioned at the start and end of structural domains are numbered according to; <http://www.uniprot.org/uniprot/Q9BY67>. Arrows indicate the position of α -, β -, and γ -shedding that produce α CTF, β CTF, and ICD, respectively. A hatched rectangle indicates the C-terminal region recognized by the CADM1 antibody used. SP, signal peptide; TM,

transmembrane domain; 4.1-BM, protein 4.1-binding motif; PDZ-BM, PDZ domain-binding motif.

Supplementary figure S4

Protein levels of full-length CADM1 and α CTF in NCI-H441 cells. **A.** Western blot analyses of human lung cancer cell lines, NCI-H441, A549 and NCI-H596, and normal lungs for CADM1. The protein levels of full-length CADM1 in these cell lines and tissues were well correlated with their mRNA levels assessed by Northern blot analyses shown in our past work.² Note that CADM1 is as abundant in NCI-H441 cells as in normal lungs, when normalized to β -actin. Considering that lung tissues contain CADM1-negative cells, it seems that full-length CADM1 in NCI-H441 cells is certainly lower than in normal lung epithelial cells in vivo. **B.** Western blot analyses of NCI-H441 cells transfected with α CTF cDNA for CADM1. Protein lysates were prepared from cells transfected individually three times (corresponding to lanes 1 to 3). The ratios of α CTF to full-length CADM1 were plotted with grey circles in **C**, together with the ratios in emphysematous lungs (closed circles; identical to the plot of figure 1C). A *P* value was calculated by the Mann–Whitney *U*-test.

Supplementary figure S5

Exogenous expression of FLAG-tagged α CTF and α CTFmut in NCI-H441 cells. Upper panel: NCI-H441 cells were untransfected (left) or transfected with p3xFLAG- α CTF (middle) or p3xFLAG- α CTFmut (right), and were fractionated into cytosolic (CS) and mitochondrial (Mit) fractions. Whole cytoplasmic lysates (CP) were extracted from aliquots of the cells. These lysates and fractions were analysed with Western blotting

using an anti-FLAG antibody. White and black arrows indicate FLAG-tagged α CTF and α CTFmut, respectively. Open and closed arrows indicate FLAG-tagged α CTF and α CTFmut, respectively. (This result was obtained by reprobng the blots in Fig. 5 of the main text.)

Lower panel: The blots were stained with silver reagents (Wako Pure Chemical Industries, Osaka, Japan) to indicate the protein loading per lane.

Supplementary figure S6

Differential interference contrast images of NCI-H441 cells stained with JC-1 dye. These pictures correspond to the differential interference contrast images of NCI-H441 cells shown in Fig. 6A of the main text. As described in the Fig. 6A legend, NCI-H441 cells were untransfected (upper) or transfected with pCX4bsr-SP- α CTF (middle) or pCX4bsr-SP- α CTFmut (lower) and were stained with JC-1 dye 48 h later. Differential interference contrast images were captured by a confocal laser microscope. Green and red fluorescence images are shown in Fig. 6A of the main text. Bar = 20 μ m.

Supplementary figure S7

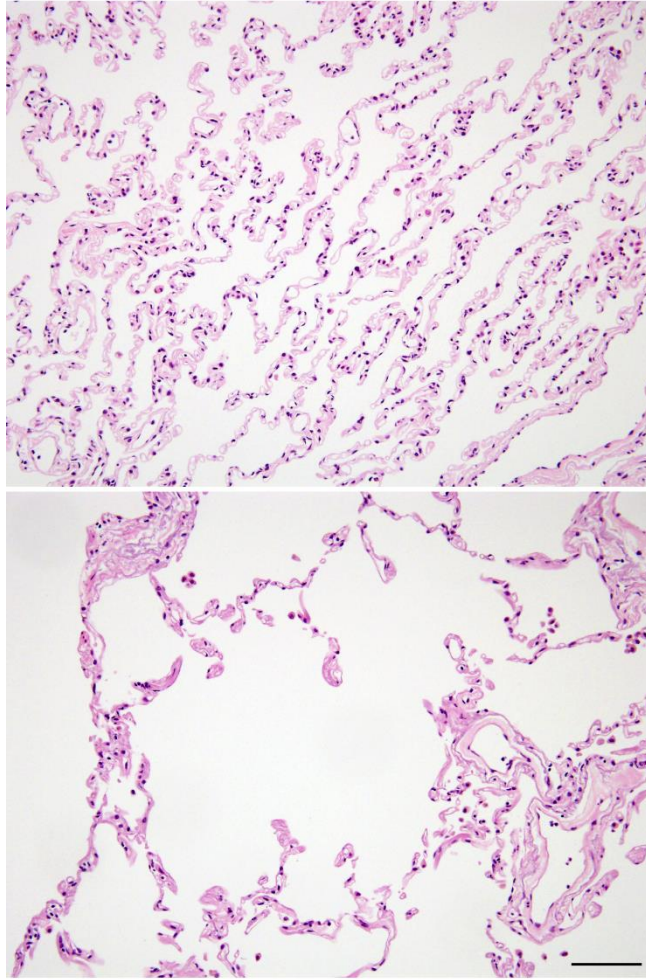
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Patient characteristics are as follows.

Lung histology	Patient No.	Age	Sex	Cause of death
Normal	1	43	M	Infectious colitis
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	3	76	M	Hepatocellular carcinoma
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	2	62	F	Peritoneal carcinoma
	3	72	M	Pancreatic cancer

Supplementary figure S8

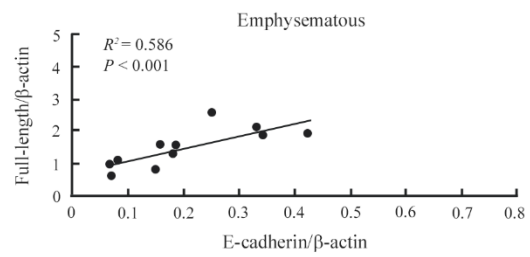
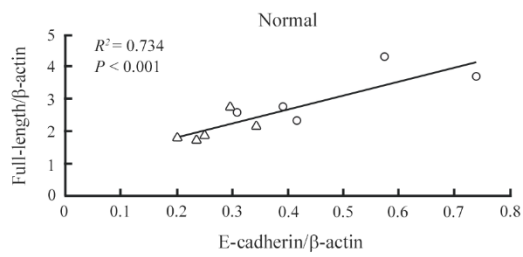
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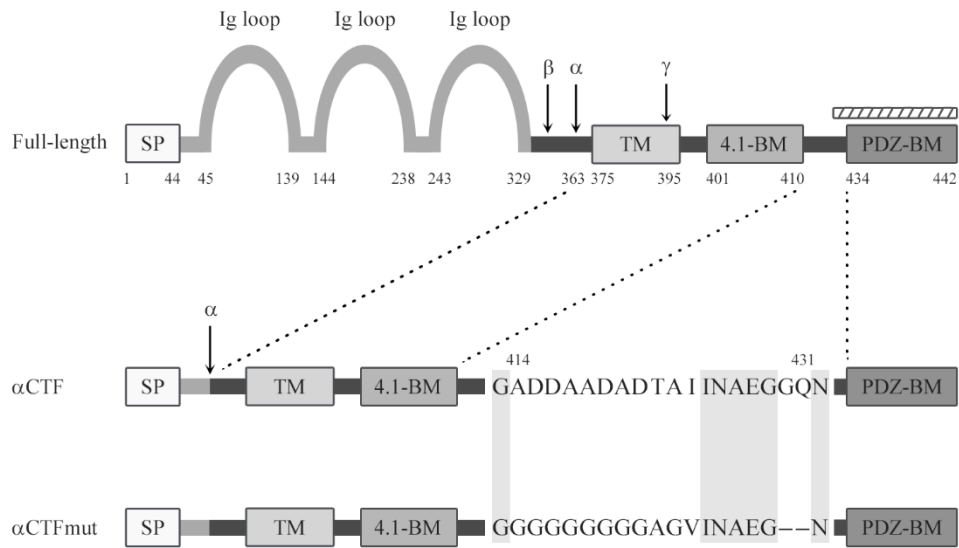
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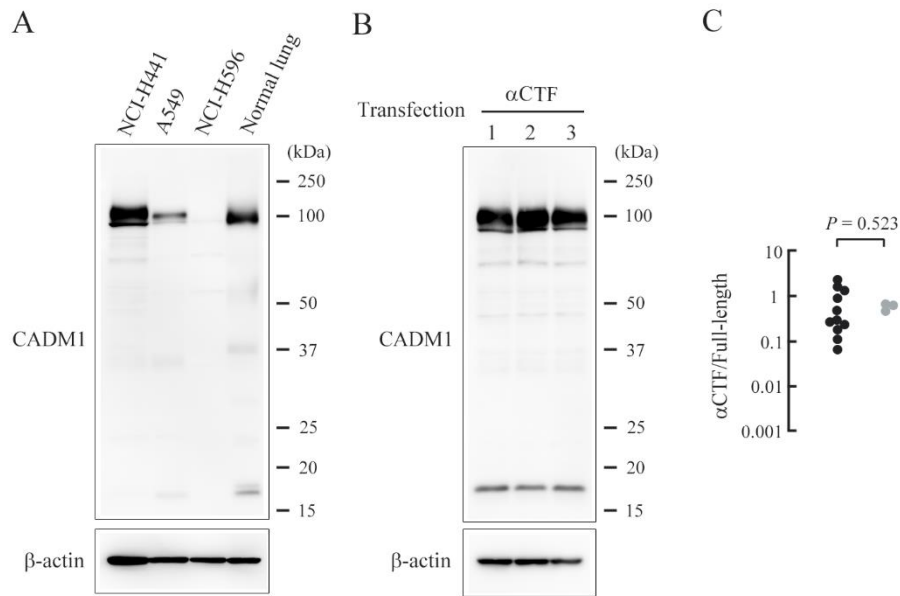
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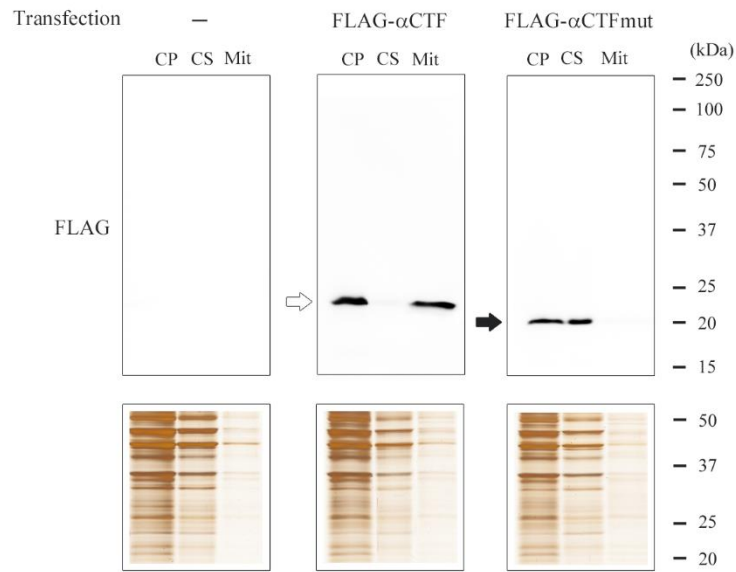
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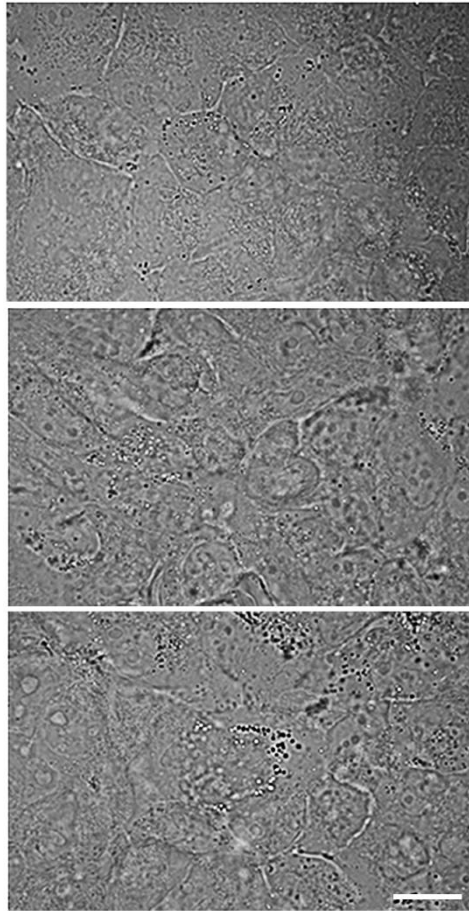


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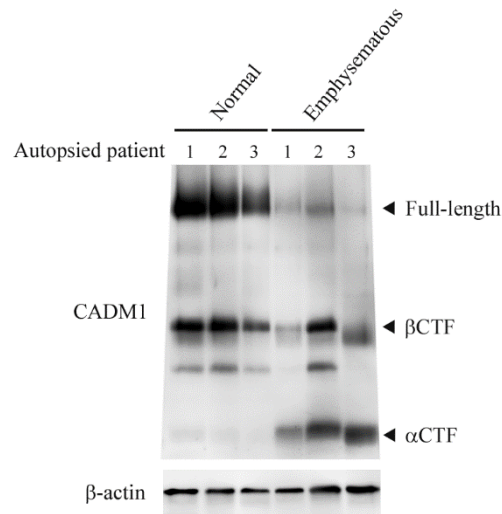
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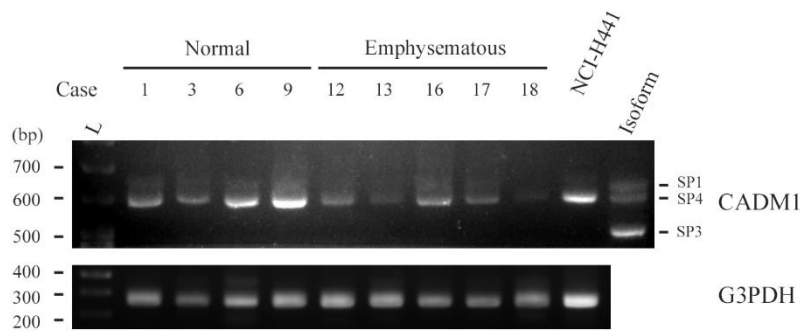


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