Thesis for the Master of Biomedical Sciences

A Study of Molecular Mechanism of CSMD1 as a Tumor Suppressor in Lung Cancer

폐암에서 종양 억제 유전자로서 기능하는 CSMD1의 분자적 기전에 대한 연구

The Graduate School

of the University of Ulsan

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A Study of Molecular Mechanism of CSMD1 as a Tumor Suppressor in Lung Cancer

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Abstract

Lung cancer accounts for a large portion of cancer patient mortality, but lung cancer treatment remained as a challenge for many people. Furthermore, most patients who use immune checkpoint inhibitors (ICIs) develop resistant tumor during or after treatment is discontinued. The CUB and Sushi multi-domain 1 (CSMD1) gene has been proposed as a tumor suppressor gene in many cancers. But the role of CSMD1 as a tumor suppressor in lung cancer is largely unknown. Thus, the objective of this study was to understand the function of CSMD1 as a tumor suppressor gene in lung cancer. CSMD1 over-expression in LLC1 shows inhibition of cell proliferation. And selected candidate genes from RNA-seq recover inhibition of cell proliferation by CSMD1 over-expression. It could possibly relate to the anti-tumor pathway of CSMD1. In BrdU/7-AAD assay, over-expression of CSMD1 decreased S phase. It suggests that the decrease in cell proliferation by CSMD1 expression possibly due to cell cycle arrest. In the tumor allograft model, induction of CSMD1 expression through doxycycline treatment tend to have less cancer metastasis than the control group. The control group showed significantly lower protein expression of pan-cytokeratin and lower ki-67 score than the doxycycline treatment group. It indicates that there were fewer lung cancer cells, which suggest that there was less lung metastasis. Considering that the gene expression level of CSMD1 was significantly higher in the doxycycline treatment group, this suggests that CSMD1 inhibits lung cancer metastasis. Taken together, our results suggest that CSMD1 could function as a biomarker for diagnosing lung cancer or be applied as a immunotherapy target.

Introduction

Lung cancer accounts for a large portion of cancer-related deaths worldwide. In 2023, out of approximately 1.95 million cancer patients, 238,340 were lung cancer patients, and 127,070 of them resulted in death. This corresponds to approximately 20.8% of the total mortality rate, and lung cancer causes serious health problems due to its high mortality rate and prevalence[1]. The poor prognosis of lung cancer is due to the difficulty in early diagnosis and the low response rate to conventional chemotherapy[2]. Current treatments for lung cancer include surgery[3], radiotherapy[4], chemotherapy[5], and immunotherapy[6]. Some patients with lung cancer experience long-term clinical benefits using immune checkpoint inhibitors (ICIs). Among immune checkpoint inhibitors, PD-1 inhibitors have brought about many changes in the treatment of lung cancer[7]. It has been shown to improve overall survival (OS) compared to chemotherapy in first-line treatment in patients whose tumors express PD-L1 in at least 50% of their cells[8]. Recently, the combination of ICIs and chemotherapy has been shown to improve survival in patients with squamous[9] and non-squamous[10] NSCLC, regardless of PD-L1 expression. However, PD-L1 and tumor mutational burden (TMB) have not proven to be indicative biomarkers[11]. And most patients develop the disease during or after treatment is discontinued[12]. Therefore, lung cancer treatment remains a challenge for many people.

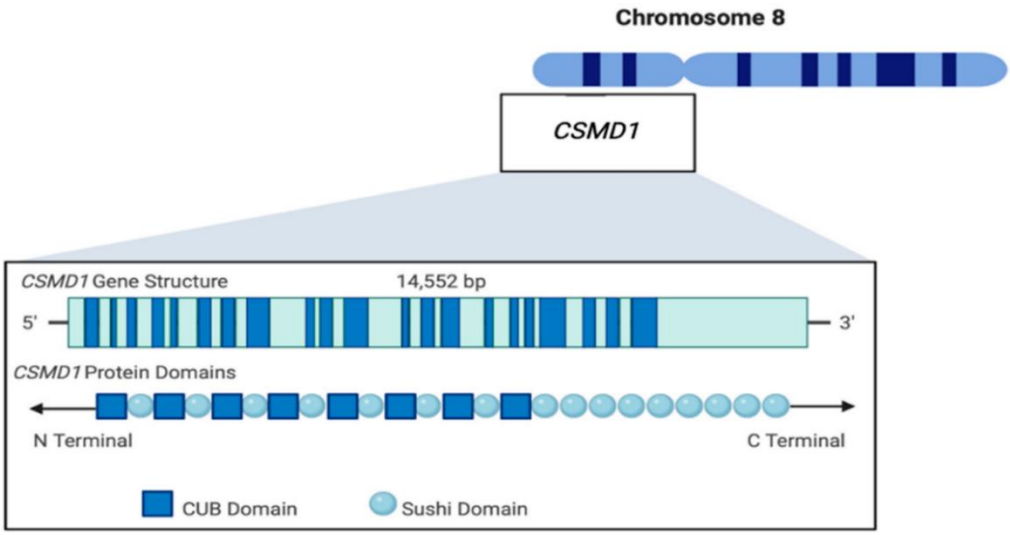


Figure 2. Schematic illustration of the CSMD1 (CUB and Sushi Multiple Domains 1) gene and protein structures[13].

The CUB and Sushi multi-domain 1 (CSMD1) genes consist of 71 exons and are present on 8p23.2 chromosomes[14] (Fig 1). The CSMD1 protein is a transmembrane protein with multiple CUB and SUSHI repeats[15]. There is a variety of evidence suggesting that CSMD1 is a tumor suppressor gene. CSMD1 is known to exhibit frequent deletions in many cancers, including lung cancer[16-19]. In the case of esophageal cancer, patients with mutations in CSMD1 had a lower survival rate and lower sensitivity to chemotherapeutic agents[20]. In addition, in breast cancer, CSMD1 expression was lower in tumor tissue than in normal tissue, and the overall survival rate and disease-free survival rate were higher in patients with high CSMD1 expression[21]. Additionally, breast cancer cells over-expressing CSMD1 were injected into mammary fat pad of SCID mice, and lymph node metastasis was relatively low compared to the control group[22]. And CSMD1 protein expression was reduced in colon cancer tissues compared to normal tissues[23], and somatic mutations in CSMD1 were found in colon cancer[24]. Additionally, over-expressing CSMD1 in melanoma cells decreased cell proliferation, increased apoptosis rate, and decreased cell migration[25].

The role of CSMD1 as a tumor suppressor gene in lung cancer is unknown. However, CSMD1 loss occurred frequently in lung carcinoma and lung adenocarcinoma. And compared with normal tissues, the expression of several exons of the CSMD1 transcript was decreased in tissues from Squamous Cell Lung Cancer patient[26]. According to unpublished data, lung cancer patients with CSMD1 mutations had a lower survival rate than patients without mutations, and patients with CSMD1 gene deletion are thought to show more resistance to immunotherapy for lung cancer.

Therefore, in this paper, we tried to find out the function of CSMD1 as a tumor suppressor gene. We would like to find out how CSMD1 suppresses lung cancer, using a Lung Cancer cell line that overexpresses CSMD1. Through these results, we would like to suggest the possibility that CSMD1 may function as a biomarker for diagnosing lung cancer or be applied as a immunotherapy target.

Materials and Method

- Reagents

The experiment was performed using the following antibodies.
CSMD1 (ab166908, Abcam, UK), Ki-67(ab16667, Abcam, UK), Anti-V5 tag (ab27671, Abcam, UK), β-actin (sc-47778, Santa-cruz biotechnology, US), Pan-Cytokeratin (sc-81714, Santa-cruz biotechnology, US).

- Vectors

We obtained the HES1 and SRPK1 over-expression vectors from the Korea Human Gene Bank. The SMC2 over-expression vector was constructed through Gateway cloning.

- Cell culture

Mouse lung cancer cell line LLC1 was cultured in Dulbecco's Minimum Essential Medium (DMEM, HyClone, US) containing 10% fetal bovine serum (HyClone, US) and 1% penicillin/streptomycin (HyClone, US). TR, TR+/CSMD1-, and TR+/CSMD1+ cells were cultured in the same media and subcultured using Trypsin-EDTA (0.05%, Gibco, US). All cells were maintained at 37°C in a 5% CO2-humidified cell incubator.

- Transfection

The day before transfection, plate 1*10^6 cells in 100mm dish. Dilute DNA and P3000 (L3000001, ThermoFisher, US) in Opti-MEM (31985062, ThermoFisher, US) and mix gently. Mix Lipofectamine 3000 (L3000001, ThermoFisher, US) gently in Opti-MEM and incubate for 10 minutes at room temperature. After 10 minutes incubation, combine the diluted DNA with the diluted Lipofectamine 3000. Mix gently and incubate for 15 minutes at room temperature. After that, Add the DNA-Lipofectamine 3000 complexes to growth medium. Change the medium after 12 hours.

- Infection

The day before transfection, plate 2*10^6 HEK-293Tx cells in 100mm dish. Dilute DNA and P3000 in Opti-MEM and mix gently. The interested DNA was packaged with psPAX2 and pMD2.G. Mix Lipofectamine 3000 gently in Opti-MEM and incubate for 10 minutes at room temperature. After

10 minutes incubation, combine the diluted DNA with the diluted Lipofectamine 3000. Mix gently and incubate for 15 minutes at room temperature. After that, Add the DNA-Lipofectamine 3000 complexes to growth medium. Change the medium after 12 hours. The next day, the lentiviral particles was added into cells 3 times every 12 hours. The lentivirus was filtered using 0.45um syringe filter (4654, Pall corporation, US) after centrifugation at 1,000 rpm for 5 minutes. Then, the supernatant was added with hexadimethrine bromide (H-9268, Sigma-Aldrich, US).

- Protein extraction and western blot analysis

Proteins were extracted using RIPA buffer (RC2002-050-00, biosesang, Republic of Korea) containing 1 tablet of protease inhibitor (05 892 791 001, Roche, Switzerland) and 1 tablet of phosphatase inhibitor (04 906 837 001, Roche, Switzerland). After adding RIPA buffer, the sample was waited on ice for 15 minutes and then centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant containing protein is mixed with sample buffer and boiled at 98°C for 10 minutes. Western blot was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

- Densitometry analysis of proteins

The densitometry analysis of protein was calculated using imageJ (v1. 54f). Blot images were imported into the imageJ. Selected the region of interest (ROI) and obtained pixel densities of each band. All pixel densities were then calculated.

- cDNA synthesis and Real-Time PCR

RNA was extracted using Tri-RNA Reagent (FATRR 001, Favorgen, Taiwan) and the manufacturer's protocol was followed. To measure the relative expression of CSMD1, real-time PCR was performed using Bio Rad CFX96 (Bio-Rad, US) and AMPIGENE qPCR Green Mix Lo-ROX (ENZ-NUC103-1000, Enzo, US). All data were relatively quantified using internal control genes.

Table 1. PCR primer list

Primer	Sequence
CSMD1 Forward 5`-3`	TCCTGCTCATAACACCTGC
CSMD1 Reverse 5`-3`	ACATTCGGTCTGTATCCCACT
RPL13a Forward 5`-3`	TATGCTGCCCCACAAAACCA
RPL13a Reverse 5`-3`	CTTCCAGCCAACCTCGTG
mGAPDH Forward 5'-3'	AACTTTGGCATTGTGGAAGG
mGAPDH Reverse 5'-3'	ACACATTGGGGGTAGGAACA
PIEZO2 Forward 5'-3'	TCATGTTCCTGGCTGACACT
PIEZO2 Reverse 5'-3'	TCTTCCTGAGGTAGAGGGCT
RNF180 Forward 5'-3'	GGTGGTGGCTCTGAAAACAG
RNF180 Reverse 5'-3'	CATCTGCCAGTCACAAGCTG
HES1 Forward 5'-3'	AAAAATTCCTCGTCCCCGGT
HES1 Reverse 5'-3'	TGCCGCGAGCTATCTTTCTT
DSCC1 Forward 5'-3'	TCCATATGAAGGACCTGACAGT
DSCC1 Reverse 5'-3'	CCGAGTTCCTGAAGGCATGT
ASPM Forward 5'-3'	TCCCGTCACCTTGGCTTATT
ASPM Reverse 5'-3'	TGCCGGAATCCTGAGTTTCT
RAD51AP1 Forward 5'-3'	TGGAAGGCAGTGATGGTGAT
RAD51AP1 Reverse 5'-3'	AGTCCACCGAAGTCACCAAA
SRPK1 Forward 5'-3'	CGGACCACTGGACAACAAAG
SRPK1 Reverse 5'-3'	CCAGTTCAAAGGCCATGCAT
SMC2 Forward 5'-3'	TCTCAGGTTCGGGCTTCTAA
SMC2 Reverse 5'-3'	CTGGTGTTGTTGGCATTGAC

- Fluorescence-activated cell sorting (FACS)

  Harvest the cells and put 5*10^5 cells into e-tube. Add 2ug of 1st Ab and incubate for 2 hours at 4 ℃. Afterwards, add 2nd Ab at 1:500 and incubate for 30 minutes at room temperature without light. Afterwards, cells are filtered using a strainer, analyze on a flow cytometer.

- Cell proliferation assay

Cells were seeded at 1,500 cells per well in a 96 well plate (30096, SPL Life Sciences, Republic

of Korea) and cultured in a final volume of 100ul (n=3). After 24 hours, add 10ul Quanti-Max™ WST-8 cell viability assay kit solution (QM1000, BioMax, Republic of Korea) according to the manufacturer's guidelines and use a multi-mode microplate reader (Synergy H1, Bio Tek, US). The absorbance was detected at a wavelength of 450 nm. In addition, 2ug/ml tetracycline was added to TR+/CSMD1- cell medium and cultured for 24 to 72 hours to induce expression of CSMD1. Afterwards, the absorbance was at a wavelength of 450 nm.

- BrdU/7-AAD staining

8,000 cells were seeded in a 60mm cell culture dish (20060, SPL Life Sciences, Republic of Korea) and treated with 10uM BrdU 60 hours later. After 72 hours, cells were harvested according to the guidelines of the BD Pharmingen™ FITC BrdU Flow Kit (559619, BD Pharmingen™, US), treated with BrdU and 7-AAD, analyze on a flow cytometer.

- Annexin V-FITC staining

Cells were seeded at 3*10^5 cells in a 100mm cell culture dish, and 2ug/ml tetracycline was added to the cell culture medium. After 72 hours, cells were harvested according to the guidelines of the Annexin V-FITC Apoptosis Staining / Detection Kit (ab14085, Abcam, UK), treated with Annexin V and PI. Analyze Annexin V-FITC binding by flow cytometry using FITC signal detector and PI staining by the phycoerythrin emission signal detector.

- Generation of tumor allograft model

For the lung allograft model, 5th week of age, male C57BL/6 mice were injected into the tail vein with LLC1 TR9+/CSMD1- cells at a concentration of 5*10^6 cells/ml at a volume of 100ul. For CSMD1 expression, the feed and water of the experimental group contained 2 mg/ml doxycycline (22586, Glentham life sciences, UK) and 20 mg/ml sucrose crystalized (S0809, Duchefa Biochemie, Netherlands). And the other group, the control group, did not receive doxycycline. After 3 weeks, two of the control mice were sacrificed and their lungs were collected. Five days later, all remaining mice were sacrificed, lungs and spleens and blood were collected.

- Immunohistochemistry (IHC)

Immunohistochemical analysis of ki-67 expression was performed with the anti-ki-67 antibody at a 1:200 dilution. paraffin sections measuring 4um were de-paraffinized in graded alcohols.

Endogenous peroxide activity was inhibited by immersing the sections in 0.5% H2O2. Antigen retrieval was carried out by immersing the sample in citrate buffer (CBB999, Scytek, US) and boiling it. Sections were incubated with the antibody for overnight at 4℃. Bound antibody was detected with VECTASTAIN® Elite® ABC-HRP Kit (PK-6200, VECTOR LABORATORIES, US). Sections were counterstained with EASYSTAIN Harris Hematoxylin (S2-5, YD Diagnostics, Republic of Korea).

Result

Table 2. List of 20 upregulated genes from RNA-seq analysis

Gene symbol	Log2FC	*p*Val
ASIC2	9.98273743	6.0551E-11
MYH14	9.8384976	1.6378E-10
IGSF21	9.71173038	4.0413E-10
SYT16	9.41892167	2.8388E-09
FER1L6	9.31372833	5.6839E-09
PTH2R	9.31372833	5.6839E-09
ADGRD1	9.24672209	8.1804E-09
SMOC1	9.22367678	9.261E-09
PIEZO2	9.20025736	1.192E-08
PLXDC2	9.20025736	1.192E-08
PKNOX2	9.12762787	1.76E-08
CFAP221	8.97038416	4.6257E-08
ADGRB3	8.91391568	7.1922E-08
SCML4	8.88483037	8.3655E-08
PTPRE	8.85514661	9.7505E-08
KCND3	8.79388151	1.3333E-07
DOK5	8.72989892	2.1689E-07
FAM178B	8.72989892	2.1689E-07
SEMA5B	8.72989892	2.1689E-07
TCERG1L	8.72989892	2.1689E-07

Table 3. List of 20 downregulated genes from RNA-seq analysis

Gene symbol	Log2FC	*p*Val
CDC14C	-2.44687411	0.00673976
HES1	-2.44492259	0.00021915
MEIOC	-2.01779139	0.00244308
DSCC1	-1.96215067	0.0040217
RNF212	-1.95682162	0.00354662
CCNE2	-1.93557529	0.00331538
TEX19	-1.88025618	0.02325024
MASTL	-1.85059985	0.00562603
RAD21	-1.80771025	0.00468603
ASPM	-1.76206744	0.00584378
TRIM39	-1.75888225	0.02209136
ANLN	-1.75172955	0.00606398
MKI67	-1.74639749	0.00615185
KIF20B	-1.72114605	0.00719847
RAD51AP1	-1.70999994	0.00758024
XRCC3	-1.65000934	0.01922579
BUB1B	-1.63523721	0.01045772
KIF23	-1.63092785	0.01035924
ESCO2	-1.62679799	0.01272271
GEM	-1.62409667	0.01255175

Identification of genes possibly related to CSMD1 tumor suppression pathway

In previous study by senior researcher, it was possible that CSMD1 is would be tumor suppressor gene in lung cancer. The reason why over-expression of CSMD1 showed inhibition of cancer cell growth and migration, and less metastasis of lung cancer in over-expressed CSMD1 in vivo model. But the anti-tumor pathway and its components have not been investigated. Therefore, RNA-seq was performed to identify the signaling pathways and involved genes in CSMD1 associated function as a tumor suppressor in lung cancer. RNA-seq was performed after confirming induction of CSMD1 gene expression (data not shown).

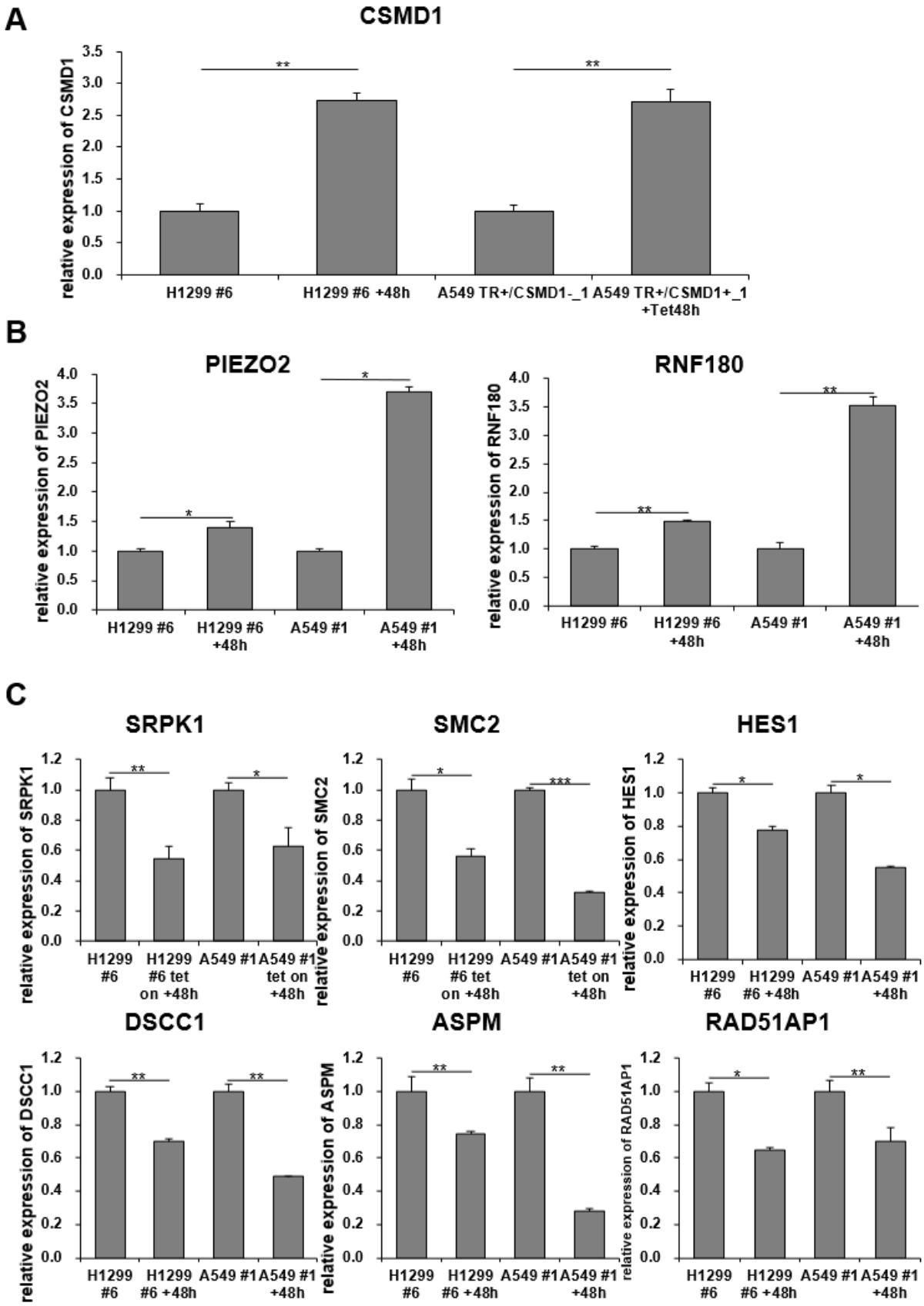
Using the Tet-on inducible CSMD1 expression system in H1299 human lung cancer cell line, the expression of CSMD1 was regulated according to tetracycline treatment. And the transcriptome was analyzed depend on CSMD1 expression during treatment of tetracycline for 24 and 48 hours were compared, since there was a difference in CSMD1 expression levels at 24 and 48h in a previous study.

Based on RNA-seq results, the gene expression level was compared with CSMD1. And 2-fold change of genes according to CSMD1 expression were compared. Then, upregulated and downregulated of 20 genes were selected (Tables 1 and 2). Also, relationship between gene expression and cancer prognosis and expression in cancer are investigated. Among these genes, those that showed significantly different expression in non-patients and cancer patients and had a significant relationship with cancer prognosis were selected.

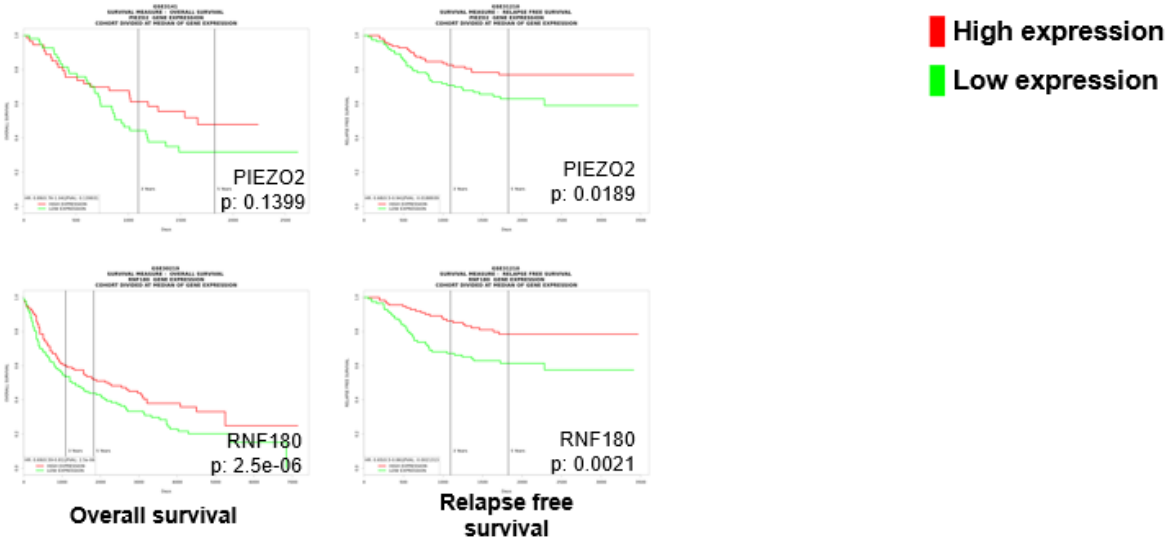
Afterwards, qRT-PCR primers were designed for the selected genes, and differential gene expression patterns in response to CSMD1 expression were verified in H1299 and A549 cells. After 48 hours of tetracycline treatment, CSMD1 was over-expressed at gene expression (Fig 2A). Notably, the qRT-PCR analysis revealed an increase in the expression levels of PIEZO2 and RNF180, consistent with the upregulation observed in the RNA-seq data upon CSMD1 over-expression (Fig 2B). Conversely, the gene expression levels of SRPK1, SMC2, HES1, DSCC1, ASPM, and RAD51AP1, which were downregulated in the RNA-seq data upon CSMD1 over-expression, exhibited decreased gene expression levels in the qRT-PCR analysis (Fig 2C).

To narrow down the list of selected genes, the overall survival and relapse free survival of lung cancer patients according to the selected genes were investigated using PROGgeneV2. The upregulated selected genes (PIEZO2, RNF180) were positively correlated with the good prognosis, with higher overall survival and relapse free survival in lung cancer patients (Fig 2D). On the other hand, the downregulated selected genes (SRPK1, SMC2, HES1, DSCC1, ASPM, RAD51AP1) were positively correlated with the poor prognosis, with lower overall survival and relapse free survival in lung cancer patients (Fig 2E).

Therefore, we identified the candidate genes involved in CSMD1 anti-tumor pathway by RNA-seq. And it could possibly have an important relationship with lung cancer prognosis.


A
CSMD1
relative expression of CSMD1
H1299 #6
H1299 #6 +48h
A549 TR+/CSMD1-_1
A549 TR+/CSMD1+_1 +Tet48h
B
PIEZO2
relative expression of PIEZO2
RNF180
relative expression of RNF180
H1299 #6
H1299 #6 +48h
A549 #1
A549 #1 +48h
C
SRPK1
relative expression of SRPK1
SMC2
relative expression of SMC2
HES1
relative expression of HES1
H1299 #6
H1299 #6 tet on +48h
A549 #1
A549 #1 tet on +48h
DSCC1
relative expression of DSCC1
ASPM
relative expression of ASPM
RAD51AP1
relative expression of RAD51AP1
H1299 #6
H1299 #6 +48h
A549 #1
A549 #1 +48h

D


High expression
Low expression
PIEZO2
p: 0.1399
PIEZO2
p: 0.0189
RNF180
p: 2.5e-06
RNF180
p: 0.0021
Overall survival
Relapse free survival

E

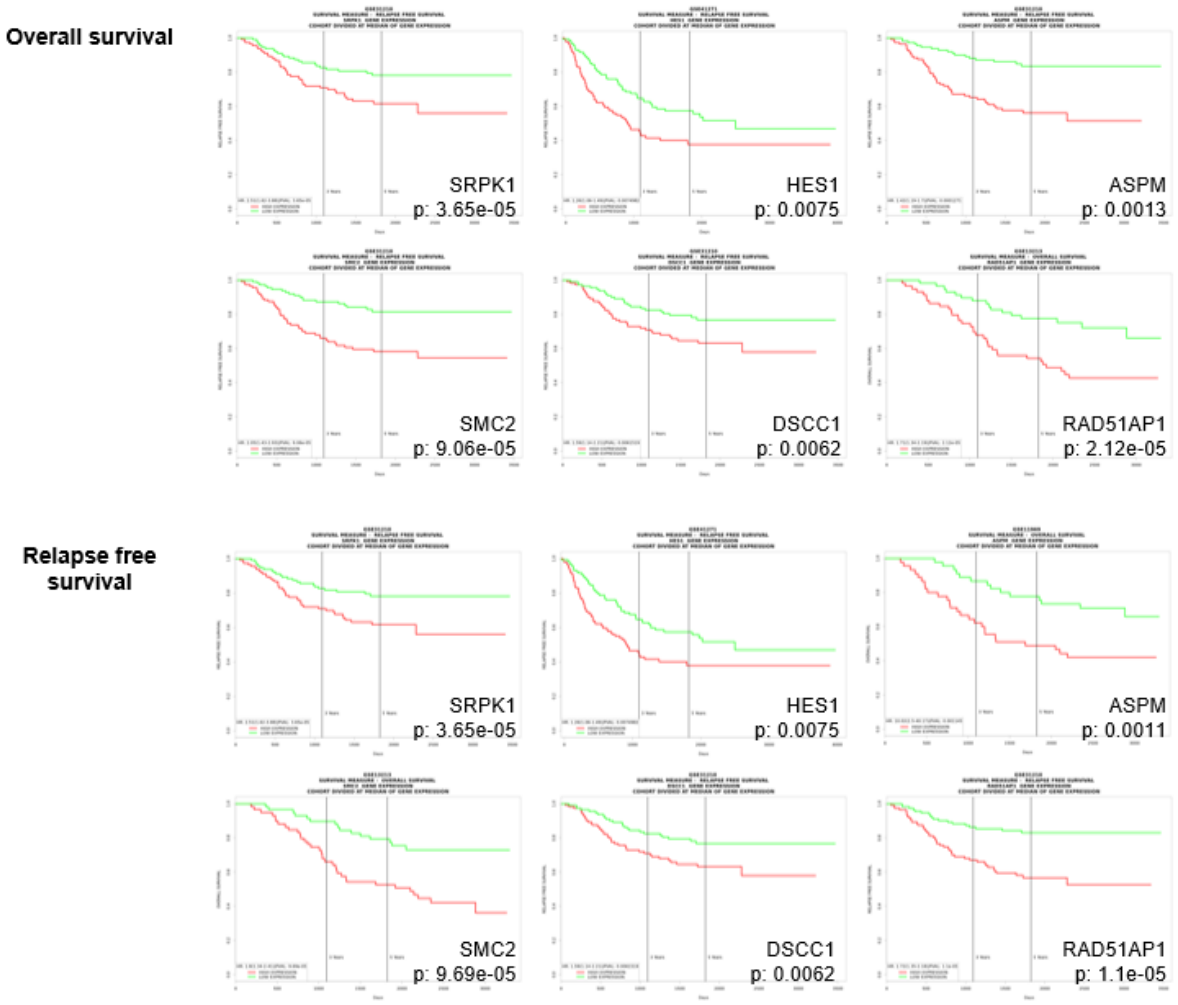

Overall survival
SRPK1
p: 3.65e-05
HES1
p: 0.0075
ASPM
p: 0.0013
SMC2
p: 9.06e-05
DSCC1
p: 0.0062
RAD51AP1
p: 2.12e-05
Relapse free survival
SRPK1
p: 3.65e-05
HES1
p: 0.0075
ASPM
p: 0.0011
SMC2
p: 9.69e-05
DSCC1
p: 0.0062
RAD51AP1
p: 1.1e-05

Figure 2. Identification of genes possibly related to CSMD1 tumor suppression pathway.

A The gene expression of CSMD1 by qRT-PCR. **B** The gene expression of PIEZO2 and RNF180 by qRT-PCR. **C** The gene expression of SRPK1, SMC2, HES1, DSCC1, ASPM, and RAD51AP1 by qRT-PCR. **D** The prognosis of up-regulated selected genes (PIEZO2 and RNF180) by PROGgeneV2. **E** The prognosis of down-regulated selected genes (SRPK1, SMC2, HES1, DSCC1, ASPM, and RAD51AP1) by PROGgeneV2. (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$)

Generation of Tet-repressor and CSMD1 stable cell line to CSMD1 Over-expression

In the previous study, the tumor xenograft model was not successful (Data not shown). This is because xenografts can cause unexpected negative reactions *in vivo* due to immune rejection. As a result, there arises a demand for an alternative *in vivo* tumor model for the investigation of how CSMD1 affects the growth and metastasis of lung cancer. Therefore, tumor allograft model was designed. And it is important to make a vector that ensures stable and continuous expression of CSMD1.

When CSMD1 was over-expressed in a lung cancer cell line, the cells died or could not make normal growth. So, it was important to make the Tet-on inducible CSMD1 expression system. Consequently, the Tet-on system was applied to LLC1, allowing the expression of CSMD1 only when cells were treated with tetracycline.

Vector that over-expresses CSMD1 was constructed and was infected into cells to study CSMD1 anti-tumor effect on lung cancer cells. This plasmid was created by senior researcher. The Gateway cloning method was used to generate the inducible CSMD1 plasmid in the Tet-On inducible system.

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When construct an expression vector by gateway cloning, there must be a Tet-operator promotor, which is the locus where the Tet-repressor binds to the destination vector. And LR recombination reaction was performed to fuse the entry clone with the CSMD1 ORF to the destination vector (Fig 3A).

Lentiviral vectors were used to ensure stable and prolonged expression of CSMD1. The Tet-on inducible CSMD1 expression system was infected into mouse lung cancer cell line LLC1. Before infection with lentiviral particle containing the ORF of CSMD1, Tet-repressor expression was necessary to inhibit the transcription of CSMD1. Therefore, Tet-repressor stable cells were generated in LLC1, and selected cells by antibiotics that strongly express Tet-repressor. These clonal cells were named LLC1 TR9. And lentivirus expressing CSMD1 was added to LLC1 TR9 with Tet Repressor. Before treatment with tetracycline, CSMD1 transcription was repressed by the Tet-repressor. And we called these cells LLC1TR9+/CSMD1-, which had both Tet-Repressor and CSMD1.

Then, it was important to check the protein expression of CSMD1. Initially, Fluorescence Activated Cell Sorting (FACS) was used to confirm over-expression of CSMD1 using CSMD1 and V5 antibodies.

CSMD1 was transfected into 293Tx, and the level of CSMD1 over-expression was confirmed 48 hours later. As a result, FACS showed low binding affinity of CSMD1 and V5 antibodies (Fig 3B), despite qRT-PCR (Fig 3C) and Western Blot (Fig 3D), which CSMD1 was over-expressed after CSMD1 transfection. The black peak represented cells treated with secondary antibody only, the red peak indicates treatment with primary and secondary antibodies on non-transfected cells, and the blue peak indicates treatment with primary and secondary antibodies on cells over-expressing CSMD1. However, there was no significant difference between the red and blue peaks, suggesting that CSMD1 and V5 antibodies didn't bind well to the antigen.

Hence, Western blot and qRT-PCR were selected as the methods for confirming the CSMD1 expression. The LLC1TR9+/CSMD1- cells were treated with 2 μg/ml of tetracycline for 24 to 48 hours. qRT-PCR (Fig 3E) and Western-blot (Fig 3F) were performed to confirm the expression of CSMD1. While the gene expression of CSMD1 was detected in LLC1TR9+/CSMD1-, no protein expression was observed. Moreover, the expression of CSMD1 was confirmed after treating LLC1TR9+/CSMD1- cells with tetracycline, and both gene and protein expressions were higher than those in LLC1 TR9 and LLC1TR9+/CSMD1- cells that were not treated with tetracycline.

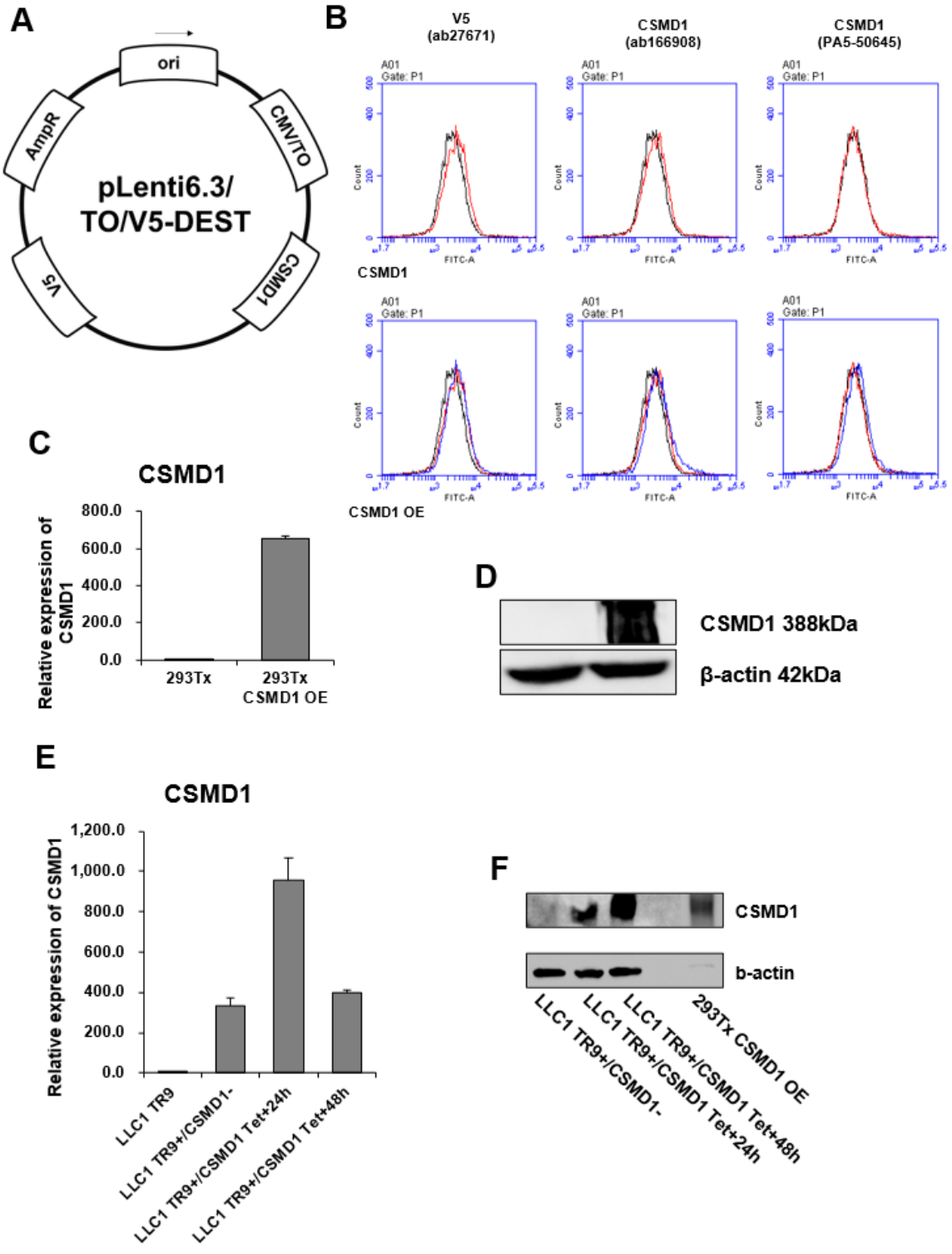


Figure 3. Generation of Tet-repressor and CSMD1 stable cell line to CSMD1 over-expression

A Schematic Tet-on CSMD1 vector map. **B** Binding analysis by FACS. Histogram labels: black: 2nd Ab only; red: Antibodies labeled; blue: Antibodies labeling on transfected cells **C** The gene expression of CSMD1 by qRT-PCR. **D** The protein expression of CSMD1 by western blot. **E** The gene expression of CSMD1 by qRT-PCR. **F** The protein expression of CSMD1 by western blot.

Inhibition of cell proliferation by CSMD1 over-expression

To investigate the function of CSMD1 as a tumor suppressor gene in lung cancer, it is necessary to observe the inhibition of mouse lung cancer cell growth upon CSMD1 over-expression. Therefore, cell proliferation assay was performed on mouse lung cancer cell line LLC1 to ascertain the impact of CSMD1 over-expression on cell growth. Tetracycline was treated with LLC1 TR9+/CSMD1- and the absorbance was detected. The results demonstrated that the over-expression of CSMD1 led to a significant reduction in cell proliferation (Fig 4A, B). A quantitative difference in absorbance was detected, and a significant difference in cell density was observed. Thus, it suggested that the growth of mouse lung cancer cells is controlled by the expression of CSMD1.

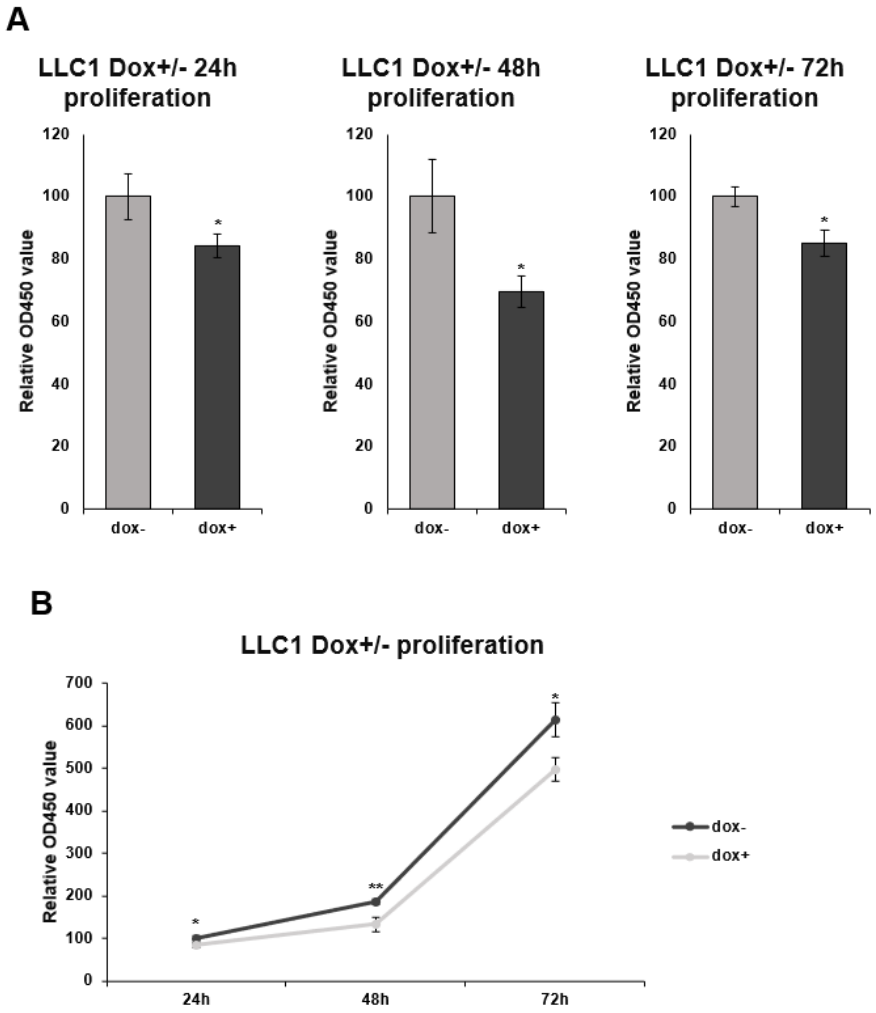


Figure 4. Inhibition of cell proliferation by CSMD1 over-expression

A Cell proliferation assay at a wavelength of 450 nm of absorbance. **B** Comparison of 24-, 48-, and 72-hours of cell proliferation. (* : $p < 0.05$, ** : $p < 0.01$)

Recovery of cell proliferation inhibition through selected gene over-expression

Based on figure 4 results, it was confirmed that cell proliferation decreases when CSMD1 is over-expressed in LLC1 TR9+/CSMD1-. To investigate whether CSMD1 tumor suppression pathway candidate genes affect cell growth, candidate genes were transfected into LLC1 TR9+/CSMD1- cells, and then treated with tetracycline to confirm changes in cell growth. Among several genes, three genes that are oncogenes and are related to lung cancer were chosen for the experiment. Transfection of RFP (negative control) significantly decreased cell proliferation due to over-expression of CSMD1, while the transfection of candidate genes showed either no significant difference in cell proliferation or an increased cell proliferation (Fig 5A). The gene over-expression of CSMD1, HES1, SRPK1, and SMC2 was confirmed by qRT-PCR (Fig 5B). In summary, the candidate genes interfere with the inhibition of lung cancer cell growth by CSMD1.

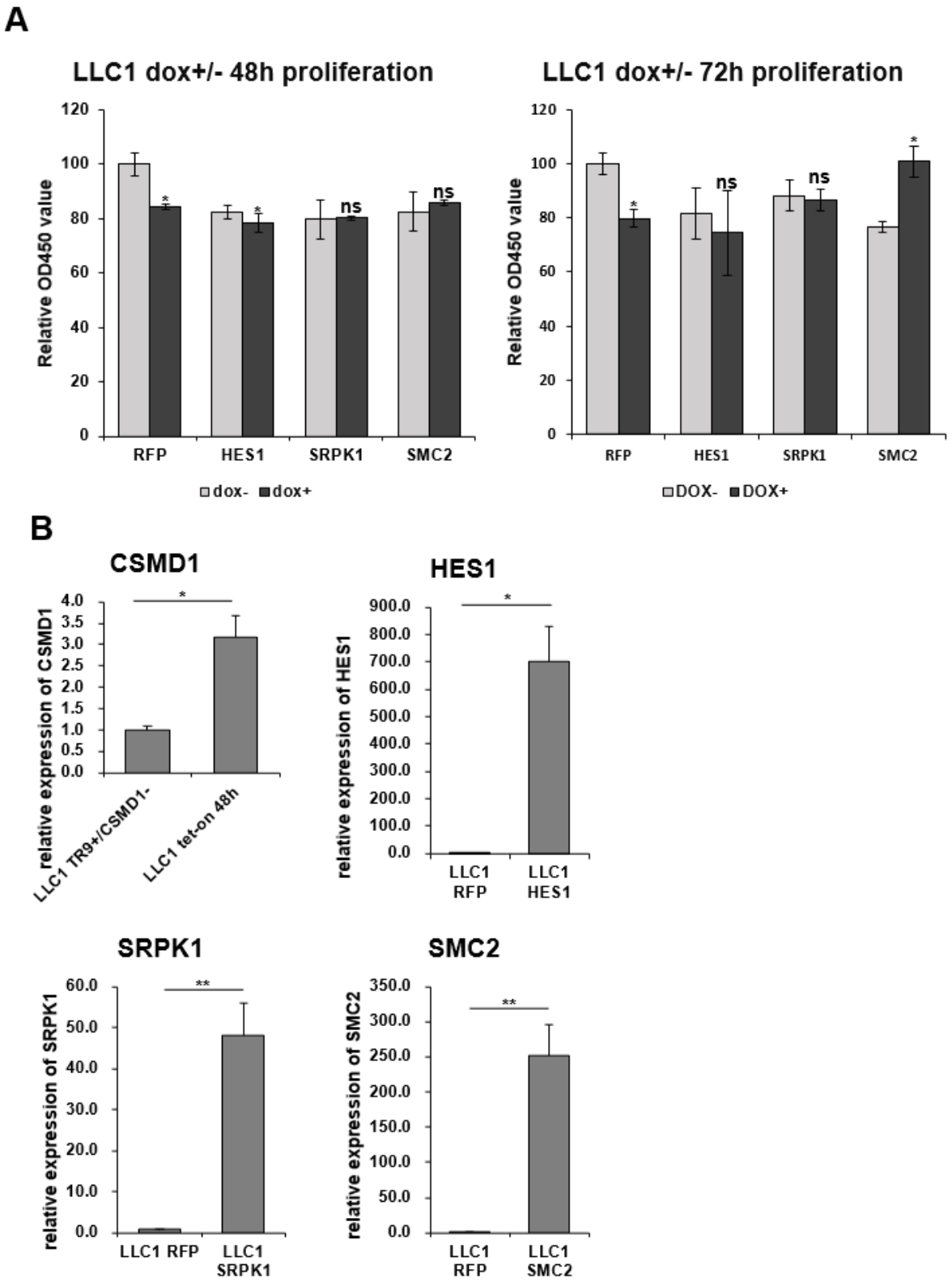


Figure 5. Recovery of cell proliferation inhibition through selected gene over-expression

A Cell proliferation assay at a wavelength of 450 nm of absorbance. **B** Confirmation of over-expressing CSMD1, HES1, SRPK1 and SMC2 by qRT-PCR. (* : $p < 0.05$, ** : $p < 0.01$)

Apoptosis assay by annexin V-FITC staining

It is a question that how CSMD1 inhibits cell proliferation as a tumor suppressor. So, it is necessary to know the mechanism by which cell proliferation decreases.

If cell apoptosis increases, the number of dead cells will increase. So, it could be considered as decrease in cell proliferation. Therefore, Annexin V-FITC staining was performed to determine whether cell proliferation was decreased due to increased cell apoptosis because of CSMD1 over-expression.
Through Annexin V-FITC staining, we confirmed whether CSMD1 over-expression caused by doxycycline treatment changes the level of apoptosis. The experiment was conducted in triplicate for statistical significance (Fig 6A). At 72 hours after treatment with doxycycline, there was no significant difference in apoptosis compared to not treated with doxycycline (Fig 6B). Over-expression of CSMD1 was confirmed by qRT-PCR (Fig 6C).
Apoptosis was not increased when CSMD1 was over-expressed. Therefore, these results showed that the inhibition of cell proliferation was not due to increased apoptosis by CSMD1 over-expression.

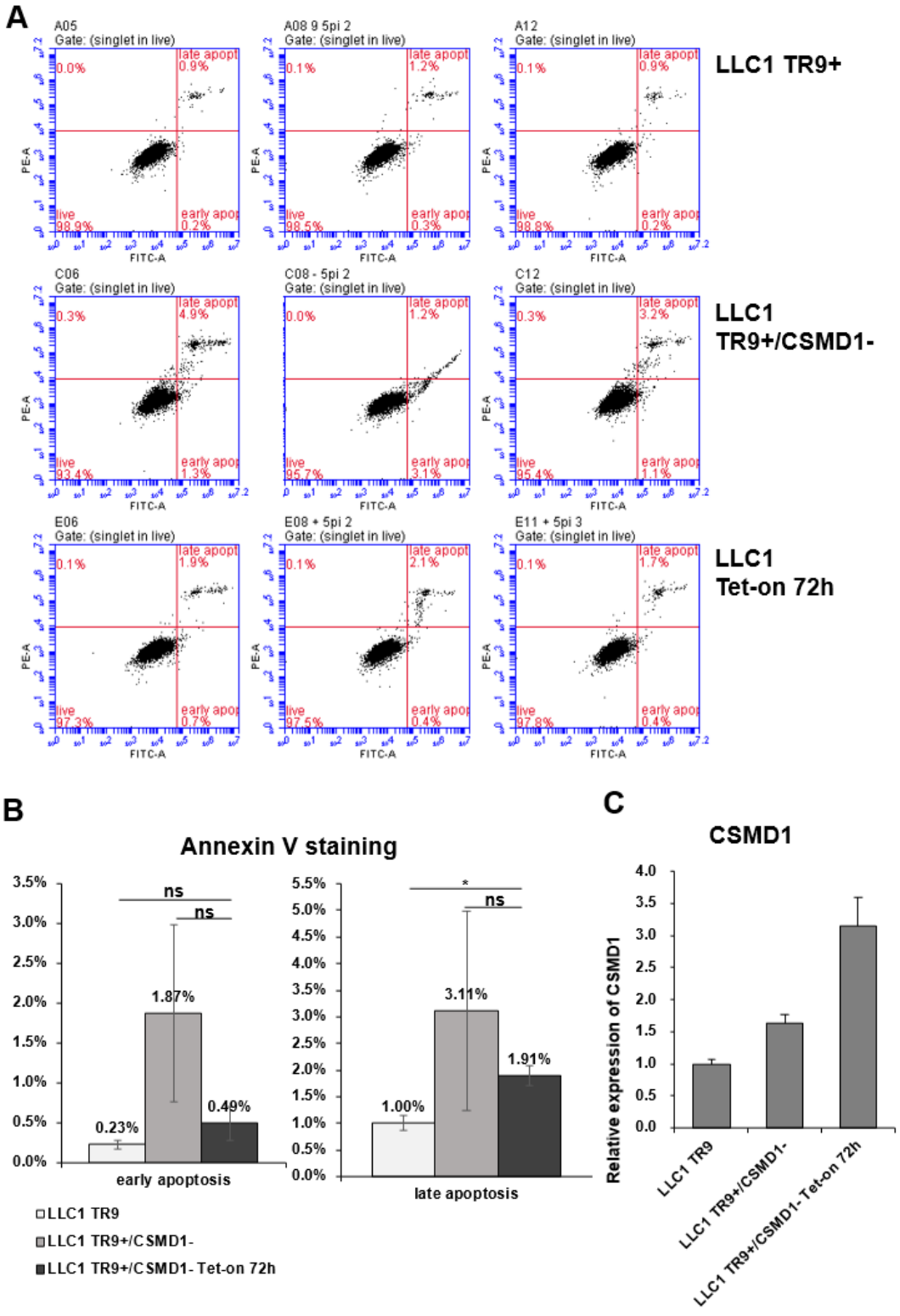


Figure 6. Apoptosis assay by annexin V-FITC staining

A Annexin V-FITC staining by flow cytometry LLC1 cells with/ without CSMD1 expression. **B** Quantification of apoptosis assay with LLC1 cells with/ without CSMD1 expression. **C** The gene expression of CSMD1 by qRT-PCR. (* : $p < 0.05$)

Cell cycle analysis by BrdU/7-AAD staining

Since the decrease in cell growth did not seem to be due to an increase in cell death, another hypothesis was proposed. If cell cycle arrest occurs, it can lead to stop the cell replication, and subsequently cell proliferation may decrease. Therefore, BrdU/7-AAD analysis was performed to prove that the decrease in cell growth was due to cell cycle arrest.

The experiment was conducted in triplicate for statistical significance (Fig 7A). After 72 hours of doxycycline treatment, compared to not treating doxycycline, the S phase significantly decreased, the G2/M phase significantly decreased, and the G0/G1 phase significantly increased (Fig 7B). And when LLC1 TR9+ and LLC1 TR9+/CSMD1- were treated with doxycycline, S phase decreased as CSMD1 was over-expressed. The over-expression of CSMD1 was confirmed by qRT-PCR (Fig 7C).

Considering that the S phase decreased when CSMD1 is over-expressed, it could be considered that DNA replication decreased because DNA synthesis occurs on the S phase.

In conclusion, it is suggested that the decrease in cell growth upon over-expression of CSMD1 is due to a decrease in S phase by cell cycle arrest.

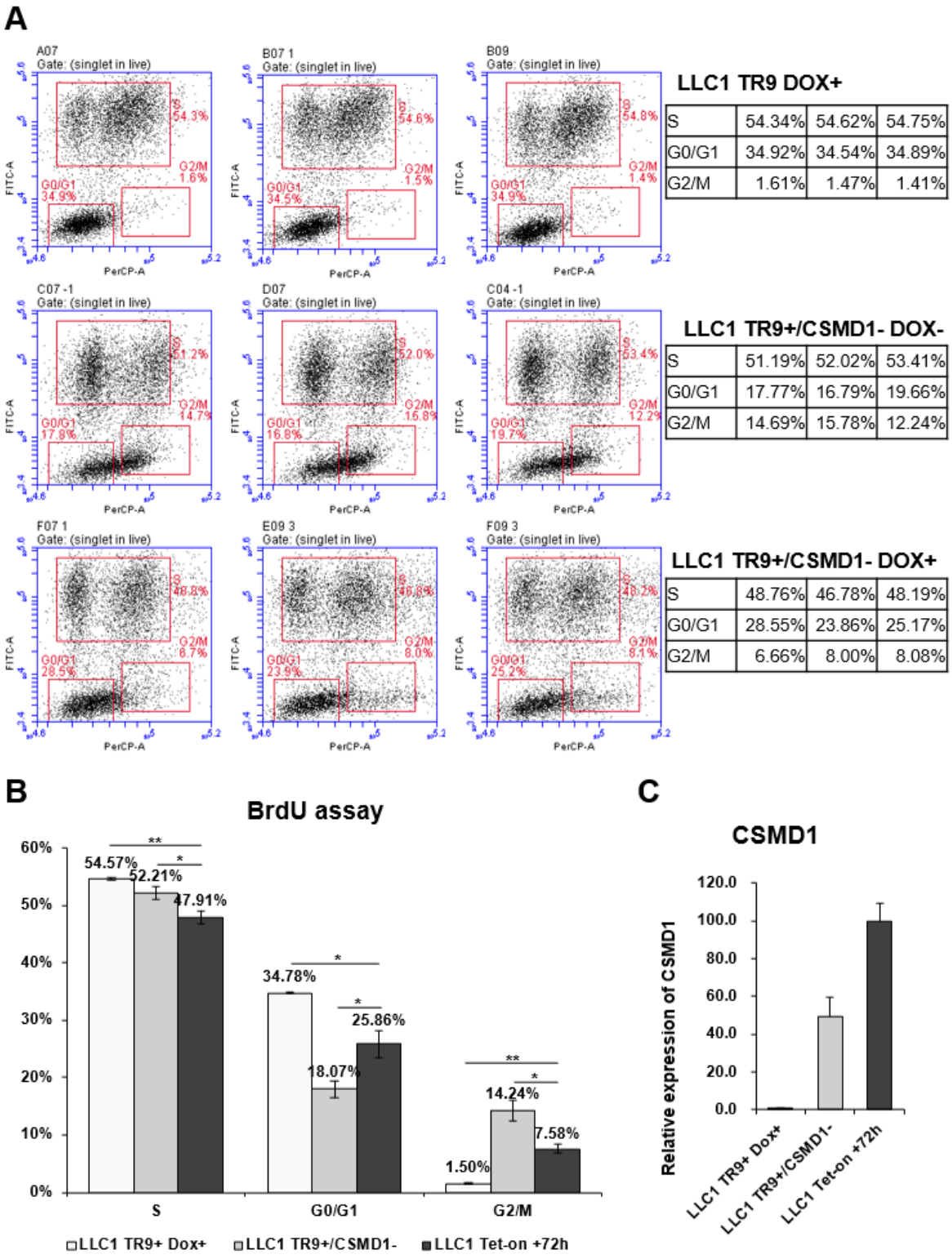


Figure 7. Cell cycle analysis by BrdU/7-AAD staining

A BrdU/7-AAD assay analyzed by flow cytometry with LLC1 cells with/ without CSMD1 expression. **B** Quantification of BrdU/7-AAD staining for apoptosis assay by flow cytometry with LLC1 cells with/ without CSMD1 expression. **C** Confirmation of CSMD1 gene expression by qRT-PCR. (* : $p < 0.05$, ** : $p < 0.01$)

Analysis of tumor allograft model according to CSMD1 expression

To confirm the CSMD1 function as a tumor suppressor gene, *in vivo* experiments are necessary. Therefore, a tumor allograft model is designed for *in vivo* experiments. LLC1 TR9+/CSMD1- cells at a concentration of 5*10^6 cells/ml were injected into the tail vein of 5th week of age, male C57BL/6 mice in a volume of 100ul for the lung allograft model (Fig 8A). The doxycycline treatment group's food and water contained 2 mg/ml doxycycline and 20 mg/ml sucrose crystallized to induce CSMD1 expression. While the control group did not receive doxycycline. Each group consisted of 7 mice, and 1 mouse was not injected. Three weeks after the injection, two mice in the control group, which had lost more than 10% of their body weight were sacrificed to check the level of tumor metastasis. At 26 days after tail vein injection, all mice were sacrificed, and their lungs (Fig 8B), spleens (Fig 8C), and blood was collected.

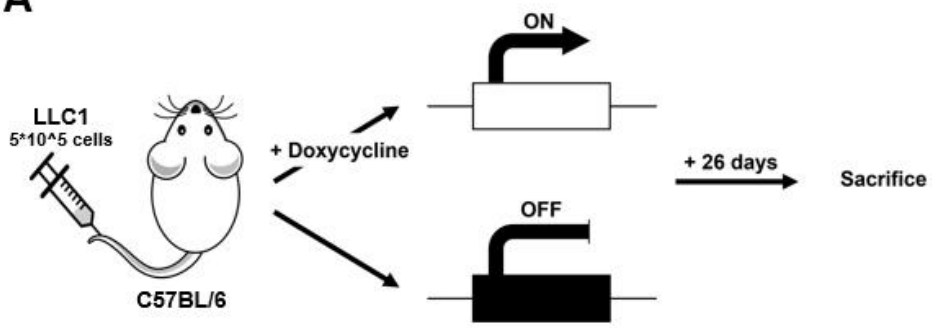
To assess the level of lung metastasis, the metastatic index in lung was calculated through H&E staining (Fig 8D). The doxycycline treatment group tended to have less cancer metastasis than the control group (Fig 8E). Furthermore, the doxycycline treatment group tended to weigh more (Fig 8F) than the control group. Conversely, lung weight (Figure 8G) tended to be lower in the doxycycline treatment group compared to the control group. However, there was no significant difference in spleen weight between the doxycycline treatment group and the control group (Fig 8H).

Additionally, the protein and RNA were extracted from the lungs to confirm the expression level of CSMD1. At the gene expression level, the doxycycline treatment group showed significantly higher expression of CSMD1 than the control group (Fig 8I). However, at the protein level, it was difficult to confirm a significant difference in CSMD1 expression between the doxycycline treatment group and the control group (Fig 8J).

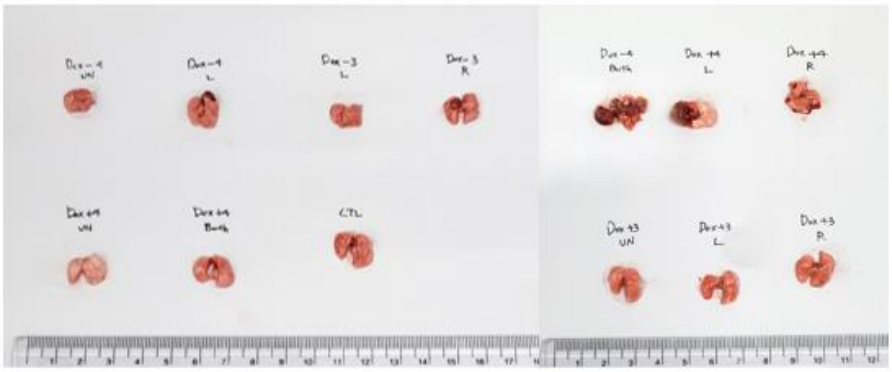
Therefore, the protein expression level of Pan-cytokeratin, an LLC1 marker, was confirmed by western Blot (Fig 8J). And the protein densitometry of Pan-cytokeratin was analyzed using ImageJ and normalized to β-actin (Fig 8K). The doxycycline treatment group showed significantly lower pan-cytokeratin protein expression than the control group, indicating a lower presence of lung cancer cells, which could suggest a lower lung metastasis.

And the expression level of ki-67 was confirmed by immunohistochemistry to compare the level of metastasis of lung cancer cells (Fig 8L). Three samples were selected and stained from the doxycycline treatment group and the control group, and five areas were randomly photographed, and the brown-stained areas were counted. As a result, the ki-67 score in the doxycycline treatment group was significantly lower than the control group. In summary, the tumor allograft model suggests that CSMD1 function as a tumor suppressor that suppresses the metastasis and proliferation of lung cancer *in vivo*.

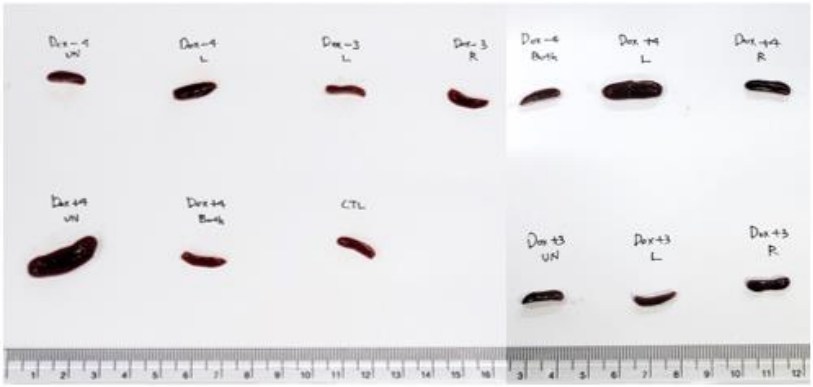
A


LLC1
5*10^5 cells
C57BL/6
+ Doxycycline
ON
OFF
+ 26 days
Sacrifice

B



C



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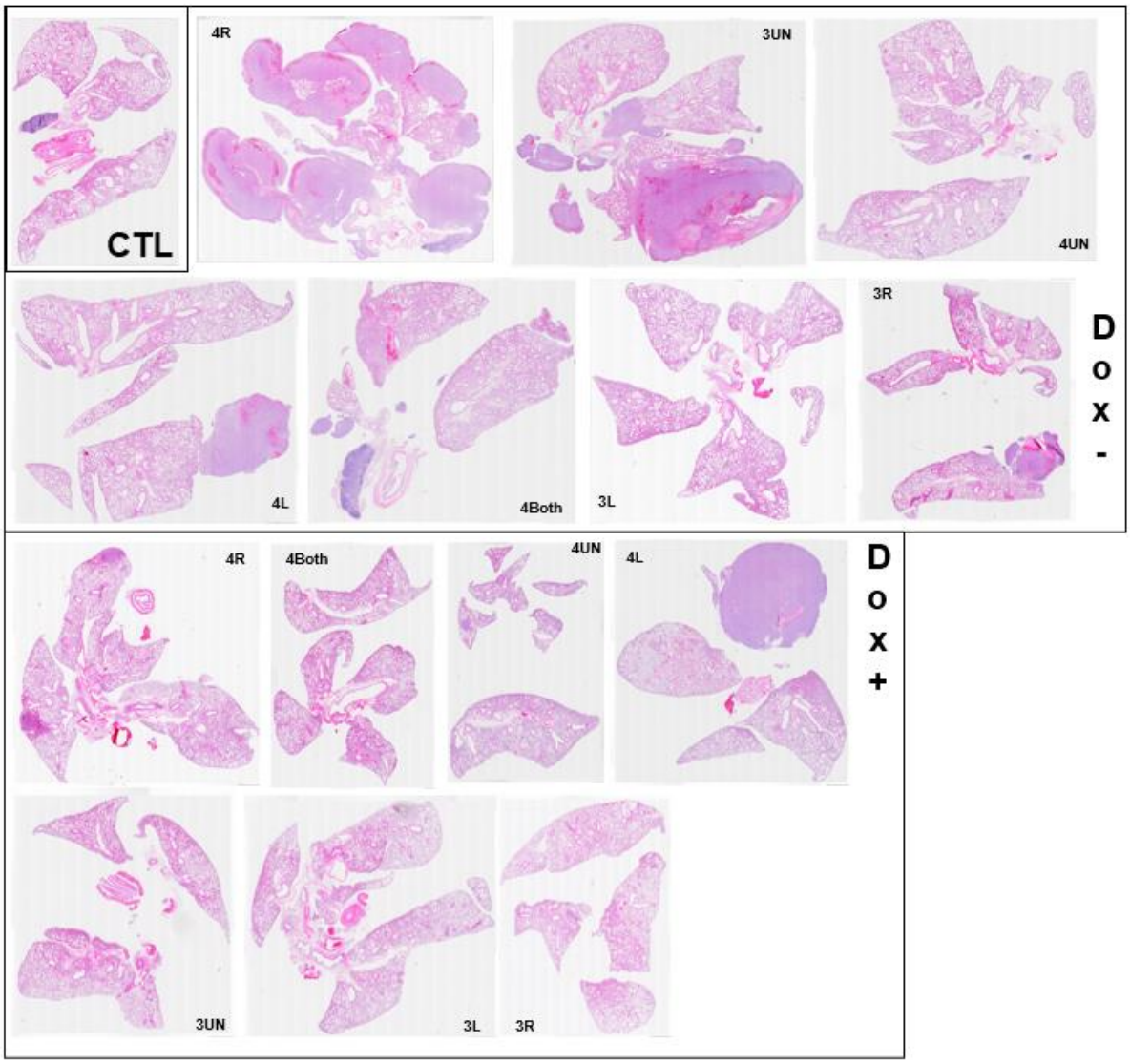
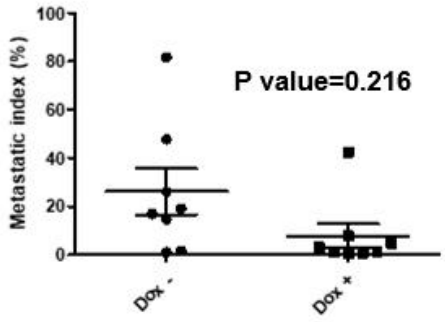
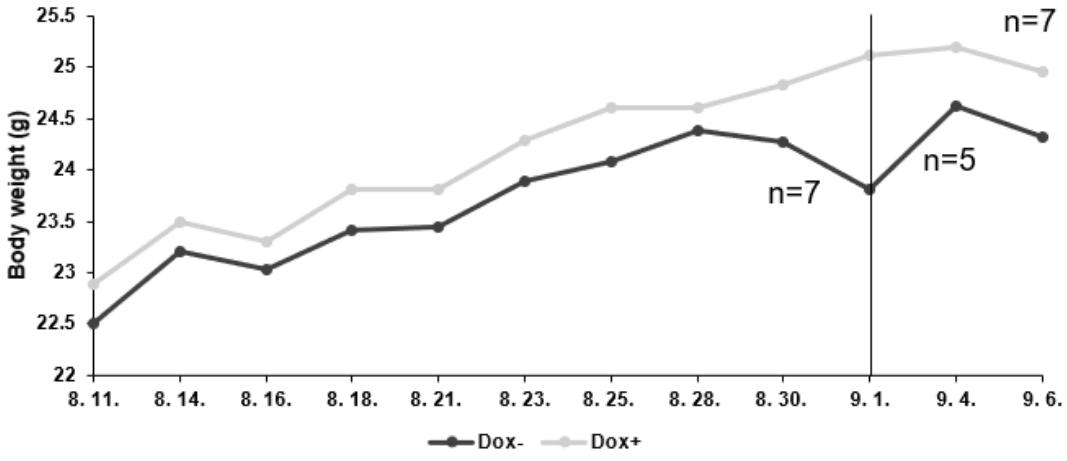

CTL
4R
3UN
4UN
4L
4Both
3L
3R
Dox -
4R
4Both
4UN
4L
3UN
3L
3R
Dox +

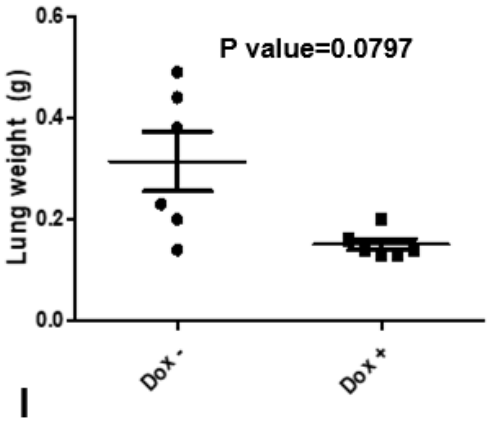
E


Metastatic index (%)
100
80
60
40
20
0
P value=0.216
Dox -
Dox +

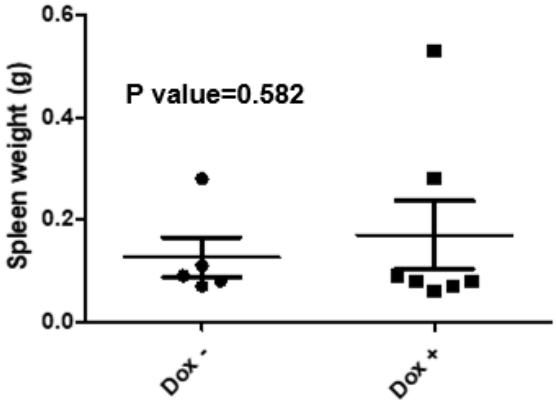
F


25.5
25
24.5
24
23.5
23
22.5
22
Body weight (g)
n=7
n=5
n=7
8. 11.
8. 14.
8. 16.
8. 18.
8. 21.
8. 23.
8. 25.
8. 28.
8. 30.
9. 1.
9. 4.
9. 6.
Dox-
Dox+

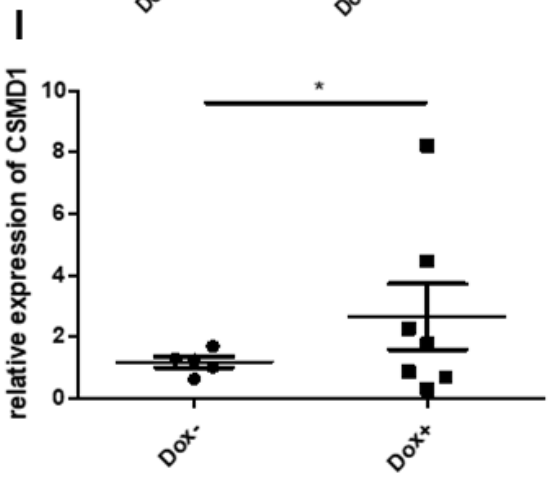
G


0.6
0.4
0.2
0.0
Lung weight (g)
P value=0.0797
Dox -
Dox +

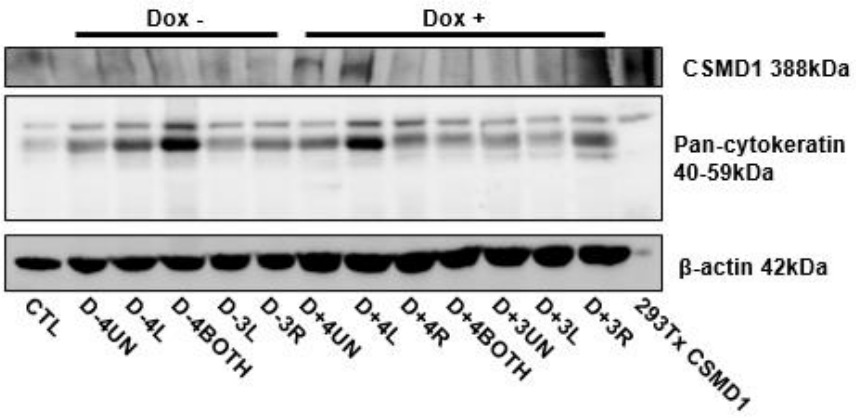
H


0.6
0.4
0.2
0.0
Spleen weight (g)
P value=0.582
Dox -
Dox +

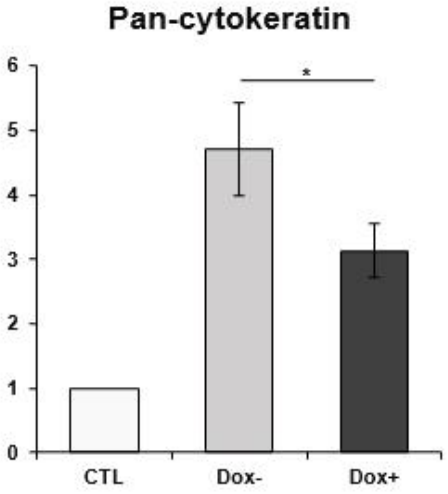
I


10
8
6
4
2
0
relative expression of CSMD1
*
Dox-
Dox+

J



K



Protein densitometry	
CTL	1.66
D-4UN	4.36
D-4L	5.24
D-4B	6.34
D-3L	3.12
D-3R	4.44
D+4UN	3.33
D+4L	4.16
D+4R	3.09
D+4B	2.73
D+3UN	2.88
D+3L	2.04
D+3R	3.67

L

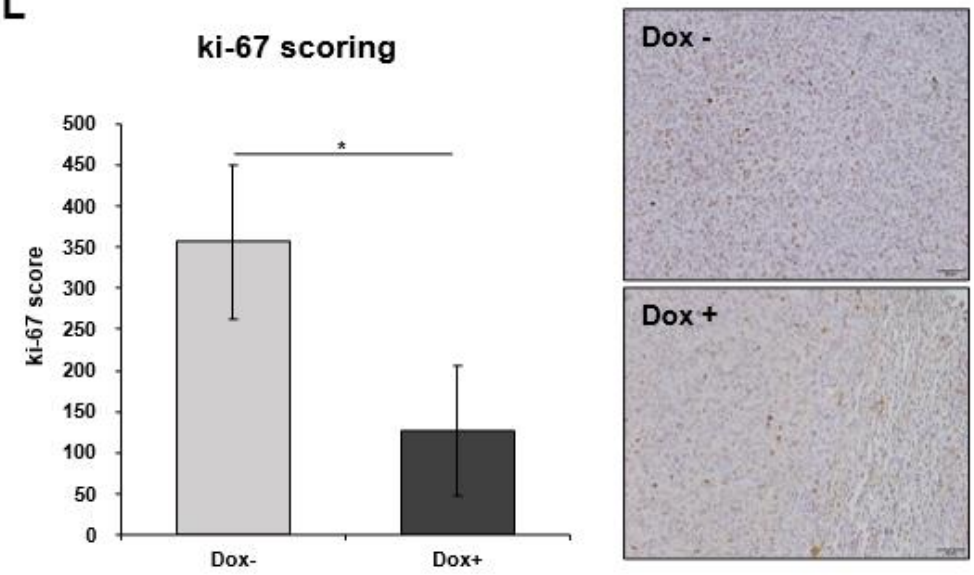


Figure 8. Analysis of tumor allograft model according to CSMD1 expression

A Schematic summary of tumor allograft model. **B** The photo of mouse lungs. **C** The photo of mouse spleens. **D** Mouse lung H&E (Hematoxylin and Eosin) staining. **E** Calculation of metastatic index from H&E staining. **F** Changes of body weight with/without CSMD1 expression. **G** Lung weight. **H** Spleen weight. **I** The gene expression of CSMD1 by qRT-PCR. **J** The protein expression of CSMD1 and Pan-cytokeratin by western blot. **K** The protein densitometry of Pan-cytokeratin by using ImageJ and normalized to β-actin. **L** Calculation of Ki-67 score from Immunohistochemistry. (* : $p < 0.05$, ** : $p < 0.01$)

Discussion

CSMD1 is frequently deleted in many cancers and various evidence suggests that it functions as a tumor suppressor gene. According to unpublished data, lung cancer patients with CSMD1 mutations had a lower survival rate than patients without mutations, and patients with CSMD1 gene deletion are thought to show more resistance to immunotherapy for lung cancer. However, the function of CSMD1 as a tumor suppressor gene in lung cancer has not been proven. Therefore, this paper aims to prove the function and mechanism of CSMD1 as a tumor suppressor gene in lung cancer.

The list of 20 up/down-regulated genes (Table 2, 3) was selected based on over-expression of CSMD1 from RNA-seq. The gene expression of selected genes when CSMD1 is over-expressed was significantly altered. Furthermore, these genes were significantly correlated with overall survival and relapse-free survival of lung cancer patients (Fig 2). This implies that the candidate genes could be associated with the prognosis of lung cancer patients. And it suggests the possibility that the candidate genes can be components of the signaling pathway in which CSMD1 functions as a tumor suppressor gene in lung cancer.

When CSMD1 was over-expressed in a mouse lung cancer cell line LLC1 using the Tet-on inducible CSMD1 plasmid (Fig 3), the growth of lung cancer cells was inhibited (Fig 4). Through this, we confirmed the potential of CSMD1 as a tumor suppressor gene. Among the gene list obtained from RNA-seq, three genes known as oncogenes and related to lung cancer were selected. They recovered the growth inhibition of lung cancer cells when CSMD1 was over-expressed (Fig 5), indicating the possibility that these candidate genes are involved in the pathway where CSMD1 functions as a tumor suppressor gene in lung cancer. To determine whether candidate genes are components of the anti-tumor pathway of CSMD1, further literature research is needed to understand the pathway the selected candidate genes function in cancer. It should be also investigated whether they are related to the anti-tumor signaling pathway of CSMD1.

It is also important to find out how CSMD1 inhibits the growth of lung cancer cells. Annexin V-FITC staining was performed to verify if cell growth inhibition was due to increased apoptosis, but it was not the case (Fig 6). Another possibility is that cell growth is inhibited due to decreased cell replication caused by cell cycle arrest. To confirm this, BrdU/7-AAD assay was performed, and it showed a decrease in the S phase when CSMD1 was over-expressed (Fig 7). It indicates that DNA replication may also decrease. Therefore, it can be suggested that the growth of lung cancer cells is influenced by cell cycle arrest.

In the tumor allograft model, the doxycycline treatment group tend to have less cancer metastasis than the control group (Fig 8). The doxycycline treatment group showed significantly lower protein expression of pan-cytokeratin than the control group, indicating a lower presence of lung cancer cells, which

suggest reduced lung metastasis. And the doxycycline treatment group showed significantly lower ki-67 score than the control group. Considering that the gene expression level of CSMD1 was significantly higher in the doxycycline treatment group, this implies that the expression of CSMD1 suppresses the lung cancer metastasis. However, there was a substantial difference in the level of lung cancer metastasis among individuals, and it was difficult to confirm CSMD1 protein expression. The cause might be the difficulty of tail vein injection and differences in doxycycline dosage among individuals. If these problems are improved, there is a possibility that the function of CSMD1 as a tumor suppressor gene in lung cancer will be investigated in-depth through future research.

Based on these results, we would like to suggest that CSMD1 may function as a biomarker for diagnosing lung cancer or be applied as a gene therapy target.

Further study

CSMD1 is known as a component3 (C3) convertase inhibitor of the complement system. The complement system is an integral part of the immune system responsible for recognizing pathogens and damaged cells, inducing inflammation, and destroying the cellular membranes of pathogens. Within this system, CSMD1 serves as a C3 convertase inhibitor, preventing the hydrolysis of C3. Consequently, we aim to investigate whether this action influences the immune system, potentially impacting tumor suppression. The expression of complement system components will be checked according to the expression of CSMD1 and observe whether the growth of lung cancer cells changes accordingly.

Korean abstract

폐암은 암 환자 사망률의 큰 부분을 차지하며 폐암 치료는 많은 사람들에게 여전히 어려운 과제로 남아 있다. 또한, 면역관문억제제 (ICIs)를 사용하는 환자 대부분은 치료 도중 또는 치료 중단 후에 암이 진행된다. CUB and Sushi multi-domain 1 (CSMD1) 유전자는 많은 암에서 종양 억제 유전자일 가능성을 많이 보여준다. 그러나 폐암에서 종양 억제 유전자로서 CSMD1 의 역할은 알려져 있지 않다. 본 연구에서는 폐암에서 종양 억제 유전자로서 CSMD1 의 기능을 입증하는 것을 목표로 한다. 마우스 폐암 세포 LLC1 에서 CSMD1 과발현은 세포증식의 억제를 보여준다. 또한 CSMD1 의 과발현에 따른 transcriptome 을 분석하여 유전자별 발현 변화를 RNA-seq 으로 확인하고, CSMD1 의 anti-tumor pathway 와 관련되어 있을 것이라 여겨지는 유전자 리스트를 선발하였다. 선택된 후보 유전자를 과발현 할 시, CSMD1 과발현에 의한 세포증식 억제를 회복한다. 이는 폐암에서 CSMD1 의 anti-tumor pathway 와 후보 유전자가 관련되어 있을 가능성을 시사한다. 또한 CSMD1 의 과발현에 의한 세포증식 억제 메커니즘을 알아보기 위해 BrdU/7-AAD 분석을 수행하였다. doxycycline 처리 72 시간 후에는 doxycycline 을 처리하지 않은 것과 비교하여 S 기 감소가 나타났다. 따라서 CSMD1 발현으로 인한 세포증식의 감소는 DNA 합성이 S 기에서 일어나기 때문에 세포주기 정지에 의한 것일 수 있음을 제시한다. 종양 동종이식 모델에서 doxycycline 투여군은 대조군에 비해 암 전이가 적은 경향을 보였다. doxycycline 투여군은 대조군에 비해 Pan-cytokeratin 단백질 발현과 ki-67 score 가 유의하게 낮은 것으로 나타났는데, 이는 폐암 세포가 적음을 의미하며, 폐 전이가 적었음을 시사한다. CSMD1 의 유전자 발현 수준이 doxycycline 투여군에서 유의하게 높았다는 점을 고려하면, 이는 CSMD1 의 발현이 폐암의 전이를 억제함을 보여준다. 이러한 발견은 CSMD1 이 폐암에서 cell cycle arrest 를 통해 종양 억제 유전자로 기능한다는 것을 시사하며, 나아가 anti-tumor pathway 에 관여할 수 있는 후보 유전자를 제안한다. 이러한 결과를 통해 CSMD1 이 폐암 진단을 위한 바이오 마커로 기능하거나 면역치료 타겟으로 활용될 수 있는 가능성을 제시하고자 한다.

Acknowledgement

논문 제출을 앞두고 지난 시간들을 돌이켜보면 감사한 분들이 참으로 많음을 느낍니다. 제가 무사히 석사를 졸업할 수 있었던 것은 저 스스로의 노력뿐만 아니라 주위 사람들의 관심과 애정이 있어서 어려움을 이겨내고 발전할 수 있었습니다.

먼저 사랑하는 가족들에게 감사드립니다. 멀리 떨어져 있지만 항상 응원해 주시고 저를 격려해 주셔서 포기하지 않고 달려올 수 있었습니다. 제가 가는 길을 믿고 지지해 주시고 어떤 일을 하더라도 행복하길 바라시는 부모님이 계셨기에 더 나은 제가 될 수 있었습니다. 그리고 늘 따뜻한 말 아끼지 않으시던 할머니, 할아버지와 막내삼촌께도 감사하다는 말씀드리고 싶습니다.

다음으로 석사과정 동안 저를 지도해 주시고 가르침을 주신 장수환 교수님께 감사드립니다. 지식과 실험기술 외에도 삶을 현명하게 살아가는 방법을 알려주셨기에 많은 지혜를 배울 수 있었습니다.

저와 학위과정을 함께 한 동기들에게도 감사드립니다. 힘든 순간을 함께 하고 서로 도움을 주고받아 여기까지 올 수 있었습니다. 배움만큼 값진 관계를 갖게 되었기에 가치 있는 시간이었습니다. 졸업 후에도 좋은 일만 있기를 바랍니다.

대학원 생활하면서 따뜻한 말씀해 주신 김승환 교수님께도 감사드립니다. 진심 어린 격려 해주신 덕분에 많은 힘을 얻을 수 있었습니다. 힘들 때마다 저의 편에 서서 다독여 주시고, 고민을 진지하게 들어주신 이종환 교수님께도 감사 인사 드립니다.

또한 LIO 실험실 멤버 모든 분들께 많은 도움을 받았습니다. 이윤경 박사님이 학위과정 동안 조언해 주시고, 지도해 주셨기에 무사히 졸업할 수 있었습니다. 이은지 선생님이 인턴 때부터 마지막까지 실험실 전반을 관리해 주시고 지원해 주셔서 랩 구성원으로써 부족함 없이 지낼 수 있었습니다. 실험실에서 가장 많은 조언을 주시고 늘 격려해주신 최은아 선생님께도 감사드립니다. 선생님이 있었기에 실험실에서 역할을 수행할 수 있는 사람이 되었다고 생각합니다. 그리고 CSMD1 프로젝트를 이끈 정은지 선생님과, LIO 후배분들께도 감사의 말씀드립니다.

마지막으로 석사 과정 동안 옆에서 도와주고 함께해 준 친구들이 많이 떠오릅니다. 가족보다 더 가족 같은 사랑하는 예빈이, 같이 있으면 언제나 즐거운 경화, 누리, 지원이, 영주. 최고의 여행 메이트이자 소울 메이트 령이, 행복을 나눠주고 항상 곁에서 응원해 준 주희랑 혜은이, 소중한 애착 인형 같은 서영이랑 지원이. 멋지고 늘 좋은 에너지를 주는 희주 언니, 같은 길을 걸으면서 훌륭한 박사가 될 세연이. 기쁜 일이 생기면 함께 나누고 싶은 승연 언니. 많이 아끼고 고마운 마음 가득한 민지 언니. 돈으로도 살 수 없는 소중한 사람들이 있었기에 지치지 않고 달려올 수 있었습니다. 정말 감사합니다.

이제 사회로 나아갈 일만 남았는데, 많은 분들께서 도와주셨기에 이만큼 성장할 수 있었습니다. 앞으로 이분들께 감사한 마음을 다 돌려줄 수 있는 사람이 되고 싶습니다. 모든 분들께 진심으로 감사드립니다.

Reference

1 Siegel, R. L., Miller, K. D., Wagle, N. S. & Jemal, A. Cancer statistics, 2023. *CA Cancer J Clin* **73**, 17-48 (2023). https://doi.org:10.3322/caac.21763

2 Mino-Kenudson, M. *et al.* Predictive Biomarkers for Immunotherapy in Lung Cancer: Perspective From the International Association for the Study of Lung Cancer Pathology Committee. *J Thorac Oncol* **17**, 1335-1354 (2022). https://doi.org:10.1016/j.jtho.2022.09.109

3 Wish, J. *et al.* Surgical Treatment at an Academic Medical Center is Associated with Statistically Insignificant Lung Cancer Survival Outcome Differences Related to ZIP Code. *World J Surg* **47**, 2052-2064 (2023). https://doi.org:10.1007/s00268-023-07006-4

4 Vinod, S. K. & Hau, E. Radiotherapy treatment for lung cancer: Current status and future directions. *Respirology* **25 Suppl 2**, 61-71 (2020). https://doi.org:10.1111/resp.13870

5 Yang, S., Zhang, Z. & Wang, Q. Emerging therapies for small cell lung cancer. *J Hematol Oncol* **12**, 47 (2019). https://doi.org:10.1186/s13045-019-0736-3

6 Lahiri, A. *et al.* Lung cancer immunotherapy: progress, pitfalls, and promises. *Mol Cancer* **22**, 40 (2023). https://doi.org:10.1186/s12943-023-01740-y

7 Zhou, F., Qiao, M. & Zhou, C. The cutting-edge progress of immune-checkpoint blockade in lung cancer. *Cell Mol Immunol* **18**, 279-293 (2021). https://doi.org:10.1038/s41423-020-00577-5

8 Herbst, R. S. *et al.* Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* **387**, 1540-1550 (2016). https://doi.org:10.1016/S0140-6736(15)01281-7

9 Sugawara, S. *et al.* Pembrolizumab plus chemotherapy in Japanese patients with metastatic squamous non-small-cell lung cancer in KEYNOTE-407. *Cancer Sci* **114**, 3330-3341 (2023). https://doi.org:10.1111/cas.15816

10 Socinski, M. A. *et al.* Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. *N Engl J Med* **378**, 2288-2301 (2018). https://doi.org:10.1056/NEJMoa1716948

11 Doroshow, D. B. *et al.* Immunotherapy in Non-Small Cell Lung Cancer: Facts and Hopes. *Clin Cancer Res* **25**, 4592-4602 (2019). https://doi.org:10.1158/1078-0432.CCR-18-1538

12 Passaro, A., Brahmer, J., Antonia, S., Mok, T. & Peters, S. Managing Resistance to Immune Checkpoint Inhibitors in Lung Cancer: Treatment and Novel Strategies. *J Clin Oncol* **40**, 598-610 (2022). https://doi.org:10.1200/JCO.21.01845

13 Ermis Akyuz, E. & Bell, S. M. The Diverse Role of CUB and Sushi Multiple Domains 1 (CSMD1) in Human Diseases. *Genes (Basel)* **13** (2022). https://doi.org:10.3390/genes13122332

14 Sun, P. C. *et al.* Transcript map of the 8p23 putative tumor suppressor region. *Genomics* **75**, 17-25 (2001). https://doi.org:10.1006/geno.2001.6587

15 Kraus, D. M. *et al.* CSMD1 is a novel multiple domain complement-regulatory protein highly

expressed in the central nervous system and epithelial tissues. *J Immunol* **176**, 4419-4430 (2006). https://doi.org:10.4049/jimmunol.176.7.4419

16 Sigbjornsdottir, B. I. *et al.* Chromosome 8p alterations in sporadic and BRCA2 999del5 linked breast cancer. *J Med Genet* **37**, 342-347 (2000). https://doi.org:10.1136/jmg.37.5.342

17 Emi, M. *et al.* Frequent loss of heterozygosity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. *Cancer Res* **52**, 5368-5372 (1992).

18 Fujiwara, Y. *et al.* Evidence for the presence of two tumor suppressor genes on chromosome 8p for colorectal carcinoma. *Cancer Res* **53**, 1172-1174 (1993).

19 Sunwoo, J. B., Holt, M. S., Radford, D. M., Deeker, C. & Scholnick, S. B. Evidence for multiple tumor suppressor genes on chromosome arm 8p in supraglottic laryngeal cancer. *Genes Chromosomes Cancer* **16**, 164-169 (1996). https://doi.org:10.1002/(SICI)1098-2264(199607)16:3<164::AID-GCC2>3.0.CO;2-X

20 Fan, X. *et al.* CSMD1 Mutation Related to Immunity Can Be Used as a Marker to Evaluate the Clinical Therapeutic Effect and Prognosis of Patients with Esophageal Cancer. *Int J Gen Med* **14**, 8689-8710 (2021). https://doi.org:10.2147/IJGM.S338284

21 Kamal, M. *et al.* Loss of CSMD1 expression is associated with high tumour grade and poor survival in invasive ductal breast carcinoma. *Breast Cancer Res Treat* **121**, 555-563 (2010). https://doi.org:10.1007/s10549-009-0500-4

22 Escudero-Esparza, A. *et al.* Complement inhibitor CSMD1 acts as tumor suppressor in human breast cancer. *Oncotarget* **7**, 76920-76933 (2016). https://doi.org:10.18632/oncotarget.12729

23 Zhang, R. & Song, C. Loss of CSMD1 or 2 may contribute to the poor prognosis of colorectal cancer patients. *Tumour Biol* **35**, 4419-4423 (2014). https://doi.org:10.1007/s13277-013-1581-6

24 Farrell, C. *et al.* Somatic mutations to CSMD1 in colorectal adenocarcinomas. *Cancer Biol Ther* **7**, 609-613 (2008). https://doi.org:10.4161/cbt.7.4.5623

25 Tang, M. R., Wang, Y. X., Guo, S., Han, S. Y. & Wang, D. CSMD1 exhibits antitumor activity in A375 melanoma cells through activation of the Smad pathway. *Apoptosis* **17**, 927-937 (2012). https://doi.org:10.1007/s10495-012-0727-0

26 Ma, C. *et al.* Characterization CSMD1 in a large set of primary lung, head and neck, breast and skin cancer tissues. *Cancer Biol Ther* **8**, 907-916 (2009). https://doi.org:10.4161/cbt.8.10.8132