의학 석사 학위논문

Establishment and characterization of 28 human lung cancer cell lines derived from pleural effusions: Key mutations and fusion genes associated with drug sensitivity

흉수 유래 폐암 세포주 28종의 수립과 특성 분석 : 주요 돌연 변이와 약제 반응성

2019 년 8 월

서울대학교 대학원

의과학과 의과학 전공

서 하 영

Establishment and characterization of 28 human lung cancer cell lines derived from pleural effusions: Key mutations and fusion genes associated with drug sensitivity

Ha-Young Seo

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at

Seoul National University College of Medicine

June, 2019

Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Ha-Young Seo

Major in Biomedical Sciences

The Graduate School

Seoul National University

Although lung cancer is a common disease, its diagnosis remains poor. While the incidence of lung cancer has decreased in western countries, lung cancer rates are predicted to increase generally in Asia. Studies investigating the mutation patterns of Asian cases compared with Caucasian types, lack adequate resources. Especially, lung cancer cell lines that are acquired from pleural effusion exudates are rare in cell line models. Pleural effusions in patients with NSCLC show progressive status and malignant features. In our study, 28 lung cancer cell lines derived from pleural effusions were newly established and their cellular and molecular characteristics were analyzed. Representative mutations in BRAF, EGFR, ERBB2, FGFR4, KRAS and TP53 as well as fusions such as ALK, CD74 and RET genes were detected and validated. Based on mutation profiles, drug sensitivities to gefitinib, erlotinib and crizotinib were measured. Cell lines that carry target mutations to each drug are generally sensitive. Cell lines originating from pleural

effusions are relatively more resistant than cell lines derived from tissues that harbor similar mutation. Analysis of mutational pattern and drug sensitivity of these cell lines can facilitate database construction for case study. Expression levels of specific oncoproteins such as ERK, ERBB2, c-MET and PTEN were detected in our cell lines. EMT markers such as EpCAM, E-cadherin, N-cadherin and Vimentin were also detected. These 28 well characterized lung cancer cell lines originating from malignant pleural effusions support studies associated with sensitivity to anti-cancer drugs and representative mutations in lung cancers. the few experimental results and small patient numbers still limit our knowledge of the acquired resistance to crizotinib. To meet this need, we established crizotinib-resistant sublines. Their parental cell lines were derived from a pleural effusion exudate which was thought to be more aggressive and resistant to anti-cancer drugs.

Keywords : Pleural effusion origin, characterization analysis, Drug sensitivity, Crizotinib-resistance

학 번 : 2016-27013

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1. Introduction

Lung cancer is one of the most common causes of cancer-related death worldwide, with an estimated one million deaths annually. In Korea, lung cancer is the most lethal disease and the third common cancer. Although there are several other types, non-small cell lung carcinoma (NSCLC) constitutes about 85% of lung cancers (1,2). A number of driver gene mutations have been found, including epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) fusion with other domains, yet numerous other genetic alterations remain undefined (3). Aside from driver mutations, various other gene mutations determine the response of lung cancer cells to anti-cancer drugs. For instance, mutations in KRAS are known to induce resistance to tyrosine kinase inhibitors targeting EGFR (1,4). These complexities emphasize the need for more biological tools to investigate the functional roles of genetic alterations and their potential effect on clinical outcomes. Specifically, lung cancer cells which evade the pleural cavity have been reported to show more aggressive behavior than primary tumor cells (5,6). Nevertheless, the characteristics of lung cancer cells derived from pleural effusion exudates make them controversial biologic models. In this study, 28 lung cancer cell lines acquired from a pleural effusion exudate were established and

characterized with regard to the association between gene mutations and drug responses.

ALK-positive lung cancer patients are treated with the ALK inhibitor, crizotinib. Even though the initial response of ALK-positive lung cancer to crizotinib is notable, acquired resistance eventually develops in most patients (7,8). There are several reported mechanisms to the resistance, including ALK amplification (9), EGFR or c-KIT activation (10), and the acquisition of KRAS mutations (11). Nevertheless, the few experimental results and small patient numbers still limit our knowledge of the acquired resistance to crizotinib. To meet this need, we established crizotinib-resistant sublines. Their parental cell lines were derived from a pleural effusion exudate which was thought to be more aggressive and resistant to anti-cancer drugs.

2. Materials and Methods

2.1. Establishment and maintenance of human NSCLC cell lines

Cell lines from pathologically proven Non-small-cell lung cancers were established. 28 pleural effusion exudate samples are obtained from patients in Seoul national university hospital. Cells from malignant pleural effusion of lung were gathered by centrifuging the medium. Gathered cells were then seeded into T-75cm^2 flasks. Tumor cells were initially cultured in Opti-MEM I (Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal bovine serum. Confined-area trypsinization or scraping method was used to attain a pure tumor cell population when stromal cells like mesothelial cells or fibroblasts grew in the initial culture. Established cell lines were sustained in RPMI 1640 medium with 10% fetal bovine serum and 1% (v/v) penicillin and streptomycin (10,000U/ml). Cultures were maintained in humidified incubators at 37℃ in an atmosphere of 5% CO2 and 95% air. The initial passage was assigned when substantial tumor cell growth was detected, and successive passages were given at sub-confluence after trypsinization. When one culture population contains both floating and adherent cells, floating cells were gathered by centrifuging the medium and dispersed by pipetting. Established cell lines were deposited to the Korean Cell

Line Bank (Seoul, Korea)

2.2. Growth properties and morphology in vitro

To acquire each tumor population's doubling time, $5x10^4$ to $2x10^5$ viable cells from each cell line were seeded into 12–24 identical well of 96 well plate and cell viability was calculated daily for 7-14 days. Since the first cell seeding, in every 24 hours, 10ul EZ-Cytox solution (Daeil Lab, Seoul, Korea) was added to well of each seeded lung cancer cells in triplicate. After 2 hour-incubation at 37 ℃, Optical density of EZ-Cytox-treated cells was calculated by Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA). Growth rate values were measured by GraphPad Prism 5 (GraphPad Software, CA, USA). Growth rates are values to multiply 10 by days that cell population were duplicated. To observe cell line's morphology, each cell line was cultured in $75cm^2$ culture flasks and then pictured daily by phase-contrast microscopy. Mycoplasma contamination was checked by the 16S-rRNA-gene-based polymerase chain reaction (PCR) amplification method using e-Myco Mycoplasma PCR Detection Kit (Intron Biotechnology, Gyeonggi, Korea).

2.3. Genomic DNA extraction and DNA fingerprinting analysis

Genomic DNA (gDNA) extraction was performed by using QIAamp DNA Mini kit (Qiagen). gDNA extracted from each lung cancer cell

line was amplified using an AmpFlSTR identifiler Polymerase Chain Reaction (PCR) Amplification Kit (Applied Biosystems, CA, USA). A single cycle of PCR amplified 15 short tandem repeat markers (CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA) and an Amelogenin gender-determining marker containing highly polymorphic microsatellite markers. Amplified PCR products were analyzed by an ABI 3500XL Genetic analyzer (Applied Biosystems).

2.4. RNA extraction and Reverse Transcriptase (RT)-PCR

To obtain RNA, TRIzol (ambion by Invitrogen, CA, USA) was added to cell pellet acquired from cultured lung cancer cell line. After cell lysis was happened, chloroform was added. Then, the sample was vortexed and 4˚C, 12,000 g centrifugation. Each tube containing the sample was divided into aqua phase, interphase, and organic phase. In those aqua phase was transferred into new tube. Isopropanol alcohol was mixed with an equal part of the aqua phase. Mixed sample was centrifuged at 4˚C, 12,000 g after incubated at -20˚C. RNA was acquired from pellet. The pellet was precipitated by ethanol. RNA was melted in 4th Distilled water containing RNase inhibitor (DEPC). Complementary DNA (cDNA) was synthesized from extracted RNA by reverse transcription

kit (Qiagen, MD, USA). Generally measured concentration of RNA was adjusted to 1ug diluted with gDNA wipeout buffer. Extracted RNA concentration was measured nano-spectrometer (Thermo Fisher Scientific, MA, USA). Each 1ug RNA was mixed with RT buffer, RT primer mix, and RTase. Those compounds were heated 42 ˚C for 30-45 min purposed on annealing, extension. Heated compound was additionally heated 95˚C for 3 min to denature synthetic cDNA.

2.5. Polymerase Chain Reaction (PCR) and mutation analysis

cDNA acquired from lung cancer cell lines was performed by the PCR system 9700 (Applied Biosystems) using PCR kit (Intron biotechnology). To detect genome rearrangement and mutation, these primers sequences are summarized in Table 1.(12,13) PCR products were sequenced by ABI 3500XL Genetic analyzer (Applied Biosystems)

Table 1. Used primer sequences

	Forward primer	Reverse primer	Annealing temperature
KIF5B exon 15 - RET exon 12 fusion	TAAGGAAATGACCAACCACCAG	GAATTTGGAAAAGTGGTCAAGG	60℃
KIF5B exon 16 - RET exon 12 fusion	GTGAAACG GTGAAACGTTGCAAGCAGTTAG		
EML4 exon 6 - ALK exon 20 fusion	GCATAAAGATGTCATCATCAACCAAG	TCTTGCCAGCAAAGCAGTAGTTGGCAGTAGT	60℃
EML4 exon 13 - ALK exon 20 fusion	GTGCAGTGTTTAGCATTCTTGGGG		
EML4 exon 18 - ALK exon 20 fusion	GTGGTTTGTTCTGGATGCAGAAACCAGAGATCT		63℃
EML4 exon 20 - ALK exon 20 fusion	GGACATTCCAGCTACATCACACAC		61℃
CD74 exon 5 - ROS1 exon 34 fusion	CCTGAGACACCTTAAGAACACCA	TGAAACTTGTTTCTGGTATCCAA	57℃
EGFR exon 19 deletion	TTGTGGAGCCTCTTACACCC	AGCAGGTACTGGGAGCCAAT	60℃
EGFR c.2602T>G (p.L858R)	ATGTCCGGGAACACAAAGAC	CATCCAGCACTTGACCATGA	60℃
KRAS exon1 codon12-13	TGACTGAATATAAACTTGTGGTAGTTG	TCGTCCACAAAATGATTCTGAA	59℃
BRAF c.1799T>A (p.V600E)	TTGCATCTAAGAGGAAAGAT	GGCCAAAAATTTAATCAGTG	61℃
ERBB2 c.1963A>G (p.I655V)	GCACCCACTCCTGTGTGGAC	TGCCAAAAGCGCCAGATCCA	62℃
FGFR4 c.1162G>A (p.G388R) c.991C>T (p.Q331*)	CGAGGCCAGGTATACGGACA	CAAAGGCCTCTGCACGTACT	63℃
TP53 exon4	TCCCCTGCCCTCAACAAGAT	AACCTCCGTCATGTGCTGTG	60℃
TP53 exon7	CTGGTGGTGCCCTATGAGCC	AGGAGCTGGTGTTGTTGGGC	61℃
TP53 exon8	CCTCTTTCCTAGCACTGCCC	CAAATGCCCCAATTGCAGGT	60℃
ALK kinase domain	GGAGGTGTATGAAGGCCAGG	TCGGAGGAAGGACTTGAGGT	61℃
ALK kinase domain (S1206Y, G1269A mutation target)	ACCTCAAGTCCTTCCTCCGA	CACTTAACTGGCAGCATGGC	61℃
ROS1 kinase domain	CTGCCTTCCCTCGGGAAAAA	GCTGCCAGATCCCTGTGAAT	63℃
β-actin	GACCACACCTTCTACAATGA	GCATACCCCTCGTAGATGGG	60℃

2.6. CCP analysis, targeted gene sequencing

CCP (Comprehensive Cancer Panel) is technology using Ion Ampliseq (Thermofisher Scientific). CCP panel could detect 409 genes mutations on exomes. There are various mutation types about driver-genes. According to the manufacturer's instructions as described, gDNA was extracted from cell pellet specimen using QIAamp DNA Mini kit. Quantity and quality of obtained DNA samples was evaluated using the Qubit fluorometer and the Qubit dsDNA HS assay kit (Life Technologies, Gent, Belgium). AmpliSeq library was assembled with NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB #E7370S/L) and the Ion AmpliSeqTM Library Kit 2.0 (Life Technologies, Part #4480441 Rev. 5.0). And then, the amplicons were purified with AMPure XP reagent (Beckman Coulter, Brea, CA, USA). The products were processed in end repair by NEBNext® End Prep kit (NEB #E7442). NEBNext® Multiplex Oligos for illumina (NEB #E7335 or NEB #E7500) were ligated with DNA ligase. Adapter-ligated products were then purified by AMPure XP reagent (Beckman Coulter, Brea, CA, USA), and PCR-amplified for a total of 7 cycles. The complete library was purified by AMPure XP reagent (Beckman Coulter) and GeneRead size selection kit(Qiagen). Size and concentration of the library were analyzed by Agilent 2100 BioAnalyzer and Agilent BioAnalyzer DNA High-Sensitivity LabChip (Agilent Technologies). The amplified

libraries's quality was proved by capillary electrophoresis (Bioanalyzer, Agilent). Qualified libraries were mixed in index tagged in equimolar amounts of the pool, after QPCR that was taken by using SYBR Green PCR Master Mix (Applied Biosystems). Sequencing was completed using an Illumina NextSeq 500 system following referred protocols for 2x150bp sequencing.

2.7. Drug sensitivity test

$2x10^5$ to $4x10^5$viable cells from each cell line were seeded into well of 96 well plate in triplicate to measure drug sensitivity of Crizotinib, Erlotinib, and Gefitinib. A day after, all cell lines was respectively treated for proper concentration of Crizotinib, Erlotinib, and Gefitinib. If the cell line was adherent cell, adherent state was confirmed. After 72 hours-incubation at 37 ℃, 10ul EZ-Cytox solution was added to well of each seeded lung cancer cells. After 2 hour-incubation at 37℃, optical density of EZ-Cytox-treated cells was calculated by Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). These steps were repeated in triplicate. The half maximal effective concentration (EC_{50}) were measured by GraphPad Prism 5 (GraphPad Software).

2.8. Protein extraction and Western blotting

Cultivated cells that had full confluency were harvested with cell scrapper. Cell pellet was treated by EzRIPA buffer (ATTO Co., Tokyo, Japan) after washed by cool PBS. Whole protein was extracted by this step. Protein concentration of each cell line was determined by SMART micro BCA protein assay kit (Intron biotechnology). Proteins that fixed into equal concentration were loaded on a 4-12% Bis-Tris gel (Invitrogen) at 70 volts for 3 hours and then proteins on loaded gel were transferred to a PVDF membrane (Invitrogen) by electro-blotting in condition under a constant current of 80mA at 4 °C overnight. Proteins of transferred membrane was blocked by incubating in 1.5% to 2.0% skim milk and 0.05% Tween 20-TBS buffer including 1mM $MgCl_2$ for an hour at room temperature. Primary antibodies were used against EGFR, exon 19 E746-A750 deleted EGFR, ERK1, ERK2, pERK1/2, PTEN, c-MET, EpCAM, E-cadherin, N-cadherin and Vimentin and □-actin. Those antibodies were Abcam products (Abcam, Cambridge, UK) except for exon 19 E746-A750 deleted EGFR that was Cell signaling product (Cell signaling, MA, USA). Mouse or rabbit IgG 2nd antibody (Jackson Immunoresearch, PA, USA) (1:5000) conjugated with peroxidase that matched with used 1st antibody was added to membrane. After chemiluminescent working solution, WESTZOL^{TM} (Intron biotechnology) was treated to the membrane, the membrane was exposed to Fuji RX film (Fujifilm, Tokyo, Japan) for 1-5 minutes.

2.9. Immunocytochemistry

Cells were seeded on chambered coverglass (Thermo Fisher Scientific, MA, USA) with a desirable cell confluency. The chambered coverglass was designed to be hydrophilic and no ECM component was treated before seeding. 72 hours after cell seeding, cells were washed with cold DPBS for 15 minutes three times. Then, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ (BD science, CA, USA). After cells were washed with washing solution (BD science), DPBS containing 2% FBS (GE Healthcare Life Sciences, Buckinghamshire, UK) was applied for an hour for blocking. After cells were washed with cold DPBS, DKK1 (Santa Cruz Biotechnology, CA, USA) and β-catenin antibody (Abcam, Cambridge, United Kingdom) diluted in 0.05% of PBS.T was applied for an 1.5 hours in room temperature. Thereafter, cells were washed with 0.05% of PBS.T, and Alexa 488 and Alexa 568 secondary antibodies (Thermo Fisher Scientific, MA, USA) diluted in 0.05% of PBS.T were applied for an hour in room temperature. 1x DAPI and Rhodamine-conjugated Phalloidin (Sigma-Aldrich, MO, USA) were diluted in distilled water and applied for 30 minutes in room temperature. The cells were washed with DPBS three times, and pictured under confocal microscope. LSM800 Confocal Laser Scanning Microscope and ZEN software (Carl Zeiss, Oberkochen, Germany) was used to examine cells. Diverse magnifications were used for different growth

patterns and sizes of cells. The intensity of each channel was fixed for the comparison of target protein expression between samples. Digital resolution, scan speed and the number of pictures averaged were set to 1024 x 1024, 40 seconds per one channel, and 8 pictures respectively. The pictures were focused on the very bottom of the fixed cells for investigating protruding region of cell colonies and the location of DKK1 and β-catenin.

2.10. RNA and Direct Sequencing

Total RNA was isolated from cell lysate using Trizol (Qiagen) and Qiagen RNeasy Kit (Qiagen). Sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA Library Prep Kit. Fifty-one million reads were obtained from the cell lysates. Following base-calling and alignment with the Tuxedo Suite, rejected reads were analyzed using FusionMap, ChimeraScan, and Defuse with default parameters for RNA and alignment to GRCh37.72. The output was filtered to include in-frame fusions, with at least one rescued read and two unique seed reads, and exclude known, recurrent artifacts. Sequencing of RNA isolated from early onset colorectal cancer cell lines was used to identify RNA sequences that spanned two different regions in a chromosome.

2.11. Whole Exome Sequencing

SureSelect sequencing libraries were prepared according to manufacturer's instructions (Agilent sureselect all Exon kit 50 Mb) using the Bravo automated liquid handler. Three micrograms of genomic DNA were fragmented to a median size of 150 bp using the Covaris-S2 instrument (Covaris, Woburn, MA). The adapter ligated DNA was amplified by PCR, and the PCR product quality was assessed by capillary electrophoresis (Bioanalyzer, Agilent). The hybridization buffer and DNA blocker mix were incubated for 5 minute at 95℃ and then for10 minutes at 65℃ in a thermal cycler. The hybridization mixture was added to the bead suspension and incubated for 30 minutes at RT while mixing. The beads were washed, and DNA was eluted with 50 ml SureSelect elution buffer (Agilent). The flow cell loaded on HISEQ 2500 sequencing system (Illumina).

2.12. Establishement of crizotinib-resistant lung cancer cell lines

We established two crizotinib-resistant sublines (SNU-2550CR and SNU-2563CR), derived from the parental SNU-2550 and SNU-2563 cell lines by continuously exposing them to 5μ of crizotinib for 90 days.

2.13. Statistical analysis

Statistical analysis was performed using R program version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) with various

packages including maftools, PerformanceAnalytics, survminer, survival, iplot, gplot, and lattice. The relationship between gene mutation or expression and clinical data of pancreatic adenocarcinoma was analyzed by the Chi-square test and Pearson correlation method. Fisher's exact test was used to analyze GO analysis of various genes. All tests were performed using bilateral 95% confidence intervals (CI). A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Culture characteristics

The *in vivo* information for patient SNU-2612A was reported previously. (12) The general *in vivo* information of the cell lines are summarized in Table 2. The morphologies of the established cell lines were categorized into four types: polygonal, oval, fibroblast-like, and round (Figure 1, Table 3). Two cell lines (SNU-2708.1 and SNU-3023) formed floating and adherent aggregates exhibiting round morphologies. Even though the cells were derived from a lung pleural effusion exudate, which was expected to have resistance to anoikis and to form floating aggregates, only two cell lines maintained the floating nature. This observation may explain the mechanism of how cells invade the lung pleural cavity. The growth rate of SNU-2708.1 could not be measured due to its aggregate growth pattern and slow growth. The culture conditions for SNU-2708.1 might not be adequate for the SNU-2708.1 cell line. DNA fingerprinting revealed a heterogeneous distribution of 15 tetranucleotide repeat loci and an Amelogenin gender determining marker in each cell line. It confirmed 28 unique and unrelated cell lines, without cross-contamination (Table 4). All cell lines were confirmed to be free of contamination from either bacteria or mycoplasma (Figure 2).

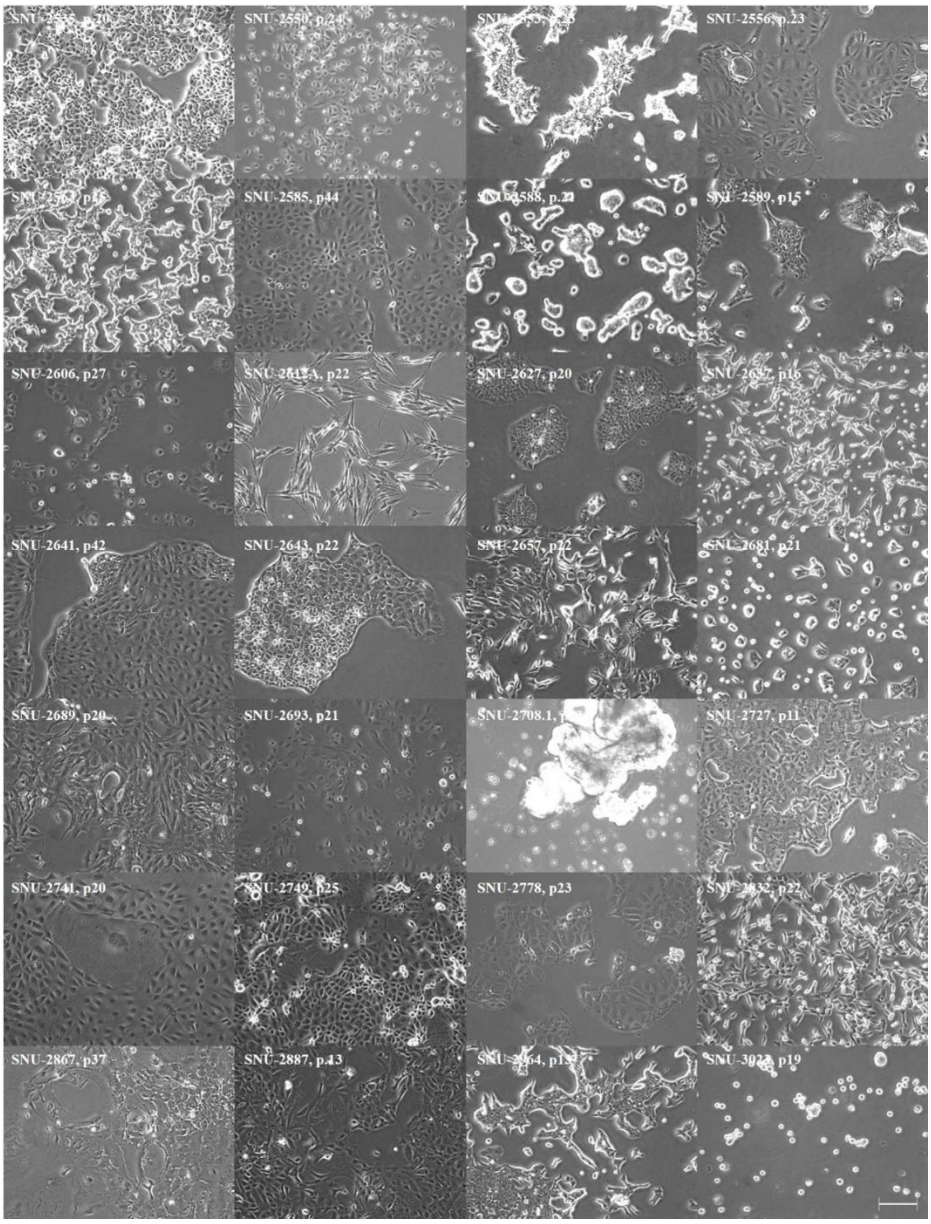


Figure 1. Phase-contrast microscopy of newly established 28 SNU lung cancer cell lines. Scale bar in microscopy of SNU-2681 represent 50μm and all microscopy have same magnification.

Table 2. Origin and *in vivo* characteristics of 28 SNU human lung cancer cell lines

No	Cell-Name	Culture initiation date	Origin	Sex	Age	Pathology
1	SNU-2535	2010-11-10	pleural effusion	F	56	ADC
2	SNU-2550	2011-02-21	pleural effusion	F	35	ADC
3	SNU-2553	2011-03-11	pleural effusion	M	40	ADC
4	SNU-2556	2011-03-29	pleural effusion	F	72	ADC
5	SNU-2563	2011-04-19	pleural effusion	M	49	ADC
6	SNU-2585	2011-10-10	pleural effusion	M	75	ADC
7	SNU-2588	2011-10-26	pleural effusion	M	43	ADC
8	SNU-2589	2011-10-27	pleural effusion	F	52	ADC
9	SNU-2606	2012-03-07	pleural effusion	F	51	ADC
10	SNU-2612A	2012-03-23	pleural effusion	M	33	ADC
11	SNU-2627	2012-06-05	pleural effusion	F	46	NOS
12	SNU-2637	2012-09-06	pleural effusion	M	42	ADC
13	SNU-2641	2012-09-24	pleural effusion	M	64	NOS
14	SNU-2643	2012-09-27	pleural effusion	F	57	NOS
15	SNU-2657	2012-11-27	pleural effusion	F	70	ADC
16	SNU-2681	2013-03-05	pleural effusion	M	63	ADC
17	SNU-2689	2013-03-21	pleural effusion	F	74	ADC
18	SNU-2693	2013-04-11	pleural effusion	M	60	ADC
19	SNU-2708.1	2013-06-10	pleural effusion	M	56	ADC
20	SNU-2727	2013-07-08	pleural effusion	F	39	ADC
21	SNU-2741	2013-08-19	pleural effusion	M	57	ADC
22	SNU-2749	2013-09-25	pleural effusion	M	67	ADC
23	SNU-2778	2013-12-11	pleural effusion	M	54	ADC
24	SNU-2832	2014-03-05	pleural effusion	F	58	ADC
25	SNU-2867	2014-04-11	pleural effusion	M	57	ADC
26	SNU-2887	2014-05-20	pleural effusion	F	56	ADC
27	SNU-2964	2014-09-03	pleural effusion	F	78	ADC
28	SNU-3023	2014-12-04	pleural effusion	F	70	ADC

*ADC : adenocarcinoma

Table 3. *in vitro* characteristics of 28 SNU human lung cancer cell lines

No.	Cell-Name	Growth pattern	Doubling time	Cell morphology
1	SNU-2535	Adherent	61	Polygonal
2	SNU-2550	Adherent	32	Oval
3	SNU-2553	Adherent	61	Fibroblast-like
4	SNU-2556	Adherent	35	Oval
5	SNU-2563	Adherent	47	Polygonal
6	SNU-2585	Adherent	31	Oval
7	SNU-2588	Adherent	39	Polygonal
8	SNU-2589	Adherent	33	Polygonal
9	SNU-2606	Adherent	28	Oval
10	SNU-2612A	Adherent	39	Fibroblast-like
11	SNU-2627	Adherent	40	Polygonal
12	SNU-2637	Adherent	30	Polygonal
13	SNU-2641	Adherent	44	Oval
14	SNU-2643	Adherent	27	Polygonal
15	SNU-2657	Adherent	27	Polygonal
16	SNU-2681	Adherent	34	Round / Polygonal
17	SNU-2689	Adherent	25	Fibroblast-like / Oval
18	SNU-2693	Adherent	34	Polygonal / Oval
19	SNU-2708.1	floating	-	Round
20	SNU-2727	Adherent	102	Polygonal
21	SNU-2741	Adherent	55	Polygonal
22	SNU-2749	Adherent	31	Polygonal
23	SNU-2778	Adherent	35	Polygonal
24	SNU-2832	Adherent	25	Fibroblast-like
25	SNU-2867	Adherent	87	Fibroblast-like / Polygonal
26	SNU-2887	Adherent	102	Polygonal
27	SNU-2964	Adherent	46	Polygonal
28	SNU-3023	floating	29	Round

Table 4. DNA fingerprinting analysis using 16 STR loci for newly established 28 SNU human lung cancer cell lines

No.	Cell-Name	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539
1	SNU-2535	13,15	28,29	11	11	14,15	7,9.3	9,11	9,11
2	SNU-2550	14,15	29,30	8,12	10	15	9	8,11	9
3	SNU-2553	12,13	30.2,31	9,11	11,12	16	7	8	11
4	SNU-2563	11,15	28,29	10,11	10	15	7,9.3	11,13	12,13
5	SNU-2556	11,14	29,32.2	9,10	10,11	16	9	12,13	9
6	SNU-2585	12,16	31,31.2	10,12	12	16	7	9,10	9,12
7	SNU-2588	10,13	32.2	10,12	10,11	17	7,9	8,12	12
8	SNU-2589	14,16	30	8,12	12,13	15	6,8	11,12	9
9	SNU-2606	13	29	9,12	12,13	15	7,9	9	8,9
10	SNU-2612A	13,14	30	8,10	10	15,16	9	8,12	9,13
11	SNU-2627	10,11	30,32.2	10,11	12,13	17	7,9	12	12
12	SNU-2637	12,13	30,31.2	8,9	9,13	16,17	9	9,11	9
13	SNU-2641	13	30,31.2	11,13	12,13	15	9	8,10	10,13
14	SNU-2643	13	29,31	8,11	10,12	15,17	6,7	8	12
15	SNU-2657	10,12	29	9,10	11,12	14	6,7	8	9,12
16	SNU-2681	11,12	29.2,30	11	10,12	15	9	10	12
17	SNU-2689	11,14	30	11	12,14	15,17	6	11	9
18	SNU-2693	14,15	29,30	8,10	10	15,18	9	13	11,12
19	SNU-2708.1	14	29,30	8,10	10,11	18	9	11	9,12
20	SNU-2727	11	29,31.2	11,14	10	16	6,8	9	9
21	SNU-2741	14	30,32.2	8,12	9,12	15	7,9	8	9,11
22	SNU-2749	14	30	9,12	12	16,17	6,7	10,12	10,12
23	SNU-2778	13,16	32.2	12	10,11	15,17	8	8	9,10
24	SNU-2832	15,16	30,31	8,11	7,11	15,19	6	10,11	9,13
25	SNU-2867	15	29,30	9,10	12	15,16	6,9	8,11	9
26	SNU-2887	13	29,31	10,11	12	17	7	12	10
27	SNU-2964	13,15	29.2,30	10,11	10,12	15,16	7,9	12	9,11
28	SNU-3023	15,16	31	8,11	12	14	6,9.3	12	11

Continued

No.	Cell-Name	D2S1338	D19S433	Vwa	TPOX	D18S51	Amelogenin	D5S818	FGA
1	SNU-2535	20,23	10.2,14	16,17	11	15	X	10,12	24,27
2	SNU-2550	23,24	12,15,15.2	14,18	11	8,17	X	11,12	19
3	SNU-2553	OL,23	13,14	14,15	8,9	16	X,Y	9,11	21,24
4	SNU-2563	18,23	13.2,15	17,18	8,11	15	X,Y	10,11	22,23
5	SNU-2556	20,26	14,14.2	14	8,11	15	X,Y	11	23,24
6	SNU-2585	19,20	13	18	8,9	13,18	X,Y	10,11	22
7	SNU-2588	24,25	13.2,15	18	8	13,17	X,Y	11,13	24
8	SNU-2589	17,20	13,17.2	17	8,11	13,14	X	11,12	21,22
9	SNU-2606	24,26	13,15.2	17,19	8,11	16	X	9,13	21,26
10	SNU-2612A	19,21	13,14	18	8,10	13,16	X,Y	9	18,21
11	SNU-2627	25	13,14.2	17,19	8,11	21	X	11,12	OL
12	SNU-2637	18,23	14.2,15.2	18,19,20	8	13,21	X,Y	11	22,25
13	SNU-2641	17,23	13	16,17	8,11	14,15	X,Y	11	17,21
14	SNU-2643	19,24	13.2,15	14,17	8,11	20	X	11	22,24
15	SNU-2657	24	13,15	16	11,12	13,22	X	11,12	22,23
16	SNU-2681	17,23	14,15.2	17	8	13	X,Y	9,10	21
17	SNU-2689	22	14.2,15.2	17	11	14,17	X	10,11	20
18	SNU-2693	18,24	13	16	10,11	13	X	10	27
19	SNU-2708.1	24	14	17	9	17	X,Y	11	20,23
20	SNU-2727	19	13,14	14	8	17	X	11	23
21	SNU-2741	23	10.2,14	17	8	14	X	10,11	22
22	SNU-2749	18	13,14	14,18	8,11	12	X,Y	11	22,23
23	SNU-2778	23,26	14,15.2	14	8	20	X	10,12	22,23
24	SNU-2832	18,24	14	14	8	14,15	X	11,12	21,22
25	SNU-2867	23	13,14	20	11	16	X,Y	11	24
26	SNU-2887	23	16.2	18	8	14	X	11	24,25
27	SNU-2964	17	14	17	8,9	16	X	9,12	21,22.2,23.2
28	SNU-3023	19,25	14,15	14,16	8	15	X	12	23,24

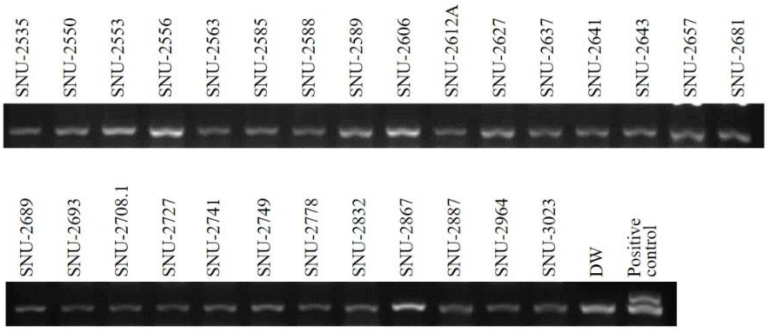


Figure 2. Mycoplasma test by the 16S-rRNA-gene-based polymerase chain reaction (PCR) amplification method

3.2. Mutational traits

To investigate the representative genetic aberrations in the newly-established NSCLC cell lines, targeted gene sequencing was performed. The mutation profiles of six genes involved in drug responses are summarized in Table 5. When the detected mutation had been already reported in the Clinvar database (https://www.ncbi.nlm.nih.gov/clinvar), its predicted effect was specified. The effect of an EGFR R521K polymorphism was listed as benign in the Clinvar database. However, several studies indicated its association with the occurrence of colorectal cancer (14) and the response to cetuximab in lung cancer (15). Since targeted sequencing may omit large deletions, direct Sanger sequencing was performed on six genes (BRAF, EGFR, ERBB2, FGFR4, KRAS, and TP53) (Figure 3). Although targeted gene sequencing has revealed the presence of previously reported oncogenic mutations, a few significant oncogenes and tumor suppressor genes, such as KMT2D, RBM10, and U2AF1, were not included. To establish more a comprehensive mutational context, whole exome sequencing (WES) was performed. The general information, including variant classifications and SNV classes, are summarized in Figure 4a. SNU-2832 had the largest number of variants, whereas SNU-2612A had the smallest number of variants. The median number of variants per sample was 244. Mutational load and the response to checkpoint immunotherapy have been closely associated in colorectal and lung cancer. Environmental

factors, such as UV radiation or smoking, or germline mutations in the mismatch repair genes affect the hypermutation rate in tumors (16). We analyzed the exclusive mutations in the hyper-mutated and hypo-mutated groups (Figure 5). The mutational signatures, characterized by a specific pattern of nucleotide substitutions, were extracted by decomposing a matrix of nucleotide substitutions, then comparing them to the public database presented by Alexandrov et al. The newly-established lung cancer cell lines showed a pattern of signature 5 (Figure 4b). Since the signature 5 pattern doesn't have specific etiology, we visualized the p-values of the top 30 signatures (Figure 4c). Signature 6 had similar p-values as signature 5. Signature 6 is associated with high numbers of small (shorter than 3bp) insertions and deletions at mono/polynucleotide repeats. It is associated with defective DNA mismatch repair and is found in microsatellite unstable tumors. The prevalence of aberrations in key driver genes including fusion genes is shown in Figure 4d and Figure 6. All cell lines were verified by PCR and targeted gene sequencing according to representative genetic variations in NSCLC (Table 6) (3,17). Cell lines carrying fused genes were confirmed by PCR using specific primer sets targeting break junctions. SNU-2535, SNU-2550, SNU-2553, SNU-2563, and SNU-2637 carried an EML4-ALK fusion. Cell lines carrying an EML4-ALK fused gene showed no mutation in the ALK kinase domain, with the exception of SNU-2535, which carried a G1269A mutation in the kinase domain (Figure 7). In

contrast, SNU-2606 and SNU-2832 carried a CD74-ROS1 fusion. The expression of ALK and the ROS1 kinase domain were detected by RT-PCR (Figure 8). Further, SNU-2612A and SNU-2778 harbored a RET fusion gene. Standing mutations were also detected by targeted gene sequencing of each cell line. The mutational statuses and proposed functions of such genes are summarized in Table 7. Many such driver genes in cancer co-occur or show exclusiveness in their mutation patterns. We performed pair-wise Fisher's exact test to detect such significant pairs of genes. For instance, mutations in the KRAS and TSC1 genes were co-occurring (Figure 4e). Mutations were further analyzed for gene set enrichment analysis to find representative aberrant pathways in the established lung adenocarcinoma cell lines. Genes of the MAPK family signaling cascade and transcriptional regulation by TP53 were mostly mutated (Figure 4f). In association with the aberrant pathway analysis, we also predicted which pathway could be targeted by drugs. The results showed that kinase and the DNA repair pathways were potential drug gene categories (Figure 4g). Other mutations, such as specific protein over-expression related to drug sensitivity, were detected by Western blotting, including EGFR, ERK1/2, PTEN, and c-MET (Figure 9). The EGFR protein was highly expressed in SNU-2589, SNU-2606, SNU-2681, SNU-2693, SNU-2708.1, SNU-2727, SNU-2741, SNU-2867, and SNU-2964. The EGFR protein expression of SNU-2606 was the highest in the study, whereas the overall ERK1 protein level was poorly

expressed.

Table 5. Mutation profiles by targeted gene sequencing

Cell line	BRAF			EGFR		
	nt	nt change (a.a. change)	effects	nt	nt change (a.a. change)	effects
SNU-2535	wt			1591	aGg>aAg(R521K)	Benign
SNU-2550	wt			1591	aGg>aAg(R521K)	Benign
SNU-2553	wt			wt		
SNU-2556	wt			1591	aGg>aAg(R521K)	Benign
SNU-2563	wt			1591	aGg>aAg(R521K)	Benign
SNU-2585	wt			1591	aGg>aAg(R521K)	Benign
				1611	Aat>Gat(N528D)	-
SNU-2588	wt			1591	aGg>aAg(R521K)	Benign
SNU-2589	wt			1591	aGg>aAg(R521K)	Benign
				2053	cGg>cAg(R675Q)	-
SNU-2606	wt			1591	aGg>aAg(R521K)	Benign
SNU-2612A	wt			wt		
SNU-2627	wt			wt		
SNU-2637	wt			wt		
SNU-2641	wt			wt		
SNU-2643	wt			1591	aGg>aAg(R521K)	Benign
SNU-2657	1799	gTg>gAg (V600E)	Pathogenic	1591	aGg>aAg(R521K)	Benign
SNU-2681	wt			wt		
SNU-2689	wt			1591	aGg>aAg(R521K)	Benign
				2602	cTg>cGg(L858R)	Drug response
SNU-2693	wt			1591	aGg>aAg(R521K)	Benign
SNU-2708.1	wt			1591	aGg>aAg(R521K)	Benign
				3079	cTc>cAc(L1017H)	-
				2262-2279	aaggaattaagagaagca>aaa (ELREA701del)	Drug response
SNU-2727	wt			wt		
SNU-2741	wt			1591	aGg>aAg(R521K)	Benign
SNU-2749	1799	gTg>gAg (V600E)	Pathogenic	1591	aGg>aAg(R521K)	Benign
SNU-2778	wt			1591	aGg>aAg(R521K)	Benign
SNU-2832	wt			1591	aGg>aAg(R521K)	Benign
SNU-2867	wt			1591	aGg>aAg(R521K)	Benign
SNU-2887	wt			1591	aGg>aAg(R521K)	Benign
SNU-2964	wt			1591	aGg>aAg(R521K)	Benign
SNU-3023	wt			2602	cTg>cGg(L858R)	Drug response

Continued

Cell line	ERBB2			FGFR4		
	nt	nt change (a.a. change)	effects	nt	nt change (a.a. change)	effects
SNU-2535	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2550	wt			wt		
SNU-2553	wt			wt		
SNU-2556	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2563	wt			wt		
SNU-2585	wt			wt		
SNU-2588	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2589	1963	Atc>Gtc (I655V)	Pharmacogenic	wt		
SNU-2606	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2612A	wt			wt		
SNU-2627	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2637	wt			wt		
SNU-2641	wt			wt		
SNU-2643	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2657	1963	Atc>Gtc (I655V)	Pharmacogenic	1162	Ggg>Agg(G388R)	Pathogenic
SNU-2681	1963	Atc>Gtc (I655V)	Pharmacogenic	wt		
SNU-2689	1963	Atc>Gtc (I655V)	Pharmacogenic	1162	Ggg>Agg(G388R)	Pathogenic
				1251	caG>caA(Q417Q)	Splice site
SNU-2693	wt			wt		
SNU-2708.1	wt			wt		
SNU-2727	wt			wt		
SNU-2741	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2749	1963	Atc>Gtc (I655V)	Pharmacogenic	1162	Ggg>Agg(G388R)	Pathogenic
SNU-2778	wt			1162	Ggg>Agg(G388R)	Pathogenic
SNU-2832	1963	Atc>Gtc (I655V)	Pharmacogenic	wt		
SNU-2867	wt			wt		
SNU-2887	wt			wt		
SNU-2964	wt			wt		
SNU-3023	wt			1162	Ggg>Agg(G388R)	Pathogenic

Continued

Cell line	KRAS			TP53		
	nt	nt change (a.a. change)	effects	nt	nt change (a.a. change)	effects
SNU-2535	wt			wt		
SNU-2550	wt			wt		
SNU-2553	wt			wt		
SNU-2556	wt			wt		
SNU-2563	wt			839	aGa>aAa(R280K)	Pathogenic
SNU-2585	35	gGt>gTt (G12V)	Pathogenic	wt		
SNU-2588	wt			wt		
SNU-2589	wt			438	tgG>tgA(W146*)	-
SNU-2606	wt			817	Cgt>Tgt(R273C)	Pathogenic
SNU-2612A	wt			wt		
SNU-2627	wt			wt		
SNU-2637	wt			wt		
SNU-2641	wt			wt		
SNU-2643	wt			818	cGt>cAt(R273H)	Pathogenic
SNU-2657	wt			wt		
SNU-2681	34	Ggt>Tgt (G12C)	Pathogenic	329	cGt>cTt(R110L)	Probable-pathogenic
SNU-2689	wt			wt		
SNU-2693	wt			wt		
SNU-2708.1	wt			wt		
SNU-2727	wt			wt		
SNU-2741	wt			wt		
SNU-2749	wt			991	Cag>Tag(Q331*)	-
SNU-2778	wt			wt		
SNU-2832	wt			wt		
SNU-2867	wt			wt		
SNU-2887	wt			wt		
SNU-2964	wt			wt		
SNU-3023	wt			wt		

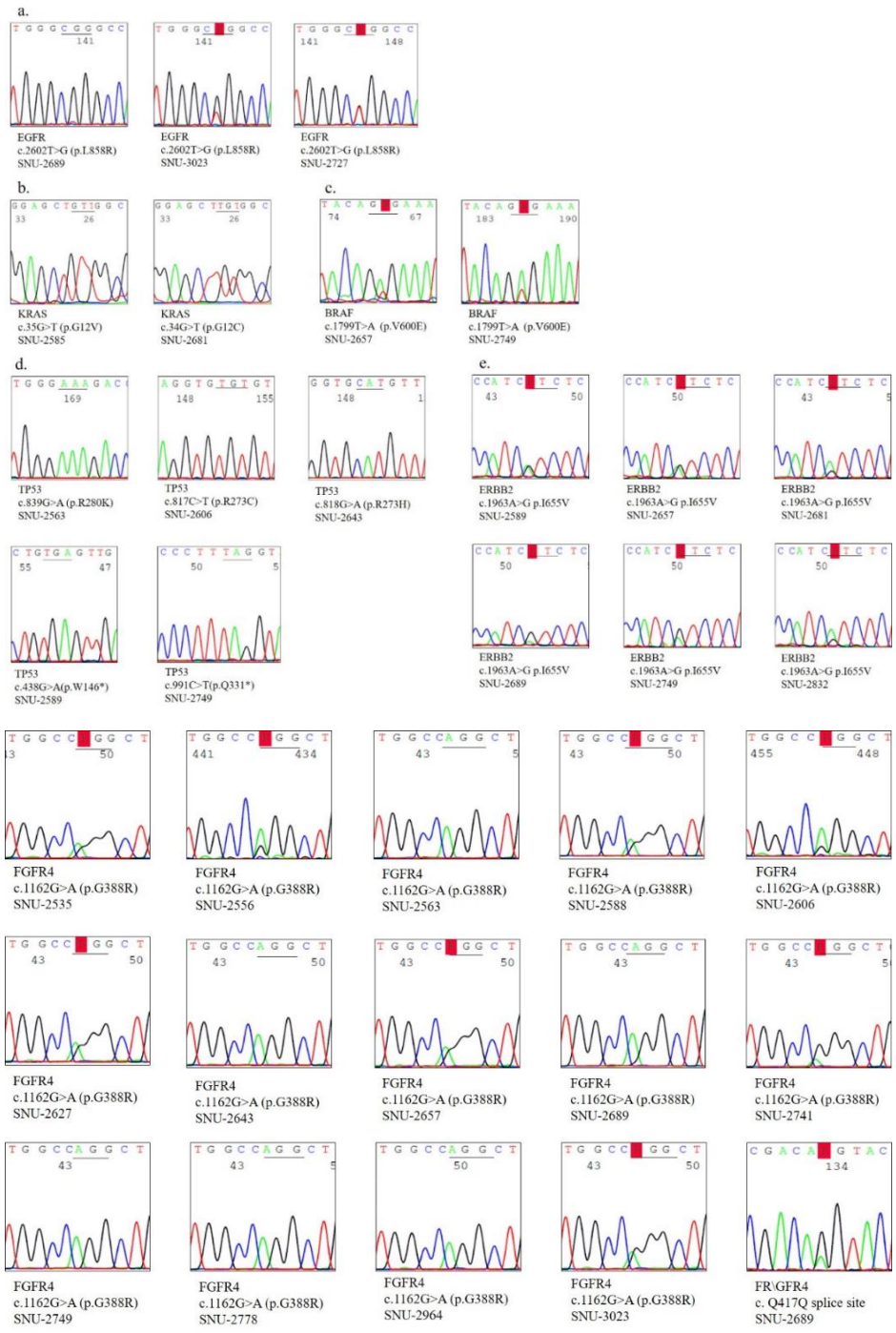

a.
EGFR
c.2602T>G (p.L858R)
SNU-2689
EGFR
c.2602T>G (p.L858R)
SNU-3023
EGFR
c.2602T>G (p.L858R)
SNU-2727
b.
KRAS
c.35G>T (p.G12V)
SNU-2585
KRAS
c.34G>T (p.G12C)
SNU-2681
c.
BRAF
c.1799T>A (p.V600E)
SNU-2657
BRAF
c.1799T>A (p.V600E)
SNU-2749
d.
TP53
c.839G>A (p.R280K)
SNU-2563
TP53
c.817C>T (p.R273C)
SNU-2606
TP53
c.818G>A (p.R273H)
SNU-2643
TP53
c.438G>A(p.W146*)
SNU-2589
TP53
c.991C>T(p.Q331*)
SNU-2749
e.
ERBB2
c.1963A>G p.I655V
SNU-2589
ERBB2
c.1963A>G p.I655V
SNU-2657
ERBB2
c.1963A>G p.I655V
SNU-2681
ERBB2
c.1963A>G p.I655V
SNU-2689
ERBB2
c.1963A>G p.I655V
SNU-2749
ERBB2
c.1963A>G p.I655V
SNU-2832
FGFR4
c.1162G>A (p.G388R)
SNU-2535
FGFR4
c.1162G>A (p.G388R)
SNU-2556
FGFR4
c.1162G>A (p.G388R)
SNU-2563
FGFR4
c.1162G>A (p.G388R)
SNU-2588
FGFR4
c.1162G>A (p.G388R)
SNU-2606
FGFR4
c.1162G>A (p.G388R)
SNU-2627
FGFR4
c.1162G>A (p.G388R)
SNU-2643
FGFR4
c.1162G>A (p.G388R)
SNU-2657
FGFR4
c.1162G>A (p.G388R)
SNU-2689
FGFR4
c.1162G>A (p.G388R)
SNU-2741
FGFR4
c.1162G>A (p.G388R)
SNU-2749
FGFR4
c.1162G>A (p.G388R)
SNU-2778
FGFR4
c.1162G>A (p.G388R)
SNU-2964
FGFR4
c.1162G>A (p.G388R)
SNU-3023
FR\GFR4
c. Q417Q splice site
SNU-2689

Figure 3. Validation of results of targeted gene sequencing by Sanger sequencing analysis.

a. EGFR c.2602T>G (p.L858R) variation was detected in SNU-2689, SNU-2727 and SNU-3023. b. KRAS c.35G>T (p.G12V) mutation was validated in SNU-2585 and c.34G>T (p.G12V) was also validated in SNU-2681. c. BRAF c.1799T>A (p.V600E) variation was detected in SNU-2657 and 2749. d. TP53 c.839G>A (p.R280K) missense mutation that is located in exon 7 was detected in SNU-2563. In same exon, SNU-2606 and SNU-2643 harbored pathogenic single nucleotide polymorphism. SNU-2606 had c.817C>T (p.R273C) mutation and SNU-2643 had c.818C>T (p.R273H) mutation. SNU-2589 harbored c.438G>A (p.W146*) nonsense mutation in exon 4. And SNU-2749 had c.991C>T (p.Q331*) nonsense mutation in exon 8. PCR substrate that was used in validating Nonsense mutation was genomic DNA differed from other substrate that was cDNA. e. ERBB2 c.1963A>G (p.I655V) variation was detected in SNU-2589, SNU-2657, SNU-2681, SNU-2689, SNU-2749 and SNU-2832. f. Validation of results of FGFR4 genes mutation regions sequencing by Sanger sequencing analysis. FGFR4 c.1162G>A (p.G388R) mutation was detected in total 14 cell lines of 28 cell lines (SNU-2535, SNU-2556, SNU-2563, SNU-2588, SNU-2606, SNU-2627, SNU-2643, SNU-2657, SNU-2689, SNU-2741, SNU-2749, SNU-2778, SNU-2964 and SNU-3023). SNU-

2689 was additionally c.991C>T (p.Q331*) nonsense mutation by using genomic DNA as PCR substrate.

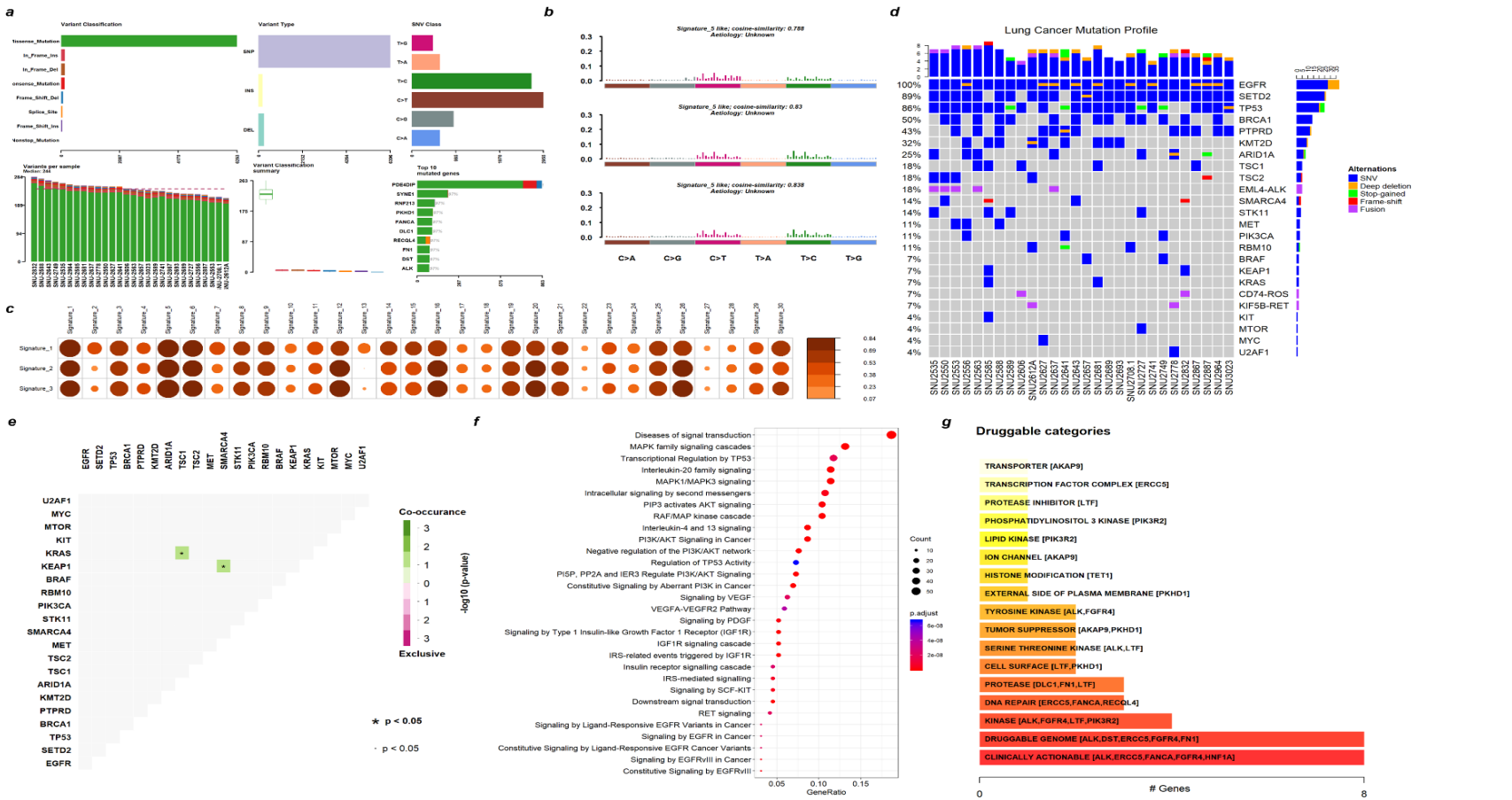

a
Variant Classification
Missense_Mutation
In_Frame_Ins
In_Frame_Del
Nonsense_Mutation
Frame_Shift_Del
Splice_Site
Frame_Shift_Ins
Nonstop_Mutation
Variant Type
SNP
INS
DEL
SNV Class
T>G
T>A
T>C
C>T
C>G
C>A
Variants per sample
Median: 240
Variant Classification summary
Top 10 mutated genes
PDE4DIP
SYNE1
RNF213
PKHD1
FANCA
DLC1
RECQL4
FN1
DST
ALK
b
Signature_5 like; cosine-similarity: 0.788
Aetiology: Unknown
Signature_5 like; cosine-similarity: 0.83
Aetiology: Unknown
Signature_5 like; cosine-similarity: 0.838
Aetiology: Unknown
C>A
C>G
C>T
T>A
T>C
T>G
c
Signature_1
Signature_2
Signature_3
d
Lung Cancer Mutation Profile
EGFR
SETD2
TP53
BRCA1
PTPRD
KMT2D
ARID1A
TSC1
TSC2
EML4-ALK
SMARCA4
STK11
MET
PIK3CA
RBM10
BRAF
KEAP1
KRAS
CD74-ROS
KIF5B-RET
KIT
MTOR
MYC
U2AF1
Alternations
SNV
Deep deletion
Stop-gained
Frame-shift
Fusion
e
Co-occurance
Exclusive
-log10 (p-value)
★ p < 0.05
· p < 0.05
f
Diseases of signal transduction
MAPK family signaling cascades
Transcriptional Regulation by TP53
Interleukin-20 family signaling
MAPK1/MAPK3 signaling
Intracellular signaling by second messengers
PIP3 activates AKT signaling
RAF/MAP kinase cascade
Interleukin-4 and 13 signaling
PI3K/AKT Signaling in Cancer
Negative regulation of the PI3K/AKT network
Regulation of TP53 Activity
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
Constitutive Signaling by Aberrant PI3K in Cancer
Signaling by VEGF
VEGFA-VEGFR2 Pathway
Signaling by PDGF
Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)
IGF1R signaling cascade
IRS-related events triggered by IGF1R
Insulin receptor signalling cascade
IRS-mediated signalling
Signaling by SCF-KIT
Downstream signal transduction
RET signaling
Signaling by Ligand-Responsive EGFR Variants in Cancer
Signaling by EGFR in Cancer
Constitutive Signaling by Ligand-Responsive EGFR Cancer Variants
Signaling by EGFRvIII in Cancer
Constitutive Signaling by EGFRvIII
GeneRatio
Count
p.adjust
g
Druggable categories
TRANSPORTER [AKAP9]
TRANSCRIPTION FACTOR COMPLEX [ERCC5]
PROTEASE INHIBITOR [LTF]
PHOSPHATIDYLINOSITOL 3 KINASE [PIK3R2]
LIPID KINASE [PIK3R2]
ION CHANNEL [AKAP9]
HISTONE MODIFICATION [TET1]
EXTERNAL SIDE OF PLASMA MEMBRANE [PKHD1]
TYROSINE KINASE [ALK,FGFR4]
TUMOR SUPPRESSOR [AKAP9,PKHD1]
SERINE THREONINE KINASE [ALK,LTF]
CELL SURFACE [LTF,PKHD1]
PROTEASE [DLC1,FN1,LTF]
DNA REPAIR [ERCC5,FANCA,RECQL4]
KINASE [ALK,FGFR4,LTF,PIK3R2]
DRUGGABLE GENOME [ALK,DST,ERCC5,FGFR4,FN1]
CLINICALLY ACTIONABLE [ALK,ERCC5,FANCA,FGFR4,HNF1A]
Genes

Figure 4. Mutational context of the established lung cancer cell lines. **a**. Summarization of variants. **b**. Mutational signatures characterized by a specific pattern of nucleotide substitutions. **c**. Visualization of p-values of mutational signature. **d**. Mutational landscape of the established lung cancer cell lines. **e**. Co-occurring or exclusiveness in the mutation patterns of lung cancer cell lines. **f**. Gene set enrichment analysis to find representative aberrated pathways **g**. Targetable genes were identified in reference with the number of genes that are involved in drug target pathways.

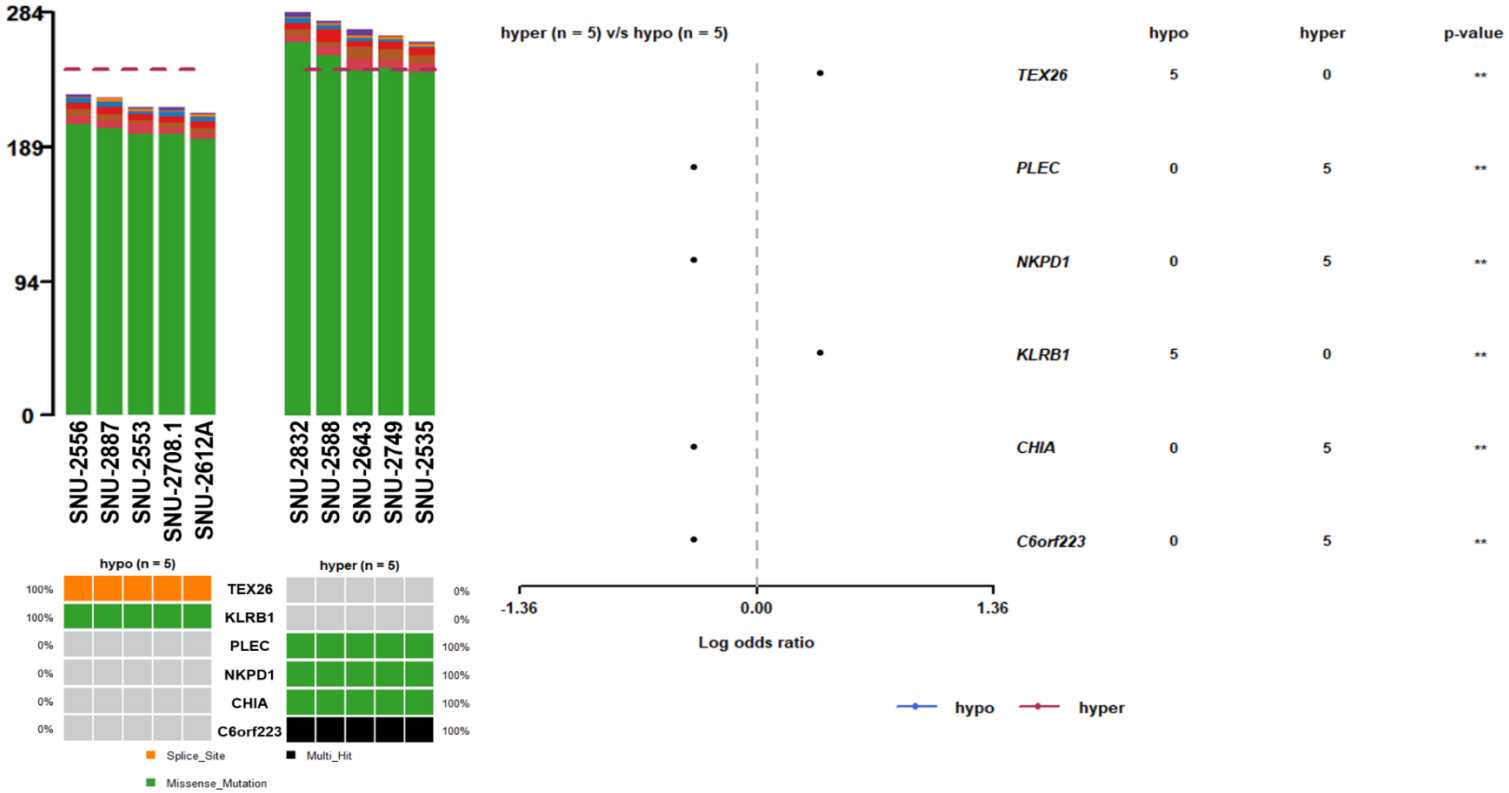


Figure 5. Mutations that are present exclusively in five hyper-mutated groups and hypo-mutated groups

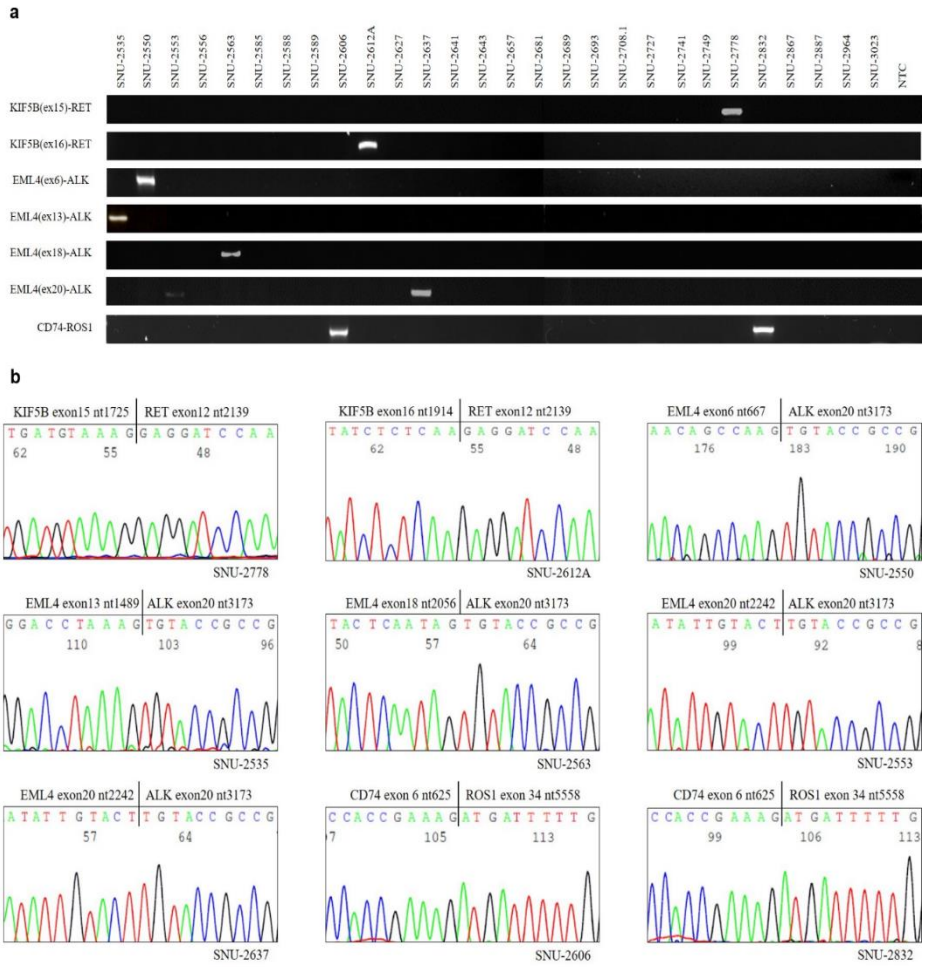

a
SNU-2535
SNU-2550
SNU-2553
SNU-2556
SNU-2563
SNU-2585
SNU-2588
SNU-2589
SNU-2606
SNU-2612A
SNU-2627
SNU-2637
SNU-2641
SNU-2643
SNU-2657
SNU-2681
SNU-2689
SNU-2693
SNU-2708.1
SNU-2727
SNU-2741
SNU-2749
SNU-2778
SNU-2832
SNU-2867
SNU-2887
SNU-2964
SNU-3023
NTC
KIF5B(ex15)-RET
KIF5B(ex16)-RET
EML4(ex6)-ALK
EML4(ex13)-ALK
EML4(ex18)-ALK
EML4(ex20)-ALK
CD74-ROS1
b
KIF5B exon15 nt1725 | RET exon12 nt2139
SNU-2778
KIF5B exon16 nt1914 | RET exon12 nt2139
SNU-2612A
EML4 exon6 nt667 | ALK exon20 nt3173
SNU-2550
EML4 exon13 nt1489 | ALK exon20 nt3173
SNU-2535
EML4 exon18 nt2056 | ALK exon20 nt3173
SNU-2563
EML4 exon20 nt2242 | ALK exon20 nt3173
SNU-2553
EML4 exon20 nt2242 | ALK exon20 nt3173
SNU-2637
CD74 exon 6 nt625 | ROS1 exon 34 nt5558
SNU-2606
CD74 exon 6 nt625 | ROS1 exon 34 nt5558
SNU-2832

Figure 6. Fusion gene detection by PCR and validation

a. PCR result of each fusion gene set primer. About KIF5B-RET rearrangement, reverse primer was RET exon 12 and then forward primer were KIF5B exons 15 and exon 16. Only SNU-2778 had expression of KIF5B exon 15-RET exon 12 and SNU-2612A had expression of KIF5B exon 16-RET exon 12. In EML4-ALK rearrangement, reverse primer of all set was ALK exon 20. Forward primers were exons 6, 13, 18 and 20 of EML4. SNU-2550 had EML4 exon 6-ALK fusion and SNU-2535 had EML4 exon 13-ALK fusion. The cell line that had EML4 exon 18-ALK fusion was SNU-2563 only. About EML4 exon 20-ALK fusion, SNU-2553 and SNU-2637 had these fusions. The other fusion set, CD74-ROS1 fusion was detected in SNU-2606 and SNU-2832. Those cell lines have CD74 exon 6 and ROS1 exon 34 rearrangement. To finely detect PCR result, this fusion forward primer was CD74 exon 5 region. *b.* Validation of PCR results by Sanger sequencing analysis of each cell line that had fusion gene detected by PCR

Table 6. Mutation profiles by targeted gene sequencing (not confirmed by Sanger sequencing)

Cell line	ATM		EPHA3		ERBB4	
	nt	nt change (a.a. change)	nt	nt change (a.a. change)	nt	nt change (a.a. change)
SNU-2535	wt		wt		wt	
SNU-2550	wt		wt		wt	
SNU-2553	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-2556	wt		wt		wt	
SNU-2563	6497	gTg>gCg(V2166A)	wt		wt	
SNU-2585	wt		wt		1744	Cat>Gat(H582D)
SNU-2588	wt		wt		wt	
SNU-2589	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-2606	wt		wt		wt	
SNU-2612A	wt		wt		wt	
SNU-2627	wt		wt		wt	
SNU-2637	wt		wt		wt	
SNU-2641	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-2643	4138	Cat>Tat(H1380Y)	wt		wt	
SNU-2657	wt		wt		wt	
SNU-2681	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-2689	wt		wt		wt	
SNU-2693	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-2708.1	2948	aAt>aGt(N1983S)	wt		wt	
SNU-2727	wt		wt		wt	
SNU-2741	wt		wt		wt	
SNU-2749	323	gCa>gGa(A108G)	wt		wt	
SNU-2778	1208	tCa>tTa(S403L)	2770	Tgg>Cgg(W924R)	wt	
SNU-2832	wt		wt		wt	
SNU-2867	wt		wt		wt	
SNU-2887	wt		wt		wt	
SNU-2964	wt		2770	Tgg>Cgg(W924R)	wt	
SNU-3023	5948	aAt>aGt(N1983S)	wt		wt	

Continued

Cell line	FGFR2		FGFR3		KDR	
	nt	nt change (a.a. change)	nt	nt change (a.a. change)	nt	nt change (a.a. change)
SNU-2535	wt		wt		wt	
SNU-2550	wt		wt		wt	
SNU-2553	wt		wt		wt	
SNU-2556	wt		wt		1416	caA>caT(Q472H)
SNU-2563	17	cGt>cCt(R6P)	wt		wt	
SNU-2585	wt		wt		1416	caA>caT(Q472H)
SNU-2588	wt		1352	aCg>aTg(T451M)	1416	caA>caT(Q472H)
SNU-2589	wt		wt		wt	
SNU-2606	wt		wt		wt	
SNU-2612A	wt		wt		2443	Gaa>Taa(E815*)
SNU-2627	wt		wt		wt	
SNU-2637	wt		wt		wt	
SNU-2641	17	cGt>cCt(R6P)	wt		wt	
SNU-2643	17	cGt>cCt(R6P)	1352	aCg>aTg(T451M)	1416	caA>caT(Q472H)
SNU-2657	wt		wt		1416	caA>caT(Q472H)
SNU-2681	wt		wt		wt	
SNU-2689	wt		wt		1416	caA>caT(Q472H)
SNU-2693	wt		wt		wt	
SNU-2708.1	wt		wt		1416	caA>caT(Q472H)
SNU-2727	wt		wt		wt	
SNU-2741	wt		1352	aCg>aTg(T451M)	1416	caA>caT(Q472H)
SNU-2749	17	cGt>cCt(R6P)	wt		1416	caA>caT(Q472H)
SNU-2778	wt		wt		wt	
SNU-2832	wt		wt		wt	
SNU-2867	wt		wt		wt	
SNU-2887	wt		wt		1416	caA>caT(Q472H)
SNU-2964	17	cGt>cCt(R6P)	wt		wt	
SNU-3023	wt		wt		1416	caA>caT(Q472H)

Continued

Cell line	NOTCH1		PAK3		PDGFRA	
	nt	nt change (a.a. change)	nt	nt change (a.a. change)	nt	nt change (a.a. change)
SNU-2535	wt		wt		wt	
SNU-2550	wt		wt		wt	
SNU-2553	wt		wt		wt	
SNU-2556	wt		wt		wt	
SNU-2563	wt		wt		wt	
SNU-2585	wt		wt		wt	
SNU-2588	wt		wt		1432	Tcc>Ccc(S478P)
SNU-2589	wt		wt		wt	
SNU-2606	wt		wt		wt	
SNU-2612A	wt		wt		wt	
SNU-2627	wt		wt		wt	
SNU-2637	wt		1033	Gat>Aat(D345N)	wt	
SNU-2641	wt		wt		wt	
SNU-2643	wt		wt		wt	
SNU-2657	wt		wt		wt	
SNU-2681	wt		wt		wt	
SNU-2689	wt		wt		1432	Tcc>Ccc(S478P)
SNU-2693	wt		1438	Gaa>Taa(E480*)	wt	
SNU-2708.1	7174	Atg>Ctg(M2392L)	wt		wt	
SNU-2727	wt		wt		wt	
SNU-2741	wt		wt		wt	
SNU-2749	wt		wt		1432	Tcc>Ccc(S478P)
SNU-2778	wt		wt		wt	
SNU-2832	wt		wt		wt	
SNU-2867	wt		wt		wt	
SNU-2887	wt		wt		1432	Tcc>Ccc(S478P)
SNU-2964	wt		wt		wt	
SNU-3023	7174	Atg>Ctg(M2392L)	wt		wt	

Continued

Cell line	PTPRD		STK11		TSC1	
	nt	nt change (a.a. change)	nt	nt change (a.a. change)	nt	nt change (a.a. change)
SNU-2535	wt		1062	ttC>ttG(F354L)	wt	
SNU-2550	wt		wt		wt	
SNU-2553	wt		wt		wt	
SNU-2556	wt		wt		wt	
SNU-2563	wt		wt		2075	cGa>cAa(R692Q)
SNU-2585	wt		wt		1335 965	gaA>gaG(E445E) aTg>aCg(M322T)
SNU-2588	wt		wt		wt	
SNU-2589	wt		wt		wt	
SNU-2606	wt		wt		1335 965	gaA>gaG(E445E) aTg>aCg(M322T)
SNU-2612A	wt		wt		wt	
SNU-2627	wt		wt		wt	
SNU-2637	wt		wt		wt	
SNU-2641	5004	tgT>tgA(C1668*)	wt		wt	
SNU-2643	wt		wt		wt	
SNU-2657	wt		wt		wt	
SNU-2681	wt		wt		2075	cGa>cAa(R692Q)
SNU-2689	wt		wt		wt	
SNU-2693	wt		wt		wt	
SNU-2708.1	wt		wt		wt	
SNU-2727	wt		wt		wt	
SNU-2741	wt		wt		wt	
SNU-2749	wt		wt		wt	
SNU-2778	wt		wt		wt	
SNU-2832	wt		wt		wt	
SNU-2867	1339	Cag>Gag (Q447E)	wt		wt	
SNU-2887	wt		wt		wt	
SNU-2964	wt		wt		wt	
SNU-3023	2983	Cgt>Tgt(R995C)	wt		wt	

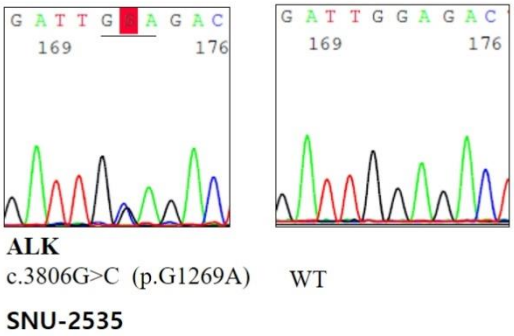


Figure 7. Validation of ALK mutation in SNU-2535. Sanger sequencing to ALK kinase domain. Cell lines that have EML4-ALK fused gene don't have any mutation in ALK kinase domain except for SNU-2535 in our cell lines. SNU-2535 harbors G1269A mutation in ALK kinase domain.

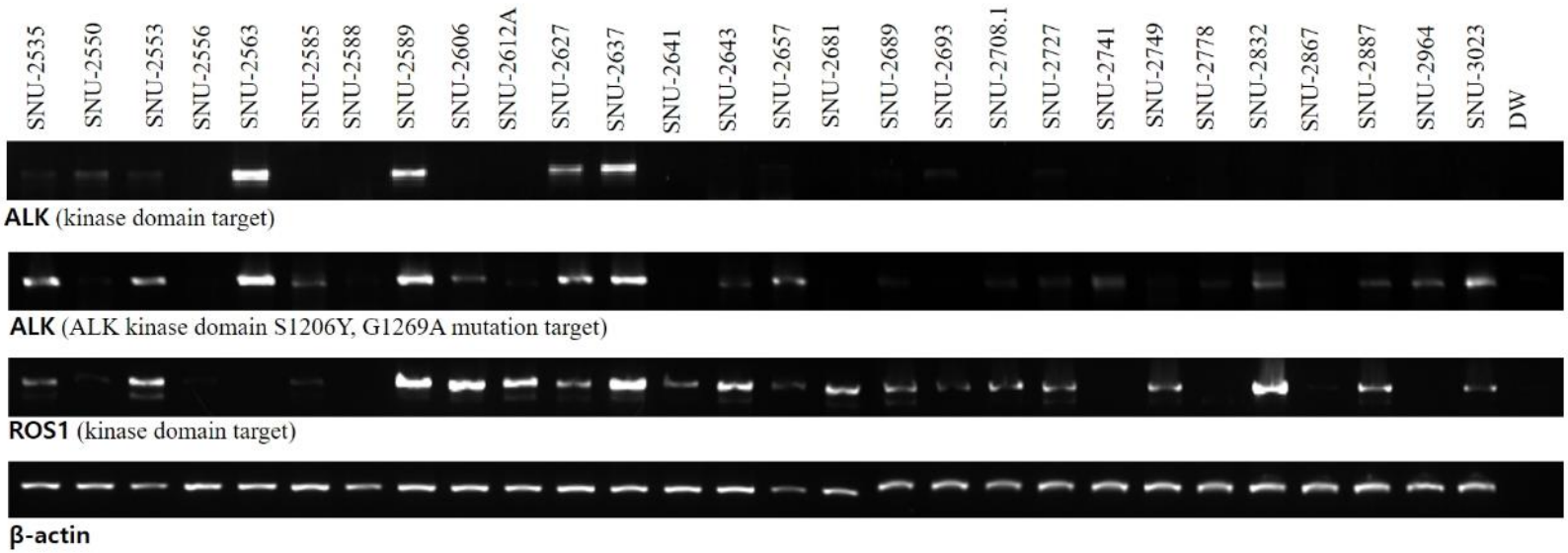
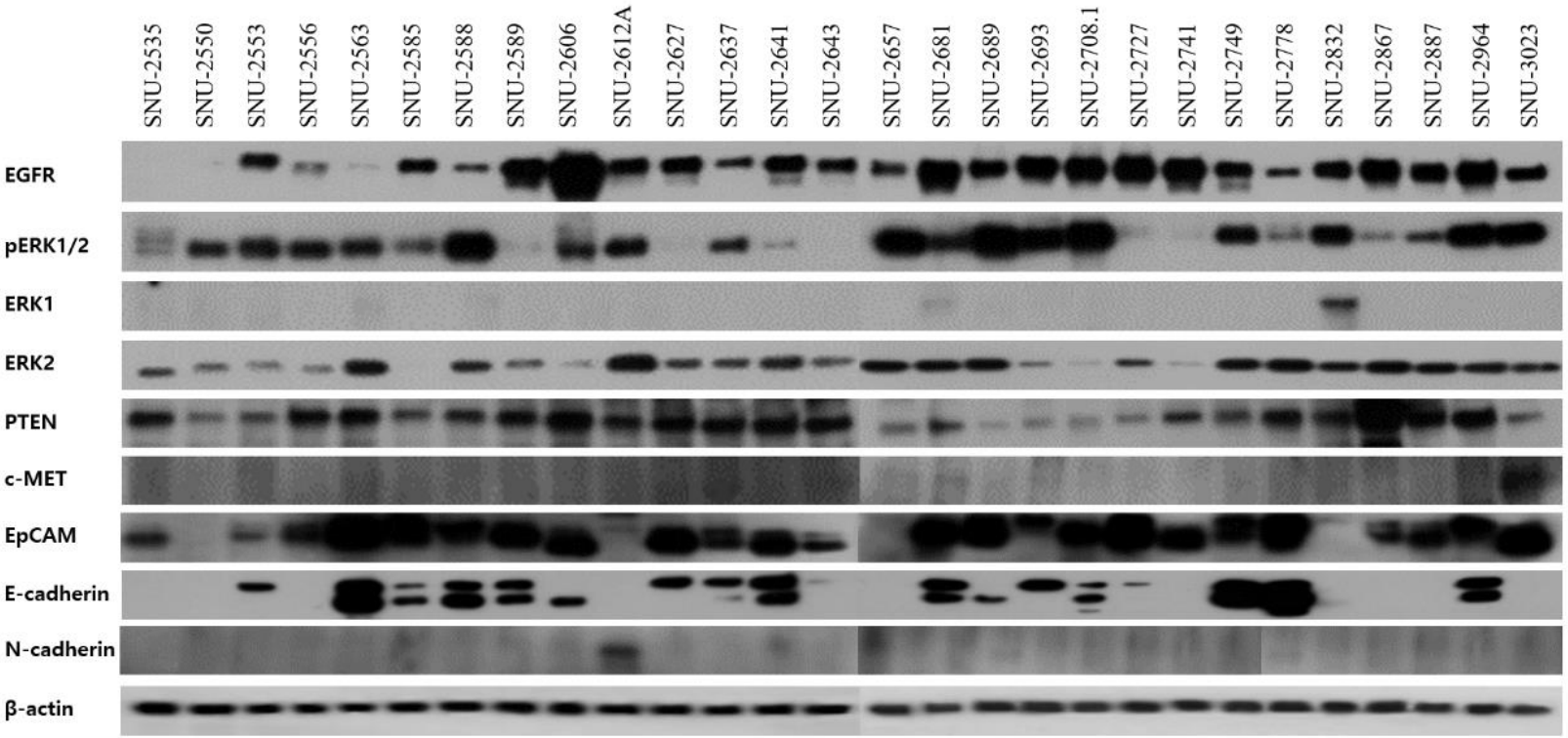

SNU-2535
SNU-2550
SNU-2553
SNU-2556
SNU-2563
SNU-2585
SNU-2588
SNU-2589
SNU-2606
SNU-2612A
SNU-2627
SNU-2637
SNU-2641
SNU-2643
SNU-2657
SNU-2681
SNU-2689
SNU-2693
SNU-2708.1
SNU-2727
SNU-2741
SNU-2749
SNU-2778
SNU-2832
SNU-2867
SNU-2887
SNU-2964
SNU-3023
DW
ALK (kinase domain target)
ALK (ALK kinase domain S1206Y, G1269A mutation target)
ROS1 (kinase domain target)
β-actin

Figure 8. Expressions of ALK and ROS1 kinase domain by RT-PCR. SNU-2535, SNU-2550, SNU-2553, SNU-2563, SNU-2589, SNU-2627 and SNU-2637 had expression of ALK kinase domain in mRNA levels. Specifically, SNU-2563, SNU-2589 and SNU-2637 were overexpressed, despite SNU-2589 didn't have EML4-ALK fused gene. SNU-2553, SNU-2589, SNU-2606, SNU-2637 and SNU-2832 had high expression level in RT-PCR targeting the ROS1 kinase domain.

Table 7. Summary of mutations by Sanger sequencing in each cell line

No.	**Cell-Name**	**Data**
1	SNU-2535	EML4(exon13)-ALK(exon20) fusion, FGFR4 G388R, ALK G1269A
2	SNU-2550	EML4(exon6)-ALK(exon20) fusion
3	SNU-2553	EML4(exon20)-ALK(exon20) fusion
4	SNU-2556	EGFR exon19 deletion, FGFR4 G388R
5	SNU-2563	EML4(exon18)-ALK(exon20) fusion, TP53 R280K
6	SNU-2585	KRAS G12V
7	SNU-2588	FGFR4 G388R
8	SNU-2589	ERBB2 I655V, TP53 W146* nonsense mutation
9	SNU-2606	CD74(exon6)-ROS1(exon34) fusion, FGFR4 G388R, TP53 R280K
10	SNU-2612A	KIF5B(exon16)-RET(exon12) fusion
11	SNU-2627	FGFR4 G388R
12	SNU-2637	EML4(exon18)-ALK(exon20) fusion
13	SNU-2641	PTPRD C1668* (targeted gene sequencing data)
14	SNU-2643	EGFR exon19 deletion, FGFR4 G388R, TP53 R273C
15	SNU-2657	BRAF V600E, ERRB2 I600V, FGFR4 G388R
16	SNU-2681	ERBB2 I655V, KRAS G12C, TP53 R273C
17	SNU-2689	EGFR L858R, ERBB2 I655V, FGFR4 G388R
18	SNU-2693	PAK3 E480* (targeted gene sequencing data)
19	SNU-2708.1	EGFR exon19 deletion
20	SNU-2727	EGFR L858R
21	SNU-2741	FGFR4 G388R, EGFR exon19 deletion
22	SNU-2749	BRAF V600E, ERBB2 I655V, TP53 Q331* nonsense mutation, FGFR4 G388R
23	SNU-2778	KIF5B(exon15)-RET(exon12) fusion, FGFR4 G388R
24	SNU-2832	CD74(exon6)-ROS1(exon34) fusion, ERBB2 I655V
25	SNU-2867	EGFR exon19 deletion
26	SNU-2887	EGFR exon19 deletion
27	SNU-2964	EGFR exon19 deletion
28	SNU-3023	EGFR L858R, FGFR4 G388R


SNU-2535
SNU-2550
SNU-2553
SNU-2556
SNU-2563
SNU-2585
SNU-2588
SNU-2589
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SNU-2612A
SNU-2627
SNU-2637
SNU-2641
SNU-2643
SNU-2657
SNU-2681
SNU-2689
SNU-2693
SNU-2708.1
SNU-2727
SNU-2741
SNU-2749
SNU-2778
SNU-2832
SNU-2867
SNU-2887
SNU-2964
SNU-3023
EGFR
pERK1/2
ERK1
ERK2
PTEN
c-MET
EpCAM
E-cadherin
N-cadherin
β-actin

Figure 9. Western blot analysis for EGFR, pERK1/2, ERK1, ERK2, PTEN, c-MET, Mesenchymal and Epithelial cell marker.

Expressions of EGFR, pERK1/2, PTEN and c-MET in 28 lung cancer cell lines were analyzed by Western blot. The EGFR expression was elevated in our cell lines including SNU-2589, SNU-2606, SNU-2681, SNU-2693, SNU-2708.1, SNU-2727, SNU-2741, SNU-2867, SNU-2887 and SNU-2964. Among the cell lines harboring EGFR L858R mutation (SNU-2689, SNU-2727 and SNU-3023), SNU-2727 overexpressed the EGFR level. Furthermore, SNU-2708.1, SNU-2741, SNU-2867, SNU-2887 and SNU-2964 were amplified among the 7 cell lines exhibiting EGFR exon19 deletions (SNU-2556, SNU-2643, SNU-2708.1, SNU-2741, SNU-2867, SNU-2887 and SNU-2964). Only SNU-2832 showed a high ERK1expression. ERK2 protein level was expressed generally significantly, whereas ERK2 expression in in SNU-2585 was limited. Expression of phosphorylated ERK1 was generally pathetic in phosphorylated ERK1/2. Generally, the level of pERK2 was highly expressed. Especially pERK2 were extremely expressed in SNU-2588, SNU-2657, SNU-2689, SNU-2689 and SNU-2708.1. PTEN expression was high in SNU-2867. c-MET expression level was overall weak except for SNU-3023. Vimentin expression levels were high in SNU-2550, SNU-2612A, SNU-2637, SNU-2657, SNU-2832 and SNU-2887. N-cadherin expression was high in SNU-2612A. The expression

of E-cadherin and EpCAM was opposite to that of vimentin and N-cadherin.

3.3. Anticancer drug response with mutational contexts

The log EC_{50} values of the 27 lung cancer cell lines were estimated for erlotinib, gefitinib (tyrosine kinase inhibitors targeting EGFR), and crizotinib (anti-cancer drug targeting ALK and ROS1) (18,19). SNU-2708.1 was excluded due to its slow growth rate. Quartiles were calculated to classify the established cell lines into relatively sensitive and resistant groups with respect to each drug. The results are summarized in Table 8. To confirm whether the mutational status of EGFR or ALK influenced the drug response of the newly-established lung cancer cell lines, widely used lung cancer cell lines harboring identical driver mutations were obtained. The NCI-H522 cell line has neither an EML4-ALK fusion nor a mutation in the EGFR kinase domain and was selected as a non-sensitive model for the drugs used. The HCC827 cell line was derived from a lung adenocarcinoma carrying a large deletion in the EGFR exon 19 (E746 - A750 deletion). Among the newly-established lung cancer cell lines, seven (SNU-2556, SNU-2643, SNU-2708.1, SNU-2741, SNU-2867, SNU-2887, and SNU-2964) harbored a large deletion in EGFR exon 19. As expected, the NCI-H522 cells didn't respond to erlotinib compared to other cell lines, and HCC827 was notably sensitive to erlotinib compared to the newly-established lung cancer cell lines. To further inspect the mutational variations, the mutation data of NCI-H522 and HCC827

were obtained from the cancer cell line encyclopedia (CCLE) website (https://portals.broadinstitute.org/ccle). We extracted the CCLE genes from the WES data of the seven lung cancer cell lines and compared their mutational status with that of HCC827 (Figure 10, Table 9.). The average number of mutations found in the lung cancer cell lines was 859, whereas the number of mutations in HCC827 was only 280. Moreover, stop gain mutations in the PDE4DIP, PRDM18, and DPYD genes were only present in the lung cancer cell lines, which may account for the resistance of the newly-established lung cancer cell lines (Table 10).

The NCI-H2228 cell line originated from a lung adenocarcinoma carrying an EML4 exon 6-ALK exon 20 fusion. Among the newly-established lung cancer cell lines, five (SNU-2535, SNU-2550, SNU-2553, SNU-2563, and SNU-2637) carried an EML4-ALK fusion. The NCI-H2228 cells exhibited a sensitive crizotinib response compared to other cell lines harboring an EML4-ALK fusion, except for SNU-2563 which contained an EML4 exon 18-ALK exon 20 fusion (Figure 10, Table 9). The average number of CCLE gene mutations found in the lung cancer cell lines was 711, whereas the number of mutations in HCC827 was only 451. Accordingly, our cell lines which originated from a pleural effusion carried more mutations in the CCLE genes and were more resistant than those derived from primary lung cancer tissues.

Table 8. Drug sensitivity about gefitinib, erlotinib and crizotinib of newly established 28 SNU human lung cancer cell lines

No	Cell-Name	Gefitinib (Log EC50, μM)	Erlotinib (Log EC50, μM)	Crizotinib (Log EC50, μM)
1	SNU-2535	0.844±0.018	0.8±0.074	0.33±0.306
2	SNU-2550	0.966±0.101	1.172±0.053	0.114±0.1
3	SNU-2553	1.161±0.017	1.352±0.022	0.592±0.154
4	SNU-2556	0.671±0.238	0.504±0.238	0.586±0.106
5	SNU-2563	0.837±0.061	1.154±0.129	-1.343±0.382
6	SNU-2585	1.035±0.076	0.642±0.268	0.611±0.345
7	SNU-2588	0.501±0.145	0.658±0.048	0.509±0.095
8	SNU-2589	1.208±0.084	1.462±0.067	0.707±0.21
9	SNU-2606	0.749±0.168	0.938±0.038	0.477±0.17
10	SNU-2612A	0.369±0.282	-0.246±0.091	1.107±0.132
11	SNU-2627	1.05±0.023	0.962±0.038	-0.011±0.072
12	SNU-2637	1.119±0.067	1.255±0.092	0.356±0.136
13	SNU-2641	0.915±0.041	0.563±0.397	0.935±0.066
14	SNU-2643	0.546±0.348	-0.086±0.122	0.969±0.028
15	SNU-2657	1.251±0.134	0.911±0.266	0.805±0.252
16	SNU-2681	1.012±0.054	1.096±0.052	0.587±0.076
17	SNU-2689	0.799±0.079	1.033±0.027	1.048±0.089
18	SNU-2693	0.796±0.371	0.841±0.276	0.49±0.093
19	SNU-2708.1	-	-	-
20	SNU-2727	1.786±0.199	1.067±0.23	1.29±0.031
21	SNU-2741	1.123±0.132	0.976±0.399	0.911±0.2
22	SNU-2749	0.833±0.057	0.707±0.07	0.554±0.043
23	SNU-2778	0.718±0.018	0.676±0.025	0.585±0.022
24	SNU-2832	0.906±0.181	1.042±0.306	-1.391±0.138
25	SNU-2867	-0.034±0.573	0.079±0.262	0.768±0.158
26	SNU-2887	0.928±0.147	0.922±0.205	0.794±0.21
27	SNU-2964	0.959±0.064	0.931±0.032	0.643±0.038
28	SNU-3023	0.641±0.137	0.57±0.118	-1.769±0.088
	Lower Quartile	0.734	0.650	0.417
	Upper Quartile	1.043	1.055	0.800

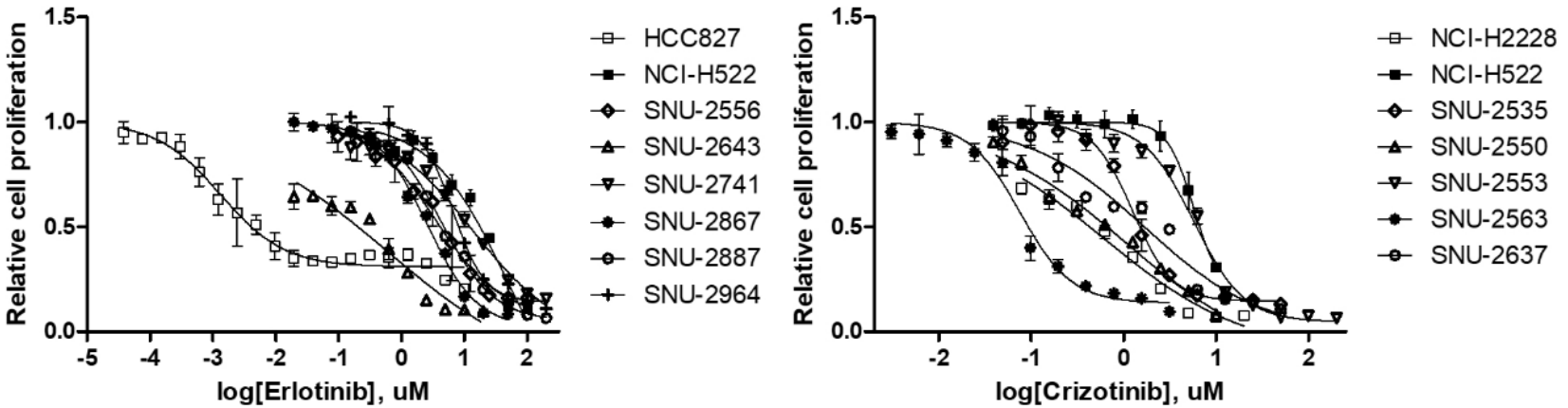
Relative cell proliferation
1.5
1.0
0.5
0.0
-5
-4
-3
-2
-1
0
1
2
log[Erlotinib], uM
HCC827
NCI-H522
SNU-2556
SNU-2643
SNU-2741
SNU-2867
SNU-2887
SNU-2964
Relative cell proliferation
1.5
1.0
0.5
0.0
-2
-1
0
1
2
log[Crizotinib], uM
NCI-H2228
NCI-H522
SNU-2535
SNU-2550
SNU-2553
SNU-2563
SNU-2637

Figure 10. Drug sensitivity for erlotinib and crizotinib compared with other cell lines according to mutational traits.

a. HCC827 showed the most sensitive erlotinib response compared with cells with a pleural effusion origin. Except for HCC827, SNU-2643 that carried EGFR exon19 homodeletion was most sensitive to erlotinib among our cell lines. In fact, NCI-H522 cells are the most resistant compared with cells that have EML4-ALK fusion, and deletion or mutation in EGFR kinase domain.

b. NCI-H2228 cells were compared with our cell lines that carried EML4-ALK fusion. NCI-H2228 carry EML4 exon 6-ALK exon20 fused gene. In the meantime, HCC827 cells were compared with our cell lines carrying a deletion in EGFR kinase domain. As a result, NCI-H2228 cells showed a sensitive crizotinib response compared with other cell lines that exhibit EML4-ALK fusion except for SNU-2563 cell lines that harbor EML4 exon 18-ALK exon20.

Table 9. EC50 values of Erlotinib and Crizotinib, compared with other cell lines according to mutational traits

Cell-Name	Erlotinib (Log EC50, μM)
HCC827	-2.87±0.209
NCI-H522	1.598±0.119
SNU-2556	0.504±0.238
SNU-2643	-0.086±0.122
SNU-2741	0.976±0.399
SNU-2867	0.079±0.262
SNU-2887	0.922±0.205
SNU-2964	0.930±0.032

Cell-Name	Crizotinib (Log EC50, μM)
NCI-H2228	-0.211±0.076
NCI-H522	0.742±0.026
SNU-2535	0.33±0.306
SNU-2550	0.114±0.1
SNU-2553	0.592±0.154
SNU-2563	-1.343±0.382
SNU-2637	0.356±0.136

Table 10. Common mutations in Erlotinib resistant lung cancer cell lines among EGFR exon 19 homodeletion cell lines

Gene_Name	CHROM	POS	REF	ALT	HGVS.c	HGVS.p
PDE4DIP	chr1	144,852,390	C	T	c.7053G>A	p.Trp2351*
PDE4DIP	chr1	145,075,683	C	T	c.180G>A	p.Trp60*
DPYD	chr1	228,469,903	A	T	c.9754A>T	p.Arg3252*
PRDM18	chr1	144,915,561	G	A	c.1864C>T	p.Arg622*

3.4. Amplified DKK1 induces resistance to crizotinib

We validated that cell lines derived from a pleural effusion exudate had more mutations and were resistant to targeted drugs compared to cell lines originating from tumor tissue. To evaluate the molecular alterations when cell lines which do not respond to targeted drugs acquire more resistance, we established two crizotinib-resistant sublines (SNU-2550CR and SNU-2563CR), derived from the parental SNU-2550 and SNU-2563 cell lines by long-term exposure to increasing concentrations of crizotinib. Even though the SNU-2550 and SNU-2563 cell lines harbored an EML4-ALK fusion, they were more resistant to crizotinib compared to NCI-H2228. The acquired resistance was confirmed by crizotinib treatment at varying concentrations (Figure 11a, 11b). Epithelial-to-mesenchymal transition (EMT) has been previously observed in targeted drug-resistant lung cancer cells (20,21). Consistent with prior findings, morphological changes to the spindle-like cell shape were noted in both the SNU-2550CR and SNU-2563CR cells (Figure 11c). DKK1 has been previously reported to promote the migration and invasion of non-small cell lung cancer by inhibiting the phosphorylation of β-catenin (22,23). The elevated expression of DKK1 was confirmed by both qRT-PCR (Figure 11d) and Western blotting (Figure11e). To further analyze if the secondary mutations in the crizotinib-resistant sublines affected their response to other drugs, eight

lung cancer drugs approved by the National Cancer Institute (NCI) and one chemical compound which inhibits the canonical Wnt pathway were used to treat the crizotinib-resistant sublines, along with the cell lines carrying an EGFR exon 19 deletion and an EML4-ALK fusion. The cell lines carrying an EGFR exon 19 deletion and an EML4-ALK fusion were clustered into different groups. Although the crizotinib-resistant sublines clustered with the EML4-ALK groups, they showed cross-resistance to both ceritinib and alectinib (a second-generation ALK inhibitor) and trametinib (MEK inhibitor) (Figure 11f). Interestingly, the SNU-2563CR subline exhibited resistance to all tested drugs. To further analyze the molecular alteration of the crizotinib-resistant sublines, WES and RNA sequencing (RNA-seq) were performed on both parental and crizotinib-resistant sublines. WES revealed a novel nonsense mutation in the PRSS1 gene in both crizotinib-resistant sublines. A germline mutation in PRSS1 has been associated with hereditary pancreatitis and familial pancreatic cancer (24), yet its function in crizotinib resistance has not been elucidated. SPIA two-way analysis was performed on the RNA-seq to pinpoint the aberrant pathways. Aberrant focal adhesion signaling was found in both crizotinib-resistant sublines (Figure 12a, b). Among the various genes involved in focal adhesion signaling, the mRNA expression of the Dickkopf-related protein 1 (DKK1) was significantly increased in both

crizotinib-resistant sublines (Figure 11d). We further investigated the potential role of amplified DKK1 expression in crizotinib-resistance by immunocytochemistry (ICC). ICC indicated that the nuclear localization of β-catenin was significantly increased in both crizotinib-resistant cell lines (Figure 11b, 11c). Accordingly, these results indicate that the EMT-derived crizotinib resistance was accompanied by augmented DKK1 expression. In conclusion, we have established 28 NSCLC cell lines from a malignant pleural effusion and associated their mutational traits with targeted drug responses. The cell lines were relatively resistant compared to cell lines originating from other tissues exhibiting similar mutational features. We also established two crizotinib-resistant sublines from cell lines carrying an EML4-ALK fusion gene. Comprehensive molecular analysis revealed that a novel nonsense mutation in the PRSS1 gene was present in the crizotinib-resistant sublines and that the expression of DKK1 was significantly augmented in the crizotinib-resistant sublines. Such analyses facilitate studies associated with drug treatment and representative mutations.

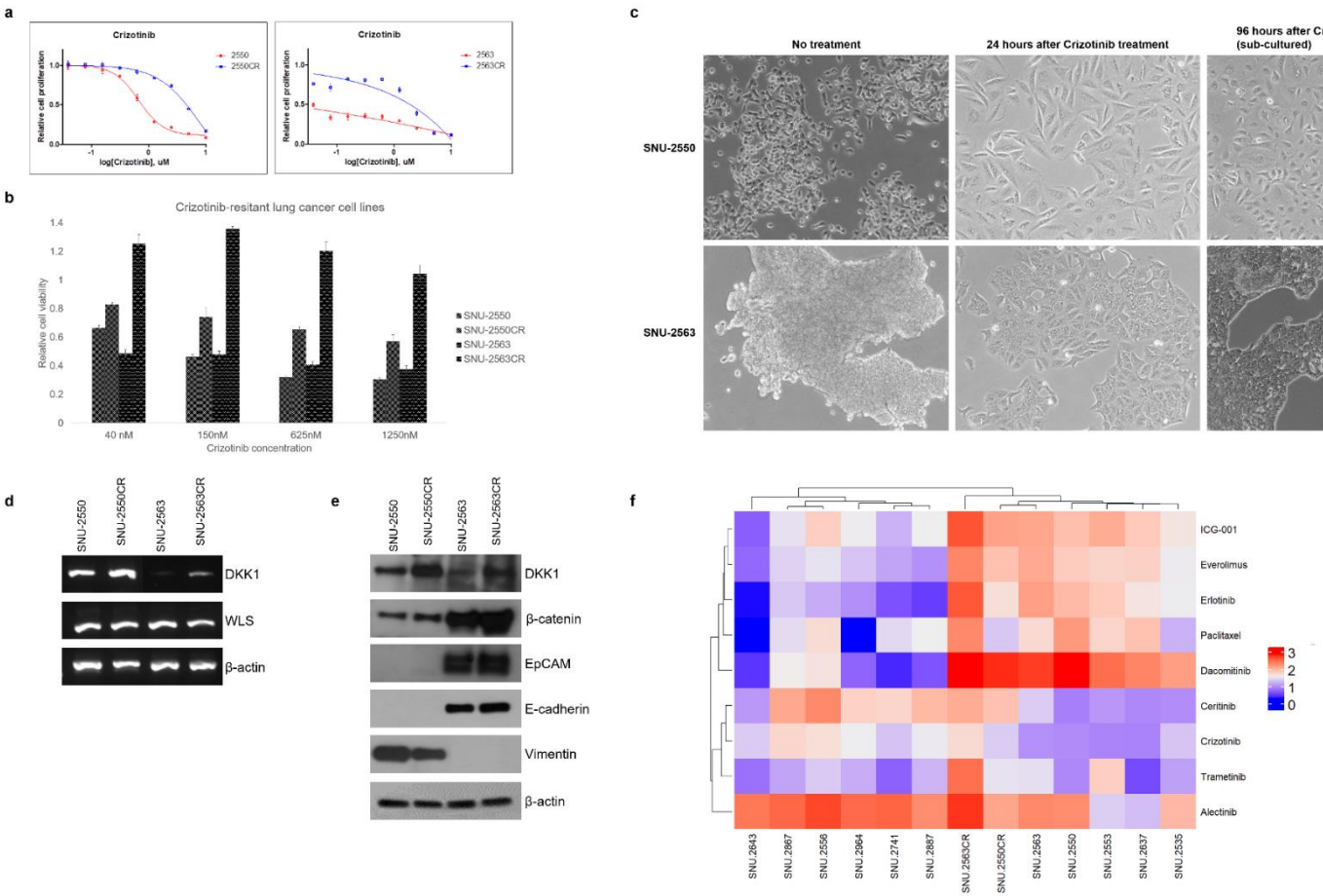
a
Crizotinib
2550
2550CR
Relative cell proliferation
log[Crizotinib], uM
Crizotinib
2563
2563CR
b
Crizotinib-resitant lung cancer cell lines
Relative cell viability
SNU-2550
SNU-2550CR
SNU-2563
SNU-2563CR
40 nM
150nM
625nM
1250nM
Crizotinib concentration
c
No treatment
24 hours after Crizotinib treatment
96 hours after Crizotinib treatment (sub-cultured)
SNU-2550
SNU-2563
d
SNU-2550
SNU-2550CR
SNU-2563
SNU-2563CR
DKK1
WLS
β-actin
e
SNU-2550
SNU-2550CR
SNU-2563
SNU-2563CR
DKK1
β-catenin
EpCAM
E-cadherin
Vimentin
β-actin
f
ICG-001
Everolimus
Erlotinib
Paclitaxel
Dacomitinib
Ceritinib
Crizotinib
Trametinib
Alectinib
SNU.2643
SNU.2867
SNU.2556
SNU.2964
SNU.2741
SNU.2887
SNU.2563CR
SNU.2550CR
SNU.2563
SNU.2550
SNU.2553
SNU.2637
SNU.2535
3
2
1
0

Figure 11. Establishment of two crizotinib resistant sublines. **a, b**. The acquired resistance was confirmed with crizotinib treatment in various concentrations. **c**. Morphological changes to the spindle-like cell shape were noted in both SNU-2550CR and SNU-2563CR cells. **d**. The mRNA expression of DKK1 was augmented in crizotinib resistant sublines. e. The protein expression of EMT-related genes and DKK1. **e**. Drug treatment of two crizotinib resistant sublines along with cell lines that carry EGFR exon 19 deletion and EML4-ALK fusion.

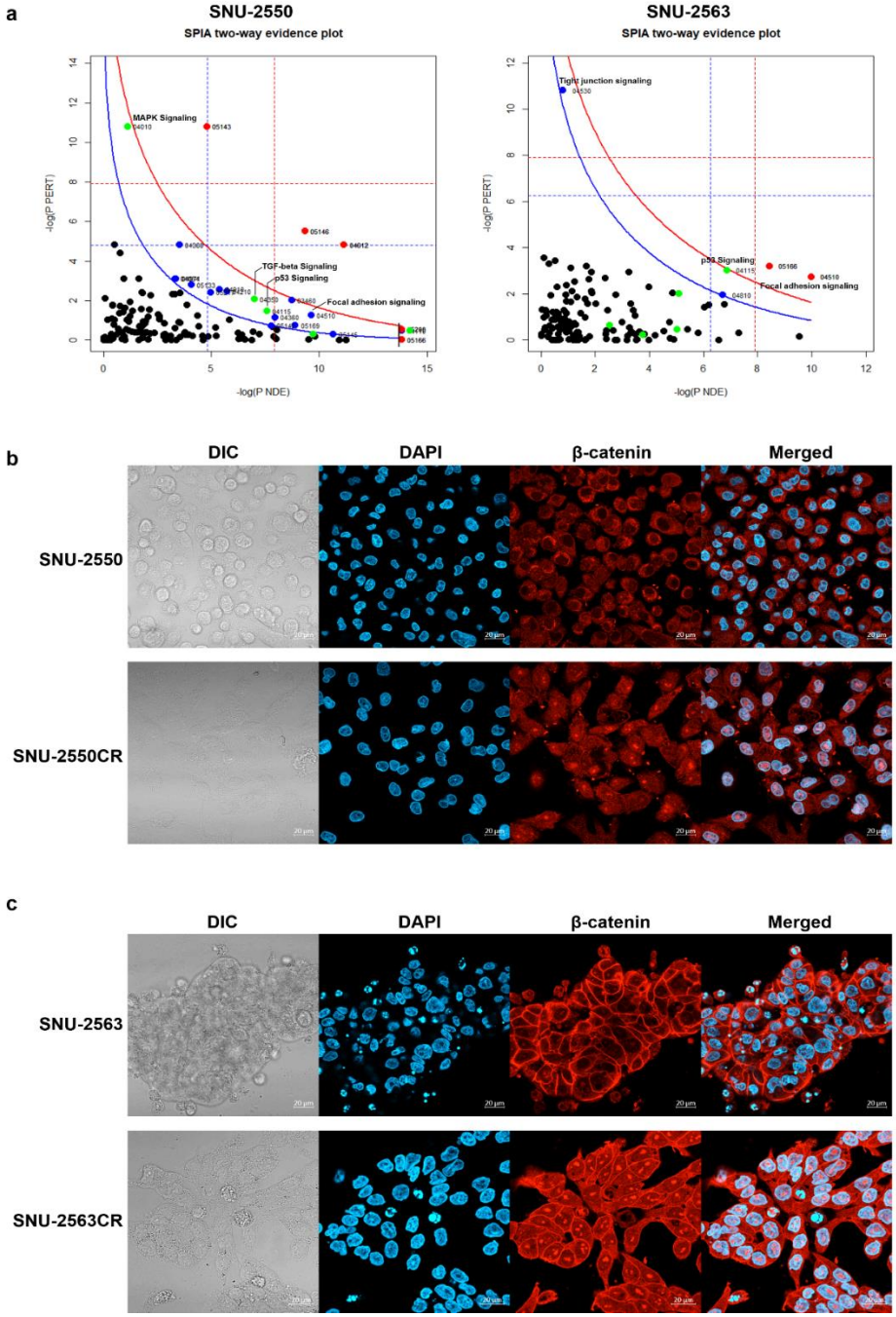
a
SNU-2550
SPIA two-way evidence plot
-log(P PERT)
-log(P NDE)
MAPK Signaling
04010
05143
05146
04012
04068
TGF-beta Signaling
p53 Signaling
Focal adhesion signaling
04115
04510
05166
SNU-2563
SPIA two-way evidence plot
Tight junction signaling
04530
p53 Signaling
04115
05166
04510
Focal adhesion signaling
04810
b
DIC
DAPI
β-catenin
Merged
SNU-2550
SNU-2550CR
c
DIC
DAPI
β-catenin
Merged
SNU-2563
SNU-2563CR

Figure 12. Molecular analysis of crizotinib resistant sublines a, b. SPIA analysis indicating aberrated pathways of two crizotinib resistant sublines. c, d. Nuclear localization of beta-catenin is significantly increased in both crizotinib resistant sublines.

4. Discussion

Lung cancer is main cause of death related to cancer. However, diagnosis remained poor except for some specific mutation target-drugs.(3,17) There are various mutations accounting for development of lung cancer, and some cases are not explained with known causes. Lung cancer is the most lethal disease and 3rd common cancer in Korea.(2) Especially, our 28 lung cancer cell lines were originated from lung pleural effusion exudate not from tissue directly. Migrated cancer cells indicate that cancer cells in lung tissues break out into pleural cavity and lung tissues are no longer normal. Pleural effusion in patients with NSCLC (Non-small cell lung carcinoma) is known to have prognostic value.(5) Therefore, the mutational status of established lung cancer cell lines derived from pleural effusion exudate is accorded to the drug response of the cell lines.

NSCLC is about 85% of all lung cancer.(1) As mentioned earlier, mutations in epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) fusion with other domain is related to high probability of NSCLC (non-small-cell lung carcinoma) risk.(4,7,13) Variations of EGFR are well-known driver mutations. Overexpression of EGFR takes 6% of NSCLC and mutations in tyrosine kinase domain are occupying up to 15%.(4) EGFR exon19 deletion are also occupying about 10% in USA and 35% in Asia.(4,25,26) Because of high

proportion of EGFR mutations in NSCLC, detection of mutated EGFR genes and its protein expression level is examined in recent studies. In ERBB family, overexpression of ERBB2 also takes about 6% and mutations in tyrosine kinase domain take 5%.(27,28) Gene translocation of ALK that is frequently fused with EML4 is also well-known mutation of NSCLC. Those rearrangements occupy about 5% of total NSCLC causes. In addition, KRAS and BRAF also are more usual in smokers' cases and develop resistance tyrosine kinase inhibitors associated EGFR. In NSCLC, mutations of KRAS and BRAF occupy 5-15% and 2% each.(29-31)

Patients who have mutations mentioned above are treated with tyrosine kinase inhibitor such as Erlotinib and Gefitinib. Those patients are also treated with Crizotinib in case of harboring fusion gene like ALK rearrangement.(18,32,33) Crizotinib also targets proto-oncogene tyrosine-protein kinase ROS encoded by ROS1 gene and MET, so called hepatocyte growth factor receptor (HGFR) encoded by MET gene.(33,34) NSCLC have been found in harboring driver mutations continuously. Driver mutations in our cell lines were detected and validated. First of all, fusion gene detection was conducted with RT-PCR. It is occasional that NSCLC has Anaplastic lymphoma kinase (ALK) gene rearrangement with other gene.(35) Besides ALK fusion is tend to be found in all ages.(1) In 28 lung cancer cell lines, SNU-2535,

SNU-2550, SNU-2553, SNU-2563 and SNU-2637 have EML4-ALK fusion. EML4-ALK fusion is not to be found in lung tumors that have EGFR or KRAS mutation.(36) Actually SNU-2535, SNU-2550, SNU-2553, SNU-2563 and SNU-2637 don't have pathogenic mutation of EGFR or KRAS, and they have effectively increased sensitivity to Crizotinib, ALK target inhibitor according to drug test. Especially, SNU-2563 has ALK gene fusion with relatively infrequent EML4 18^{th} exon, and it is very sensitive to Crizotinib. In our cell lines, SNU-2535 has G1269A mutation in ALK. G1269A mutation located in ALK kinase region induce Crizotinib resistance.(37), and SNU-2535 was the most resistant to Crizotinib in cells harboring ALK rearrangement. Recently, G1269A mutation in ALK kinase domain is treated by specific target-drug, Alectinib.(38) ALK amplification are frequent in lung cancer cells with rearrangement of ALK genes, and they are sensitive to ALK inhibitors.(39) The mRNA level of ALK in SNU-2563, SNU-2589 and SNU-2637 were highly overexpressed. They were sensitive to ALK inhibitor despite SNU-2589 don't have EML4-ALK fusion. This may imply the amplified ALK expression is responsible for sensitivity of ALK inhibitors even without EML4-ALK fusion. SNU-2563 was the most sensitive to ALK inhibitor and other ALK overexpressed cell lines also have sensitive characteristics on Crizotinib. Other Crizotinib target, ROS1 is also receptor tyrosine kinase. ROS1

fusion have increased sensitivity to Crizotinib likewise EML4-ALK fusion.(1) In our cell lines, SNU-2606, SNU-2832 was found to have CD74-ROS1 fusion. Actually SNU-2606 and SNU-2832 were sensitive to Crizotinib. SNU-2553, SNU-2589, SNU-2606, SNU-2637 and SNU-2832 had high mRNA expression level in RT-PCR targeting the ROS1 kinase domain. Generally, CD74-ROS1 fusion doesn't co-exist with ALK fusion as well as pathogenic EGFR or KRAS mutation.(40) KIF5B-RET fusion is also well known gene rearrangement in NSCLC.(12) SNU-2612A and SNU-2778 have KIF5B-RET fusion gene. Relative to ALK fusions, KIF5B-RET fusion is less frequent. Although SNU-2612A was sensitive group to Gefitinib as well as Erlotinib, KIF5B-RET fusion generally has no distinguished characteristics about Erlotinib, Gefitinib and Crizotinb. Vandetanib is generally used to target KIF5B-RET fusion.(41) We suggest that SNU-2612A and SNU-2778 are sensitive to RET rearrangement targeting drug like Vandetanib.

EGFR mutations as well as fusion genes are important factors of lung cancer.(36) Representative mutational regions of EGFR are exon 18-20 regions in which EGFR kinase domain exists. Especially, EGFR exon 19 deletion as well as rearrangement of gene like ALK, ROS1 and RET are important characteristics of lung cancer.(42) Lung adenocarcinoma cell lines that have EGFR exon 19 deletion are generally sensitive to

Gefitinib and Erlotinib.(43) In our 28 lung adenocarcinoma cell lines, total 7 cells (SNU-2556, SNU-2643, SNU-2708.1, SNU-2741, SNU-2867, SNU-2887, and SNU-2964) had EGFR deletion in exon 19. SNU-2556, SNU-2887 and SNU-2964 that had EGFR deletion in E746-A750 weren't relatively sensitive in our cell lines. Interestingly, SNU-2643 had homo-deletion in EGFR exon 19, logEC_{50} of SNU-2643 was highly sensitive to Gefitinib and Erlotinib compared to other cell lines that have EGFR exon 19 hetero-deletion. Except for SNU-2708.1 that had slow growth rate, SNU-2741 that had deletion in L747-T751 weren't sensitive, neither. Though SNU-2741 had additional deletion at 753^{th} amino acid, it didn't present high sensitivity. Rather SNU-2741 was the most resistant cell lines to Gefitinib among 7 lung cancer cell lines that had EGFR exon19 deletion. SNU-2867 had L747-S752 deletion of amino acid as well as E746V substitution. 20 nucleotides were largely deleted. SNU-2867 that had these variation was the most sensitive to Gefitinib and highly sensitive to Erlotinib, too. SNU-2741 also had large deletions but was the most resistant cell lines to Gefitinib of 7 lung cancer cell lines. Genomic location of deleted sequences might be more important to drug response than the number of deletion amino acid. Our cell lines were tended to have resistance to TKI although there are relatively sensitive or resistant in our cell lines obtained from pleural effusion exudate. Except for EGFR T790M mutation, acquired

resistance mechanism about TKI is not well-known and these target drugs also are not verified. If cell lines that acquired resistance to TKI because of T790M mutation in EGFR, Osimertinib is recently treated.(44,45) Nevertheless, our cell lines that harbor EGFR deletion or mutation don't have T790M mutation in EGFR. Further study for various TKI resistance mechanism and overcoming resistance is suggested. The results for mutation analysis for driver mutations (BRAF, EGFR, ERBB2, FGFR4, KRAS and TP53) were paralleled to the drug-response data. Cells that have somatic mutations of EGFR c.2602T>G (p. L858R) variation are also generally sensitive to Gefitinib and Erlotinib. (25,43) SNU-2689, SNU-2727 and SNU-3023 had L858R mutation and they were sensitive to Gefitinib and Erlotinib except for SNU-2727. Even though SNU-2727 has EGFR L858R substitution, it was resistant to TKIs without T790M missense mutation. This may imply that SNU-2727 develop acquired resistance. SNU-3023 was sensitive to drug treated. In contrary to EGFR deletion or L858R mutation, cells that have KRAS 12^{th} amino acid Glycine variation decreased sensitivity to TKI.(29) SNU-2585 have G12V variation and SNU-2681 had G12C variation. In Erlotinib treatment, SNU-2585 was exceptionally sensitive. Of the remaining cell lines, there are no clear evidence to sensitivity targeting to TKI. Even if lung cancer cells that have resistance to EGFR inhibitors like Erlotinib sporadically harbor

BRAF mutations, and this is not shown in our cell lines.(30) SNU-2657 and SNU-2749 harbored c.1799T>A (p.V600E) variation, but there were no special patterns to TKI. Other interesting point in those cell lines was that they all had c.1963A>G (p. I655V) mutations in ERBB2 gene. I655V variation in ERBB2 is well-known as pathogenic driver mutation in breast cancer.(46,47) In our analysis for driver mutations, the cells that had I655V variation were genetically instable. Those cells had many other mutations like nonsense mutations in TP53 genes. I655V variation in ERBB2 might be used as lung cancer prognosis marker for Asian. Furthermore, we suggest that harboring V600E variation in BRAF might be marker for I655V mutation in Asian. G388R polymorphism in FGFR4 also could be potential indicator for clinical stage in Asian. In Italian lung adenocarcinoma cases, survival of patients with G388R allele significantly decreased.(48) G388R polymorphism in FGFR4 can expect prognosis in lung adenocarcinoma.(49) In fact, 12 cells (SNU-2535, SNU-2556, SNU-2588, SNU-2606, SNU-2627, SNU-2643, SNU-2657, SNU-2689, SNU-2741, SNU-2749, SNU-2778 and SNU-3023) of total 28 cells harbored G388R mutation in FGFR4. Half of our cell lines that were originated from pleural effusion had G388R mutation could indicate that G388R mutation in FGFR4 might be marking highly progressed stage in lung cancer with pleural effusion.

In protein expression level, overexpression or decreased expression of specific oncoproteins like ERK, ERBB2, c-MET and PTEN can affect to cancerous features. pERK1/2 is commonly activated in NSCLC and associated with progressive cancers.(50) pERK1/2 were highly expressed in SNU-2588, SNU-2657, SNU-2689, SNU-2689 and SNU-2708.1. In our cell lines, ERK1/2 was activated in about 70% cell lines. Such results support that ERK1/2 activation indicating progressive status is applied to Korean cases. Representative cancer repressor, PTEN expression level is important to understand characteristics of cell lines. PTEN loss or reduced PTEN expression level affect to TKI sensitivity.(51,52) PTEN expression of SNU-2689, SNU-2693 and SNU-2727 was reduced. Those cell lines were not sensitive to Erlotinib. SNU-2693 was resistant group but, these results didn't correspond with Gefitinib. SNU-2693 was sensitive to Gefitinib. Generally, increased copy number or overexpression of c-MET induce resistance to Gefitinib in non-small cell lung cancer.(53,54) Because Crizotinib also targets MET, cells that have increased expression of MET are generally sensitive to Crizotinib.(33) SNU-3023 had overexpressed c-MET level in our western blot results. SNU-3023 was sensitive to all drugs that were treated. About characteristics resisting against TKI, SNU-3023 was not matched. We assumed that these features were affected by floating growth pattern and EGFR c.2602T>G (p. L858R) variation of

SNU-3023.

Comparison of drug sensitivity with other cell lines according to mutational traits could be important because cancer cell lines in pleural effusion are relatively resistant compared with cell lines originated from lung cancer tissue. HCC827 had the most sensitive Erlotinib response compared with cells that have pleural effusion origin. Except for HCC827, SNU-2643 that has EGFR exon19 homo-deletion is most sensitive to Erlotinib among our cell lines. And NCI-H2228 cells had sensitive Crizotinib response compared with other cell lines that have EML4-ALK fusion except for SNU-2563 that have EML4 exon18-ALK exon20. Such considerable difference between pleural effusion origin and tissue origin could suggest that needs for other approach to treat of anti-cancer drugs in lung cancer. It might be significant that cancer cells from pleural effusion have progressive status and malignant features.(5,6)

We also established a crizotinib-resistant subline (SNU-2550CR and SNU-2563CR), which was derived from the parental SNU-2550 and SNU-2563 cell lines by long-term exposure to increasing concentrations of crizotinib. Characteristics associated with EMT including morphology, EMT marker proteins, and cellular mobility, were analyzed. Compared with parental cells, the growth of crizotinib-resistant subline cells was independent of EML4-ALK, and crizotinib-

resistant subline cells showed cross-resistance to both ceritinib and alectinib (a second-generation ALK inhibitor). An ALK inhibitor is the standard treatment for advanced non–small cell lung cancer (NSCLC) patients harboring the anaplastic lymphoma kinase (ALK) fusion gene. However, secondary ALK mutations or alternative pathway changes cause a fraction of the tumors to be resistant to ALK inhibitors (10,11). We validated that cell lines derived from a pleural effusion exudate had more mutations and were resistant to targeted drugs compared to cell lines originating from tumor tissue. To evaluate the molecular alterations when cell lines which do not respond to targeted drugs acquire more resistance, we established two crizotinib-resistant sublines (SNU-2550CR and SNU-2563CR), derived from the parental SNU-2550 and SNU-2563 cell lines by long-term exposure to increasing concentrations of crizotinib. Even though the SNU-2550 and SNU-2563 cell lines harbored an EML4-ALK fusion, they were more resistant to crizotinib compared to NCI-H2228. The acquired resistance was confirmed by crizotinib treatment at varying concentrations (Fig 7a, 7b). Aberrant focal adhesion signaling was found in both crizotinib-resistant sublines. Among the various genes involved in focal adhesion signaling, the mRNA expression of the Dickkopf-related protein 1 (DKK1) was significantly increased in both crizotinib-resistant sublines. DKK1 has been previously reported to promote the migration and invasion of non-

small cell lung cancer by inhibiting the phosphorylation of β-catenin (27,28). The elevated expression of DKK1 was confirmed by both qRT-PCR (Fig 7d) and Western blotting (Fig 7e). We further investigated the potential role of amplified DKK1 expression in crizotinib-resistance by immunocytochemistry (ICC). ICC indicated that the nuclear localization of β-catenin was significantly increased in both crizotinib-resistant cell lines (Fig 8b, 8c). Accordingly, these results indicate that the EMT-derived crizotinib resistance was accompanied by augmented DKK1 expression. Comprehensive molecular analysis revealed that a novel nonsense mutation in the PRSS1 gene was present in the crizotinib-resistant sublines and that the expression of DKK1 was significantly augmented in the crizotinib-resistant sublines.

In conclusion, we have established 28 NSCLC cell lines from a malignant pleural effusion and associated their mutational traits with targeted drug responses. The cell lines were relatively resistant compared to cell lines originating from other tissues exhibiting similar mutational features. We also established two crizotinib-resistant sublines from cell lines carrying an EML4-ALK fusion gene. Such analyses facilitate studies associated with drug treatment and representative mutations. Those results could mean that cancer cells in pleural effusion exudate might be group of harboring more malignant population than group of sustaining in tissue, and that could affect drug

sensitivity. We focused on that established cell lines gathered from pleural effusion is resistant to target drug and those cell's information should be considered.

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국문 초록

폐암은 흔한 종류의 암이나 진단 방법은 여전히 미비하다. 최근 서양에서는 폐암 발병률이 감소한 반면에 아시아에서는 폐암 발병률이 전반적으로 증가하고 있다. Caucasian type 에 비해 Asian type 의 돌연변이 패턴을 조사한 연구는 참고할 만한 적절한 자원이 부족하다. 특히 흉수에서 암세포를 얻어 수립된 폐암 세포주는 세포주 모델에서는 상대적으로 드문 경우이다. 비소 세포 폐암 환자의 흉수로 전이된 암세포는 그 진행이 빠르며 악성 종양의 경향을 보이는 경우가 많다. 본 연구에서는 28 개의 흉수 유래 폐암 세포주를 새로이 확립하여 세포 및 분자생물학적 특성을 분석 하였다. BRAF, EGFR, ERBB2, FGFR4, KRAS 및 TP53 의 대표적인 돌연변이 및 ALK, CD74 및 RET 유전자와 같은 fusion gene 이 검출되고 확인되었다. 세포주가 가진 대표적인 돌연변이에 따라 gefitinib, erlotinib and crizotinib 에 대한 약물 감수성을 측정 하였다. 각 약물에 표적 돌연변이를 갖는 세포주는 일반적으로 표적 약물에 민감하게 반응하였다. 흉수 유래의 세포주는 유사한 돌연변이가 있는 조직 유래의 세포주보다 상대적으로 내성이 있는 경향을 보였다. 이러한 세포주의 돌연변이 패턴 및 약물

감수성 분석은 여러 사례가 누적된 데이터베이스 구축을 돕는다. 또한 ERK, ERBB2, c-MET 및 PTEN 과 같은 특정 oncoprotein 의 발현 정도를 수립한 세포주에서 관찰하였다. 마찬가지로 EpCAM, E-cadherin, N-cadherin 및 Vimentin 과 같은 EMT marker 도 관찰하였다. 흉수에서 유래된 28 종의 폐암 세포주는 항암제 감수성 및 폐암에서 대표적인 돌연변이와 관련된 연구를 뒷받침하도록 돕는다. 현재까지도 crizotinib 에 대한 저항성에 대한 사례는 여전히 제한적이다. 이러한 요구를 충족시키기 위해서 우리는 crizotinib 내성 세포주를 확립했다. 그들의 유래 세포주는 EML4-ALK fusion gene 을 보유한 세포주 였으며 우리가 수립한 세포주들 안에서는 상대적으로 crizotinib 에 민감하였으나. 내성이 유도된 후 민감하게 반응하지 않게 되었다. 우리는 그들의 특성과 내성 기작에 대한 연구를 진행하였다.

주요어: 흉수 유래 폐암 세포주, 특성 분석, 약제 감수성, Crizotinib-resistance

학 번 : 2016-27013