의학박사 학위논문

비소세포폐암에서 EMT 관련 EGFR 표적치료 내성 극복을 위한 CDK7 억제제의 적용

The application of CDK7 inhibitors to overcome EMT-associated EGFR-TKIs resistance in non-small cell lung cancer

울산대학교대학원

의 학 과

지 원 준

비소세포폐암에서 EMT 관련 EGFR 표적치료 내성 극복을 위한 CDK7 억제제의 적용

지도교수 이재철, 최창민

이 논문을 의학박사 학위 논문으로 제출함

2020년 2월

울산대학교 대학원
의 학 과
지 원 준

지원준의 의학박사학위 논문을 인준함

심사위원 김형렬 (인)

심사위원 이재철 (인)

심사위원 장승훈 (인)

심사위원 최창민 (인)

심사위원 황대욱 (인)

울 산 대 학 교 대 학 원

2020 년 2 월

국문 요약

연구 목적: EMT (Epithelial to mesenchymal transition) 현상은 EGFR 표적치료제에 대한 내성과 관련 있는 기전으로 알려져 있다. 하지만 3 세대 EGFR 타이로신 키나제 억제제에 대한 내성 기전으로써 EMT 현상을 극복하기 위한 방안에 대한 자료는 매우 미흡한 상태이다. 본 연구에서는 비소페포폐암 세포주에서 EMT 현상과 관련한 3 세대 EGFR 타이로신 키나제 억제제 내성 극복을 위한 CDK7 (Cyclin dependent kinase 7) 억제제의 효과에 대하여 확인해 보고자 하였다.

연구재료와 연구방법: 본 연구를 위하여 H1975 세포주를 활용하여 Osimertinib 과 WZ4002 에 대한 EGFR 표적치료제 내성 세포주를 구축하였다. EMT 발현 여부를 확인하기 위한 형태학적 변화를 확인하고, 침습/이동성 분석 및 면역탁본검사를 시행하였다. 또한 EMT 로 인한 유전적 변화를 확인하기 위하여 RNA 분석을 시행하였으며, CDK7 억제제로는 THZ1 와 QS1189 를 사용하였다.

연구결과: 실험을 통하여 구축된 H1975 내성 세포주는 형태학적으로 방추형 모양 및 위족 형성을 포함한 EMT 변화가 관찰되었다. 면역탁본검사에서 E-cadherin 의 감소와 Vimentin 의 증가가 관찰되었으며, RNA 분석에서 EPCAM 과 CDH1 의 감소가

확인되었다. 침습 및 이동성 분석에서도 현저히 증가된 침습성과 이동성을 확인할 수 있었다. 이러한 EMT 변화가 나타난 내성 세포주는 모세포보다 THZ1 에 보다 민감한 약제 반응을 보여주었다. 이러한 현상은 TGF-β 에 의하여 유도된 EMT 세포주에서도 유사하게 관찰되었으며, HCC827 과 PC9 세포주를 이용하여 구축한 gefinitib 내성 세포주에서도 같은 현상을 확인하였다.

결론: EMT 현상은 EGFR 타이로신 키나제 억제제에 대한 약제 감수성 감소와 관련이 있는 것으로 보이며, 이러한 EMT 에 의하여 생성된 내성세포주는 CDK7 억제제에 보다 높은 민감도를 보여주었다. 이러한 결과는 비소세포폐암에서 EMT 관련 3 세대 EGFR 표적치료제 내성을 극복하기 위한 전략으로서 CDK7 억제제의 활용 가능성을 시사한다.

중심단어: 비소세포폐암, EGFR 타이로신 키나제 억제제 획득 내성, EMT, CDK7 억제제

Index

Tables and Figure Legends

Introduction

Lung cancer is the leading cause of cancer mortalities globally[1, 2]. Resistance to lung cancer therapies contributes to higher death rates in these patients. A targeted therapy utilizing an Epidermal Growth Factor Receptor (EGFR) mutation improved the survival rate of patients until resistance began to develop[3]. First-generation EGFR-tyrosine kinases (TKIs) therapy exhibits efficacy for a short time (<2 years); however, inevitably, resistance gradually develops, leading to disease progression[4]. Various resistance mechanisms for EGFR-TKIs have been reported in previous research, including T790M point mutations, EGFR amplification, ERBB2 amplification, small cell lung cancer transformation, MET amplification, PI3K mutation, epithelial mesenchymal transition (EMT), BRAF mutation, and KRAS mutation[4-7]. The T790 mutation is considered the most prevalent type (40~50% of cases) of resistance to the first-generation EGFR-TKIs. Recently, a third generation drug osimertinib has been developed to overcome T790M associated resistance[8]. However, most of the mechanisms involved in third-generation EGFR-TKI resistance have not yet been

elucidated. As such, various attempts to overcome acquired resistance to 3rd generation EGFR inhibitors are critical for improving the prognosis of patients.

EMT is a biological process via which cells undergo a switch from the polarized epithelial phenotype to the mesenchymal fibroblastoid phenotype and considered to be one of the resistance mechanisms. EMT is involved in several diverse processes, including embryonic development, chronic inflammation, fibrosis[9, 10], tumorigenesis, invasion, metastasis and drug resistance[11-13]. As a result of EMT, cells downregulate the expression of epithelial proteins containing E-cadherin and upregulate the expression of mesenchymal proteins, including vimentin. In addition, cells undergoing EMT are characterized by the loss of apico-basal polarity and intact cell-cell junctions followed by the acquisition of front-rear polarity and morphologic change to a spindle shape with remodeling of the cytoskeleton[14]. This EMT program enhances the invasive capacity, therapeutic resistance, and cancer stem-cell-like properties[13]. EMT has also been reported as one of the mechanisms of EGFR-TKI resistance[7]. However, studies to overcome EMT related EGFR-TKI resistance are

still insufficient.

Cyclin-dependent kinases (CDKs) are a family of serine-threonine kinases that play an important role in cell cycle progression. Over 90% of tumors have been found to be upregulated in the CDKs due to changes in the expression and genetic variation of CDKIs. Based on this, CDKs inhibitors are likely to be developed as anticancer agents that inhibit the growth of various cancers. In particular, CDK7 acts as a master regulator of transcription and is known to modulate RNA polymerase II activity[15]. Recently, CDK7 inhibitors have been reported to inhibit abnormal cell growth associated with hematologic malignancy[16], breast[17], esophageal[18], and small cell lung cancer[19]. Specifically, small cell lung cancer studies have shown that the action of THZ1 significantly reduces the activity of super-enhancers and associated oncogene transcription factors[19], which suggests that CDK inhibitors could potentially be used as anticancer agents.

This study evaluated whether EMT was expressed in the resistant cell lines generated by treatment with 3rd generation EGFR-TKIs, and analyzes the effect of CDK7

inhibitors on EMT-associated resistance cells to evaluate a potential therapeutic strategy to overcome EMT related EGFR-TKI resistance.

Materials and Methods

2.1. Cell culture and reagents

The H1975 and HCC827 cell lines were obtained from the American Type Culture Collection (Rockville, MD), and the PC-9 cell line was provided by Dr. Kazuto Nishio (National Cancer Center Hospital, Tokyo, Japan). Cells were cultured in 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere with 5% CO_2. Osimertinib, WZ4002, and THZ1 were purchased from Selleck Chemicals (Houston, TX). The 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and TGF-β1 was purchased from Sigma (St. Louis, MO) and R&D Systems (Minneapolis, MN), respectively. QS1189 was kindly provided by Orient Bio (Seongnam, Korea).

2.2. Establishment of a 3rd generation EGFR-TKI resistant cell line

H1975/WR and H1975/OR cells were subjected to repeated exposure to WZ4002 or

osimertinib, as reported in the previous studies[20]. In all studies, resistant cells were then cultured in a drug-free medium for >1 week to eliminate the effects of each drug. The resistant cell lines were authenticated using STR analysis and confirmed to be mycoplasma free using standard methods.

2.3. Immunoblot analysis

Whole-cell lysates were prepared using EBC lysis buffer (50 mM Tris-HCl [pH, 8.0], 120 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, and 5 U/mL aprotinin) and then centrifuged. The resultant supernatant (20 μg) was separated on an 8% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Invitrogen). The membranes were probed with antibodies against p-EGFR (Tyr1173), p-Erk (Thr202/Tyr204), Erk, Akt, E-cadherin, β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), p-Akt (Ser473), β-catenin, EpCAM,

Desmoplakin, cytokeratin-8/18, (all from Cell Signaling Technology, Beverly, MA), and vimentin was used as the first antibody (Cell Signaling, Beverly, MA). Then the membranes were treated with horseradish peroxidase-conjugated secondary antibody. All membranes were developed using ECL kits (PerkinElmer, Waltham, Mass).

2.4. Invasion and Migration assays

The cell migration and invasion assays were done using Transwell (6.5 mm diameter, 8 mm pore size polycarbonate membrane), which was obtained from Corning (Cambridge, MA). The cells (1×10^5) in 200 μL medium were placed in the upper chamber, and the lower chamber was filled with 1 mL of serum-free media supplemented with 0.1% bovine serum albumin. Following incubation for 24 h, the cells that migrated to the lower surface of the filters were stained with a Diff-Quick kit (Fisher Scientific, Pittsburgh, PA), and then they were counted under a microscope. The migration assay was conducted using the same procedure with filters that were coated with extracellular matrix on the upper surface (BD

Biosciences, Bedford, MA). The mean represents the results in triplicate (standard deviation).

2.5. RNA sequencing

Each cell was pelleted, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was eliminated, and RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA Sequencing was performed by a specialized company (Macrogen, Seoul, Korea) using the TruSeq RNA Sample Prep Kit v2 and HiSeq. 2500 platform (Illumina, San Diego, CA, USA). Sequenced reads were trimmed using TrimMomatic 0.32. The trimmed reads were mapped to the hg19 reference genomes using HISAT (version 2.0.5) and Bowtie2. Expression levels were measured as fragments per kilobase for map reads (transcript per million) using StringTie (version 1.3.3b).

2.6. Cellular viability assays

Cells (1×10^4) were seeded into 96-well sterile plastic plates overnight and then treated with the relevant drugs. Following 72 h, 15 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 h. Crystalline formazan was solubilized in 100 μL of 10% (w/v) SDS solution for 24 h. Absorbance at 595 nm was read spectrophotometrically using a microplate reader. Cell counting was determined using an ADAM-MC automatic cell counter (NanoEnTek, Seoul, Korea) according to the manufacturer's instructions. The results represent at least three independent experiments, and the error bars signified the standard deviation from the mean. The IC_{50} values were determined using GraphPad Prism software (GraphPad, Inc., La Jolla, CA).

2.7. Statistics

Data are presented as the mean ± standard deviation. *P* values ≤ 0.05 were determined using unpaired or paired t-tests between groups using the GraphPad Prism software.

Results

3.1. The induction of EMT in acquired resistance to 3rd-generation EGFR-TKIs

To investigate the mechanisms of acquired resistance to 3rd-generation EGFR-TKIs, we established H1975/WR and H1975/OR through stepwise selection in WZ4002 or osimertinib, as described in previous studies[20]. Both types of cells exhibited resistance more than 10 times to each drug compared with the parent cells (WZ4002 IC_{50} = 106.9 nM in H1975 and 1494 nM in H1975/WR, osimertinib IC_{50} = 39.9 nM in H1975 and 1352 in H1975/OR; Figure 1 and Table 1). These resistant cells exhibited an increase in spindle-shaped cells that were similar to the spindle-shaped cells produced by EMT (Figure 2).

3.2. Results of immunoblot analysis used to evaluate EMT in the acquired resistance to 3rd-generation EGFR-TKIs

In order to evaluate the induction of EMT in both of the resistant cells, we analyzed the expression of marker proteins for epithelial or mesenchymal phenotypes by using

western blotting (Figure 3). Compared with the H1975 cells, epithelial marker proteins containing E-cadherin, β-catenin, EpCAM, desmoplakin, and cytokeratin-8/18 were significantly reduced in both of the resistant cells, whereas vimentin expression was increased. Moreover, the expression and activity of EGFR were reduced in both of the resistant cells; while the activity of Akt was significantly upregulated.

3.3 Results of Invasion and Migration assays of EMT associated acquired resistance cell lines to 3rd-generation EGFR-TKIs

We investigated the ability of migration and invasion, which are considered functional hallmarks of EMT. We found that the abilities of migration and invasion were significantly enhanced in both of the resistant cells (Figure 4). Thus, these data suggested that the acquisition of resistance to 3rd-generation EGFR-TKIs induced molecular changes that were consistent with EMT.

3.4. Results of RNA sequencing for the evaluation of genetic changes attributed to EMT during acquired resistance to 3rd-generation EGFR-TKIs

To determine the genetic changes caused by EMT associated markers in the resistant cell lines, we performed RNA sequencing. The top 15 genes with increasing or decreasing patterns at the RNA level are shown in Table 2. EPCAM and CDH1 were significantly reduced, and this suggested that EMT was induced at the genetic level.

3.5. Efficacy of CDK7 inhibitors on the EMT acquired resistance cell lines for the 3rd-generation EGFR-TKIs

Previous studies showed that CDK7 was associated with EMT; however, it was controversial whether targeting CDK7 could overcome EMT [21-24]. Therefore, to determine the effect of CDK7 inhibition in both types of resistant cells, we used THZ1 and QS1189 as CDK7 inhibitors. QS1189 was developed as a novel CDK7 inhibitor in a previous study[16]. As shown in Figure 5 and Table 3, both resistant cells were more sensitive to CDK7 inhibitors than the parental cells (THZ1 IC_{50} = 379 nM

in H1975, 83.4 nM in H1975/WR, 125.9 nM in H1975/OR; QS1189 IC_{50} = 755.3 nM in H1975, 232.8 nM in H1975/WR, 275.3 nM in H1975/OR). Next, CDK7 kinase activity has been involved in phosphorylation of the CTD of RNAPII, which played a role in transcription initiation and RNAPII procession[15, 25, 26]. We performed western blotting following treatment of THZ1 (Figure 6) to evaluate the inhibitory effect of CDK7 substrate in the parental and resistant cells,. The inhibitory effect of THZ1 on the activity of RNAPII-CTD was similar for H1975 and H1975/OR cells. However, H1975/WR cells showed the inhibition of RNAPII-CTD phosphorylation at Ser2, Ser5, and Ser7 at a lower concentration of THZ1. In addition, THZ1 treatment failed to inhibit the activity of EGFR and Akt, while dose-dependent induction activity was associated with Erk.

3.6. Efficacy of CDK7 inhibitor on the TGF-ß induced EMT cell lines

To determine whether induction of EMT could affect sensitivity to the CDK7 inhibitor, we assessed the response of THZ1 to TGF-β1-induced EMT. When cells

were treated with TGF-β1 for 24 h, cells with mutant EGFR were scattered with an observed loss of E-cadherin and increased vimentin. The sensitivity to THZ1 was increased in cells with TGF-β1-induced EMT (Figure 7). In addition, TGF-β1 treatment did not affect the inhibition of RNAPII-CTD phosphorylation by THZ1. Therefore, the induction of EMT enhanced sensitivity to the CDK7 inhibitor.

3.7. Efficacy of CDK7 inhibitor on the TGF-ß induced EMT using T790M naïve cell lines

Following induction of EMT using TGF-ß in T790M negative cell lines (HCC827 and PC-9), the effects of CDK inhibitors were evaluated. The sensitivity to THZ1 increased in the cells with TGF-β1-induced EMT, while the sensitivity to osimertinib had decreased (Figure 8). This pattern was consistent with the experimental results for the efficacy of CDK7 inhibitors on EMT associated resistant cell lines created with 3rd-generation EGFR-TKIs.

Discussion

We established two 3rd generation EGFR-TKI resistant cell lines (H1975/WR, H1975/OR) via repeated exposure to Osimertinib and WZ4002. The resistant cells showed phenotypical changes similar to EMT, including a spindle-cell shape and increased pseudopodia formation. Decreased E-cadherin and increased vimentin were observed in the immunoblot assay, and decreased EPCAM and CDH1 were found during RNA sequencing. The abilities of invasion and migration were increased in the resistant cells. The EMT related resistance cells showed higher sensitivity to THZ1 than the mother cells. This phenomenon was observed in the TGF-ß induced EMT cell lines and the T790M negative resistant cell lines (HCC827, PC-9).

EMT is associated with poor prognosis for non-small cell lung cancer patients [27) 28)], and previous studies have shown that the mesenchymal phenotype was more resistant to the 1st generation EGFR-TKI treatment than the epithelial phenotype[29-31)].

Previous studies have revealed that EMT was a potential mechanism involved in

first-generation EGFR-TKI resistance. Moreover, there have been a few reports of EMT related third-generation EGFR-TKI resistance[32]. Li X et al. reported that C797S was the most common cause of third-generation EGFR-TKI resistance, but EMT is also a possible cause of acquired resistance[33]. This study found that EMT was related to acquired resistance caused by 3rd generation EGFR-TKI. These phenomena were confirmed by (a) the alteration of morphology such as formation of spindle-shaped cells and pseudopodia, (b) changes in the molecular marker proteins loss of E-cadherin and gain of vimentin, (c) increased invasive and migratory ability, and (d) decreased RNA levels which were related to the epithelial markers.

Previous studies have reported that continuous exposure of tumor cells to TGF-ß induced EMT through the SMAD and MAPK pathways[11, 34]. Therefore, to determine whether EMT led to increased sensitivity to the CDK7 inhibitors apart from EGFR-TKI exposure, we established TGF-ß induced EMT cell lines and then measured the response to the CKD7 inhibitors and EGFR-TKIs. In the current study, we found that drug sensitivity for EGFR-TKIs decreased, while sensitivity to CDK7 inhibitors

increased for the TGF-induced EMT cells. These data suggested that EMT might be a potential mechanism of acquired resistance to the 3rd generation EGFR-TKIs in non-small cell lung cancer.

An interesting part of this study was that the drug sensitivity to CDK7 inhibitors was higher in the EMT associated EGFR-TKI resistant cell lines than in the parental cells. Cyclin-dependent kinase (CDK)7 is necessary for transcription and acts by phosphorylating the C-terminal domain (CTD) for RNA polymerase II (PolII), which enables transcription initiation. Increases in the transcription factors have been reported for various carcinomas, and there were a number of studies that reported pharmacological modulation of the CDK7 kinase activity, which might provide potential anti-cancer activities [17, 18, 35-37]. In this study, we found that treatment using CDK7 inhibitors inhibited the RNA polymerase II activity in EMT associated EGFR-TKI resistance cells. This suggested that EMT induced resistant cells might have transcriptional addiction, which increased the sensitivity of the CDK7 inhibitors. These results suggested a potential utility of CDK7 inhibitors as a strategy to

overcome 3rd generation EGFR-TKI resistance.

A few limitations of this study should be taken into consideration. Firstly, while our study found that EMT was correlated with acquired resistance to EGFR-TKI, the potential mechanism involved is not yet clear. Various extracellular signal factor stimuli and the activation of corresponding intracellular signaling pathways ultimately resulted in the downregulation of E-cadherin, which is considered as the hallmark of EMT[14]. Further research is required to identify the specific mechanisms of action involved. Secondly, it is still unclear what proportion of EGFR-TKI resistance was associated with EMT expression in the patients who used 3rd generation EGFR-TKIs. In a previous study on acquired resistance mechanisms to 1st generation EGFR-TKIs, EMT associated drug resistance was reported to be about 1-2%[4]. However, the proportion of resistance mechanisms by EMT expression is unclear when 3rd generation EGFR-TKI were used. This could be evaluated by using patient samples obtained in clinical practice settings. Thirdly, specific mechanisms could not be confirmed as to whether EMT induced EGFR-TKI resistant cell lines

produced higher sensitivity to the CDK7 inhibitors. Our results suggested that EMT induced resistant cells showed higher transcriptional activity and might affect increased sensitivity to CDK7 inhibitors; further research is required to elucidate this mechanism.

Conclusions

In conclusion, EMT was associated with decreased drug sensitivity to EGFR-KTI, and EMT related resistance cells showed increased sensitivity to the CDK7 inhibitors. This suggested that CKD7 inhibitors could potentially be used as a therapeutic strategy to overcome EMT associated EGFR-TKI resistance in NSCLC.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: a cancer journal for clinicians. 2018;68(1):7-30.

2. Jung KW, Won YJ, Kong HJ, Lee ES. Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2015. Cancer research and treatment : official journal of Korean Cancer Association. 2018;50(2):303-16.

3. Greenhalgh J, Dwan K, Boland A, Bates V, Vecchio F, Dundar Y, et al. First-line treatment of advanced epidermal growth factor receptor (EGFR) mutation positive non-squamous non-small cell lung cancer. Cochrane Database Syst Rev. 2016. doi: 10.1002/14651858.CD010383.pub2.(5):Cd010383.

4. Westover D, Zugazagoitia J, Cho BC, Lovly CM, Paz-Ares L. Mechanisms of acquired resistance to first- and second-generation EGFR tyrosine kinase inhibitors. Ann Oncol. 2018;29(suppl_1):i10-i9.

5. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al.

Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res. 2013;19(8):2240-7.

6. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med. 2011;3(75):75ra26.

7. Zhu X, Chen L, Liu L, Niu X. EMT-Mediated Acquired EGFR-TKI Resistance in NSCLC: Mechanisms and Strategies. Front Oncol. 2019;9:1044.

8. Lamb YN, Scott LJ. Osimertinib: A Review in T790M-Positive Advanced Non-Small Cell Lung Cancer. Target Oncol. 2017;12(4):555-62.

9. Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol. 2003;4(8):657-65.

10. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003;112(12):1776-84.

11. Buonato JM, Lazzara MJ. ERK1/2 blockade prevents epithelial-mesenchymal transition in lung cancer cells and promotes their sensitivity to EGFR inhibition. Cancer research. 2014;74(1):309-19.

12. Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol. 2017;14(10):611-29.

13. Ye X, Weinberg RA. Epithelial-Mesenchymal Plasticity: A Central Regulator of Cancer Progression. Trends Cell Biol. 2015;25(11):675-86.

14. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178-96.

15. Glover-Cutter K, Larochelle S, Erickson B, Zhang C, Shokat K, Fisher RP, et al. TFIIH-associated Cdk7 kinase functions in phosphorylation of C-terminal domain Ser7 residues, promoter-proximal pausing, and termination by RNA polymerase II. Mol Cell Biol. 2009;29(20):5455-64.

16. Choi YJ, Kim DH, Yoon DH, Suh C, Choi CM, Lee JC, et al. Efficacy of the novel CDK7 inhibitor QS1189 in mantle cell lymphoma. Sci Rep.

2019;9(1):7193.

17. Li B, Ni Chonghaile T, Fan Y, Madden SF, Klinger R, O'Connor AE, et al. Therapeutic Rationale to Target Highly Expressed CDK7 Conferring Poor Outcomes in Triple-Negative Breast Cancer. Cancer research. 2017;77(14):3834-45.

18. Jiang YY, Lin DC, Mayakonda A, Hazawa M, Ding LW, Chien WW, et al. Targeting super-enhancer-associated oncogenes in oesophageal squamous cell carcinoma. Gut. 2017;66(8):1358-68.

19. Christensen CL, Kwiatkowski N, Abraham BJ, Carretero J, Al-Shahrour F, Zhang T, et al. Targeting transcriptional addictions in small cell lung cancer with a covalent CDK7 inhibitor. Cancer cell. 2014;26(6):909-22.

20. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na, II, Yang SH, et al. Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. Lung cancer (Amsterdam, Netherlands). 2009;63(2):219-26.

21. Zhou Y, Lu L, Jiang G, Chen Z, Li J, An P, et al. Targeting CDK7 increases the stability of Snail to promote the dissemination of colorectal cancer. Cell Death Differ. 2019;26(8):1442-52.

22. Ning J, Ma X, Long C, Mao Y, Kuang X, Huang Z, et al. Anti-tumor Drug THZ1 Suppresses TGFbeta2-mediated EMT in Lens Epithelial Cells via Notch and TGFbeta/Smad Signaling Pathway. J Cancer. 2019;10(16):3778-88.

23. Zhang J, Zhu J, Yang L, Guan C, Ni R, Wang Y, et al. Interaction with CCNH/CDK7 facilitates CtBP2 promoting esophageal squamous cell carcinoma (ESCC) metastasis via upregulating epithelial-mesenchymal transition (EMT) progression. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015;36(9):6701-14.

24. Wang Y, Liu F, Mao F, Hang Q, Huang X, He S, et al. Interaction with cyclin H/cyclin-dependent kinase 7 (CCNH/CDK7) stabilizes C-terminal binding protein 2 (CtBP2) and promotes cancer cell migration. The Journal of

biological chemistry. 2013;288(13):9028-34.

25. Akhtar MS, Heidemann M, Tietjen JR, Zhang DW, Chapman RD, Eick D, et al. TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. Molecular cell. 2009;34(3):387-93.

26. Roy R, Adamczewski JP, Seroz T, Vermeulen W, Tassan JP, Schaeffer L, et al. The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell. 1994;79(6):1093-101.

27. Bremnes RM, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, et al. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. J Clin Oncol. 2002;20(10):2417-28.

28. Deeb G, Wang J, Ramnath N, Slocum HK, Wiseman S, Beck A, et al. Altered E-cadherin and epidermal growth factor receptor expressions are associated with patient survival in lung cancer: a study utilizing high-density tissue microarray and immunohistochemistry. Mod Pathol. 2004;17(4):430-9.

29. Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N, et al. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer research. 2005;65(20):9455-62.

30. Yauch RL, Januario T, Eberhard DA, Cavet G, Zhu W, Fu L, et al. Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients. Clin Cancer Res. 2005;11(24 Pt 1):8686-98.

31. Weng CH, Chen LY, Lin YC, Shih JY, Lin YC, Tseng RY, et al. Epithelial-mesenchymal transition (EMT) beyond EGFR mutations per se is a common mechanism for acquired resistance to EGFR TKI. Oncogene. 2019;38(4):455-68.

32. Uchibori K, Inase N, Nishio M, Fujita N, Katayama R. Identification of Mutation Accumulation as Resistance Mechanism Emerging in First-Line Osimertinib Treatment. J Thorac Oncol. 2018;13(7):915-25.

33. Le X, Puri S, Negrao MV, Nilsson MB, Robichaux J, Boyle T, et al. Landscape of EGFR-Dependent and -Independent Resistance Mechanisms to Osimertinib and Continuation Therapy Beyond Progression in EGFR-Mutant NSCLC. Clin Cancer Res. 2018;24(24):6195-203.

34. Yoshida T, Song L, Bai Y, Kinose F, Li J, Ohaegbulam KC, et al. ZEB1 Mediates Acquired Resistance to the Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors in Non-Small Cell Lung Cancer. PLoS One. 2016;11(1):e0147344.

35. Kwiatkowski N, Zhang T, Rahl PB, Abraham BJ, Reddy J, Ficarro SB, et al. Targeting transcription regulation in cancer with a covalent CDK7 inhibitor. Nature. 2014;511(7511):616-20.

36. Augert A, MacPherson D. Treating transcriptional addiction in small cell lung cancer. Cancer cell. 2014;26(6):783-4.

37. Ali S, Heathcote DA, Kroll SH, Jogalekar AS, Scheiper B, Patel H, et al. The development of a selective cyclin-dependent kinase inhibitor that shows

antitumor activity. Cancer research. 2009;69(15):6208-15.

Tables

Table 1. Results of drug sensitivity to the 3rd generation EGFR-TKIs

Drug	IC50 values (nM, mean ± SD)	
	H1975	**H1975/R**
WZ4002	106.9 (± 127.9)	1494 (± 1102)
Osimertinib	39.9 (± 68.4)	1352 (± 233.2)

Table 2. Top 15 up & down regulated genes in the EMT induced EGFR-TKI resistance cell lines

A) H1975/WR

Up-regulated genes		Down-regulated genes	
Gene Symbol	**Fold change**	**Gene Symbol**	**Fold change**
TAGLN	388.8	KRT19	-244.4
CCL2	227.7	UCA1	-168.0
IL8	95.7	SPINT2	-110.3
IL32	94.0	EPCAM	-94.5
TPM2	66.5	MAL2	-85.7
CNN1	65.3	MT1A	-81.4
WBP5	63.5	IGFBP2	-80.0
S100A4	51.7	CD74	-74.8
C3	47.8	PROM2	-57.9
PTX3	43.2	CDH1	-50.2
FLNC	40.5	ST14	-45.6
LOC645638	37.9	ACP5	-38.9
MLLT11	37.4	PHLDA3	-38.2
ANKRD1	35.3	FHL2	-37.9
NNMT	32.8	MLPH	-37.6

B) H1975/AR

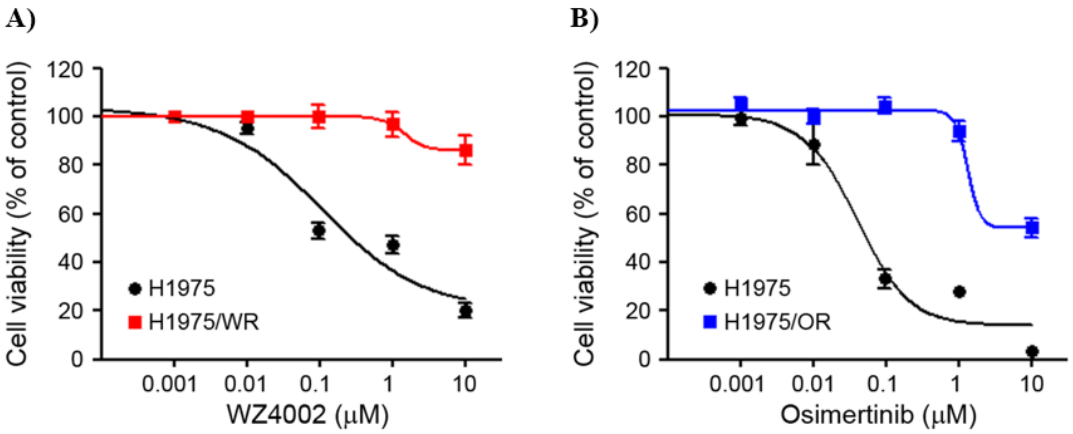
Up-regulated genes		Down-regulated genes	
Gene Symbol	**Fold change**	**Gene Symbol**	**Fold change**
TAGLN	125.0	EPCAM	-260.1
NNMT	71.6	UCA1	-194.5
GNG11	70.5	CLDN4	-134.5
C3	63.3	SPINT2	-109.2
COL12A1	56.2	MAL2	-90.0
ACTG2	53.5	CDH3	-83.0
CCL2	40.0	CD74	-77.5
PLAC8	37.7	CLDN7	-75.5
LARP6	37.4	GPNMB	-62.0
TPM2	36.2	CDH1	-61.1
QPRT	26.6	PHLDA3	-50.4
IGFBP7	25.1	IGFBP2	-47.3
ZEB1	22.4	ST14	-46.6
ACTA2	21.5	F11R	-45.6
MYL9	21.0	ACP5	-44.4

Table 3. Results of drug sensitivity to CDK7 inhibitors in the EMT related EGFR-TKI resistance cell lines

Drug	IC50 values (nM, mean ± SD)		
	H1975	**H1975/WR**	**H1975/OR**
THZ1	379.8 (± 135.3)	83.4 (± 13.2)	125.9 (± 20.1)
OS1189	755.3 (± 55.8)	232.8 (± 37.1)	275.3 (± 41.2)

Figures

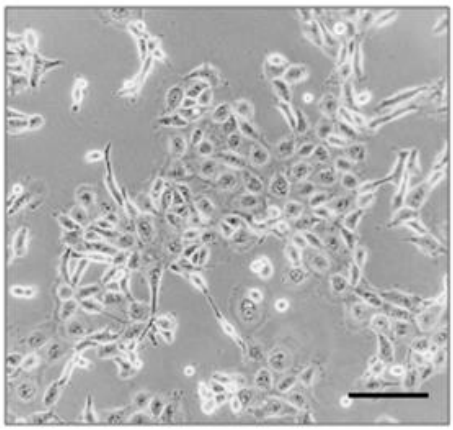
Figure 1. Establishment of the 3rd generation EGFR-TKI resistance cell line



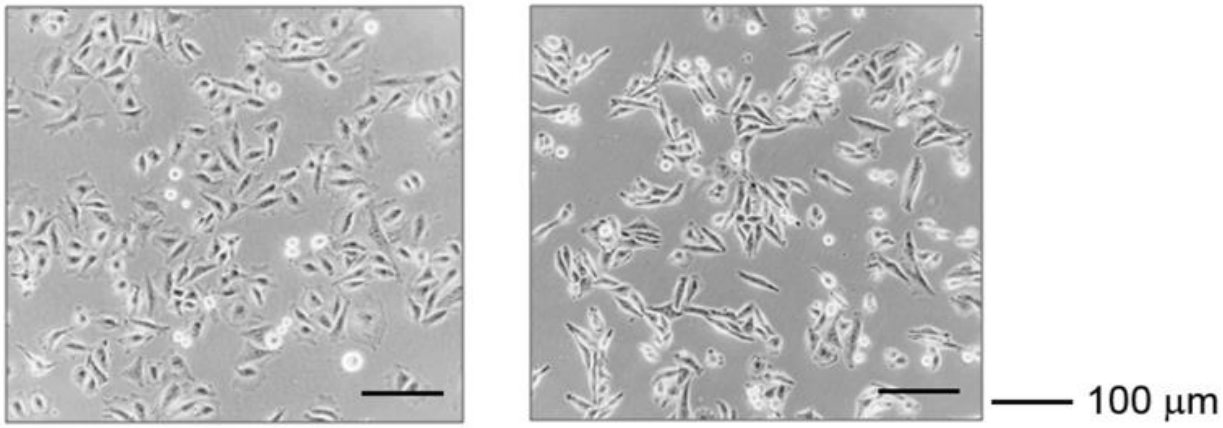
Induction of EMT in cells with acquired resistance to WZ4002 or osimertinib. Cells were treated with the indicated doses of WZ4002 or osimertinib for 72 h, and cell viability was determined by the MTT assay. IC_{50} values were calculated with GraphPad software through three independent experiments. Both resistant cells exhibited the resistance more than 10 times to each drug compared with parent cells. A) WZ4002 IC_{50} = 106.9 nM in H1975 and 1494 nM in H1975/WR, B) osimertinib IC_{50} = 39.9 nM in H1975 and 1352 in H1975/OR.

Figure 2. Phenotypic changes between the mother cell and the EMT induced EGFR-TKI resistance cell lines

A) H1975

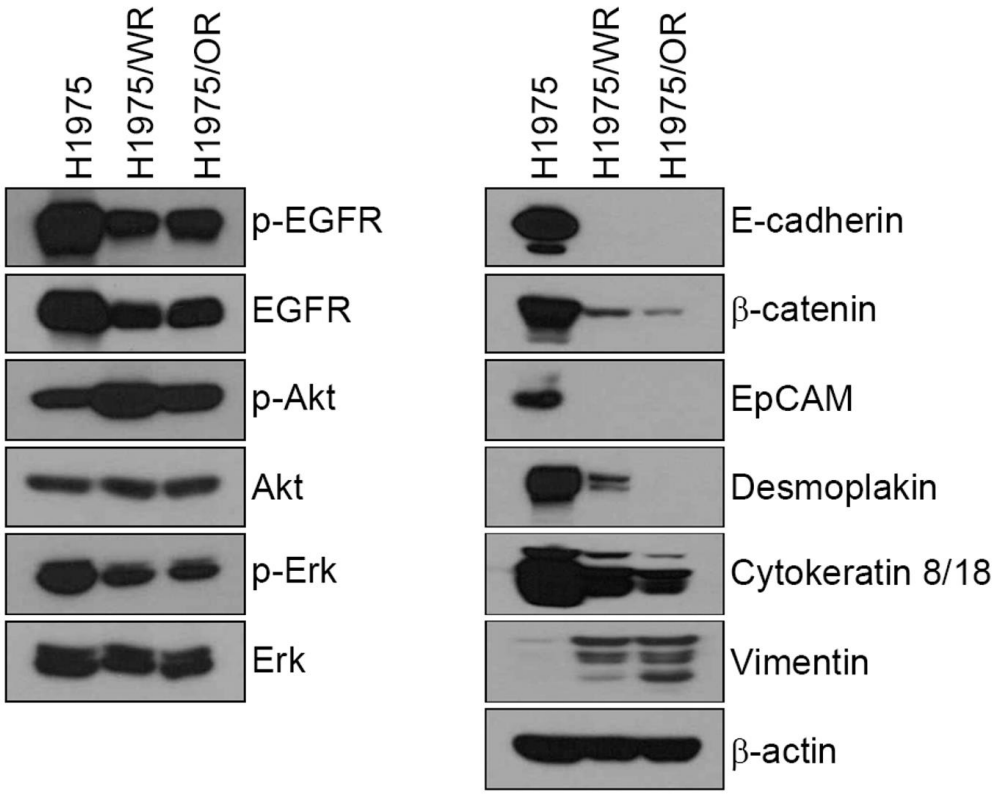


B) H1975/WR **C) H1975/OR**



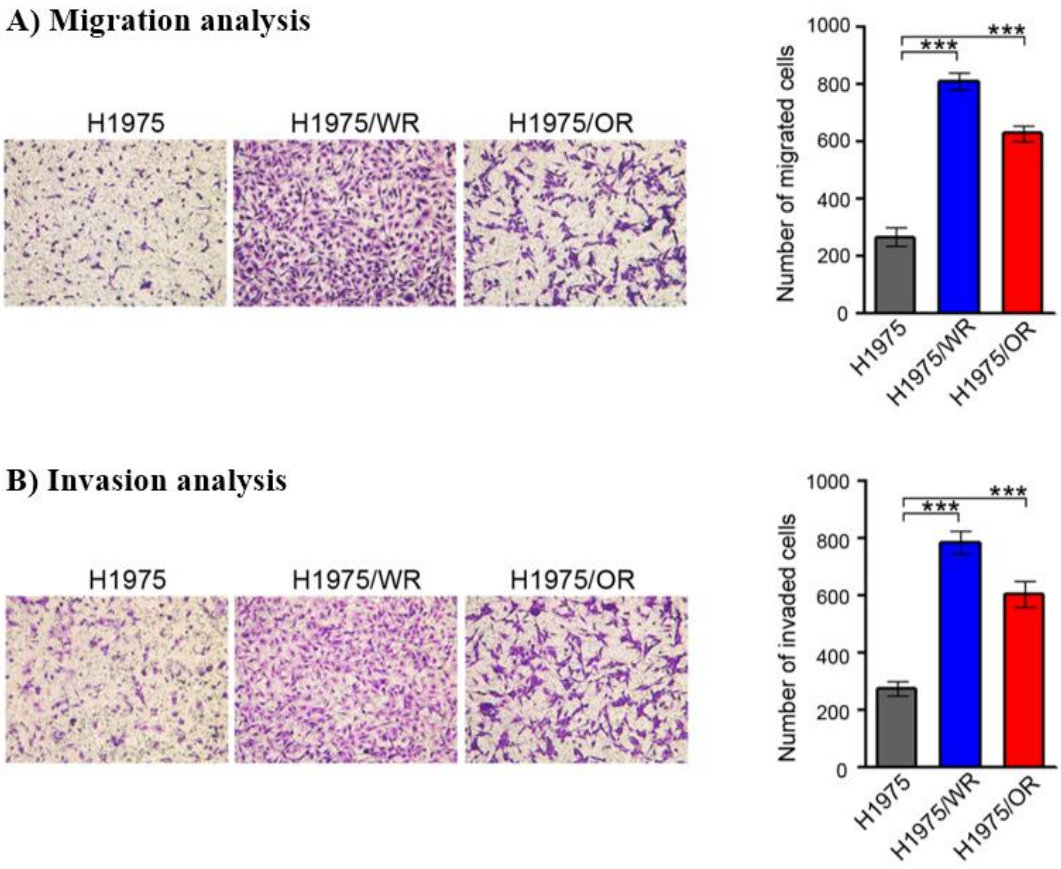
H1975 parental cells and both resistant cells (H1975/WR and H1975/OR) were evaluated for morphologic changes that were consistent with EMT using a light microscope. B), C) The resistant cells showed the increase of spindle-shaped cells that was like to EMT-like changes.

Figure 3. Immunoblot analysis of EMT expression



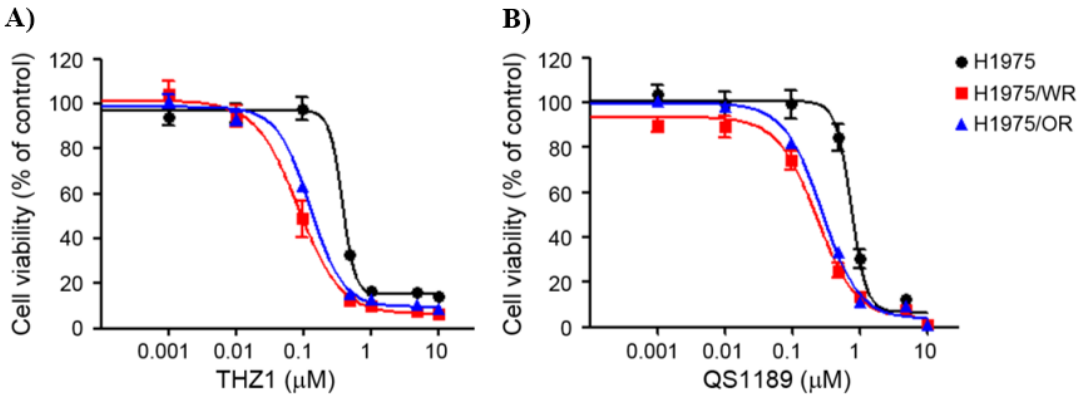
EGFR signaling and EMT-related molecules were analyzed by Western blotting. Epithelial marker proteins containing E-cadherin, β-catenin, EpCAM, desmoplakin and cytokeratin-8/18 were significantly reduced in both resistant cells (H1975/WR and H1975/OR), whereas vimentin expression was increased.

Figure 4. Invasion and Migration analysis on the EMT related EGFR-TKI resistance cell lines



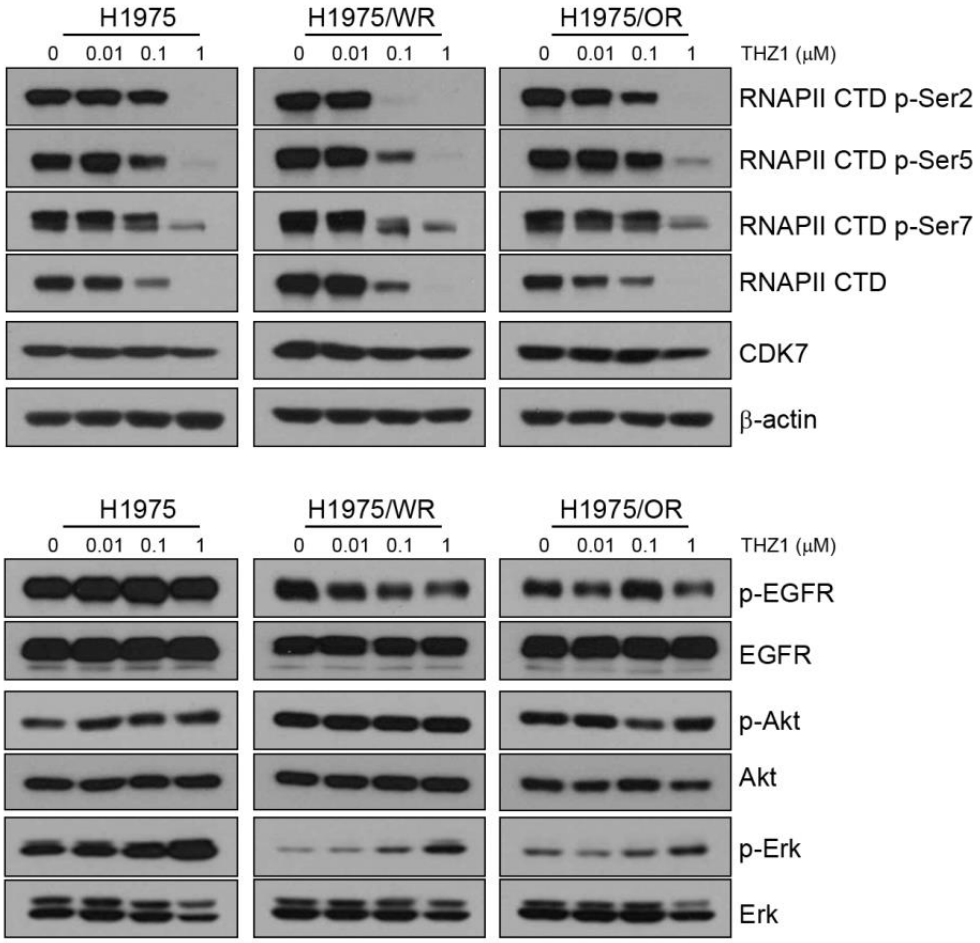
Cells were seeded onto either collagen or Matrigel-coated polycarbonate filters to determine their migratory and invasive potentials. Cells were incubated in modified Boyden chambers for 24 h, and the cells that penetrated the filter were stained and counted using a light microscope. Experiments were repeated in triplicate. Bars represent the standard deviations. ***$P < 0.0005$ compared with H1975 cells. A) migration and B) invasion were significantly enhanced in both resistant cells.

Figure 5. Effect of CDK7 inhibitors on the EMT induced EGFR-TKI resistance cell lines



Cells were treated with the indicated doses of THZ1 and QS1189, CDK7 inhibitors for 72 h, and cell viability was determined by the MTT assay. Both resistant cells were more sensitive to CDK7 inhibitors than parental cells. A) THZ1 IC_{50} = 379 nM in H1975, 83.4 nM in H1975/WR, 125.9 nM in H1975/OR, B)QS1189 IC_{50} = 755.3 nM in H1975, 232.8 nM in H1975/WR, 275.3 nM in H1975/OR).

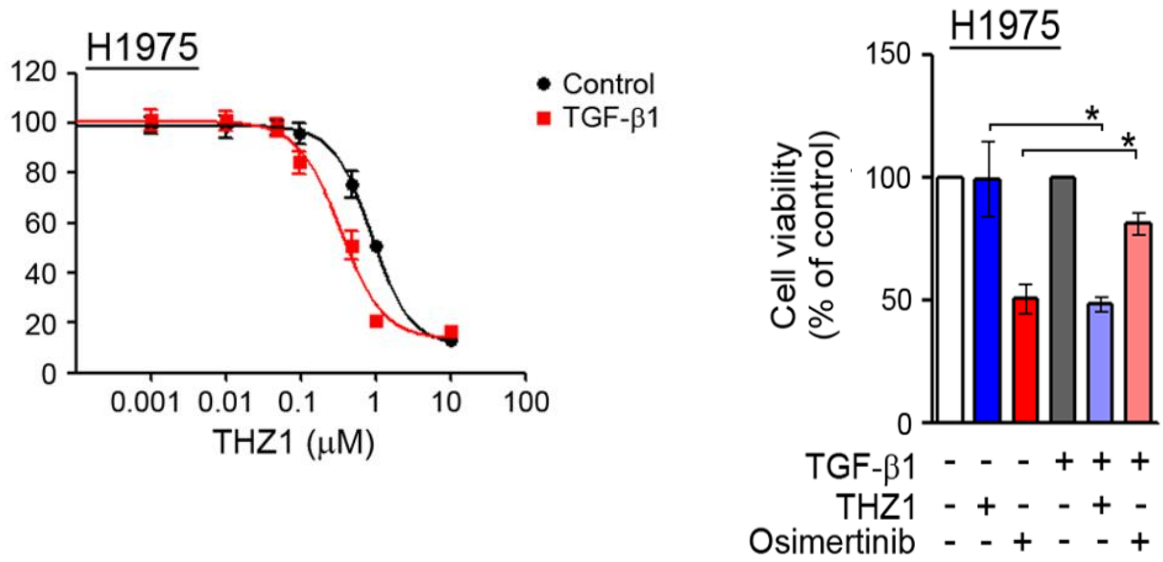
Figure 6. Immunoblot analysis according to CDK7 inhibitor treatment on the EMT induced EGFR-TKI resistance cell lines



EGFR signaling and CDK7 related molecules were analyzed by Western blotting. The inhibitory effect of THZ1 on activity of RNAPII-CTD was similar in H1975 and H1975/OR cells. However, H1975/WR cells showed the inhibition of RNAPII-CTD phosphorylation at Ser2, Ser5 and Ser7 at lower concentration of THZ1.

Figure 7. Effects of THZ1 on the TGF-β induced EMT

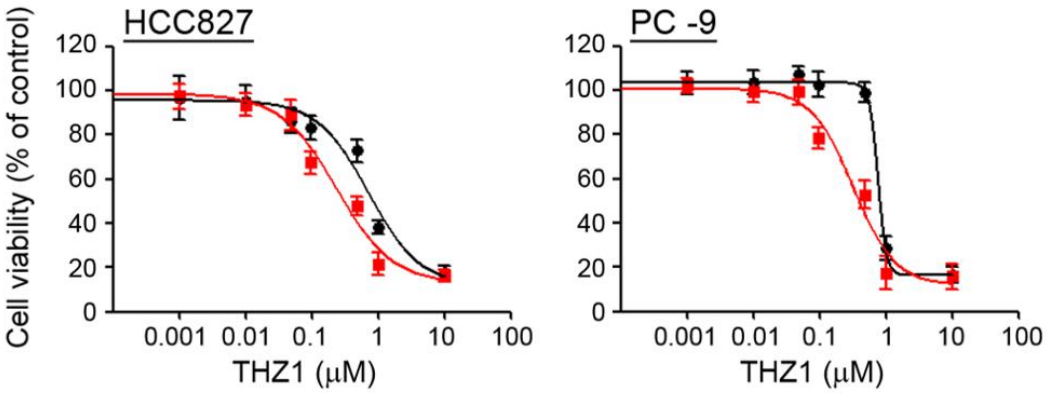
A) **B)**



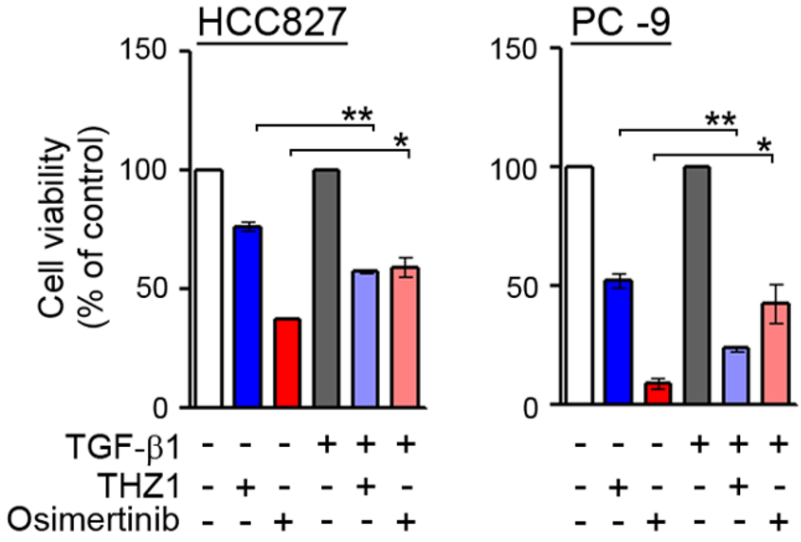
Cells were treated with the indicated doses of THZ1, CDK7 inhibitors for 72 h, and cell viability was determined by the MTT assay. A) & B) the sensitivity to THZ1 was increased in cells with TGF-β1-induced EMT. Bars represent the standard deviations. *$P < 0.05$ compared to pre-exposure of TGF-β.

Figure 8. Effects of THZ1 on the TGF-β induced EMT in T790M negative EGFR mutant cell lines

A)



B)



Cells were treated with the indicated doses of THZ1 and Osimertinib for 72 h, and cell viability was determined by the MTT assay. A) & B) The sensitivity to THZ1 was increased in cells with TGF-β1-induced EMT compared to the sensitivity to osimertinib was decreased. Bars represent the standard deviations. *$P < 0.05$ and **$P < 0.005$ compared to pre-exposure of TGF-β.

영문요약

Title:

The application of CDK7 inhibitors to overcome EMT-associated EGFR-TKIs resistance in non-small cell lung cancer

Name and Affiliation:

Wonjun Ji

Department of Pulmonary and Critical Care Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea.

Background: Epithelial to mesenchymal transition (EMT) is associated with resistance during EGFR tyrosine kinase inhibitor (EGFR-TKI) therapy. This study investigated the effects of cyclin-dependent kinase 7 (CDK7) inhibitors on EMT-associated EGFR-TKI resistance in non-small cell lung cancer (NSCLC).

Methods: In this study, we established an EGFR-TKI resistant cell line (H1975/WR, H1975/OR) via repeated exposure to Osimertinib and WZ4002. Morphologic analysis, invasion and migration assays, and immunoblot analysis were performed to evaluate the changes that occur during EMT. RNA sequencing was used to assess genetic changes caused by EMT. THZ1 was used as a CDK7 inhibitor.

Results: The established EGFR-TKI resistant cell lines (H1975/WR, H1975/OR) showed phenotypic changes in EMT, including a spindle-cell shape and increased pseudopodia formation. Decreased E-cadherin and increased vimentin were observed in the immunoblot assay. RNA sequencing revealed decreased EPCAM and CDH1. Invasion and migration increased in the resistance cells. The EMT related resistance cells showed higher sensitivity to THZ1 than the mother cells. This phenomenon was observed in TGF-ß induced EMT cell lines and gefitinib resistant cell lines (HCC827, PC-9)

Conclusion: In conclusion, EMT was associated with decreased drug sensitivity to EGFR-KTI; while, EMT related resistance cells were more sensitive to CDK7

inhibitors. This suggested that CKD7 inhibitors could potentially be used as a therapeutic strategy to overcome EMT associated EGFR-TKI resistance in NSCLC.

Key words: Non-small cell lung cancer, EGFR-TKI acquired resistance, EMT, CDK7 inhibitor