学 位 論 文 の 要 旨

氏名　河野　謙人

学 位 論 文 名　Microtubule Inhibitors Induce Cross-Resistance to Osimertinib Through CaMKII Activation in EGFR-Mutated NSCLC

発 表 雑 誌 名（巻，初頁～終頁，年）　Cancer Science (in press, 2025) (10.1111/cas.70274.)

著 者 名　Kento Kono, Ryosuke Tanino, Yukari Tsubata, Eshat Fahmida Haque, Takeshi Isobe, Tamio Okimoto

論 文 内 容 の 要 旨

INTRODUCTION

Epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) is commonly treated with EGFR tyrosine kinase inhibitors (EGFR-TKIs), and osimertinib, an EGFR-TKI, has become a key drug. The current standard postoperative adjuvant therapy for patients with EGFR-mutated NSCLC includes chemotherapy with traditional microtubule inhibitors, especially vinorelbine (VNR) and paclitaxel (PTX), prior to EGFR-TKI administration. Importantly, various mechanisms of multidrug resistance following treatment with microtubule inhibitors have been reported, and the optimal sequence of drug administration for EGFR-mutated NSCLC remains undefined. We hypothesized that prolonged exposure to microtubule inhibitors could induce acquired cross-resistance to EGFR-TKIs. In this study, we investigated whether prior treatment with two microtubule inhibitors, VNR and PTX, induces acquired cross-resistance to osimertinib in EGFR-mutated NSCLC cells in vitro.

MATERIALS AND METHODS

Cell Culture and Adjuvant Therapy Period Exposure to Microtubule Inhibitors. EGFR-mutated NSCLC cell lines, PC-9 (delE746_A750) and H1975 (L858R/T790M), derived from human lung adenocarcinoma, were used. Cells were seeded at a density of 4000 cells/cm^2 following each passage and treated with VNR or PTX dissolved in DMSO. The concentration was incrementally increased when proliferating cells reached confluence within three days of

each seeding. VNR or PTX treatment was continued for 18 weeks without interruption.

Cell Viability Assay. Cells were seeded at a density of 3000 cells/cm^2 in 96-well tissue culture plates. Following treatment, 10 μL of Cell Counting Kit-8 (CCK-8) reagent was added to each well and incubated at 37°C with 5% CO_2 for 2 h. Absorbance was measured at 450 nm. Synergistic inhibitory effects were evaluated using the SynergyFinder software with the zero interaction potency (ZIP) model to evaluate the additive and synergistic effects of microtubule inhibitors and the calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93.

Immunoblotting. Cells were lysed after 24-h of treatment. Lysate samples were loaded onto a Tris–glycine gel. Following electrophoresis and transfer to nitrocellulose membranes, the membranes were blocked with 5% non-fat milk for 1 h, followed by 14–18 h incubation at 4°C with primary antibodies in a diluent of 5% non-fat milk or 1% bovine serum albumin in 0.05% Tween-20 containing Tris-buffered saline. Target proteins were imaged using the Amersham ImageQuant 800 system.

RNA Sequencing (RNA-seq) Analysis. Total RNA of cells was extracted using the RNeasy Mini Kit, according to the manufacturer's instructions. RNA-seq samples were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit, which transcribes mRNAs into cDNAs. Paired-end 150-base reads were sequenced using the NovaSeq 6000.

Cell Cycle Analysis. Cell fixation, permeabilization, DNase treatment, fluorescein-conjugated anti-BrdU antibody labeling, and 7-aminoactinomycin D DNA staining were performed using the BrdU Flow Kit. Flow cytometry was performed using the CytoFLEX Flow Cytometer.

Colony Formation Assay. After 1 week of preculture on a 6-well plate, the cells were treated with KN-93 or DMSO at 37°C with 5% CO_2 for 72 h. Colonies were fixed with 4% formaldehyde at 20–25°C for 10 min, and stained with 0.5% crystal violet in 20% methanol. Crystal violet was dissolved by 30% acetic acid and absorbance was measured at 595 nm.

Cell Invasion Assay. The membranes of the TC Inserts with 8-μm pores were coated with 10% Matrigel Matrix in PBS and incubated at 20–25°C for 1 h. Cells were seeded at a density of 4.5×10^4 cells per insert in FBS-free culture medium. Each insert was placed into a well of a 24-well culture plate containing FBS-supplemented culture media. Following incubation at 37°C for 48 h, invaded cells on the underside of the membrane were fixed with 4% paraformaldehyde at 20–25°C for 10 min and stained with Diff-Quik.

siRNA Transfection. siRNA transfection was performed using the ScreenFect A plus reagent, according to the manufacturer's instructions. The cells were incubated for 72 h and subsequently harvested for immunoblotting and CCK-8 assays.

RESULTS AND DISCUSSION

Eighteen-week treatments with VNR and PTX induced cross-resistance to osimertinib in PC-9-derived cell lines (PC-9/VNR and PC-9/PTX) compared to the parental PC-9 in the CCK-8

assay. Although osimertinib inhibited EGFR phosphorylation in all cell lines, AKT and ERK phosphorylation was elevated in PC-9/VNR and PC-9/PTX cells, and ERK phosphorylation was not suppressed by osimertinib to the same extent as that in PC-9 cells. These results indicate that prolonged exposure to tubulin inhibitors not only induces resistance to the respective agents but also leads to cross-resistance to osimertinib, potentially through sustained ERK pathway activation.

To investigate the signaling pathways contributing to the acquired resistance to osimertinib in PC-9/VNR and PC-9/PTX cells, we performed gene expression analysis using RNA-seq. The PID_WNT_NONCANONICAL_PATHWAY gene set was significantly enriched in PC-9/VNR cells, while both PID_WNT_SIGNALING_PATHWAY and PID_WNT_NONCANONICAL_PATHWAY were enriched in PC-9/PTX cells. These findings suggest that the activation of the non-canonical Wnt/Ca^{2+} signaling pathway may contribute to the acquired cross-resistance observed in tubulin inhibitor-resistant PC-9 cells.

To evaluate the functional relevance of this pathway in PC-9/VNR and PC-9/PTX cells, we used KN-93, a selective, cell-permeable, and competitive CaMKII inhibitor. Phosphorylation of CaMKII was increased in PC-9/VNR and PC-9/PTX cells but was inhibited by KN-93 treatment to levels similar to those in PC-9 cells. Phosphorylation of EGFR and ERK was downregulated by KN-93 treatment in PC-9, PC-9/VNR, and PC-9/PTX cells. KN-93 inhibited cell viability, formation, and invasion more strongly in PC-9/VNR and PC-9/PTX cells than in PC-9 cells. CaMKII inhibition by siRNA also exhibited similar results. Together, these results indicate that CaMKII plays a critical role in sustaining the proliferation and invasive potential of tubulin inhibitor–resistant cells, and that its inhibition selectively impairs these phenotypes in drug-resistant PC-9/VNR and PC-9/PTX cells.

Finally, we evaluated the potential synergy between KN-93 and osimertinib. The combination treatment reduced cell viability more in PC-9/VNR and PC-9/PTX cells than that in the parental PC-9 cells, with synergistic or additive effects between EGFR inhibition and CaMKII inhibition.

CONCLUSION

Long-term exposure of EGFR-mutated PC-9 cells to the microtubule inhibitors vinorelbine or paclitaxel resulted in acquired cross-resistance to the EGFR-TKI osimertinib. This study provides evidence that prior treatment with microtubule inhibitors induces acquired cross-resistance to osimertinib in EGFR-mutated NSCLC cells. These findings suggest that the non-canonical Wnt/Ca^{2+} pathway and CaMKII play critical roles in drug resistance to microtubule inhibitors and EGFR-TKIs. This study underscores the importance of optimizing the timing of EGFR-TKI administration in the therapeutic sequence for EGFR-mutated NSCLC.