Concurrent Pemetrexed With EGFR-TKI Slows the Accumulation of De Novo Mutations During In Vitro Exposure

ESHAT F. HAQUE, RYOSUKE TANINO, TAMIO OKIMOTO, TAKAMASA HOTTA, TAKAE OKUNO, KENTO KONO, YUKARI TSUBATA and TAKESHI ISOBE

Department of Internal Medicine, Division of Medical Oncology & Respiratory Medicine, Shimane University Faculty of Medicine, Izumo, Japan

Abstract

Background/Aim: Resistance to targeted therapy limits its efficacy in non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations. Although concurrent administration of pemetrexed (PEM) and EGFR-tyrosine kinase inhibitors (TKIs) has yielded clinical benefits, it remains unclear whether concurrent PEM influences de novo mutations in the EGFR-TKI therapy. To address this gap, we aimed to compare the time to acquired resistance and accumulation of de novo tumor mutational burden (TMB) during in vitro exposure to osimertinib (OSI) and gefitinib (GEF), as well as their respective combinations with PEM. Materials and Methods: EGFR-mutated PC-9 cells were continuously exposed to EGFR-TKIs, alone or in combination with PEM, at equimolar concentrations. The drug concentration gradually increased to 1 and 3 μM for OSI and GEF, respectively. Whole-exome sequencing and quantitative PCR were performed to measure TMB and gene expression, respectively. Results: Concurrent PEM with either OSI or GEF extended the treatment duration compared to single EGFR-TKIs, decreased the de novo accumulated TMB per treatment time, and increased the expression of POLE2, POLQ, MLH1, BRCA1, BRCA2, RAD51, and FEN1 compared to that of single EGFR-TKIs. Conclusion: Concurrent PEM with EGFR-TKI treatment slowed TMB accumulation rate and resistance acquisition in EGFR-mutated NSCLC compared to single EGFR-TKIs in vitro.

Keywords: Concurrent therapy, EGFR-TKI, pemetrexed, tumor mutational burden.

Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of all reported lung cancer cases and is the leading cause of cancer-related deaths worldwide (1, 2). Approximately 20% of NSCLC cases harbor activating mutations in the epidermal growth factor receptor (EGFR) (3). Oncogene driver-targeting EGFR-tyrosine kinase inhibitors (TKIs) are the standard treatment that yield a high response rate in patients with EGFR-mutated NSCLC. However, their efficacy is hampered by acquired drug resistance during therapy, attributed to the emergence of tumor cells with variants in different genes, such as MAP2K1, ERBB2, JAK2, and MET (4). Previous studies suggested that the concurrent administration of EGFR-TKIs and pemetrexed (PEM), an antifolate agent that inhibits thymidine synthesis, significantly prolongs the time to acquired resistance compared to that of single EGFR-TKIs (5-12). The efficacy of concurrent combination therapy is supported by the targeting of multiple components in complex tumor cell populations, with variability in drug efficacy owing to inter-patient and intra-tumor heterogeneity (13). The synergistic efficacy derived from different drug actions is one of the primary differences between sequential and concurrent therapies, including the PLK1-mediated pro-apoptotic effect (14). However, establishing the long-term efficacy of adding PEM to EGFR-TKIs in prolonging therapeutic duration is challenging, particularly in terms of identifying optimal cancer subtypes for selecting regimens, due to the significantly different mechanisms of EGFR-TKIs and PEM. Furthermore, experimental studies investigating the mechanisms underlying resistance acquisition during long-term EGFR-TKI therapy with concurrent PEM remain limited.

Drug-induced stress can cause cell death or cycle arrest on subpopulations of drug-induced quiescent cells. However, cancer cells that escape the quiescent state often survive through drug resistance. Alterations in the DNA replication system and error-prone mitotic DNA synthesis have been reported in these resistant cells (15). Moreover, similar to the adaptive activation of error-prone DNA polymerases during tumorigenesis (16), targeted therapy can reduce DNA replication fidelity and induce adaptive mutability by increasing de novo mutations via alternative DNA repair pathways, including AXL-related error-prone DNA polymerase activation (17-19). Moreover, direct damage and DNA depletion by anticancer agents targeting DNA synthesis, such as PEM, can enhance DNA replication stress and induce DNA repair responses (20, 21), leading to increased mutational accumulation. However, concurrent PEM with EGFR-TKIs can lead to longer survival (12), although the long-term additional effects of PEM on EGFR-TKIs on de novo mutations for acquiring drug resistance remain unclear. Therefore, we hypothesized that adding PEM to EGFR-TKI therapy affects DNA replication fidelity and de novo mutation rate in cancer cells until drug resistance is developed.

Therefore, this study aimed to compare the time to acquired resistance and tumor mutational burden (TMB) accumulation as indicators of DNA replication fidelity during long-term in vitro treatments with osimertinib (OSI) and gefitinib (GEF). This study also assessed combinations of PEM with OSI (OP) or GEF (GP) using a monoclonal cell line to avoid cell proliferation from pre-existing resistant cells. Moreover, the effects of additional PEM exposure were evaluated by assessing DNA damage response and transcriptional changes in DNA polymerase and DNA damage repair proteins, which alter the DNA replication system during treatment.

Materials and Methods

Cell culture. Three human EGFR-mutated lung adenocarcinoma cell lines, PC-9 (EGFR delE746_A750), 11-18 (EGFR L858R), and H3255 (EGFR L858R), were used as parental cell lines. PC-9 cells were kindly provided by Dr. Yoshio Honma and authenticated by short tandem repeat profiling using the PowerPlex 16 System (Promega, Madison, WI, USA). The 11-18 and H3255 cells were kindly provided by Prof. Kazuko Sakai. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS and 50 μg/ml gentamicin in a humidified incubator at 37°C with 5% CO_2. GEF, OSI, and PEM were purchased from Selleck Chemicals (Houston, TX, USA). OSI and GEF were dissolved in DMSO, and PEM was dissolved in PBS.

In vitro treatment until acquisition of drug resistance. EGFR-mutated NSCLC PC-9 cells were continuously exposed to OSI or GEF, either alone or in combination with PEM, at equimolar concentrations. The initial concentrations were 10 and 100 nM of OSI and GEF, respectively, which gradually increased, following at least two treatments at each concentration, by 3.16 times after the cells reached ≥70% confluency within ≤3 days after passage, as a progressive growth threshold. The maximum concentrations of OSI and GEF were set at 1 and 3 μM, respectively, to limit off-target cytotoxicity. The treatment was stopped when the cells grew above the threshold at the respective maximum concentrations.

Cell viability assay. Cells were seeded at a density of 1,000-1,500 cells/well in a 96-well culture plate with the indicated drug concentrations. After 96 h of exposure, 10 μl of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to each well. Absorbance was measured at 450 and 640 nm using a microplate reader (Sunrise Remote; Tecan, Männedorf, Switzerland), and the difference was used as the absorbance value. Cell viability (%) was calculated using the following formula: 100 × (absorbance value of the treated sample/absorbance value of the vehicle control).

Bliss synergy score. Synergistic effects were evaluated using the SynergyFinder (version 3.0) software (https://synergyfinder.fimm.fi/) with the Bliss model to calculate the Bliss synergy score

(22). A Bliss synergy score below −10 indicates an antagonism between the drugs, between −10 and 10 indicates an additive effect, and greater than 10 indicates synergy.

Whole-exome sequencing. Genomic DNA was extracted from the cells using the DNeasy Mini Kit (Qiagen, Hilden, Germany) and sequenced using NovaSeq X Plus PE150 (Illumina, San Diego, CA, USA). The SureSelect Human All Exon V6 exon capture kit (Agilent, Santa Clara, CA, USA) was used for library construction, generating insert fragments of 180-220 bp. Illumina PE150 paired-end sequencing was performed. Data cleaning was performed by removing adapters, N-rich, and low-quality reads. The sequence data were aligned against a reference genome assembly (GRCh37) using the BWA-MEM method (23) and Picard tools (https://github.com/broadinstitute/picard). The Genome Analysis Toolkit software (24) was used to perform Base Quality Score Recalibration on the duplicate-marked BAM file. Only samples with >95% of bases with a quality score of ≥30 out of the total bases were used. The mutect2 toolkit in the Genome Analysis Toolkit package was used for somatic variant detection in paired with parental PC-9 as a germline sample, and FilterMutectCalls with default parameters was used to filter artifacts. The number of somatic non-synonymous single-nucleotide variants, insertions, and deletions per megabase pair (Mb) in protein-coding base mutations in treated cells compared to parental PC-9 cells was measured as de novo TMB. Similarly, MSIsensor-pro (25) was used to measure the percentage of de novo microsatellite instability (MSI) sites (100 × number of somatic sites/total number of sites) using default parameters to detect microsatellites with sizes ranging from five to 40 bp.

Immunoblotting. Cells were lysed using M-PER (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1% (v/v) Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan) and 1% (v/v) Protease Inhibitor Cocktail (Thermo Fisher Scientific), after a 48-h treatment with the indicated agents. Cell lysates were incubated in a tube shaker at 1,000 rpm for 5 min, followed by centrifugation at 14,000 × g for 10 min, and the supernatant was used as the protein sample. Protein samples were mixed with LDS Sample Buffer and Sample Reducing Agent (both from Thermo Fisher Scientific) and heated at 95°C for 5 min. Samples were loaded onto Tris-glycine gels. Following electrophoresis and transfer to nitrocellulose membranes, the membranes were blocked with 2% ECL Prime (Cytiva, Tokyo, Japan) at 20°C-25°C for 1 h, followed by overnight incubation at 4°C with primary antibodies diluted in 1% bovine serum albumin in 0.05% Tween-20 containing Tris-buffered saline (TBS-T). The membrane was washed three times for 10 min in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody diluted in TBS-T [anti-rabbit IgG (Cytiva; NA934, 1:30,000)] in TBS-T at 20°C-25°C for 1 h and washed four times with TBS-T. Target proteins were visualized using ECL Select Detection Reagent

(Cytiva) and imaged using an ImageQuant 800 system (Cytiva). The primary antibodies used were anti-phospho-CHK1 [Cell Signaling Technology (CST), Danvers, MA, USA; #2348, 1:1,000], anti-phospho-CHK2 (CST; #2197, 1:1,000), anti-phospho-H2AX (CST; #9718, 1:1,000), and anti-GAPDH (CST; #8884, 1:2,000).

Quantitative reverse transcription polymerase chain reaction (PCR). Following a 48-h treatment period, total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Quantitative PCR (qPCR) samples were prepared using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) and KOD SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions. Following incubation at 98°C for 2 min, qPCR was performed using the Thermal Cycler Dice Real Time System II (Takara Bio, Kusatsu, Japan) with 40 cycles of conditions: 98°C for 10 s, 60°C for 10 s, and 68°C for 37 s. The primer sequences used for qPCR are listed in Table I.

Statistical analysis. Two-way analysis of variance (ANOVA) followed by Šidák's multiple comparisons tests were performed using Prism (version 10.6.1; GraphPad Software, Boston, MA, USA). A two-sided p-value <0.05 was considered statistically significant.

Results

Concurrent treatment of PEM and EGFR-TKI showed additional or synergistic effects in NSCLC cells. To evaluate the susceptibility of NSCLC cells to each drug and determine methods for long-term combination exposure, we first measured the viability of PC-9, 11-18, and H3255 cells following treatment with OSI, GEF, or PEM (Figure 1). All cell lines were sensitive to OSI and GEF; however, 11-18 and H3255 cells were less sensitive to PEM treatment than PC-9 cells. Next, to evaluate the synergistic and additive efficacy of additional PEM in EGFR-TKI treatment, we determined the Bliss synergy scores (Figure 2). OP treatment had a synergistic effect on PC-9 and 11-18 cells. Although 11-18 and H3255 cells were less sensitive to PEM alone, GP treatment had an additive effect on all cell lines. Due to their initial susceptibility to EGFR-TKIs and PEM, PC-9 cells were selected for long-term concurrent combination treatments to evaluate de novo mutational accumulation.

Comparison of duration until the emergence of drug-resistant cell populations during long-term exposures to EGFR-TKIs with or without concurrent PEM. To compare the time to drug resistance under treatment conditions with single EGFR-TKI treatment or concurrent combination with PEM, we continuously exposed PC-9 cells to each treatment and measured the total time until resistance acquisition (Figure 3). The durations of OSI and GEF treatments were 11.0 and 16.0

weeks, respectively, and those of OP and GP treatments were 61.9 and 43.6 weeks, respectively. The time difference between each TKI alone and each TKI with PEM was 50.9 weeks for OSI and OP and 27.6 weeks for GEF and GP. However, the duration of OSI with GEF (OG) treatment was 11.1 weeks. We observed no differences between the treatments of OSI and OG. Moreover, the longest exposure time within a concentration of OSI was 42.2 weeks at 1.0 μM OP and 20.9 weeks at 100 nM GP (Figure 3). The results indicated that concomitant PEM increased the time to acquired drug resistance compared to that of EGFR-TKI alone, even under monoclonal in vitro conditions. However, concurrent exposure to the two EGFR-TKIs did not prolong the duration.

Additional concurrent PEM to EGFR-TKI slowed de novo accumulation of TMB and MSI sites in drug-exposed cells. We next evaluated de novo TMB and MSI sites in drug-exposed cells following continuous exposure to EGFR-TKIs with or without PEM. The mean depth of coverage for exome targets (%coverage of target region with ≥10× depth) in the whole-exome sequencing were reported as follows: parental PC-9, 108.11 (98.49); GEF, 89.51 (98.22); OSI, 145.82 (98.81); GP, 107.79 (98.47); OP, 122.10 (98.56); OG, 102.60 (98.38). The total de novo TMB differed from 4.8 (OG) to 13.7 (OP) (Figure 4A), as well as the de novo MSI sites between treatments, ranging from 0.06% (OG) to 0.36% (OP) (Figure 4A). However, when we analyzed the mutational accumulation per total exposure time for each treatment, lower rates were observed in the concurrent PEM treatment groups than those observed with EGFR-TKI alone (Figure 4B). Specifically, the de novo TMB/week was lower in combination-treatment groups (PC-9/OP, 0.22; PC-9/GP: 0.16) than that in single-treatment groups (PC-9/OSI, 0.70; PC-9/GEF, 0.65). Similarly, the percentage of de novo MSI sites/week was lower in the combination treatment groups (PC-9/OP, 0.0059; PC-9/GP, 0.0015) than that in the single-treatment groups (PC-9/OSI, 0.013; PC-9/GEF, 0.012).

Adding PEM induced single-strand breaks and increased expression of polymerase and DNA damage repair genes. To evaluate the DNA damage effects of additional PEM, we measured the phosphorylation levels of CHK1, CHK2, and H2AX, which are markers for single- (CHK1) and double- (CHK2 and H2AX) strand breaks (Figure 5). All treatments with concurrent PEM increased phosphorylated CHK1 in PC-9 cells and decreased phosphorylated CHK2 compared with the baseline levels of the vehicle control but did not affect phosphorylated H2AX. Next, to investigate the de novo mutational-related effects of additional PEM, we performed gene expression analysis (Figure 6). PC-9 cells exhibited relatively lower gene expression of DNA polymerase epsilon, catalytic subunit (POLE), DNA polymerase epsilon 2, accessory subunit (POLE2), DNA polymerase theta (POLQ), BRCA1, BRCA2, RAD51, and flap structure-specific endonuclease 1 (FEN1) following EGFR-TKI treatment than that following vehicle control

treatment. However, additional PEM increased their expression. Notably, both OP and GP treatments significantly increased POLE2 (OP, p=0.0181; GP, p=0.0009), POLQ (OP, p=0.0465; GP, p=0.0036), MLH1 (OP, p=0.0058; GP, p=0.0192), BRCA1 (OP, p<0.0001; GP, p<0.0001), BRCA2 (OP, p<0.0001; GP, p<0.0001), RAD51 (OP, p<0.0001; GP, p<0.0001), and FEN1 (OP, p=0.0002; GP, p<0.0001) expression compared with those of single EGFR-TKI treatment.

Discussion

To understand the concurrent use of PEM in EGFR-TKI treatment against EGFR-mutated NSCLC cells, in this study, we evaluated the efficacy and mutability during long-term concurrent PEM exposure compared to those of EGFR-TKI alone in monoclonal NSCLC cells. Concurrent treatment with PEM prolonged the time to acquired resistance to over 40 weeks compared to the less than 16 weeks observed with EGFR-TKIs alone. The time differences between concurrent and monotherapy (50.9 and 27.6 weeks between OSI and OP and between GEF and GP, respectively) are similar to or greater than the 16-42 weeks (3.6-9.8 months) difference in the median PFS observed in previous clinical trials (5-7, 10, 26). Notably, both OP and GP treatments significantly increased the total exposure time compared with that of OSI or GEF treatments, even though only synergistic effects per the Bliss synergy scores were observed following short-term OP exposure. The synergistic effect of OP may be attributed to the longer total exposure time of OP (62 weeks) than that of GP (44 weeks). In contrast, the concomitant EGFR-TKI treatment (OG) did not change the time to resistance acquisition (11 weeks) compared to each EGFR-TKI alone, indicating that doubling the amount of EGFR-TKI in the concentration range and combining two EGFR-TKIs were not effective. The findings of this study demonstrated that adding PEM slowed the accumulation of de novo TMB and MSI sites during exposure compared to single EGFR-TKIs, particularly with OSI. To the best of our knowledge, this is the first evidence indicating that thymidine deficiency induced by PEM does not increase adaptive mutability when combined with EGFR-TKIs, unlike chemical mutagens, such as N-ethyl-N-nitrosourea (27-29).

During tumorigenesis, error-prone DNA polymerases contribute to mutational accumulation through replication errors (16), and this accumulation depends on the number of genome replication cycles. However, when combined with EGFR-TKIs, PEM should further reduce the replication rate of cells sensitive to both treatments, and this can be a long-lasting effect (9). Conversely, thymidylate synthase inhibitors, such as PEM and 5-fluorouracil, cause uracil-induced replication stress due to thymidine deficiency (30, 31), which should increase the mutational accumulation per replication. Hence, when selecting an agent to be combined with EGFR-TKIs, the agent-induced slowing of DNA replication and inhibition of error-prone

replication should outweigh the agent-induced mutational accumulation. Overall, this study provides additional evidence that PEM is a favorable agent for concurrent use with EGFR-TKIs.

Although this is a preliminary evaluation, the qPCR results suggest that PEM affects DNA replication fidelity by increasing the transcription of DNA polymerases and DNA damage repair proteins. POLE2, one of the four subunits of polymerase epsilon, functions as a high-fidelity DNA polymerase in the leading strand and has proofreading exonuclease activity (32). High-fidelity DNA polymerases are essential for maintaining genomic integrity, and deficiencies in these polymerase genes drive hypermutations (33), particularly in gene mutants of polymerase epsilon, which affect both TMB and MSI sites (34, 35). PEM-induced POLE2 expression may indicate the activation of extended leading-strand replication in the absence of thymidine. BRCA1, BRCA2, and RAD51 are known to be DNA double-strand break repair proteins that play crucial roles in homologous recombination (21). Although POLQ is an error-prone polymerase often upregulated in cancer cells and may contribute to mutagenesis (36, 37), a lack of POLQ also leads to defective DNA repair and increase in double-strand breaks, owing to its crucial role in repairing double-strand breaks (38, 39). Although detectable double-strand break formations through CHK2 and H2AX markers are limited against various forms of DNA damage, not only PEM-induced single-strand breaks but also replication stress-induced DNA damage might be repaired by POLQ, BRCA1, BRCA2, and RAD51, which are consistently expressed under concurrent PEM treatment. Collectively, this indicates that the PEM-induced expression response of these proteins may explain the reduced mutability and extended DNA replication to compensate for thymidine inhibition. Conversely, this delays the accumulation of de novo TMB via mechanisms that maintain genomic integrity and/or limit replication-dependent mutations.

Alterations in DNA damage repair pathways are associated with immune checkpoint therapy responses (40). The data on total de novo TMB, which increased following EGFR-TKI exposure in all types of treatments, can aid in translating the results of this study into clinical practice, since increased TMB is highly related to immune-inflamed “hot” tumors that are more responsive to immune checkpoint therapy (41). Although this study suggests that the potential benefit of concurrent PEM administration is the suppression of de novo mutations in tumor cells, thereby delaying resistance acquisition, it may also lead to a lower efficacy of subsequent immune checkpoint therapy based on the TMB increase with the duration of previous therapy. In contrast, paclitaxel, a microtubule inhibitor, has been reported to induce responsiveness to immune checkpoint therapy (42, 43). Moreover, sacituzumab tirumotecan, an antibody-drug conjugated topoisomerase I inhibitor, showed better survival outcomes than platinum-based chemotherapy in patients with EGFR-TKI-resistant NSCLC (44). Therefore, these data suggest that the use of these

agents after concurrent PEM with EGFR-TKIs may be useful for inducing a “hot” tumor microenvironment prior to immune checkpoint therapy in patients with EGFR-TKI-resistant NSCLC. Our results suggest that considering the effects of mutational changes in tumor cells by the combined agents with EGFR-TKIs is essential for selecting the subsequent regimens.

This study has certain limitations. First, we did not directly measure the genetic and epigenetic mechanisms underlying mutability during concurrent PEM exposure. Second, the de novo accumulation rates of TMB and MSI sites were estimated to be constant during the drug exposure period and did not consider doubling time. Third, only the PC-9 cell line was used to measure the time to resistance acquisition and mutability. Fourth, we only performed in vitro evaluation, which does not fully reflect the complexity of the tumor microenvironment in patients, such as interactions with immune cells that can also increase TMB (45).

Conclusion

Our findings suggest that long-term concurrent PEM with EGFR-TKI treatment does not increase the mutational accumulation rate of EGFR-mutated NSCLC cells through PEM-induced modulation of DNA polymerase and DNA repair gene expression. Thus, our work provides evidence that concurrent PEM reduces mutability, prolonging the time to acquired resistance during EGFR-TKI therapy in patients with EGFR-mutated NSCLC. Further research is required to generalize the PEM-induced mechanisms that slow the rate of mutational accumulation in EGFR-mutated NSCLC cells.

Supplementary Material

The original immunoblotting images can be found at the following DOI: 10.6084/m9.figshare.30830912. The whole-exome sequencing data were deposited in the NCBI BioProject (accession number: PRJNA1380009).

Conflicts of Interest

Yukari Tsubata has received personal fees from AstraZeneca K.K., outside of the submitted work. Takeshi Isobe has received personal fees from AstraZeneca K.K. and Boehringer Ingelheim GmbH, outside of the submitted work. All other Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization and methodology, R.T.; validation, E.F.H. and R.T.; formal analysis, E.F.H. and R.T.; investigation, E.F.H.; resources, T.Okimoto, Y.T. and T.I.; data curation, E.F.H. and R.T.; writing – original draft preparation, E.F.H.; writing – review and editing, R.T., T.Okimoto, T.H., T.Okuno, K.K., Y.T. and T.I.; visualization, E.F.H. and R.T.; supervision, T.Okimoto, Y.T. and T.I.; project administration, T.Okimoto; funding acquisition, E.F.H. All Authors have read and approved the final version of the manuscript.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools were used in the preparation, analysis, or presentation of this manuscript.

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Table I. The primer sequences used in qPCR.

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
POLA1	CACGGCGACGACTGTGAGATAG	CCATCTTCCACATAGCCAATACCATC
POLD1	GGTTACAACATCCAGAACTTCGAC	GGACGTCGTAGTACCCTTTGAG
POLD2	GGAAGTTTCTGGTGGAGGACTATTG	GCCTTATTGATAGAATCCCTGCTCTG
POLD3	CGGACCAAAACAAGATCGTGACATAC	CTGTCCTTTAGCATGGCTTTCTGGA
POLE	CATCCTGAACTCCTTCTATGGCTATGTC	CATATACCATCTGTGTCCAGCTCTAAGG
POLE2	CTACTGGAACAGTCCAACTAGACCTTAG	CTCTGGACCAGGTACAAACACAAAAC
POLI	GCACATCAGACTACTTGTTGGATCTC	CTGAGAGTATCACAGGGGAGTTATC
POLK	GATAGCACAAAGGAGAAGTGTGACAG	GACAGCATACTCATTGATCCTACAGC
POLL	GGCTGAGAAAATCATAGAGATCCTGGAG	CCAAGTACTTCTGTTGCTGACCATTC
POLM	CCTGATGTGCAGTGTCTCATTAGTTG	GAAAATTATCTTCCTCCCAGCTCCTACC
POLN	CTAAGACTTGGGGAAAGAGTACAGAG	TGGTGATTGAGTCTTCCTGTCTTC
POLQ	ACACTAGTAGGAGAAAGCCCACCTT	CTCCTGAAAACATCAGCTCCAGTGT
REV1	CTATGCAGAAGGATGGGACTTCATCTAC	GCGGAAGGATCTGTGTATCCATTAAC
MLH1	GGCCAGCTAATGCTATCAAAGAGATG	GTTTAGGAGGGGCTTTCAGTTTTC
MSH2	GCATTTTCTTCAACCAGGAGGTG	CTTCAAACTGAGAGAGATTGCCAGG
MSH6	GGTAGATGCGGTGCTTTTAGGAG	CCTCCTTAGTGTCTGGCTTAAATTCC
PMS2	GATTCAAGAGTTTGCCGACCTAAC	GTAAGACCTGGACCATTTTGGCATAC
BRCA1	CATCTGTCTGGAGTTGATCAAGGAAC	GAGTTCTCACAGTTCCAAGGTTAGAGAG
BRCA2	CATACCCTATACAGTGGATGGAGAAG	CTGAAATAACCCTCAAGGTAAGCTGG
RAD51	AGAGAAGTGGAGCGTAAGCCAG	CCACAGTATGGAATCCAGCTTCTTC
MBD4	GAGAGCAGAGGACTCTCTAACTTTAC	CCTCATACTTCTCGTTGTGTTCTGAG
APEX1	AGGGCAACGCGGTAAAAATATTGC	CTTGGATCGAGCATTCATCATATAAGTCC
FEN1	CCCTTAAGAGCTACAGCTAGAGAAAC	GTGAACATCACCATCTTACTCCTCTG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

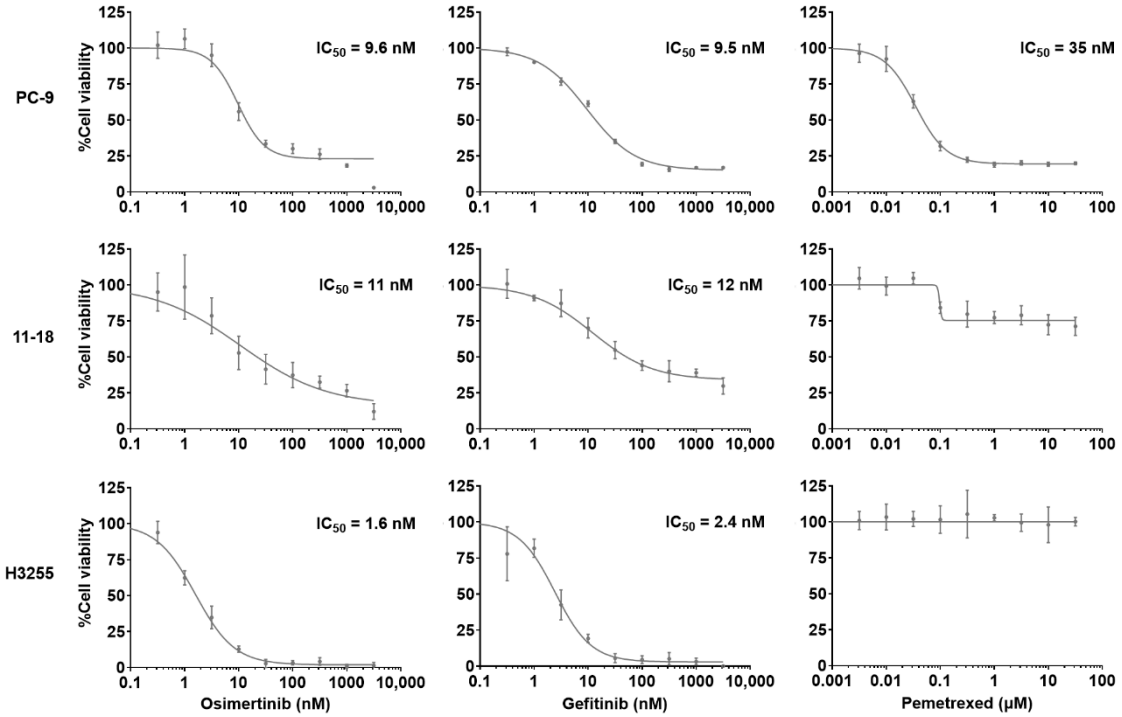

Figure 1. PC-9 cells were more sensitive to pemetrexed than 11-18 and H3255 cells. Cell viability of epidermal growth factor receptor (EGFR)-mutated human non-small cell lung cancer (NSCLC) cells with EGFR activation variants (PC-9, delE746_A750; 11-18, L858R; H3255, L858R) and half-maximal inhibitory concentration (IC50) were evaluated using the WST-8 assay following treatment with osimertinib, gefitinib, or pemetrexed at the indicated concentrations for 96 h (mean±standard deviation, n=4).

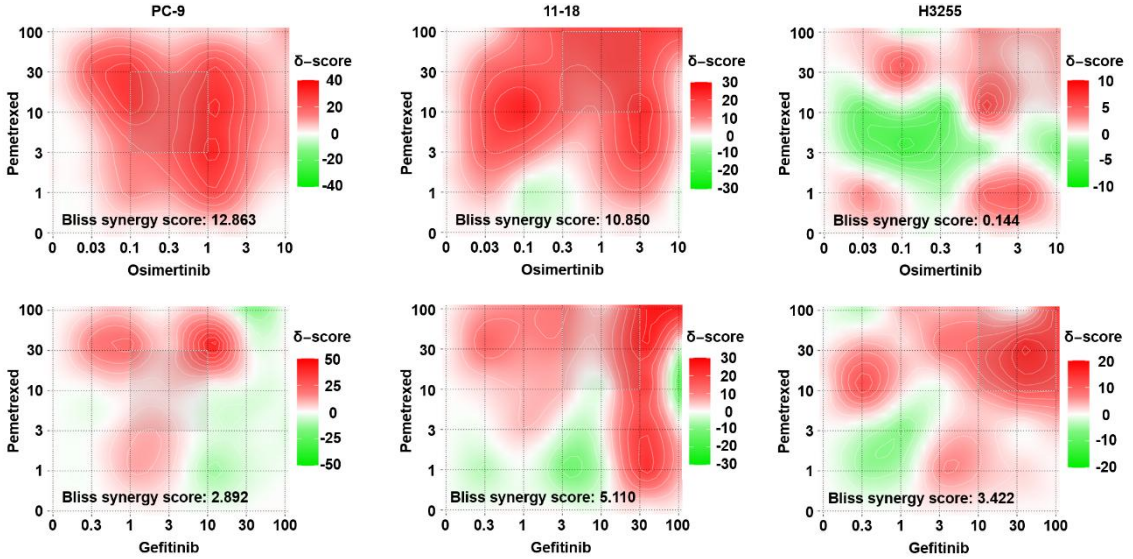

Figure 2. Combined pemetrexed with osimertinib induced synergistic effects in PC-9 and 11-18 cells, while combined pemetrexed with gefitinib induced additive effects in all epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) cells. The synergy plots show the Bliss synergy score and drug interaction matrix in cells treated with combinations of pemetrexed and EGFR-tyrosine kinase inhibitors (TKIs) after 96 h of exposure to the indicated drugs.

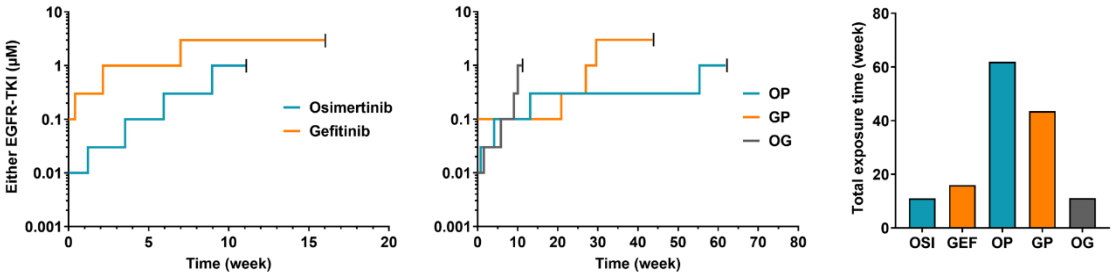

Figure 3. Concurrent combination of pemetrexed with epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) [osimertinib (OSI) and gefitinib (GEF)] prolonged treatment time and resistance acquisition in PC-9 cells. Treatments with pemetrexed and OSI (OP) or GEF (GP) were more effective than single EGFR-TKIs in long-term treatment, whereas a combination of two EGFR-TKIs (OG) had an efficacy similar to that of single EGFR-TKIs.

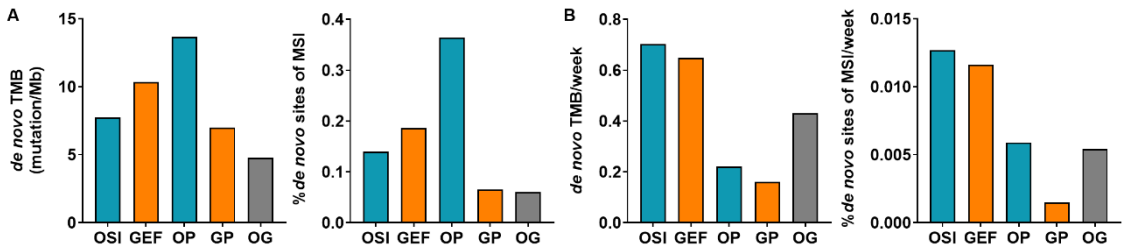

Figure 4. Concurrent combination of pemetrexed with epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) delays de novo mutations in PC-9 cells. (A) After long-term exposure to EGFR-TKIs treatment with or without pemetrexed, the total de novo mutation rate increased in the tumor mutational burden (TMB) and microsatellite instability (MSI) sites. (B) OP and GP treatments decreased the rate of emergence of de novo TMB and MSI sites per drug exposure duration. OP, Pemetrexed with osimertinib; GP, pemetrexed with gefitinib; OG, osimertinib with gefitinib; OSI, osimertinib; GEF, gefitinib.

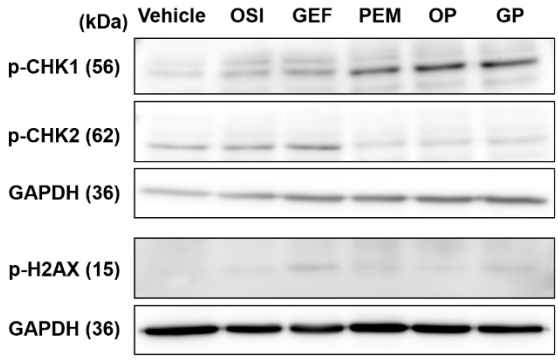

Figure 5. Combined pemetrexed (PEM) induced single-strand breaks but not double-strand breaks in PC-9 cells. The phosphorylation levels of CHK1, CHK2, and H2AX proteins, extracted from lysed PC-9 cells after 48h treatment with epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) (30 nM OSI and 100 nM GEF) and/or 300 nM PEM, were determined via immunoblotting, with GAPDH as a loading control. The images are representative of two independent experiments (shown in Supplementary data). OP, Pemetrexed with osimertinib; GP, pemetrexed with gefitinib; OG, osimertinib with gefitinib; OSI, osimertinib; GEF, gefitinib.

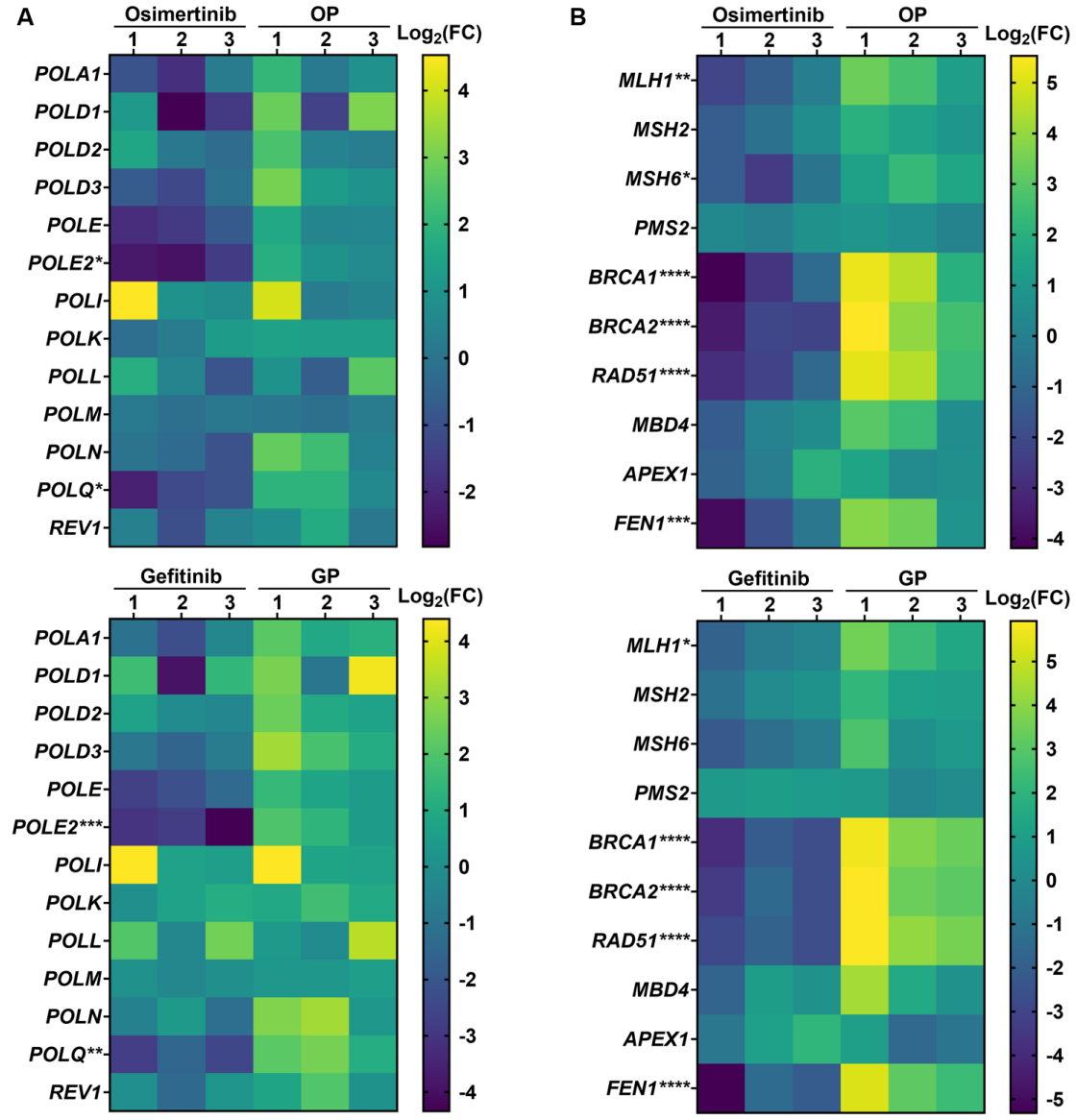

Figure 6. Combination treatments of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) with pemetrexed (PEM) increased the expression levels of (A) DNA polymerase (POLE2 and POLQ) and (B) DNA damage repair (MLH1, BRCA1, BRCA2, RAD51, and FEN1) genes compared to those of single EGFR-TKIs in PC-9 cells. Fold changes (FC; n=3 independent experiments) from the treatment with vehicle control for the expression levels of polymerase genes in non-small cell lung cancer (NSCLC) cells after 48h EGFR-TKI treatment (30 nM OSI and 100 nM GEF) with or without 300 nM PEM were determined using reverse-transcription quantitative PCR. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$; two-way analysis of variance (ANOVA) followed by Šidák's multiple comparisons tests. OP, Pemetrexed with osimertinib; GP, pemetrexed with gefitinib.