学 位 論 文 の 要 旨

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学　位　論　文　名	Concurrent Pemetrexed With EGFR-TKI Slows the Accumulation of De Novo Mutations During In Vitro Exposure
発　表　雑　誌　名 （巻，初頁～終頁，年）	Anticancer Research (in press)
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論 文 内 容 の 要 旨

INTRODUCTION

Non-small cell lung cancer (NSCLC) represents the majority of lung cancer cases and occasionally harbors oncogenic mutations in the epidermal growth factor receptor (EGFR), making EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as osimertinib (OSI) and gefitinib (GEF), first-line targeted therapies. Despite high initial response rates, treatment is frequently limited by acquired resistance, which is associated with the emergence of additional de novo variants across the tumor genome. Clinical and preclinical studies have reported that concurrent administration of an EGFR-TKI with pemetrexed (PEM), an antifolate that inhibits thymidine synthesis, can prolong clinical benefit compared with EGFR-TKI monotherapy; however, the mechanistic basis remains insufficiently characterized.

Targeted therapy-induced stress can involve altered DNA replication fidelity and engagement of alternative DNA repair pathways, leading to "adaptive mutability" (therapy-induced de novo mutational accumulation). Conversely, PEM can also induce replication stress through nucleotide imbalance and nucleotide pool depletion, leaving it unclear how PEM influences adaptive mutability when combined with EGFR-TKIs during long-term administration.

We tested the hypothesis that concurrent PEM with EGFR-TKI therapy modifies DNA replication/repair responses and modulates adaptive mutability during the period leading to acquired resistance. To exclude pre-existing resistant cells, we employed EGFR-mutant NSCLC cell lines and compared long-term EGFR-TKI exposure to OSI or GEF alone versus each combined with PEM (OP or GP). We measured time to resistance acquisition, de novo tumor mutational burden (TMB) and microsatellite instability (MSI), as well as markers of the DNA

damage response and expression of replication/repair genes.

MATERIALS AND METHODS

Cell culture. Three EGFR-mutated lung adenocarcinoma cell lines (PC-9: EGFR delE746_A750; 11-18 and H3255: EGFR L858R) were cultured under standard RPMI-1640 conditions. Short-term drug sensitivity to OSI, GEF, and PEM was evaluated using a 96-hour viability assay (CCK-8) over a range of concentrations. Drug interaction was quantified using SynergyFinder (Bliss model), where scores >10 indicate synergy, −10 to 10 indicate additivity, and <−10 indicate antagonism.

Drug resistance development. For long-term resistance evolution, we continuously exposed PC-9 cells to OSI or GEF alone, OSI+PEM (OP), GEF+PEM (GP), or OSI+GEF (OG), using a fixed 1:1 molar ratio (EGFR-TKI:PEM) in combination arms. Starting doses were 10 nM OSI and 100 nM GEF, escalated stepwise (×3.16) after at least two treatments per concentration when cells reached ≥70% confluency within ≤3 days, defining a progressive growth threshold. Maximum concentrations were capped at 1 μM (OSI) and 3 μM (GEF) to limit off-target cytotoxicity; treatment ended once cells grew above the threshold at the maximum concentration.

De novo mutational accumulation. Genomic DNA from each group underwent whole-exome sequencing. Variants were called with Mutect2 using parental PC-9 as the matched reference. De novo TMB was defined as the number of nonsynonymous SNVs/indels per Mb in treated cells relative to parental cells; MSI was quantified with MSIsensor-pro as the percentage of somatic MSI sites.

Drug-induced DNA damage, replication, and repair alterations. DNA damage signaling was assessed by immunoblotting (p-CHK1, p-CHK2, and p-H2AX), and transcriptional changes in replication/repair genes were measured by qPCR after 48-h treatment of parental PC-9 cells.

RESULTS AND DISCUSSION

In short-term assays, all three EGFR-mutant lines were sensitive to OSI and GEF, whereas PEM sensitivity varied, with 11-18 and H3255 showing lower susceptibility than PC-9. Bliss analyses indicated that OP was synergistic in PC-9 and 11-18, while GP showed additive effects across all lines. Therefore, we used PC-9 cells as the long-term model given their baseline susceptibility to both EGFR-TKIs and PEM.

Under continuous escalation, time to acquired resistance differed by regimen. OSI and GEF monotherapy produced resistant growth within 11.0 and 16.0 weeks, respectively. In contrast, concurrent PEM dramatically extended exposure duration: OP continued for 61.9 weeks and GP for 43.6 weeks, yielding added durations of 50.9 weeks (OSI vs OP) and 27.6

weeks (GEF vs GP). Notably, combining two EGFR-TKIs (OG) did not prolong control (11.1 weeks), suggesting that increasing EGFR-pathway inhibition within the tested range was insufficient to delay resistance, whereas adding PEM prolonged treatment duration.

Whole-exome sequencing revealed treatment-dependent de novo mutational landscapes. Total de novo TMB ranged from 4.8 (OG) to 13.7 (OP), and de novo MSI ranged from 0.06% (OG) to 0.36% (OP), indicating that mutations accumulated under long-term drug pressure. However, when normalized by total treatment duration, combination regimens exhibited substantially lower mutation accumulation rates. De novo TMB/week was 0.70 (OSI) and 0.65 (GEF) versus 0.22 (OP) and 0.16 (GP). Similarly, MSI/week was 0.013 (OSI) and 0.012 (GEF) versus 0.0059 (OP) and 0.0015 (GP). Thus, despite longer exposure times, PEM co-treatment slowed the rate of mutational accumulation per unit time, aligning with the observed delay in resistance emergence, consistent with clinical observations.

Mechanistically, in short-term testing, concurrent PEM increased p-CHK1 (a replication-stress marker) and reduced p-CHK2, without a clear increase in p-H2AX, implying a shift toward replication-stress signaling rather than overt double-strand break signaling under the tested conditions. qPCR further showed that EGFR-TKI exposure alone tended to reduce expression of several replication/repair genes (e.g., POLE/POLE2, POLQ, BRCA1/2, RAD51, FEN1), while adding PEM significantly increased key transcripts including POLE2, POLQ, MLH1, BRCA1/2, RAD51, and FEN1 compared with TKI alone. Collectively, these transcriptional changes may enhance genome maintenance and thereby reduce adaptive mutability, offering a plausible explanation for slower TMB/MSI accumulation and prolonged time to resistance. Limitations of this study include the lack of direct genetic/epigenetic causality testing, assumptions of constant mutation accrual rates, and reliance on a single long-term in vitro model.

CONCLUSION

This study demonstrates for the first time that PEM co-treatment slows the rate of de novo TMB and MSI accumulation per unit treatment time, countering concerns that antifolate-induced nucleotide stress would accelerate mutagenesis during targeted therapy. We also found that increased p-CHK1 and upregulation of DNA replication/repair-related genes under PEM co-exposure support a mechanistic hypothesis that genome maintenance responses may be reinforced, reducing adaptive mutability and delaying resistance evolution. Further multi-line, replicated, and functional validation studies are needed to generalize and mechanistically confirm these findings.