Pemetrexed induces ROS generation and cellular senescence by attenuating TS-mediated thymidylate metabolism to reverse gefitinib resistance in non-small cell lung cancer

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Abstract

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are strongly recommended for non-small cell lung carcinoma (NSCLC) patients harboring active EGFR mutations, while drug resistance inevitably makes exploring the resistance mechanisms and seeking effective therapeutic strategies urgent endeavors. Thymidylate synthetase (TYMS or TS) is a dominant enzyme in thymidylate nucleotide metabolism. In this study, based on public database analysis and examination of gene sets from 140 NSCLC patients that received EGFR-TKI therapy, we found a significantly positive correlation between TS expression and overall survival (OS) and disease-free survival (DFS) in lung adenocarcinoma. Twenty-four tissue specimens from NSCLC patients exhibited upregulated TS mRNA expression in NSCLC patients resistant to gefitinib. The human NSCLC cell line PC9, which is sensitive to gefitinib, and relatively resistant PC9/GR cells were used to demonstrate that knockdown of TS restored the sensitivity of resistant cells to gefitinib. Furthermore, pemetrexed effectively suppressed TS-mediated thymidylate metabolism and induced ROS generation and cellular senescence, thereby hampering carcinogenesis and restoring cell sensitivity to gefitinib. The combination of pemetrexed and gefitinib damaged the proliferation, migration and invasion capabilities of gefitinib-resistant cells, exhibiting a synergistic anticancer effect. Our findings illuminate the potential mechanism of TS-triggered gefitinib resistance and indicate that inhibition of TS by pemetrexed can potentiate the effect of gefitinib in NSCLC cells resistant to gefitinib. Pemetrexed combined with gefitinib has potent anti-progression potential in gefitinib-resistant NSCLC. This suggests that NSCLC patients with both high TS expression and EGFR-driving mutations might benefit more from a combination strategy of EGFR-TKIs and pemetrexed-based chemotherapy than EGFR-TKI monotherapy, which has profound clinical implications and considerable therapeutic value.

Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase that plays a significant role in the development and progression of NSCLC\(^1\). NSCLC patients with attractive EGFR driver gene mutations are strongly recommended to receive EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib, erlotinib, and afatinib. EGFR-TKIs result in considerable improvements in disease control and overall survival in advanced NSCLC. Additionally, a significantly improved quality of life is credited to minor adverse effects of EGFR-TKIs compared to traditional chemotherapy\(^2\)–\(^6\). Undeniably, despite the excellent curative effect of EGFR-TKIs, a fair proportion of NSCLC patients inevitably suffer from drug resistance within 9 to 13 months\(^7\)–\(^8\). The most common mechanism of resistance is a threonine-to-methionine acid change at position 790 (T790M) of exon 20. This mutation accounts for approximately 50–60% of cases with acquired resistance to first-line EGFR-TKIs, and osimertinib is a specific proven agent aimed at this gatekeeper mutation\(^9\)–\(^10\). Other mechanisms include second point mutations, such as D761Y\(^11\), T854A\(^12\), or L747S\(^13\), and histological and phenotypic transformations, such as SCLC transformation and epithelial–mesenchymal transition (EMT). However, several underlying mechanisms of resistance to EGFR-TKIs remain complicated and largely elusive\(^14\)–\(^16\). Studies on the
underlying resistance mechanisms of EGFR-TKIs in lung cancer and therapeutic approaches for reversing drug resistance should be extremely encouraged.

Of note, stress from the tumor microenvironment triggers alterations in metabolic preferences in cancer cells to fulfill the increased nutrient consumption required for cell survival and proliferation, which is so-called “metabolic reprogramming”\(^\text{17, 18}\). Otto Warburg first observed that cancer cells utilize aerobic glycolysis as their preferable energy resource rather than mitochondrial oxidative phosphorylation, which provides the vital energy supply in normal cells. This phenomenon in cancer cells is well known as the “Warburg effect”\(^\text{19}\). Since then, strategies targeting tumor metabolic reprogramming have attracted considerable attention from many researchers. Targeting hexosamine biosynthesis induces metabolic vulnerabilities in KRAS/LKB1 comutant lung cancer and subsequently inhibits cancer proliferation\(^\text{20}\). Impairment of mitochondrial metabolism induced by targeting BACH1 can make triple-negative breast cancer (TNBC) more sensitive to chemotherapy and targeted therapy\(^\text{21}\). Enhancement of antioxidant capacity and nucleotide precursors triggers oncogenic transformation\(^\text{22}\). Nucleotides are the dominant genetic biosynthesis building blocks and consist of pyrimidines (thymine, uracil, and cytosine) and purines (adenine and guanine). These substances are vital for DNA and RNA biosynthesis, cell fate signaling, enzyme function regulation, and cell metabolism. The synthesis and availability of large amounts of nucleotides and energy are necessary to support the growth and survival of malignant cells, and accelerated de novo nucleotide metabolism provides abundant nucleotide materials for cells to proliferate rapidly, which suggests that nucleotide metabolism is a promising target for anticancer treatment\(^\text{23}\).

Pemetrexed, a TS-targeted agent, exerts anticancer efficacy by inducing thymidylate deficiency and imbalances in the intracellular nucleotide pool, thereby leading to DNA damage and impaired DNA replication and repair\(^\text{24, 25}\). Our previous study demonstrated that EGFR-TKIs combined with pemetrexed considerably improved PFS in advanced NSCLC patients carrying EGFR driving mutations\(^\text{26}\). However, the underlying molecular and cellular mechanism of this synergistic anticancer strategy is still poorly defined. In this study, we first describe the baseline characteristics and distribution of TS single-nucleotide polymorphisms (SNPs) in NSCLC with attractive EGFR mutations\(^\text{27}\). The polymorphism located at the 5’UTR of the TS gene promoter enhancer region (TSER) is composed of a variable number tandem repeat (VNTR) sequence\(^\text{27}\). There are three major different genotypes (2R/2R, 2R/3R, and 3R/3R) in the whole population\(^\text{28}\). The 3R/3R genotype is correlated with higher TS mRNA expression and high dUMP to dTMP conversion efficacy, which is a vital precursor for DNA synthesis\(^\text{29}\). In contrast, a 6-bp variation in the 3’UTR of the TS gene promoter usually has an intimate correlation with lower TS mRNA expression in colorectal cancer tissues, as reported by Lenz et al.\(^\text{27, 30}\). In our study, in NSCLC patients, we found that a 6-bp polymorphism in the 3’UTR of the TS sequence had a positive correlation with the efficacy of EGFR-TKI therapy. Furthermore, we demonstrated that TS-mediated thymidylate metabolism drives tumorigenesis and generates gefitinib resistance in NSCLC. Additionally, the potential mechanism by
which pemetrexed induces metabolic vulnerability and reverses gefitinib resistance was explored and elucidated.

**Results**

The level of TS in LUAD and multiple other solid malignancies is significantly upregulated and correlated with decreased disease-free survival (DFS) and overall survival (OS).

First, we performed a pancancer analysis in over 4000 primary tumor samples of 31 common malignant carcinomas in the ONCOMINE database (www.oncomine.org). We found that TS mRNA expression was considerably upregulated in tumor samples compared with nontumor controls, except for acute myeloid leukemia (LAML) (Fig. 1A). Based on this result, with Gene Expression Profiling Interactive Analysis (EGPIA)\(^31\), we further analyzed the importance of the TS gene in DFS and OS in the above-related 30 tumor types, of which LAML was excluded due to its lower TS expression in contrast with normal controls. As expected, cancer patients with higher TS expression levels had shorter DFS (Fig. 1B, HR 1.4, log-rank p < 0.001) and OS (Fig. 1B, HR 1.5, log-rank p < 0.001). To explore the significance of TS in lung cancer, we further demonstrated an upregulation trend of TS protein expression in lung adenocarcinoma samples compared to normal samples in the Human Protein Atlas (HPA) database (Fig. 1C). Furthermore, high TS expression was correlated with unfavorable DFS (Fig. 1B, HR 1.3, log-rank p = 0.062) and OS (Fig. 1B, HR 1.7, log-rank p < 0.001) in LUAD. Based on the above analyses, we focused on the function of TS in EGFR-TKI resistance. The gene expression profile of gefitinib-resistant samples in two datasets (GSE114647 and GSE112274) was extracted for further analysis. The resistant samples were retrospectively redivided into two groups (TS high group and TS low group) according to the median expression level and subsequently subjected to multiple gene set enrichment analysis (GSEA) using GSEA software. As suspected, the pyrimidine metabolic pathway and purine biosynthesis pathway were upregulated in the two TS-high groups, and several other pathways, such as fatty acid metabolism, the TCA cycle and one carbon pool, were also activated in the TS-high group (Fig. 1D). All the above results indicate that TS might serve as a novel molecular marker for the prediction of disease progression and patient outcome in LUAD and many malignant tumors and may act as an effective marker to predict EGFR-TKI resistance in LUAD.

**Patients with upregulated TS expression have lower PFS during EGFR-TKI therapy**

First, we retrospectively analyzed the correlation between TS polymorphism variation and the efficacy of EGFR-TKI therapy in NSCLC patients. Between November 2014 and December 2021, a total of 140 patients pathologically confirmed to have NSCLC harboring EGFR driving mutations were included in the study, among which 68 (49%) patients had wild-type TS, and the remaining 72 (51%) patients were confirmed to have different TS polymorphism variations. Given that previous studies have demonstrated that the 3R/3R genotype is usually accompanied by a relatively higher TS mRNA level than the TS wild
type, while the 6-bp deletion in the 3’UTR of the TS gene promoter region is correlated with a lower TS mRNA level\textsuperscript{32,33}, patients with 3R/3R and 6-bp deletion comutations were excluded from our analysis because uncertainty of TS mRNA levels was induced by 3R/3R and 6-bp deletion comutations. Patients with only the 3R/3R genotype were also not included here because the sample size was too small to be representative (only 6 patients harbored the 3R/3R genotype). To analyze the relationship between the TS mRNA level and EGFR-TKI targeted therapy, 10 patients with wild-type TS and 2 patients with 6-bp deletions were excluded due to a lack of EGFR-TKI treatment information. Finally, 58 patients with wild-type TS and 39 patients with a 6-bp deletion were followed for Kaplan–Meier analysis of progression-free survival (PFS). Figure 2 shows that 31 (53.45%) patients were observed to have disease progression in the TS wild-type group, whereas 10 (25.64%) events occurred in the TS 6-bp group. The median PFS in the TS wild-type group was 21.25 months vs. 35.77 months in the TS 6-bp group (HR 2.014, log-rank p = 0.02746, 95% CI, 1.080 to 3.757). Patient details and clinical characteristics are listed in Table 1.

**High TS expression is associated with gefitinib resistance in NSCLC**

To demonstrate whether TS exerts a significant role in EGFR-TKI resistance in NSCLC, the expression of TS in biopsy specimens from 24 patients with pathologically confirmed NSCLC harboring EGFR-sensitive mutations, such as EGFR exon 19 deletion (19DEL) or exon 21 mutation (L858R), was examined. Patients who had never received EGFR-TKIs were included in the gefitinib-sensitive group, and patients experiencing disease progression during or after treatment with EGFR-TKIs were placed in the gefitinib-resistant group. qRT-PCR assays showed that patients in the gefitinib-resistant group had a tremendous increase in TS expression compared to patients in the gefitinib-sensitive group (Fig. 3A). After that, we further determined the TS expression level in gefitinib-sensitive lung cancer cells (PC9) and the corresponding gefitinib-resistant PC9/GR cells. In accordance with the above study, we found that both the mRNA and protein expression levels of TS were higher in PC9/GR cells than in PC9 cells (Fig. 3B and 3C). Thus, the results for both EGFR-TKI-resistant NSCLC patients and PC9/GR cells suggested that the upregulation of TS induces gefitinib resistance.

**TS overexpression promotes gefitinib resistance in PC9 cells**

To demonstrate the significance of TS in gefitinib resistance, PC9 cells were transfected with a TS overexpression plasmid (oe-TS). qRT-PCR assays indicated that TS mRNA expression was significantly upregulated in PC9 cells transfected with oe-TS. Likewise, western blot experiments showed that the TS protein level dramatically increased in PC9 cells transfected with oe-TS. Colony formation ability was considerably stronger after overexpression of TS in PC9 cells. CCK8 assays demonstrated that the survival rate of PC9 cells transfected with oe-TS significantly increased under gefitinib treatment at the same concentration. The IC\textsubscript{50} values of gefitinib in PC9 cells transfected with oe-TS were over 50-fold higher than those in oe-NC PC9 cells (7.468 mmol/L, 95% CI, 0.02416 to 0.4698 mmol/L vs. 0.1348 mmol/L, 95% CI, 2.377 to 25.16 mmol/L). Furthermore, ethynyl deoxyuridine (EdU) staining experiments
suggested that the upregulation of TS promoted the proliferation of PC9 cells and enhanced their tolerance to gefitinib (Fig. 4A-E).

Correspondingly, we further transfected PC9/GR cells with sh-NC or two different shRNAs targeting TS (sh-TS a/b). qRT-PCR and western blot assays were performed to validate the TS knockout efficiency (Fig. 4F, 4G). As Fig. 4H shows, the colony number of PC9/GR cells transfected with sh-TS a/b was dramatically decreased. CCK8 assays showed that the knockdown of TS made PC9/GR cells less tolerant to gefitinib. The IC50 value was 6.728 mmol/L (95% CI, 4.894 to 9.381 mmol/L) in PC9/GR cells transfected with sh-TS a and 2.506 mmol/L (95% CI, 1.460 to 3.687 mmol/L) in PC9/GR cells transfected with sh-TS b, in contrast with 12.85 mmol/L (95% CI, 9.722 to 18.14 mmol/L) in PC9/GR cells transfected with sh-NC (Fig. 4H). EdU staining experiments indicated that knockdown of TS inhibited the proliferation of PC9/GR cells and that the inhibitory effect was further enhanced in the presence of gefitinib (Fig. 4J). The above results demonstrate that upregulation of TS might induce gefitinib resistance in NSCLC and that downregulation of TS can restore sensitivity in gefitinib-resistant NSCLC.

**Pemetrexed inhibits TS-mediated thymidylate biosynthesis to restore sensitivity to gefitinib**

Pemetrexed is well known to inhibit TS-mediated nucleotide biosynthesis to exert antitumor effects and is widely used as a preferable chemotherapeutic agent for numerous malignancies. Our previous studies revealed that a proportion of advanced NSCLC patients who experience disease progression during EGFR-TKI therapy could benefit from previously administered targeted agents after cisplatin-pemetrexed chemotherapy. Thus, we hypothesized that pemetrexed might resensitize resistant NSCLC cells to EGFR-TKIs by inhibiting the biological functions of TS. Figure 5A presents a schematic showing the major procedures involved in thymidylate biosynthesis. As crucial materials necessary for DNA repair and cell proliferation, thymidylate nucleotides are synthesized from UMP through a series of complex enzymatic reactions. Abundant metabolic intermediates ensure normal nucleotide metabolism, and nucleotide precursors, such as thymidine and uridine, can promote this biosynthesis pathway. However, pemetrexed inhibits the activity of TS, which is a key enzyme in thymidylate nucleotide biosynthesis, to hamper DNA replication and damage repair.

Therefore, we first demonstrated that pemetrexed can inhibit gefitinib-resistant cell proliferation and that this effect could be rescued by the exogenous nucleotide precursors thymidine and uridine (Fig. 5B). Moreover, combination treatment with thymidine and uridine (NUC) further weakened the cytotoxic effect of pemetrexed in PC9/GR cells (Fig. 5B i). The common phenomenon was observed after knockdown of TS, and NUC supplementation made PC9/GR cells with TS knockout more tolerant to gefitinib (Fig. 5B ii). CCK8 assays illustrated that pemetrexed at the same concentration used for gefitinib treatment exerted an inhibitory effect on the survival of PC9/GR cells and that this phenomenon displayed a dose–response relationship (Fig. 5B iii). However, this anticancer effect was diminished by exogenous supplementation with NUC (Fig. 5B iv). That is, activated nucleotide metabolism promoted gefitinib...
resistance in lung cancer, whereas pemetrexed impaired cellular nucleotide metabolism by inhibiting TS, thereby diminishing the cell proliferation capacity and further resensitizing PC9/GR cells to gefitinib.

**Pemetrexed inhibits TS-mediated thymidylate biosynthesis to induce ROS generation**

Of note, TS utilizes N 10-formyl tetrahydrofolate (N 10-fTHF) as the methyl-group donor to methylate deoxyuridine-5′-monophosphate (dUMP) to deoxythymidine-5′-monophosphate (dTMP), and the latter is subsequently converted to deoxythymidine triphosphate (dTTP), which serves as a crucial precursor for DNA replication and damage repair. Accumulating evidence suggests that impaired dTTP synthesis can lead to intracellular oxidative DNA damage and ultimately induce cell death. We next detected intracellular levels of reactive oxygen species (ROS), which represent the cellular ability to clear oxidative damage and repair DNA damage. We found that intracellular ROS levels were significantly elevated in PC9/GR cells treated with either TS knockdown or pemetrexed. However, after exogenous supplementation with nucleotide precursors, the intracellular ROS level was decreased (Fig. 5C and D i-iii). In contrast, a decreased ROS level was observed in PC9 cells transfected with TS overexpression plasmid (Fig. 5C iv and 5D iv).

**Pemetrexed inhibits TS-mediated thymidylate biosynthesis to promote cellular senescence and SASP**

Given that cellular senescence is widely recognized as a potent tumor-suppressing mechanism and that impaired DNA replication and excessive ROS levels are characteristic of cellular senescence, we examined senescence-associated β-galactosidase (SA-b-Gal) activity in PC9/GR cells after TS knockdown or pemetrexed treatment. SA-b-Gal staining showed that the proportion of SA-b-Gal-positive cells was higher after TS knockdown or pemetrexed treatment (Fig. 6A and 6B). The picture marked with ## in Fig. 6A ii visually displays the morphological changes related to the senescence response in PC9/GR cells, such as enlarged cell size, more irregular protrusions and intensive cytoplasmic vacuolization. In addition, many inflammatory cytokines referred to as the senescence-associated secretory phenotype (SASP), such as IL-1α, IL-1β, IL-6, and IL-8, showed a similar expression pattern in PC9/GR cells (Fig. 6C). However, the effect of cellular senescence triggered by pemetrexed or TS knockdown was eliminated by exogenous supplementation with the nucleotide precursor NUC. These results demonstrate that damaged nucleotide biosynthesis induced by pemetrexed leads to a lack of nucleotide materials and higher intercellular replication stress; thus, cells fail to defend against ROS-based DNA damage and undergo cellular senescence.

**Gefitinib combined with pemetrexed attenuates PC9/GR cell progression**

Based on the above results, we attempted to explore the synergistic anticancer activity of pemetrexed and gefitinib (GP) in gefitinib-resistant cells. EdU staining assays illustrated that GP treatment significantly hampered DNA proliferation to a greater extent than pemetrexed or gefitinib alone. The anticancer
efficacy of GP treatment was attenuated by supplementation with NUC (Fig. 7A). Functionally, we subsequently verified the invasion and migration potential of resistant cells treated with GP. Wound healing assays suggested that the cells displayed a decreased migration rate after GP treatment compared with no treatment or gefitinib or pemetrexed treatment alone (Fig. 7B). Most importantly, the synergistic anticancer effect of the GP combination was rescued by NUC supplementation. Transwell assays showed the same results, indicating that GP can inhibit cell invasion to a greater extent than single drug treatment (Fig. 7C). As expected, NUC partially restored the cell invasion capacity of resistant cells even after GP treatment. These data reveal that pemetrexed can inhibit TS-mediated nucleotide biosynthesis, thereby inhibiting cell proliferation, migration and invasion. Pemetrexed combined with gefitinib still exhibited synergistic antioxidant effects in gefitinib-resistant cells.

Discussion

As reported, there were almost 2.2 million new lung cancer cases and 1.8 million related deaths in 2020, and lung cancer remains the predominant cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for the vast majority of all pathological classifications in malignant lung carcinomas. As an EGFR-targeted small molecule agent, gefitinib has brought unprecedented therapeutic benefits in NSCLC patients with driving mutations in the EGFR gene, including exon 19 deletion and L858R mutation. Unfortunately, the efficacy of gefitinib is inevitably diminished due to the emergence of acquired resistance during the course of treatment. The molecular mechanism determining EGFR-TKI resistance in lung cancer remains incompletely understood, although numerous related mechanisms have been elucidated in previous studies. Exploring novel therapeutic strategies for reversing drug resistance in lung cancer has been a long-standing topic.

In recent years, the pivotal role of metabolic reprogramming in tumorigenesis and cancer development has attracted the attention of a growing number of researchers. Tumor metabolic reprogramming refers to the adaptive changes that occur in cancer cells to meet the excessive nutrient and energy requirements to fuel the bioenergetic and biosynthetic demands of malignant cell proliferation. Net production of intracellular biomass, such as proteins, lipids and nucleotide acids, is essential for successful cell replication and division. The biosynthesis of these diverse macromolecules is achieved through an intricate metabolic network that directs the acquisition and utilization of nutrients from different sources. During cell proliferation, a plentiful pool of pyrimidine and purine nucleotides is of particular importance owing to the demand for ribosomal RNA synthesis, duplication of the genomic DNA and maintenance of the RNA transcriptome. More importantly, nucleotide biosynthesis is intimately associated with multiple metabolic processes. The pathways leading to pyrimidine and purine nucleotide production require energy and biomass input from various metabolic processes, including the tricarboxylic acid (TCA) cycle, one-carbon-unit cycle, electron donors in the electron transport chain (ETC), and the pentose phosphate pathway. Given these extensive metabolic interactions, many antimetabolites exploiting the nucleotide biosynthesis process are currently used in anticancer strategies and are considered novel treatments to specifically impair cell proliferation. Zhou et al. illustrated that inhibiting CDC-like kinase 3 (CLK3), a dual
specificity protein kinase regulated by C-MYC, can block the progression of cholangiocarcinoma by reprogramming nucleotide biosynthesis\(^ {41}\). The inhibition of deoxyuridine 5′-triphosphate nucleotidohydrolase made triple-negative breast cancer (TNBC) cell lines sensitive to fluoropyrimidines and anthracyclines by decreasing nucleotide pools and inducing DNA damage to enhance the efficacy of these chemotherapeutic agents\(^ {42}\). Halbrook et al. focused on the molecular mechanisms of gemcitabine resistance. They demonstrated that tumor-associated macrophages (TAMs) programmed by pancreatic ductal adenocarcinoma (PDA) could release a spectrum of pyrimidine species that had specific potential to competitively inhibit the level of gemcitabine uptake and metabolism\(^ {43}\). The antimitobolic agents fluoropyrimidines can be converted into 5′-fluorodeoxyuridine monophosphate (FdUMP). Consequently, FdUMP forms a stable complex with the thymidylate synthase (TS) enzyme and inhibits its function, thereby preventing the de novo synthesis of dTMP\(^ {44}\). Pemetrexed, a multitargeted antifolate that inhibits folate-dependent enzymes involved in de novo thymidine and purine nucleotide biosynthesis, such as TS and GART, has been widely used as the preferred chemotherapeutic agent in NSCLC\(^ {45,46}\). Several studies have indicated that high TS expression is associated with pemetrexed resistance\(^ {47-50}\). However, these observations cannot fully explain the phenomena observed in clinical NSCLC patients\(^ {51}\), hinting that other mechanisms responsible for pemetrexed resistance might exist.

In our study, we illustrated the role of TS-mediated thymidylate nucleotide biosynthesis in the development of gefitinib resistance. Pemetrexed, a classical TS-targeted inhibitor, showed the potential to promote ROS generation and cellular senescence in gefitinib-resistant NSCLC cells in our study. The lack of thymidine nucleotide production after TS knockdown or pemetrexed treatment diminished cell proliferation, migration and invasion. Furthermore, this anticancer effect was reversed by exogenous nucleotide precursors (thymidine and uridine). The therapeutic strategy of using gefitinib in combination with pemetrexed exhibited an excellent anticancer effect in gefitinib-resistant cell lines, indicating that pemetrexed might reverse gefitinib resistance by damaging TS-mediated thymidine nucleotide metabolism. Our results were consistent with the outcomes of several previous studies. As Ken Takezawa et al. demonstrated, gefitinib itself possesses the potential to inhibit TS protein, while this ability is lost during the development of gefitinib resistance\(^ {52}\). These study findings support the results of our study indicating that the combination of pemetrexed and gefitinib hampers gefitinib-resistant cell progression by synergistically damaging the thymidine nucleotides synthesized by TS. Zhang et al. discovered that upregulation of nucleotide production triggers oncogenic transformation in murine and human cells\(^ {22}\). An exogenous nucleoside mix of four ribonucleosides (A, G, U, and C) and four deoxyribonucleosides (dA, dG, dT, and dC) promoted the colony formation ability of immortalized human mammary epithelial (PHMLEB) cells. Depletion of the lncRNA lincNMR damaged nucleotide metabolism via the YBX1-RRM2 axis, which targets various key enzymes in nucleotide metabolism (RRM2, TK1, and TS), thereby leading to diminished tumor growth and inducing senescence in more cells\(^ {53}\). The authors observed that the supply of exogenous dNTP pools in the cell culture environments rescued the impact of lincNMR downregulation on the cell proliferation phenotype. Furthermore, another study based on a mouse lung cancer model found a correlation between the TS expression level and epithelial-to-mesenchymal
transition (EMT) phenotypes. They performed qPCR and GSEA to identify several EMT transcription factors (HM A2, HOXC6, SNAI2, SOX9, ARNTL2 and SHOX6) in NSCLC samples with upregulated TS expression. In summary, all these results point to a common conclusion, specifically, that nucleotide metabolism occupies the predominant position in tumorigenesis and cellular transformation.

In conclusion, our present study illustrated that NSCLC patients with higher TS expression are more inclined to develop resistance during EGFR-TKI targeted therapy. Pemetrexed can potentially reverse EGFR-TKI resistance by inhibiting TS-mediated thymidylate nucleotide metabolism and inducing senescence in cancer cells. Concomitant pemetrexed administration can retard the evolution of resistance or restore the sensitivity of cancer cells to EGFR-TKIs. This study provides a compelling rationale for selecting an advantageous population for targeted agents combined with chemotherapy, which has significant clinical implications and far-reaching practical value.

**Materials And Methods**

Patient characteristics and tissue samples for survival analysis

A total of 140 patients were enrolled in this study. Information about EGFR mutations, TS polymorphism variants and the clinicopathological characteristics of NSCLC patients is summarized in Table 1. For pathological sample collection, a total of 24 LUAD patients harboring either EGFR exon 19DEL or L858R were enrolled in the study. The project was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, China), and written informed consent was obtained from all patients.

Cell culture

The human NSCLC cell line PC9 (EGFR exon 19 deletion) and gefitinib-resistant cell line PC9/GR were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco-BRL) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA) at 37°C in incubators with 5% CO₂.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from tissues or cells using TRIzol reagent (Thermo Fisher Scientific, USA). Then, 1.0 µg of isolated RNA was reverse-transcribed to cDNA with PrimeScript™ RT reagent (Takara, Japan) according to the manufacturer's instructions. qRT-PCR analysis was performed with SYBR Green (Takara, Japan). In accordance with the manufacturer's instructions, qRT-PCR and data collection were carried out with a StepOnePlus RT-PCR system (Applied Biosystems, USA). GAPDH was used as the normalization control. The sequences of all primers used in this study are listed in Supplementary Materials Table S1.

Western blot analysis and antibodies
Cell proteins were lysed with RIPA buffer (Sigma, USA) and subsequently subjected to ultrasonic lysis. A BCA Protein Assay kit (Pierce, USA) was used to quantify the lysate protein concentration. Total protein was separated in a 12.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, USA). After incubation with antibodies and washing, signals were detected using a chemiluminescence system (Bio-Rad, USA). The primary antibodies used in this study were anti-rabbit TS (850 µg/mL, 1:2000 dilution, Proteintech, cat no. 15047-1-AP) and anti-β-actin (1000 µg/mL, 1:2000 dilution, Proteintech, cat no. 66009-1-IG).

Transfection of cell lines

The empty vector plasmid (oe-NC), TS-overexpressing plasmid (oe-TS) and two different shRNAs against TS (shTS a/b) were synthesized by Genscript Corporation (Nanjing, China). The ORF Sequence Information for TS and the targeted sequences for sh-TS a/b are listed in Supplementary Materials Table S2. Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used for cell transfection according to the manufacturer's instructions. After transfection for 48 hours, the cells were harvested and processed for further experiments.

CCK8 assay

The survival rate of cells treated with gefitinib was estimated using CCK8 assays (Selleck, Shanghai, China). PC9 cells transfected with empty plasmid vector or oe-TS and PC9/GR cells transfected with sh-NC or sh-TS a/b were seeded into 96-well plates at 3000 cells per well and exposed to different concentrations of gefitinib (MedChemExpress, China) for 48 hours. After treatments, 10 µL of CCK8 was added to each well and incubated with the cells at 37°C for 1 hour. The absorbance was measured using an enzyme microplate reader at 450 nm. Each treatment was applied in triplicate, and three independent experiments were performed.

Colony formation assay

Two cell lines were collected after the abovementioned treatments and reseeded into six-well plates at 1000 cells/well. Then, the cells were treated with the corresponding drug or PBS for 24 hours. The medium was changed every 3 days. After incubation for 14 days, the cells were fixed with paraformaldehyde (4%) for 30 min and subsequently stained with crystal violet (0.5%) for 30 min. After a brief wash with PBS, the plates were allowed to dry at room temperature, and colonies were photographed. Each experiment was performed in triplicate.

Ethynyl deoxyuridine (EdU) staining analysis

A BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime, Shanghai, China) was used to assess cell proliferation. The cells were seeded in 96-well plates at a density of 5000 cells/well. After transfection or different drug treatments, 10 µM EdU labeling medium was added to the 96-well plates and incubated with the cells for 2 h at 37°C under 5% CO₂. After that, the cells were treated with 4%
paraformaldehyde and 0.3% Triton X-100 and subsequently stained with anti-EdU working solution. Hoechst 33342 was used to label the cell nuclei. The percentage of EdU-positive cells was estimated after fluorescence microscopy analysis.

Wound-healing assay

The indicated cells were cultured on 6-well plates at $5 \times 10^{5}$ cells per well and grown to total confluence for 24 hours. A pipette tip (1000 µL) was used to scratch three vertical lines in each well. At 0 and 24 h, images of the scratches were taken. The healing area of the scratches was calculated using ImageJ software. The experiment was performed in triplicate, and the mean value was calculated. Five fields (100× magnification) of view were randomly selected for imaging and counting.

Transwell assay

In total, $5 \times 10^{4}$ cells in serum-free medium were placed into the upper chamber of a transwell assay insert (8-µm pore size, Millipore). Medium containing 10% FBS and 1 µmol/L gefitinib and/or pemetrexed was added to the lower chamber. Cells that invaded through the membrane were fixed with 4% paraformaldehyde for 15 minutes and stained with crystal violet for 10 minutes, while cells that had not migrated or invaded the membrane were removed with cotton swabs. Cells were imaged using an IX71 inverted microscope (Olympus, Tokyo, Japan) at 100× magnification.

ROS detection

Cells were treated with 10 µM DCFDA (2’,7’-dichlorodihydrofluorescein) for 30 min in the dark. After two washes with PBS and trypsinization for collection, the cells were immediately analyzed via flow cytometry. Intracellular DCFDA signals were measured with a flow cytometer (FACScan, BD Biosciences). ROS levels are shown as relative fluorescence units (RFU) at Ex/Em = 488/525 nm.

Senescence-associated β-Gal assay

Cells transfected with sh-TS a/b or treated with pemetrexed for 48 hours were collected and replated in 12-well plates. The chromogenic β-gal substrate X-gal (C0602, Beyotime Biotechnology, Shanghai, China) was used to stain the fixed cells for 8 hours at 37°C. The cells were washed three times with PBS, and microscopy images were taken via bright field microscopy at 200× magnification.

 Statistical analysis

The data were analyzed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). PFS was estimated using Kaplan–Meier analysis, and differences were determined with a log-rank test. Differences between groups were compared using a $t$ test, and a $P$ value $< 0.05$ was considered statistically significant.

Declarations
**Author contributions**

Y.C. and C.Z. designed the study, conducted experiments, and analyzed the data. S.D.J. and J.L. provided the tissue samples and analyzed the clinical pathological data. Y.C. and J.L.D. contributed to laboratory measurements and data analysis. Y.C. wrote the manuscript. R.H.G. and Z.H.Z. contributed to supervision of the study and evaluated the manuscript. All authors read and approved the final manuscript.

**Conflicts of interest**

The authors report no competing interests related to this study.

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Figure 1

The level of TS in LUAD and multiple other solid malignancies is significantly upregulated and correlated with decreased disease-free survival (DFS) and overall survival (OS). A, Thymidylate synthetase (TYMS or TS) mRNA expression in multiple cancer types. B, Kaplan–Meier survival analysis comparing high and low TS expression in both the pancancer cohort and the lung adenocarcinoma (LUAD) cohort in GEPIA. C, The protein level of TS in LUAD tissues and adjacent normal lungs. D, The metabolic pathways upregulated in gefitinib-resistant datasets (GSE112274 and GSE114647) with high TS expression, displayed with gene set enrichment analysis (GSEA) using GSEA software. *p < 0.05.
Figure 2

Kaplan–Meier survival analysis of PFS in 97 LUAD patients accepting EGFR-TKI treatment, comparison of the TS wild-type group and the 6-bp group.

*Figure 3*

Relative TS expression in NSCLC biopsy tissues from NSCLC patients and in cell lines. A, TS expression in NSCLC tissues from patients who had never been administered gefitinib (sensitive group) compared to patients who developed resistance during gefitinib treatment (resistant group). The TS expression level in
the two groups was measured using qPCR and normalized to GAPDH expression. The ΔCt value was calculated by subtracting the GAPDH Ct value from the TS Ct value. Smaller ΔCt values represent higher TS mRNA levels. B and C, qRT–PCR and western blot assays showing TS expression in PC9 and PC9/GR cells. The results represent the average of three independent experiments. **p < 0.01.

Figure 4

TS overexpression promoted gefitinib resistance in PC9 cells, and TS knockdown reversed the sensitivity to gefitinib (GEF) in PC9/GR cells. A and B, The TS mRNA and protein expression levels in PC9 cells transfected with oe-TS plasmid, determined using qRT–PCR and western blotting assays, respectively. C, CCK8 assays to measure the IC50 value of gefitinib in transfected PC9 cells treated with gefitinib at gradient concentrations for 48 h. D, Colony formation experiments to estimate the proliferation of PC9 cells transfected with the oe-TS plasmid and treated with GEF (1 μM). E, EdU (green) or DAPI (blue) staining assays were conducted to determine the DNA synthesis capacity of the transfected PC9 cells under the same gefitinib treatment (1 μM) for 48 h. Figure F to J present the mRNA and protein detection, CCK8, colony formation and EdU staining results in PC9/GR cells transfected with sh-TS a/b, under the same treatment conditions applied in PC9 cells. **p < 0.01, ***p < 0.001.
Figure 5

PEM induced ROS generation and cellular senescence to enhance the sensitivity of PC9/GR cells to GEF through TS-mediated thymidylate metabolism. **A**, Schematic showing the major procedures involved in thymidylate metabolism. UMP, uridine monophosphate; TMP, thymidine monophosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; TS, thymidylate synthase. **B**, PEM restored the sensitivity of PC9/GR cells to GEF by inhibiting TS-mediated thymidylate metabolism. **B i**, CCK8 assays showed the cell proliferation rate after culture with PEM and/or exogenous nucleotide precursors (uridine and thymidine) or their cocktail (NUC). **B ii**, The proliferation ability of PC9/GR cells transfected with sh-TS was rescued by NUC supplementation. **B iii**, CCK8 assays were used to measure the gefitinib IC50 in PC9/GR cells treated with GEF and PEM for 48 h. **B iv**, CCK8 assays were used to estimate the viability of PC9/GR cells cotreated with GEF and PEM for 48 h. **C and D**, PEM or TS knockdown induced ROS generation. **C i to C iii**, PC9/GR cells transfected with PEM or sh-TS for 24 h and subsequently treated with NUC for 24 h. Then, the ROS level was detected with 10 μM DCFH-DA staining and flow cytometry. **C iv**, PC9 cells transfected with oe-TS and subsequently stained with 10 μM DCFH-DA, then the ROS level was detected by flow cytometry. **D**, Histograms constructed based on the quantitative analysis of fluorescence intensity. The fluorescence signal was monitored at Ex/Em = 488/525 nm. RFU, relative fluorescence units. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 6

Pemetrexed inhibits TS-mediated thymidylate metabolism to promote cellular senescence and the senescence-associated secretory phenotype (SASP). **A and B**, SA-b-Gal staining assays in PC9/GR cells after the abovementioned treatments. The blue cells represent senescent cells, and the number of senescent cells was counted with ImageJ software. **C**, The mRNA levels of SASP-associated genes were measured according to $\triangle\triangle$Ct values. ***p < 0.001.
Figure 7

GEF and PEM combination (GP) treatment synergistically hampered resistant cell DNA proliferation and attenuated cell migration and invasion. 

A, EdU staining assays illustrating DNA proliferation in PC9/GR cells treated with GEF, PEM, GP or GP+NUC for 48 h. The number of proliferating cells was counted with ImageJ and is displayed as the percentage of proliferating cells. 

B, The migration of PC9/GR cells treated with GEF, PEM, GP or GP+NUC for 48 h was evaluated with wound healing assays. The migration rate = healing area of scratch/initial area of scratch × 100%. 

C, The invasion capacity of PC9/GR cells under the same treatments was detected using transwell assays. 

D, Histogram analysis showing the results from three independent experiments. ***, p < .001.

Supplementary Files

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- supplementarymaterials.pdf
- table1.pdf