Am J Cancer Res 2023;13(8):3517-3530
www.ajcr.us /ISSN:2156-6976/ajcr0151244

Original Article

DNMT3A governs tyrosine kinase inhibitors responses through IAPs and in a cell senescence-dependent manner in non-small cell lung cancer

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Received May 16, 2023; Accepted July 15, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Patients with non-small cell lung cancer (NSCLC) treated with tyrosine kinase inhibitors (TKIs) inevitably exhibit drug resistance, which diminishes therapeutic effects. Nonetheless, the molecular mechanisms of TKI resistance in NSCLC remain obscure. In this study, data from clinical and TCGA databases revealed an increase in DNMT3A expression, which was correlated with a poor prognosis. Using NSCLC organoid models, we observed that high DNMT3A levels reduced TKI susceptibility of NSCLC cells via upregulating inhibitor of apoptosis proteins (IAPs). Simultaneously, the DNMT3A^{high} subset, which escaped apoptosis, underwent an early senescent-like state in a CDKN1A-dependent manner. Furthermore, the cellular senescence induced by TKIs was observed to be reversible, whereas DNMT3A^{high} cells reacquired their proliferative characteristics in the absence of TKIs, resulting in subsequent tumour recurrence and growth. Notably, the blockade of DNMT3A/IAPs signals enhanced the efficacy of TKIs in DNMT3A^{high} tumour-bearing mice, which represented a promising strategy for the effective treatment of NSCLC.

Keywords: Tyrosine kinase inhibitors, DNMT3A, non-small cell lung cancer, inhibitor of apoptosis proteins, cellular senescence

Introduction

Lung cancer is one of the most prevalent and aggressive malignancies worldwide, with over 1.8 million deaths in 2021 [1]. Despite therapeutic advances for non-small-cell lung cancer (NSCLC), approximately 85% of all lung cancer cases, which represent a subtype of lung cancer, fail to achieve long-term remission [2]. Moreover, the presence of activating mutations in the kinase domain of epidermal growth factor receptor (EGFR) in patients with NSCLC provides a rationale for the clinical application of EGFR tyrosine kinase inhibitors (TKIs, such as gefitinib and erlotinib) [3, 4]. Despite promising therapeutic responses initially, patients ultimately acquire resistance to TKIs in clinical settings, leading to tumour recurrence [5]. Therefore, unravelling the molecular mechanism underlying TKI resistance is vital to the development of therapeutic regimens for patients with NSCLC.

The development of TKI resistance is a complex process mediated by multiple determinants. One of the well-investigated mechanisms is the T790M mutation, wherein threonine is replaced with methionine at position 790 in exon 20 of *EGFR*, which inhibits the binding capacity of TKIs [6]. Additionally, systematic genetic analy-

ses revealed mutations in the *PIK3CA* gene in resistant NSCLC samples, which played a role in reducing the susceptibility to gefitinib in HCC827 cells [7]. Additionally, evidence also shows that DNA methyltransferases (DNMTs) participate in regulating therapy resistance. DNMTs are a conversed family of DNA-modifying enzymes that mediate the addition of a methyl group to 5-cytosine. Canonical DNMT enzymes comprise DNMT1, DNMT3A and DNMT3B in mammals, which are responsible for maintaining and establishing DNA methylation patterns [8]. Abnormal DNMT family members have been reported in various human diseases, including neoplastic conditions [9]. In breast cancer, elevated DNMT3B expression correlated with advanced clinical stages and short overall survival [10]. Similarly, Han et al. reported that DNMT3A served as a part of a negative feedback loop that regulated chemoresistance in ovarian cancer cells [11]. Additionally, exome sequencing identified *DNMT3A* as one of the driver mutations in the pathogenesis of chronic myeloid leukaemia and TKI failure [12]. Therefore, the detection of DNMTs in tumour samples might present a promising avenue to monitor treatment response.

Cell apoptosis, a vital biological process, is essential for maintaining normal tissue development and cellular homeostasis. IAPs, acting as pivotal regulators of cell death, play a crucial role in tumor development [13]. IAPs constitute a highly conserved protein family, which includes cIAP1, cIAP2, XIAP, ML-IAP, and NAIP. These IAPs maintain tissue stability by orchestrating the balance between cell survival, proliferation, and apoptosis through diverse mechanisms [14]. Numerous studies have demonstrated a close correlation between abnormal expression of IAPs and tumor progression. Within tumor cells, IAPs have the ability to form complexes with caspases, thereby inhibiting their activity and impeding the apoptotic process, resulting in the poor prognosis in patients [15]. Elevated levels of IAP expression have been associated with the heightened tumor invasion, metastasis, and drug resistance. Additionally, excessive expression of IAPs has been linked to unfavorable prognoses in cancer patients [16]. Nevertheless, the association between DNMTs and IAPs remains ambiguous.

In this study, we aim to explore the role DNMT3A during NSCLC progression, wherein could be a predictive market of TKI response. Utilising the TCGA database, elevated DNMT3A expression was observed to present a poor outcome in patients with NSCLC. Moreover, subsequent experiments indicated that DNMT3A exacerbated the resistance to TKIs via upregulating inhibitor of apoptosis proteins (IAPs), which was further validated in organoid models derived from patients with NSCLC, which may aid in the elaboration of therapeutic strategies for NSCLC.

Materials and methods

Cell culture and organoids

Human NSCLC cancer cell line A549, NCI-H1299 and NCI-H3255 cells were obtained from the American Type Culture Collection and maintained in a Roswell Park Memorial Institute (RPMI) complete 1640 culture medium (Thermo Fisher, USA) containing 10% foetal bovine serum (Thermo Fisher, USA) in a 5% CO_2 incubator (Thermo Fisher, USA) at 37°C. For DNMT3A overexpressed NCI-H3255 cell lines, PCMV plasmids were transfected using Lipofect8000 (Thermo Fisher, USA) to induce gene overexpression. pCMV-DNMT3A-3 × FLAG vectors were obtained from Cyagen (China). The protein levels of DNMT3A in NCI-H3255 cells were assessed using western blotting.

Patient-derived organoids (P1 and P2 cells) were isolated from two patients with NSCLC. Briefly, fresh NSCLC tissues were minced into 1-3 mm^3 pieces, which were digested in an RPMI complete 1640 culture medium (Thermo Fisher, USA) containing type IV collagenase (Sigma, UK) and DNase I (Sigma UK) for 90 min. Following this, cell suspensions were treated with red blood cell lysis buffer and seeded in a 3D Matrigel containing growth factors (Coring, USA) with a determined cultured medium (1 ml 1640: 1 ml F12 medium, 0.002 ml 100 × low-glutamate mixture, Thermo Fisher, USA). The two organoids derived from patient #1 (P1, EGFR L858R, $DNMT3A^{low}$) and patient #2 (P2, EGFR L858R, $DNMT3A^{high}$) displayed proliferative characteristics in two months. Haematoxylin and eosin (H&E) staining and immunostaining of NSCLC marker carcinoembryonic antigen (CEA) were performed on days 5, 10 and 15.

Clinical specimens

Transcript profiles and clinical data of 743 NSCLC tissues and 397 normal tissues were

obtained from the Cbioportal and Sanger databases (normalized values, FPKM). NSCLC patients were divided into high and low DNMT3A expression groups according to the medium expression of DNMT3A. Paraffin sections from 30 patients with NSCLC were obtained from Shanghai Pulmonary Hospital, Tongji University School of Medicine, which were divided into recurrent and non-recurrent groups based on the follow-up visit (3 years) data. Written informed consents of all involved patients have been submitted, and this study was conducted according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects, the Declaration of Helsinki, and approved by the ethics committee of the Shanghai Pulmonary Hospital, Tongji University School of Medicine (K17-007).

Cell proliferation and migration

P2 cells and DNMT3A overexpressed NCI-H3255 cells were pre-treated with phosphate-buffered saline (PBS), erlotinib (2 μM, Sigma, UK) and afatinib (5 mM, Sigma, UK) for 48 h. Subsequently, cells were seeded in 96-well plates (2000 cells per well) and a cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Biyuntian, China) following the manufacturer's protocol.

Cell migration assay was performed using 8 μm pore membrane Transwell plates (Corning, USA) with Matrigel. 1×10^5 A549 or NCI-H1299 cells were seeded into the upper chambers with a serum-free medium. Medium containing 10% foetal calf serum was added into the lower chamber as a chemoattractant. After 24 h, the migrating cells in the lower chamber were stained with 0.1% crystal violet and visualised using an inverted microscope.

Cell apoptosis and cell cycle assay

P1, P2, vector and DNMT3A overexpressed NCI-H3255 cells were treated with PBS, erlotinib (2 μM), afatinib (5 μM), SM-1295 (20 nM, MedChemEexpress, USA), MK-2206 2HCl (10 nM, MedChemEexpress, USA), SCH772984 (50 nM, MedChemEexpress, USA) or in combination for 48 h. Following this, the cells were collected for cell apoptosis and cell cycle assays using a flow cytometer (Becton, Dickinson and Company, USA). Apoptosis was determined using an Annexin V-FITC/PI-PE cell apoptosis assay kit (Becton, Dickinson and Company, USA). The cell cycle was analysed using a PI/RNase staining buffer (Becton, Dickinson and Company, USA).

β-Galactosidase staining assay

P1, P2, vector and DNMT3A overexpressed NCI-H3255 cells were treated with PBS, erlotinib (2 μM), afatinib (5 μM), SM-1295 (20 nM) or MK-2206 2HCl (10 nM) for 48 h. Following this, the cells were rinsed thrice with PBS, and senescence-associated β-Galactosidase (SA-β-Gal) staining was performed using a β-Galactosidase Staining Kit (Solarbio, China) per the manufacturer's protocol. Random fields were selected to observe SA-β-gal positivity under a photon microscope (Leica, Germany).

Real-time quantitative polymerase chain reaction (PCR)

Total RNA of P1, P2, vector and DNMT3A overexpressed NCI-H3255 cells was extracted using Trizol reagent (Thermo Fisher, USA). Samples were then reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Takara, Japan). SYBR Green (Takara, Japan) was used for PCR per the manufacturer's instructions. The sequences of primers used in this study are designed according yo the guidance of PrimerBank.

Western blotting

P1, P2 and NCI-3255 cells were prepared using RIPA lysis buffer containing protease and phosphatase inhibitors (Biyuntian, China). A total of 20 μg proteins were loaded per well and incubated overnight at 4°C with primary antibodies. The primary antibodies included anti-DNMT3A (ab188470, Abcam, UK), anti-c-IAP1 (ab108361, Abcam, UK), anti-CDKN1A (ab-109520, Abcam, UK) and anti-β-actin (ab8226, Abcam, UK). The secondary antibodies included goat anti-Rabbit IgG HRP and goat anti-mouse IgG HRP (Thermo Fisher, USA).

Immunostaining assay

A total of 30 NSCLC samples were randomly collected and divided into recurrent and non-recurrent groups. Deparaffinized NSCLC sections were blocked and then incubated overnight at 4°C with primary antibodies (anti-DNMT3A (ab188470, Abcam, UK) and anti-c-IAP1 (ab108361, Abcam, UK)). Following this, the

sections were incubated with secondary antibodies, which included goat anti-Rabbit IgG HRP and goat anti-mouse IgG HRP (Thermo Fisher, USA). Nuclei were counterstained with H&E. The expression intensity of DNMT3A and c-IAP1 in NSCLC tissues was analysed using the Image Pro Plus 6.2 software (Media Cybernetics, USA).

Animal experiments

NOD-SCID mice (6-week-old) were obtained from HFK Bio (China). For tumorigenic assays, P1 and P2 cells were pretreated with PBS or erlotinib (2 μM) for 48 h. Following this, 1×10^5 cells (encapsulated in 50 μl Matrigel) were subcutaneously implanted into immunodeficient mice. On days 10, 20 and 30, the tumour formation was evaluated. For anti-cancer effects evaluation, 1×10^6 P1 or P2 cells (encapsulated in 50 μl Matrigel) were subcutaneously implanted into immunodeficient mice. On day 15, mice were treated with PBS, erlotinib (5 mg/kg), afatinib (5 mg/kg), SM-1295 (2 mg/kg) or in combination twice a week via a tail vein injection. Tumour tissues (n = 3 in each group) were isolated for Annexin V/PI staining, TUNEL, cell cycle and SA-β-gal activity analysis on day 25. Tumour volume and survival (n = 5 in each group) were recorded daily. Tumour volume was calculated as follows: tumour volume = length × width × width^2/2. All animal experiments were conducted according to standard procedures that were approved by the Animal Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical significance was analysed via the GraphPad Prism 5.2 software (Graphpad company, USA) using Student's t-test and ANOVA. Comparisons of survival assay were analysed by Kaplan-Meier survival analysis. $P<0.05$ was deemed as statistically significant. Each experiment was repeated at least thrice.

Results

Elevated DNMT3A expression predicted poor prognosis in patients with NSCLC

To validate the clinical relevance of DNMT3A and the prognosis of patients with NSCLC, we then assessed the transcriptome expression of DNMT3A in 513 NSCLC tissues compared with 397 normal tissues from the TCGA database, wherein DNMT3A was upregulated in NSCLC tissues compared to normal tissues (**Figure 1A**). Importantly, patients with high DNMT3A levels exhibited a shortened survival time compared to those with low DNMT3A levels (**Figure 1B**), indicating that increased DNMT3A predicted a poor prognosis in patients with NSCLC. We also speculated that DNMT3A directly stimulated cancer cell proliferation/migration to module tumour progression/metastasis, which could result in poor prognosis in patients. To verify this, we evaluated the expression of DNMT3A at the mRNA level in patients from stages T1-T4. However, no significant difference in DNMT3A expression was observed between the groups (**Figure 1C**). Additionally, a similar DNMT3A expression level was observed between non-metastatic and metastatic patients with NSCLC using the TCGA database (**Figure 1D**). To further elucidate the role of DNMT3A in NSCLC cell proliferation and migration, we silenced DNMT3A in A549 and NCI-H1299 cell lines and then determined cell proliferation and migration. However, the silencing of DNMT3A did not significantly affect cell proliferation (**Figure 1E**) and migration (**Figure 1F**). These results suggested that DNMT3A did not directly module tumour growth and metastasis to affect patient prognosis. Thus, we speculated that DNMT3A could regulate TKI resistance, resulting in an altered response to NSCLC therapy. We examined DNMT3A expression in NSCLC tumour tissues from clinical patients. All patients were treated with standard TKI therapy and divided into recurrent and non-recurrent based on a 5-year follow-up visit. Notably, increased DNMT3A levels were observed in tumour tissues from recurrent patients with NSCLC compared to those in the non-recurrent group (**Figure 1G**). Overall, these results indicated that accumulated DNMT3A predict a poor prognosis in patients with NSCLC and might be involved in the regulation of TKIs resistance.

DNMT3A reduced TKI sensitivity in DNMT3A^{high} organoids

To validate the role of DNMT3A in regulating TKI sensitivity during NSCLC progression, we established tumour organoids derived from patients with NSCLC. Two organoids derived from

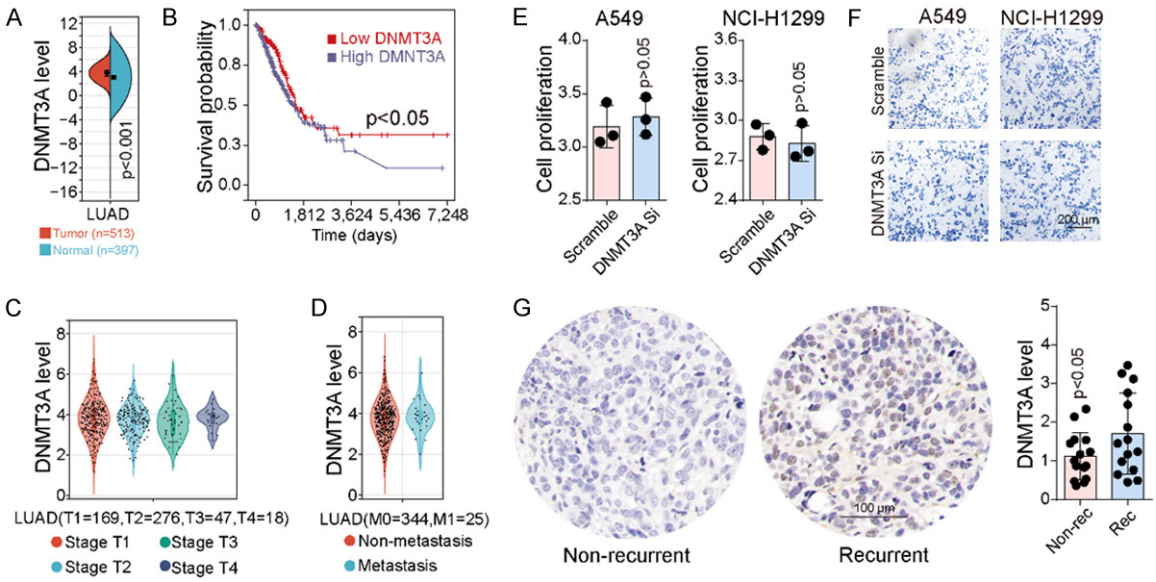

Figure 1. Elevated DNMT3A expression predicted poor prognosis in patients with non-small cell lung cancer (NSCLC). A. mRNA expression of DNMT3A in normal tissues (n = 397) and NSCLC tissues (n = 513) derived from TCGA database. The expression level, tumor vs normal, 3.77 vs 3.05, log2 (x+0.001). B. Kaplan-Meier overall survival curve of the high and low expressions of DNMT3A in patients with NSCLC (n = 513) derived from TCGA database. C. mRNA expression of DNMT3A in patients with NSCLC of T1-T4 derived from TCGA database (n = 510). D. mRNA expression of DNMT3A in non-metastatic and metastatic patients with NSCLC derived from TCGA database (n = 369). E. Cell proliferation of A549 or NCI-H1299 cells treated with scramble or DNMT3A siRNAs for 72 h. F. Transwell assay of A549 or NCI-H1299 cells treated with scramble or DNMT3A siRNAs for 24 h. Scale bar = 200 μm. G. Representative images of DNMT3A immunohistochemical staining in NSCLC tissues from recurrent and non-recurrent patients (n = 30, scale bar = 100 μm).

patient #1 (P1, EGFR L858R, DNMT3A^{low}) and patient #2 (P2, EGFR L858R, DNMT3A^{high}) were established in Matrigel and displayed proliferative characteristics in two months. An immunostaining assay was performed to examine the expression of NSCLC marker CEA in organoids (**Figure 2A**). Meanwhile, the DNMT3A expression at the protein level was determined using western blotting (**Figure 2B**). Subsequently, these cells were treated with TKIs, erlotinib and afatinib. Notably, cell apoptosis induced by TKIs was observed to be significant in P1 cells compared to P2 cells (**Figure 2C**). To further validate these results, we overexpressed DNMT3A in NCI-H3255 (EGFR L858R) (**Figure 2D**) and then treated the cells with erlotinib and afatinib. Consistently, DNMT3A overexpression induced TKI resistance in NCI-H3255 cells (**Figure 2E**). These results suggested the role of DNMT3A in regulating TKI response in NSCLC. Following this, we assessed the influence of DNMT3A in TKI response in NSCLC cells *in vivo*. Thus, P1 and P2 cells were subcutaneously implanted into immunodeficient mice and treated with erlotinib. Significant tumour suppressive effects were observed in P1-bearing mice (**Figure 2F**). Notably, annexin V/PI staining demonstrated more apoptotic cells in tumour tissues derived from P1-bearing mice, compared to the P2 group (**Figure 2G**). Together, these results suggested that DNMT3A increased TKI sensitivity in NSCLC cells.

DNMT3A modulated IAPs expression to suppress TKI-induced cell apoptosis

Previous studies have illustrated that DNMT3A-knockout oocytes exhibited a loss of methylation at dispersed IAPs repeats. Notably, IAPs serve as the inhibitors of apoptotic proteins, which play a central role during drug resistance development in cancer [17-19]. Thus, the transcriptome expression of DNMT3A and IAPs encoding genes (BIRC1-7) in 230 NSCLC tissues was downloaded from the TCGA database, and correlation analysis was performed between DNMT3A and IAPs encoding genes. Notably, DNMT3A expression was positively correlated with BIRC2, which encodes a c-IAP1 anti-apoptotic protein (**Figure 3A**). To further

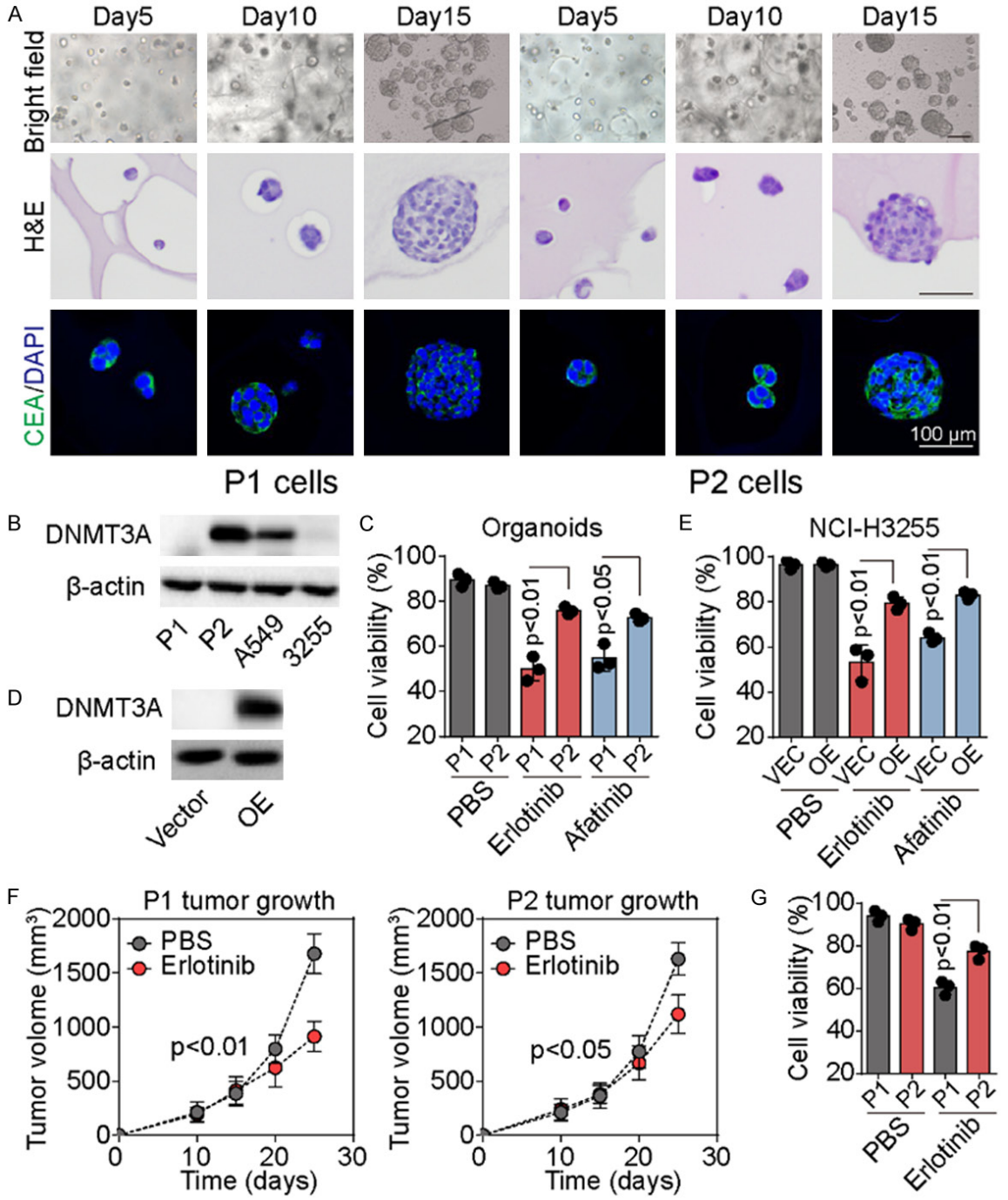

Figure 2. DNMT3A reduced tyrosine kinase inhibitors (TKIs) sensitivity in non-small cell lung cancer (NSCLC) patient-derived organoid model. A. Representative images of P1 and P2 cells in 3D Matrigel on days 5, 10 and 15. Organoids were assessed using haematoxylin and eosin staining and immunostaining of NSCLC marker CEA. Scale bar = 100 μm. B. Western blotting of DNMT3A in P1, P2, A549 and NCI-H3255 cells. C. Cell apoptosis of P1 and P2 cells treated with phosphate-buffered saline (PBS), erlotinib (2 μM) and afatinib (5 μM) for 48 h was assessed using an Annexin V/PI assay. D. Western blotting of DNMT3A in vector and DNMT3A overexpressed NCI-H3255 cells. E. Cell apoptosis of vector and DNMT3A overexpressed cells treated with erlotinib (2 μM) and afatinib (5 μM) for 48 h using an Annexin V/PI assay. F. P1 and P2 cell-bearing mice were treated with PBS or erlotinib (2 μM in 50 μl PBS) via an intratumor injection twice a week. Tumour volumes were recorded (n = 5 in each group). G. P1 and P2 cell-bearing mice (day 15) were treated with PBS or erlotinib (2 μM in 50 μl PBS) via an intratumor injection. After 48 h, tumour cells were isolated and cell apoptosis was determined (n = 3 in each group).

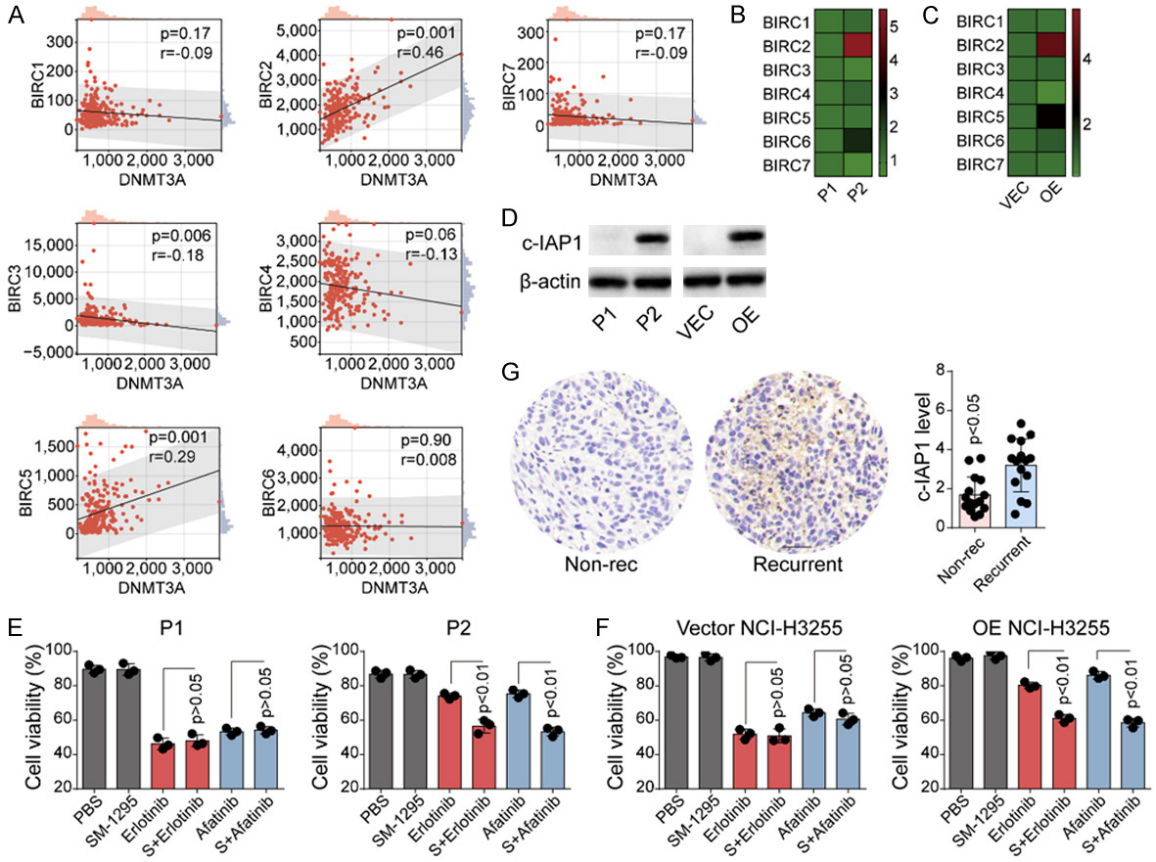

Figure 3. DNMT3A modulated IAPs expression to suppress tyrosine kinase inhibitors (TKIs)-induced cell apoptosis. A. The correlation analysis between DNMT3A expression and BIRCs (BIRC1-7) expression in 230 patients with non-small cell lung cancer (NSCLC) from the TCGA database. B. Heatmap of BIRCs (BIRC1-7) expression at the mRNA level in P1 and P2 cells. C. Heatmap of BIRCs (BIRC1-7) expression at the mRNA level in vector and DNMT3A over-expressed NCI-H3255 cells. D. Western blotting of c-IPA1 in P1, P2, vector and DNMT3A overexpressed NCI-H3255 cells. E. P1 and P2 cells were treated with SM-1295 (20 nM), erlotinib (2 μM), afatinib (5 μM) or in combination for 48 h. Then, cell apoptosis was assessed. F. Vector and DNMT3A overexpressed NCI-H3255 cells were treated with SM-1295 (20 nM), erlotinib (2 μM), afatinib (5 μM) or in combination for 48 h and then cell apoptosis was assessed. G. Representative images of c-IAP1 immunohistochemical staining in NSCLC tissues from recurrent and non-recurrent patients (n = 30, scale bar = 100 μm).

validate the role of BIRCs, we examined the mRNA level of BIRCs in P1 and P2 cells, which revealed increased BIRC2 levels in P2 cells (DNMT3A^{high}) compared to the P1 group (DNMT3A^{low}) (**Figure 3B**). Consistently, the overexpression of DNMT3A also resulted in the upregulation of BIRC2 in NCI-H3255 cells (**Figure 3C**), which was validated at the protein level using western blotting (**Figure 3D**). This indicated that DNMT3A upregulated c-IPA1 in NSCLC cells. To evaluate the role of c-IPAs in DNMT3A-induced TKIs resistance, we further treated tumour cells with IAPs inhibitor SM-1295. Notably, the inhibition of IAPs significantly increased the cytotoxicity of erlotinib and afatinib to P2 cells (DNMT3A^{high}), while limited influence was found in P1 cells (DNMT3A^{low}) (**Figure 3E**). Additionally, similar results were observed in DNMT3A overexpressed NCI-H3255 cells (**Figure 3F**). These results suggested that DNMT3A modulated TKI sensitivity via IAPs. Furthermore, we analysed the c-IPA1 expression in tumour tissues from recurrent and non-recurrent patients with NSCLC, wherein c-IPA1 was upregulated in recurrent patients (**Figure 3G**). Collectively, these data suggested that DNMT3A upregulated c-IPA1 to suppress TKI-induced cell apoptosis.

DNMT3A^{high} cells entered an early/reversible senescent-like status in the presence of TKIs

Previous studies have clarified the role of DNMT3A in modulating TKI sensitivity. However,

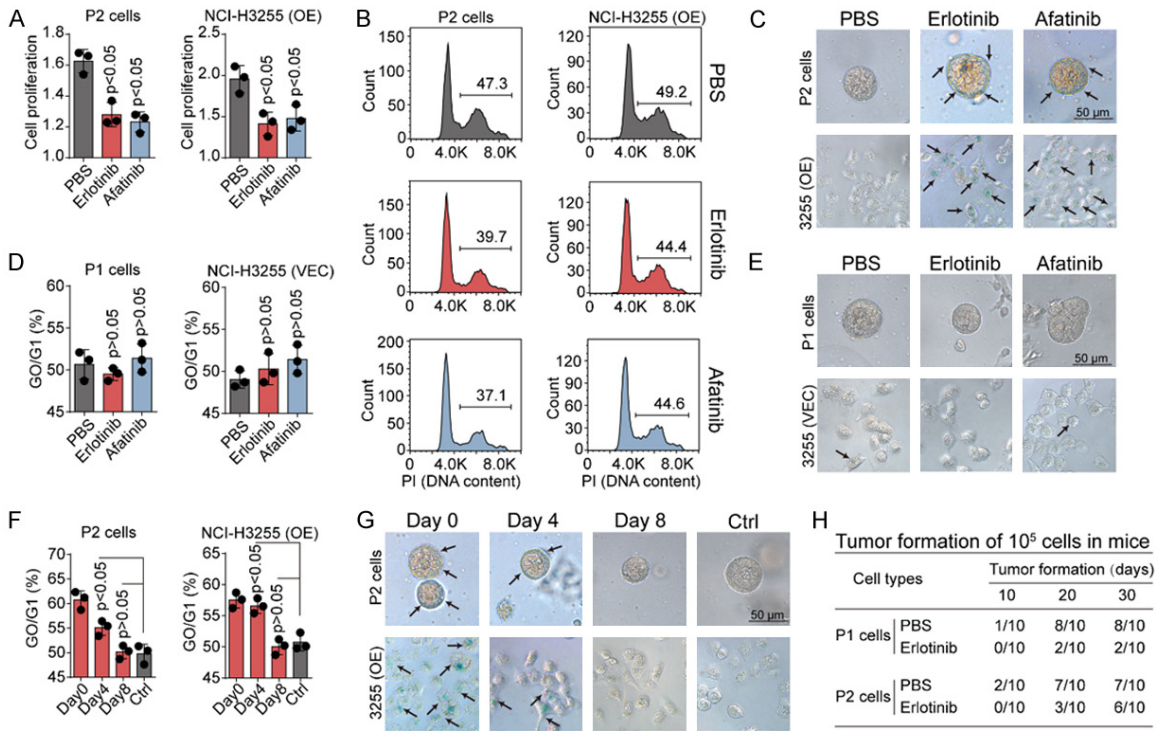

Figure 4. DNMT3A^{high} cells entered an early senescent-like status in the presence of tyrosine kinase inhibitors (TKIs). A. Cell proliferation of P2 and DNMT3A overexpressed NCI-H3255 cells treated with phosphate-buffered saline (PBS), erlotinib (2 μM) and afatinib (5 μM) for 72 h. B. Cell cycle assay of P2 and DNMT3A overexpressed NCI-H3255 cells treated with PBS, erlotinib (2 μM) and afatinib (5 μM) for 48 h using PI staining. C. SA-βgal activity staining in P2 and DNMT3A overexpressed NCI-H3255 cells treated with PBS, erlotinib (2 μM) and afatinib (5 μM) for 48 h. Scale bar = 50 μm. D. Cell cycle assay of P1 and vector NCI-H3255 cells treated with PBS, erlotinib (2 μM) and afatinib (5 μM) for 48 h using PI staining. E. SA-βgal activity staining in P1 and vector NCI-H3255 cells treated with PBS, erlotinib (2 μM) and afatinib (5 μM) for 48 h. Scale bar = 50 μm. F and G. P2 and DNMT3A overexpressed NCI-H3255 cells treated with erlotinib (2 μM) for 48 h and then, cell cycle and SA-βgal activity were analysed on days 0, 4 and 8. Scale bar = 50 μm. H. P1 and P2 cells were treated with PBS or erlotinib (2 μM) for 48 h. Following this, 10^5 cells (encapsulated in 50 μl Matrigel) were subcutaneously implanted into immunodeficient mice. On days 10, 20 and 30, the tumour formation was evaluated.

in this study, erlotinib exhibited a significant influence on tumour growth in P2 cell-bearing mice while cancer cells isolated from tumour tissues exhibited a low rate of cell apoptosis. Thus, we hypothesised that TKIs could influence the proliferation and cell cycle in DNMT3A^{high} tumour cells. To verify this hypothesis, we examined the cell proliferation of P2 cells and DNMT3A overexpressed NCI-H3255 cells in the presence and absence of TKIs. Both erlotinib and afatinib suppressed the cell proliferation in P2 and NCI-H3255 cells (**Figure 4A**). However, erlotinib and afatinib treated cells enhanced the G0/G1 cell cycle arrest (**Figure 4B**). Additionally, SA-βgal activity assay revealed an increased localisation of the senescence biomarker β-galactosidase in erlotinib/afatinib treated P2 or DNMT3A overexpressed NCI-H3255 cells (**Figure 4C**). Meanwhile, limited influence on cell cycle and the β-galactosidase location was observed in P1 cells (**Figure 4D** and **4E**). These results suggested that TKIs promoted cell senescence in DNMT3A^{high} NSCLC cells. We also speculated if the removal of TKIs could reverse cell senescence in NSCLC cells. Thus, we treated P2 and DNMT3A overexpressed NCI-H3255 cells with erlotinib for 48 h. Following this, TKIs were removed and a cell cycle/SA-βgal activity assay was performed. The cell cycle arrest and accumulation of β-galactosidase in tumour cells were reversed after 8 days in the absence of TKIs (**Figure 4F** and **4G**), suggesting that TKIs drove DNMT3A^{high} cells to enter an early senescent-like status and that these cells could revert to a proliferative status in the absence of TKIs. To validate the influence of the TKI-induced senescence in tumour recurrence, we treated P2 cells with

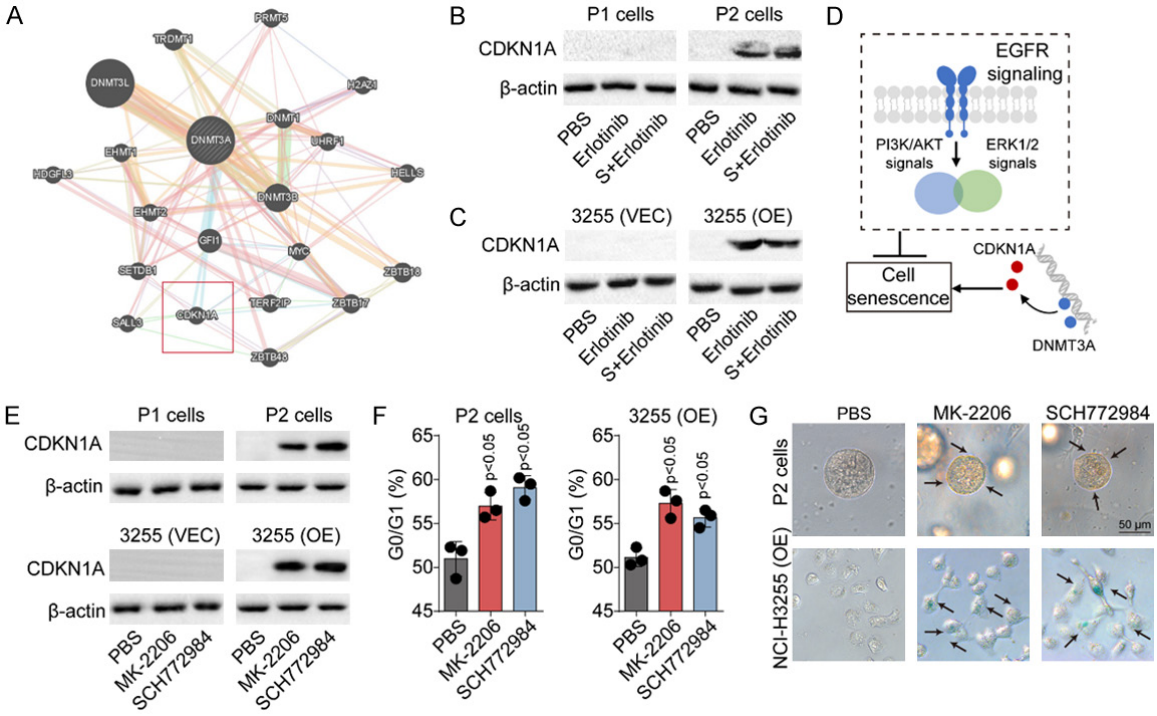

Figure 5. DNMT3A upregulated CDKN1A to promote cell senescence. A. The protein-protein network of DNMT3A was analysed using the GeneMANIA database. B. Western blotting of CDKN1A in P1 and P2 cells treated with phosphate-buffered saline (PBS), erlotinib (2 μM) and afatinib (5 μM) for 48 h. C. Western blotting of CDKN1A in vector and DNMT3A overexpressed NCI-H3255 cells treated with PBS, erlotinib (2 μM) and afatinib (5 μM) for 48 h. D. Schematic diagram of EGFR-signaling induced CDKN1A inhibition. E. P1, P2, vector and DNMT3A overexpressed NCI-H3255 cells were treated with PBS, MK-2206 2HCl (10 nM) and SCH772984 (50 nM) for 48 h. Then, the protein level of DNMT3A was determined using western blotting. F and G. P2 and DNMT3A overexpressed NCI-H3255 cells were treated with PBS, MK-2206 2HCl (10 nM) and SCH772984 (50 nM) for 48 h. Then, cell cycle and SA-βgal activities were analysed. Scale bar = 50 μm.

and without TKIs for 48 h and then incubated them in immunodeficient mice. Notably, TKI pre-treatment did not influence the tumour formation potential of P2 cells. However, the tumour formation time was prolonged in the TKI-treated group (**Figure 4H**), Importantly, TKI treatment significantly suppressed tumour formation in P1 cells, indicating that DNMT3A^{high}, senescent-like tumour cells could escape apoptosis and contribute to tumour recurrence after TKI therapy. Therefore, these results suggested that DNMT3A^{high} cells could enter a reversible senescent-like status, instead of apoptosis, in the presence of TKIs and re-acquire their tumorigenic ability in the absence of TKIs.

DNMT3A upregulated CDKN1A to promote cell senescence

To further elucidate the mechanism of DNMT3A-regulated cell senescence, we analysed the protein-protein interaction network using the GeneMANIA database and screened the proteins that interacted with DNMT3A. Notably, the cell-cycle negative regulatory protein CDKN1A, also known as P21, was co-expressed with DNMT3A (**Figure 5A**). To validate the role of CDKN1A, we then performed western blotting to examine the expression of CDKN1A in the established organoids. Interestingly, erlotinib and afatinib treatment upregulated CDKN1A expression in DNMT3A^{high} P2 cells; however, no significant effect on CDKN1A expression was observed in DNMT3A^{low} P1 cells (**Figure 5B**). Moreover, similar results were observed in NCI-H3555 cells (**Figure 5C**). Overall, these findings suggested that TKIs could promote CDKN1A expression in a DNMT3A dependent manner. As reported previously, EGFR transduces important growth factor signalling, resulting in the activation of pro-survival signalling pathways, including PI3K/AKT and ERK1/2 signals [20]. These pro-survival signals suppress cell-cycle negative regula-

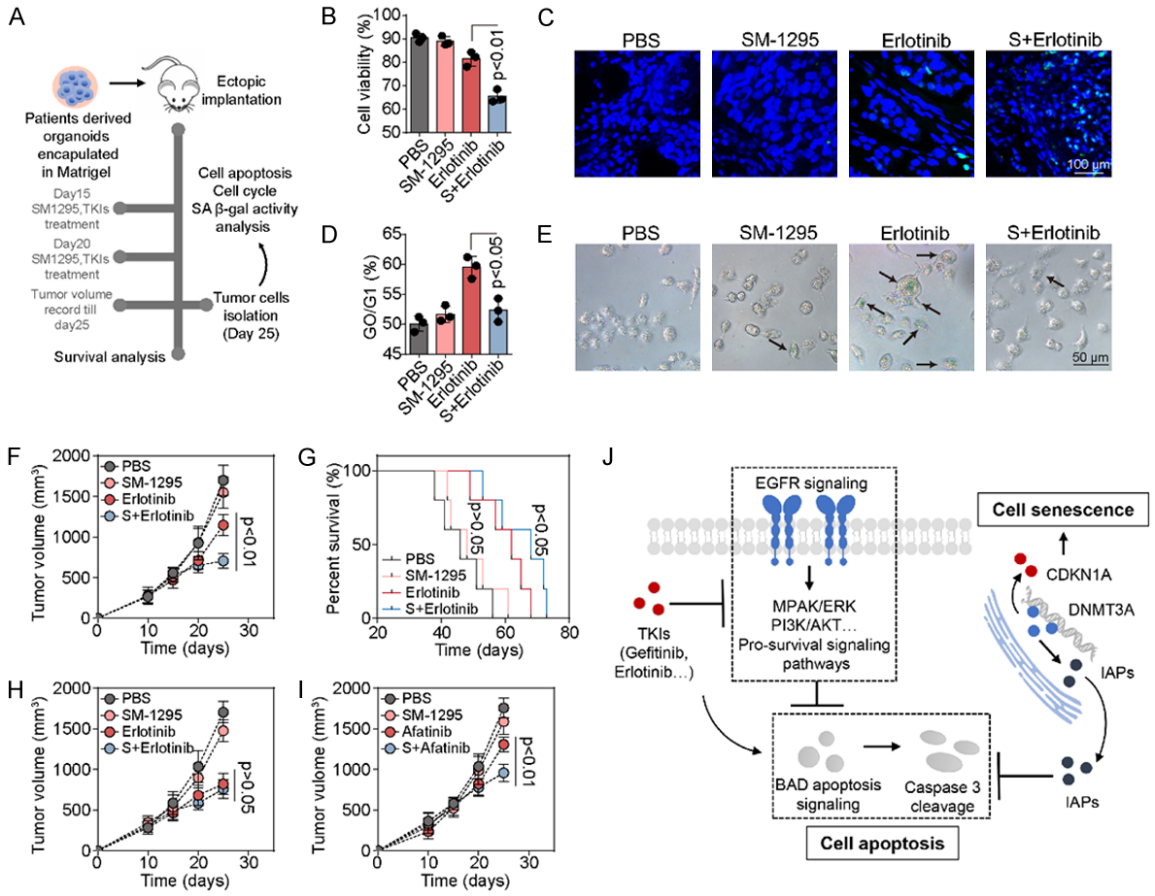

Figure 6. Blockade of DNMT3A/Inhibitor of apoptosis proteins AIPs signals improved the outcome of tyrosine kinase inhibitors (TKIs) in the patient-derived organoid model. (A-E) 1 × 10^6 P2 cells (encapsulated in 50 μl Matrigel) were subcutaneously implanted into immunodeficient mice. On day 15, mice were treated with phosphate-buffered saline (PBS), erlotinib (5 mg/kg), SM-1295 (2 mg/kg) or in combination twice a week via a tail vein injection (A). Tumour tissues were isolated for Annexin V/PI staining (B), TUNEL (C), cell cycle (D) and SA-βgal activity (E) analysis on day 25. Scale bar = 50 μm. (F) Tumour volume of mice in (A) was recorded. (G) The overall survival of mice in (A). (H) The P1-bearing mice were treated with PBS, erlotinib (5 mg/kg), SM-1295 (2 mg/kg) or in combination, and the tumour volume was recorded. (I) The P2-bearing mice were treated with PBS, afatinib (5 mg/kg), SM-1295 (2 mg/kg) or in combination, and the tumour volume was recorded. (J) The schematic diagram of crosstalk between the EGFR and DNMT3A signalling pathways.

tory proteins, such as P21 and P27 [21, 22]. Thus, we speculated that DNMT3A could mediate the activation of CDKN1A. However, EGFR upregulated AKT and ERK pro-survival signals to suppress CDKN1A. Additionally, the blockade of EGFR signalling by TKIs suppressed these pro-survival signalling pathways (AKT and ERK), resulting in the upregulation of CDKN1A induced by DNMT3A (**Figure 5D**). To validate our hypothesis, we treated P2 cells with the AKT inhibitor MK-2206 2HCl and ERK inhibitor SCH772984 and determined CDKN1A expression using western blotting. The inhibition of AKT and ERK signals upregulated CDKN1A expression in DNMT3A^{high} P2 cells but had no influence on DNMT3A^{low} P1 cells (**Figure 5E**). Similar results were also observed in NCI-H3255 cells. Consistently, AKT inhibitor MK-2206 2HCl and ERK inhibitor SCH772984 promoted cell cycle arrest (**Figure 5F**) and SA-βgal activity (**Figure 5G**) in P2 cells and DNMT3A overexpressed NCI-H3255 cells. Thus, these results suggested that DNMT3A upregulated CDKN1A to promote cell senescence.

Blockade of DNMT3A/IAPs signals improved the outcome of TKIs in a patient-derived organoid model

Previous studies report that the blockade of IAPs signals by SM-1295 increased the cell

cytotoxicity of TKIs in DNMT3A^{high} NSCLC cells. Thus, to evaluate the anti-cancer potential of SM-1295 *in vivo*, we subcutaneously implanted P2 cells into immunodeficient mice and treated them with erlotinib and SM-1295 twice a week via a tail vein injection (**Figure 6A**). Consistent with the *in vitro* results, SM-1295 increased the cell apoptosis of tumour cells isolated from tumour-bearing mice (erlotinib treatment), which was determined using Annexin V/PI assay (**Figure 6B**) and TUNEL staining (**Figure 6C**). Moreover, SM-1295 also suppressed cell cycle arrest (**Figure 6D**) and senescent-like phenotypes (**Figure 6E**) induced by erlotinib in NSCLC tumour-bearing mice. These results indicated that IAPs inhibitor suppressed cell senescence induced by TKIs and promoted tumour cells to enter an apoptotic state. Consistent with the cell cytotoxicity assay, mice treated with SM-1295 and erlotinib exhibited slower tumour growth and a prolonged overall survival time than the erlotinib group (**Figure 6F** and **6G**). However, the anti-cancer effects of IAPs inhibitor were not observed in DNMT3A^{low} P1 cell-bearing mice (**Figure 6H**), indicating that the blockade of IAPs increased the tumour suppressive effects of TKIs in DNMT3A^{high} NSCLC cells. Furthermore, the evaluation of the tumour suppressive effects of the SM-1295/afatinib combination revealed that SM-1295 enhanced the anti-cancer effects of afatinib (**Figure 6I**). Thus, these findings indicated that the blockade of DNMT3A/AIPs signals could improve the outcome of TKIs in DNMT3A^{high} NSCLC.

Discussion

Drug resistance toward TKIs is multifactorial, involving both gene mutations and epigenetic modifications. An extensive literature review established DNA methylation as a trigger of cellular events culminating in TKIs resistance. Additionally, clinical data also indicates that homeobox DNA methylation is predictive of an increased rate of EGFR-TKIs resistance in advanced lung adenocarcinoma [23]. Moreover, in a study of NSCLC, promoter hypermethylation resulted in death-associated protein kinase (DAPK) transcriptional silencing in resistant cells, and the reconstitution of DAPK sensitised these cells to erlotinib [24]. DNA methyltransferase, which catalyses the attachment of methylation markers to genomic DNA, also participates in regulating TKI response. Furthermore, Nishioka et al. reported that long-term exposure to BCR/ABL TKIs enhanced DNMT3A expression in leukaemia cells; however, the administration of DNMT inhibitor decitabine reversed drug resistance [25]. Moreover, TKIs in combination with decitabine also hindered tumour growth in xenograft mice models. In this study, the analysis of data obtained from the TCGA database showed a correlation between DNMT3A upregulation and shortened survival in patients with NSCLC. Accordingly, we ruled out the influence of DNMT3A on cellular proliferation/migration and found that DNMT3A conferred TKI resistance to NSCLC cells. Additionally, we constructed organoid models/DNMT3A overexpressed cell lines and demonstrated the role of DNMT3A in modulating TKI susceptibility *in vivo*. Additionally, we screened the DNMT3A mutations in blood samples from NSCLC patients, and found a trend of increased DNMT3A deficiency in peripheral blood samples from patients who responded to TKI treatment (not shown in data). Such markers could aid in reducing the patient's suffering caused by tumour biopsy.

To evade apoptosis, cancer cells often express high levels of IAPs and become 'addicted' to them [26]. The mammalian IAP family comprises eight members that are defined by one or more repeats of a highly conserved domain baculovirus, named the baculovirus IAP repeat [27]. A plethora of factors is implicated in the regulation of IAPs, thereby contributing to tumour resistance [28]. Moreover, collagen type XI alpha 1 (COL11A1) elicits the expression of three IAPs through the Src-PI3K/Akt-NF-kB signalling pathway, thus suppressing apoptotic phenotype in ovarian cancer [29]. Similarly, the ectopic expression of connective tissue growth factor rendered breast cancer cells more resistant to chemotherapy reagents by upregulating Bcl-xL and c-IAP1 [30]. In the present study, we broadened the role of IAPs in the response to targeted therapy, identifying c-IAP1 as a modulator of TKI resistance. We observed that DNMT3A mediated c-IAP1 upregulation to potentiate the cytotoxicity of TKIs, which could be reversed by inhibiting IAPs. Furthermore, DNMT3A^{high} cells failing to undergo apoptosis entered early senescent-like status in the presence of TKIs, which validated that TKI resistance is modulated by DNMT3A.

Senescence occurs when cells are exposed to various endogenous and exogenous stressors. Senescent cells share some common characteristics, including morphological changes, cell cycle withdrawal, DNA damage response and metabolic reprogramming [31]. Despite the incapability to propagate, senescent cells remain viable for a while and display a senescence-associated secretory phenotype [32]. Apart from its role in the critical tumour suppressive mechanism that prevents the propagation of oncogenic activated cells, cellular senescence also assists in evading the cytotoxic effect of anti-tumour interventions [33]. In breast cancer, chemotherapeutic agents were responsible for the generation of senescent cells from which the enrichment of stemlike cells occurred [34]. Furthermore, research on renal cell carcinoma demonstrated that cellular senescence occurred following the administration of sunitinib, a multitargeted inhibitor of receptor tyrosine kinases, which depended on the p53/Dec1 signalling pathway [35]. Additionally, *in vivo* experiments suggested that drug-induced senescence was related to colorectal cancer recurrence. Moreover, it was also reported that the abolition of the senescent phenotype restored the sensitivity to SN38, indicating the reversibility of chemotherapy-induced senescence [36]. In the current study, NSCLC cells with high DNMT3A levels entered an early senescence state rather than an apoptotic state in response to TKIs, which was indicated by an enhanced G0/G1 cell cycle arrest and increased β-galactosidase localisation. Furthermore, DNMT3A drove cellular senescence by increasing the expression of CDKN1A, a core inhibitor of the cell cycle. Although they acted as barriers for tumorigenesis, these senescent cells could proliferate after the removal of TKIs, leading to recurrent tumours. This role of DNMT3A in mediating senescence could provide a plausible explanation for the relapse of NSCLC, which was consistent with the upregulation of DNMT3A in recurrent samples.

Using xenograft mice models, we found that IAPs inhibitor SM-1295 synergistically promoted cellular apoptosis mediated by TKIs, thus inhibiting tumour growth. Moreover, TKIs in cooperation with SM-1295 prevented the induction of cellular senescence, which could be a potential therapeutic regimen to counteract relapse tendencies. Thus, our study illustrated that (1) Recurrent tumour tissues exhibited elevated DMNT3A expression, which could be translated into a prognostic marker for NSCLC. (2) DNMT3A enhanced IAPs expression to protect tumour cells from TKI-induced apoptosis. (3) DNMT3A^{high} tumour cells entered a reversible senescence state after TKI administration, which was dependent on CDKN1A upregulation. (4) TKIs in combination with an IAPs inhibitor delayed tumour development and arrested tumour growth in NSCLC organoids (**Figure 6J**).

In conclusion, this study suggested that DNMT3A assisted NSCLC cancer cells to avoid apoptosis and triggered an early senescent-like phenotype in NSCLC cells. Furthermore, the role of IAPs as a modulator of TKIs resistance has been explored. Thus, targeting DNMT3A/IAPs signals could reveal new directions for NSCLC intervention.

Acknowledgements

We appreciated the support of Tongji University School of Medicine, Shanghai Engineering Research Center of Pharmaceutical Translation, Shanghai Institute for Biomedical and Pharmaceutical Technologies, Key Laboratory of Systems Health Science of Zhejiang Province, Fudan University, Shanghai Jiao Tong University, and Shanghai Institute of Nutrition and Health. Meanwhile, we thank the technical help from the Smartquerier Gene Technology (Shanghai) Co., Ltd. This study was financially supported by the Key Projects of Shanghai Zhangjiang High Tech Park (2016-08).

All participants have written informed consents.

Disclosure of conflict of interest

None.

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