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FK228 potentiates topotecan activity against small cell lung cancer cells via induction of SLFN11

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The response rate of topotecan, as a second-line chemotherapeutic drug for small cell lung cancer, is ~20%. DNA/RNA helicase SLFN11 (schlafen family member 11), a member of the Schlafen (SLFN) family, is a crucial determinant of response to many DNA damaging agents, expression of SLFN11 tends to augment the antitumor effects of the commonly used DNA-targeting agents. In the present study we investigated how *SLFN11* expression regulated the sensitivity of small cell lung cancer to topotecan. We showed that *SLFN11* expression levels were positively associated with the sensitivity to topotecan in a panel of seven SCLC cell lines. Topotecan treatment induced different patterns of the DNA response network in SCLC cells: DNA damage response (DDR) was more prominently activated in *SLFN11*-deficient SCLC cell line H82 than in *SLFN11*-plentiful SCLC cell line DMS273, whereas topotecan induced significant accumulation of p-Chk1, p-RPA2 and Rad51 in H82 cells, but not in DMS273 cells. We unraveled that *SLFN11* expression was highly negatively correlated to the methylation of the *SLFN11* promoter. HDAC inhibitors FK228 and SAHA dose-dependently increased *SLFN11* expression through suppressing DNA methylation at the *SLFN11* promoter, thereby sensitizing SCLC cells to topotecan. Finally, we assessed the methylation status of the *SLFN11* promoter in 27 SCLC clinical specimens, and found that most of the clinical samples (24/27) showed DNA methylation at the *SLFN11* promoter. In conclusion, it is feasible to combine topotecan with FK228 to improve the response rate of topotecan in SCLC patients.

Keywords: small cell lung cancer; topotecan; DNA damage response; FK228; SLFN11; methylation; epigenetic modulation

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INTRODUCTION

Small cell lung cancer (SCLC) is an aggressive malignancy with high metastatic potential and poor prognosis, accounting for ~15% of all newly diagnosed lung cancers [1]. The use of platinum-based combination regimens remains the state-of-art first-line treatment for SCLC. Unfortunately, most patients with SCLC recur within months and become resistant to treatment despite the dramatic initial response to chemotherapy and radiation. After disease recurrence, topotecan is the only chemotherapeutic agent for second-line SCLC until recently FDA-approved lurbinectedin for metastatic SCLC. Consequently, the median survival time for SCLC after diagnosis is 10–12 months [2, 3].

Topotecan, a camptothecin derivative and topoisomerase I inhibitor, is the primary chemotherapy option for many years at relapse after first-line therapy, which is routinely administered by the oral or infusion route. Multiple investigations in clinical trials of topotecan treatment for relapsed SCLC have demonstrated no significant difference in clinical efficacy and tolerability between these two routes of administration [4]. The effective rate and median survival time of topotecan as a single agent were better than the cyclophosphamide, doxorubicin, and vincristine regimen and best support treatment (BSC). However, although single-agent topotecan significantly improves patient survival, the response

rate of patients with recurrent SCLC to topotecan was ~20%, no matter whether it is oral or intravenous formulations [4]. The low clinical effectiveness of topotecan dramatically limits its application, and the molecular mechanism leading to the low response rate to topotecan remains to be determined. Effective target was an unmet clinical need to optimize the efficacy of topotecan and improve patients' quality of life.

DNA/RNA helicase SLFN11 (schlafen family member 11), a member of the Schlafen (SLFN) family, is a crucial determinant of response to many DNA damaging agents, such as topoisomerase I (TOP1) inhibitors (camptothecin, topotecan, and irinotecan), topoisomerase II (TOP2) inhibitors (etoposide, mitoxantrone, and doxorubicin), alkylating agents (cisplatin and carboplatin), DNA synthesis inhibitors (gemcitabine and cytarabine), and PARP inhibitors [5–9]. Expression of SLFN11 tends to augment the antitumor effects of these commonly used DNA-targeting agents. These drugs interfere with the DNA replication process, induce DNA replication stress, and cause DNA damage. SLFN11 can act as a factor to monitor the DNA replication process by interacting with several critical components of the DNA damage response (DDR) system, such as ssDNA-binding protein RPA and DHX9 [6, 10]. Once endogenous or exogenous impediments stall DNA replication, SLFN11 immediately irreversibly blocks replication via activation of the DDR network and induction of cell cycle arrest

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[6, 10]. *SLFN11* expression is positively correlated with overall survival in ovarian cancer, and methylation of *SLFN11* at promoter could be used to predict the prognosis in colorectal cancer [11, 12].

SLFN11 has been reported as a predictor of response to PARP inhibitor monotherapy in SCLC, and a recent clinical trial has shown that a high *SLFN11* expression level is associated with a better clinical outcome in recurrent SCLC patients treated with PARP inhibitor veliparib [13, 14]. Interestingly, the expression of *SLFN11* seems to be epigenetically regulated and induced expression of *SLFN11* by EZH2 inhibitor overcomes the acquired resistance to chemotherapy in SCLC [11, 15, 16]. However, whether *SLFN11* expression confers sensitivity to topotecan in SCLC has yet to be fully understood.

In this study, we sought to illustrate the role of *SLFN11* in regulating SCLC cells' response to topotecan treatment and develop a strategy to improve the response rate of topotecan in SCLC.

MATERIALS AND METHODS

Small molecules and antibodies

Topotecan was purchased from Selleck Chemical (Shanghai, China). FK228 and SAHA were purchased from MedChemExpress (Concord, CA, USA). Antibodies against *SLFN11* (#34858), PARP (#5625), Chk1 (#2360), p-Chk1 (#12302), RPA2 (#2208), p-RPA2 (#83745), Rad51 (#8875) and γ -H2AX (Ser139) (#9718) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against β -Actin (HC201-02) was purchased from TransBionovo (Beijing, China).

GDSC and CCLE data analysis

Sequencing data (RNA-seq) and methylation status from SCLC cell lines, and general information for these cell lines, were downloaded from <https://sites.broadinstitute.org/ccle/datasets>. The GDSC (Genomics and Drug Sensitivity in Cancer; Massachusetts General Hospital-Wellcome Sanger Institute) database was downloaded from <https://www.cancerxgene.org/downloads/anova>. The JNCI dataset was obtained from Polley et al., 2016 [17]. Expression data for *SLFN11*, the methylation status of CpG in the promoter region of *SLFN11*, and IC₅₀ values from SCLC cell lines were retrieved, analyzed, and displayed in scatter plots.

Human SCLC tumor specimens and ethics statement

Twenty-seven formalin-fixed, paraffin embedded human primary SCLC tumor tissue samples were collected. And the study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hefei Institutes of Physical Science, Chinese Academy of Sciences, and written informed consent was obtained from all patients.

Cell culture and maintenance

The human SCLC cell lines H82, H526, DMS79, DMS273, H196, H446, and H69, were grown in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were sub-cultured when they reached 80% of confluence. All cell lines used in this study were routinely confirmed as being mycoplasma negative.

Cell viability assays

SCLC cells were plated in a 96-well white plate (Costar, Kennebunk, ME, USA) in 100 μ L medium at an initial density of 3000 cells/well and incubated 24 h prior to the treatment. Then, either vehicle control or different concentrations of topotecan were added into each well of the plate in triplicate, and the plate was continued to incubate for 72 h. Relative cell viability was

determined by the CellTiter-Glo Luminescent assay (Promega, Madison, WI, USA). A multilabel plate reader (Envision PerkinElmer, USA) was used to collect the luminescent signals.

Protein extraction and Western blotting

After drug treatment, the whole-cell protein was extracted by resuspending cell pellets in RIPA buffer supplemented with protease inhibitor cocktail as previously described [18]. Equal amounts of proteins were subjected to SDS/PAGE and transferred to PVDF membrane for immunoblotting. Protein bands were visualized after incubation with primary and secondary antibodies using ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence staining

SCLC cells (1×10^6 cells per well in a six-well plate) were treated with topotecan or vehicle control (DMSO) for 24 h. After drug treatment, cells were fixed with freshly prepared 4% paraformaldehyde for 10 min and then subjected to permeabilization with 0.01% Triton X-100 in TBS for 5 min. The permeabilized cells were then incubated with primary rabbit anti-phospho-histone H2AX (Ser139) antibody (1:200) or anti-Rad51 antibody (1:500) at 4 °C overnight, followed by the anti-rabbit-FITC secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. After washing, samples were stained with DAPI for 1 min, mounted, and examined by Leica fluorescence microscopy (Leica, Wetzlar, Germany). Cells with more than 5 foci were considered positive, and at least 50 cells for each condition were scored.

Measurement of *SLFN11* mRNA expression

The cellular RNA of the treated cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was reverse transcribed from total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. For reverse transcriptase-polymerase chain reaction analysis, PCR amplification was carried out in triplicate using FastStart Essential DNA Green Master Mix (Roche, Mannheim, Germany) on the Roche LightCycler 96 Real-Time PCR System. β -Actin was utilized as the internal control. The primers for *SLFN11* [19] and β -Actin were as follows. *SLFN11*-F: 5'-ATTATTAGTAGCGTGACGGTTATC-3'; *SLFN11*-R: 5'-CGACAAATATACAAATTAACCGCG-3'; Actin-F: 5'-CATGTACGTTGCTATCCAGGC-3'; Actin-R: 5'-CTCCTTAATGTCACGCACGAT-3'.

Cell apoptosis assay

After 48 h topotecan exposure, cells were harvested and then stained with FITC Annexin V and PI using a FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. Next, the stained cells were subjected to flow cytometry analysis by FACSCalibur (BD Pharmingen, San Diego, CA, USA). Then, the data were analyzed using FlowJo software (BD Pharmingen, Ashland, OR, USA).

Virus production and establishment of SCLC cells with stable knockdown of *SLFN11*

Three lentiviral shRNAs (shSLFN11-1: 5'-CGACTGGTTGTATCTCTTTA-3'; shSLFN11-2: 5'-ATCCAATAGCCAAGTACTTA-3'; shSLFN11-3: 5'-CCAGAATACGTCCCTGCATT-3') targeting human *SLFN11* were cloned into the pLKO.1 vector. The *SLFN11* shRNA-containing pLKO.1 vector was co-transfected with psPAX2 and pMD2.G into HEK-293T cells using Effectene Transfection Reagent (QIAGEN, Hilden, Germany). Viral supernatant was recovered 48 h after transfection. DMS273 cells were infected with lentiviral particles in the presence of 8 μ g/mL polybrene (Sigma-Aldrich, Steinheim, Germany). Puromycin (Solarbio, Beijing, China) at 1 μ g/mL was added to the medium for selection at 48 h post-infection.

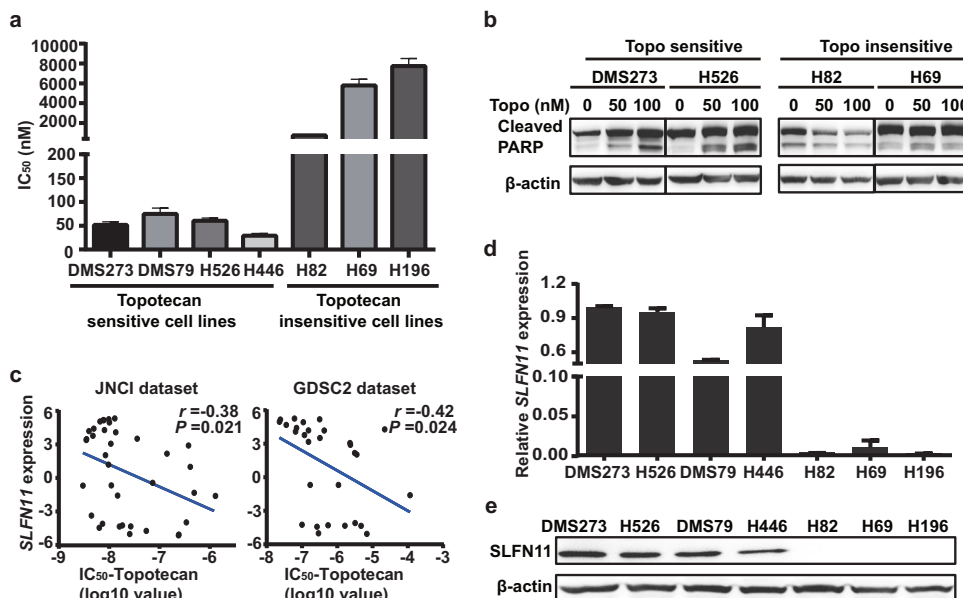


Fig. 1 The sensitivity of topotecan is positively correlated to SLFN11 expression in SCLC. **a** The IC₅₀ values of topotecan in a panel of SCLC cell lines. The SCLC cells were incubated with different concentrations of topotecan for 72 h. The cell viability was determined by the CellTiter Glo assay. **b** Western blotting analysis of PARP cleavage in SCLC cells treated with different concentrations of topotecan. The cells were treated with a range of concentrations of topotecan for 24 h. β-Actin was used as a loading control. **c** IC₅₀ to topotecan is negatively correlated with SLFN11 expression. Pearson correlation analysis of SLFN11 expression and IC₅₀ values of topotecan from the JNCI dataset (left panel) and GDSC2 dataset (right panel). **d**, **e** RT-qPCR (**d**) and Western blot analysis (**e**) showing the expression levels of SLFN11 across seven SCLC cell lines.

Ectopic expression of SLFN11 in SCLC cells

The entire length of SLFN11 was cloned into vector pCMV-3-flag with primer pair SLFN11-F (5'-CGCGGATTCATGGAGCAAATCAGTGCCC-3') and SLFN11-R (5'-GGGAAGCTTCTAATGGCCACCCACGGAA-3'). The resulting vector was confirmed by DNA sequencing. Then, the resulting vector was transfected into H82 cells using Effectene Transfection Reagent (QIAGEN, Hilden, Germany). The efficiency of overexpression was determined by Western blotting [20].

Chromatin immunoprecipitation (ChIP) assays

SCLC cells were treated with DMSO or 2 nM FK228 for 48 h. The cells were cross-linked for 10 min with 1% formaldehyde in the medium at room temperature and then lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) with proteasome inhibitor cocktail. The lysates were sonicated to shear the DNA to an appropriate size of between 100 and 1000 bp. The sonicated chromatin was then incubated with 2.5 μg H3K9Ac antibody (Cell Signaling Technology, Danvers, MA, USA) or with equal amounts of immunoglobulin G (IgG) as a negative control at 4 °C overnight. After reversing cross-link and proteinase K treatment, the immunoprecipitated genomic DNA was purified for quantitative-PCR (qPCR) detection. Primer pairs were designed against the SLFN11 CpG promoter region [19].

Bisulfite modification and methylation-specific PCR (MSP)

After 24 h of treatment with DMSO and FK228/SAHA, genomic DNA was extracted and treated with sodium bisulfite as previously described [19]. Briefly, 2 μg of DNA was denatured by 3 M sodium hydroxide in a volume of 50 μL for 10 min at 37 °C. Freshly prepared hydroquinone (20 mM, 30 μL) and 520 μL of 3.6 M freshly prepared sodium bisulfite were mixed with the denatured DNA and incubated at 42 °C for 30 min. The reaction mixture was incubated at 50 °C in the dark for 16 h. After that, the modified DNA was purified and recovered. The purified modified DNA was detected by MSP (methylation-specific PCR). The primers used for MSP detection were as follows [19].

Me-SLFN11-F: 5'-ATTATTAGTAGCGTGACGGTTATC-3'; Me-SLFN11-R: 5'-CGACAAATATACAAATTAACCGCG-3'; UnMe-SLFN11-F: 5'-TATATTATTAGTAGTGTGATGTTATT-3'; UnMe-SLFN11-R: 5'-ATACAACAAATATACAAATTAACCCACA-3'.

Statistical analysis

Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for data processing and statistical analyses. The differences were considered statistically significant using a two-tailed unpaired *t*-test when the *P* value is <0.05.

RESULTS

The sensitivity of topotecan is positively correlated with the expression of SLFN11 in SCLC

A panel of SCLC cell lines were treated with different concentrations of topotecan, and the sensitivity of the SCLC cell lines was characterized using the CellTiter Glo assay. Based on the difference of the half-maximal inhibitory concentration (IC₅₀) values in seven SCLC cell lines, the seven cell lines were designated as either belonging to a topotecan-sensitive group, consisting of DMS273, DMS79, H526, and H446, or a topotecan-insensitive group, comprising of H82, H69, and H196 (Fig. 1a). Next, the levels of PARP cleavage were examined by Western blotting after the cells were treated with 50 or 100 nM topotecan for 48 h; the results demonstrated that the accumulation of cleaved PARP was only observed in topotecan-sensitive cells, but not in topotecan-insensitive cells (Fig. 1b). Accumulating evidence showed that SLFN11 expression is associated with the sensitivity of DNA damage reagents in various cancers [5–8]. We extracted the SLFN11 expression data and IC₅₀ values of topotecan from the CCLE (Cancer Cell Line Encyclopedia) database, JNCI and GDSC2 (Genomics of Drug Sensitivity in Cancer 2) datasets [17], respectively, and analyzed the correlation by Pearson correlation. The results showed that the expression level of SLFN11 was significantly negatively correlated with the IC₅₀ values of topotecan in SCLC cells (Fig. 1c, *n* = 36 for the JNCI dataset; *n* = 28

for the GDSC2 dataset). To further confirm the correlation between the sensitivity of topotecan and *SLFN11* expression, we performed RT-qPCR assays to examine *SLFN11* expression in the panel of SCLC cell lines. The results demonstrated that the topotecan-sensitive SCLC cell lines indeed tended to have a high level of *SLFN11* expression (Fig. 1d). Moreover, Western blot analysis of *SLFN11* expression in all topotecan-sensitive cell lines showed high expression. In contrast, a very low or undetectable level of *SLFN11* was observed in topotecan-insensitive cells (Fig. 1e). Altogether, *SLFN11* expression has a strong positive association with the sensitivity of topotecan in SCLC cells.

Topotecan induces different patterns of the DNA response network in SCLC in a dose-dependent manner

We then sought to investigate the mechanisms underlying the different effects of topotecan in SCLC cells. Given that topotecan treatment tends to cause double-strand breaks (DSBs), topotecan-sensitive DMS273 and topotecan-insensitive H82 were chosen to assess the changes in the DDR network. Both DMS273 and H82 were incubated with 50 nM topotecan for 24 h and subjected to immunofluorescence staining for γ -H2AX and Rad51. The immunofluorescence test indicated that topotecan led to the substantial accumulation of γ -H2AX in the nucleus of DMS273 but not in H82 cells. On the contrary, prominent nuclear Rad51 foci were seen in H82, while no noticeable nuclear Rad51 signals were detected in DMS273 cells (Fig. 2a). These results suggested that the DNA damage repair system is activated in topotecan-insensitive cells, but not in topotecan-sensitive cells in the presence of 50 nM topotecan. Next, p-Chk1, a replication stress response (RSR) marker, and p-RPA2 and Rad51 were chosen to evaluate the RSR and homologous recombination (HR) repair pathways' changes. Western blot results showed that topotecan treatment induced dose-dependent accumulation of p-Chk1, p-RPA2, and Rad51 in insensitive cell lines H82 and H69. In contrast, a dose-dependent accumulation pattern was not seen in the SCLC cells with high *SLFN11* expression (Fig. 2b). We also examined the effect of topotecan on the DDR network using time-course analyses. The accumulation of p-Chk1, p-RPA2, and Rad51 were gradually increased over time in H82 and H69 cells, but not in DMS273 and H526 cells (Fig. 2c). Concomitantly, the accumulation of γ -H2AX, a well-established DSB sensor, was significantly increased as early as 12 h in DMS273 and H526 cells with high *SLFN11* expression, but not in H82 and H69 cells without *SLFN11* expression. In conclusion, these data suggest that the DDR network is quickly activated upon topotecan treatment in *SLFN11*-deficient SCLC cells, and the DSBs are repaired promptly; in contrast, the DSBs are accumulated due to the failure to evoke the DDR network in *SLFN11*-proficient cells.

The maintenance of the DNA damage checkpoint and HR repair is suppressed in the presence of *SLFN11*

Previous studies have shown that *SLFN11* suppresses the maintenance of Chk1 phosphorylation in response to camptothecin treatment in several types of cancer cells [21]. To test whether a similar phenomenon occurs in SCLC, the cells were incubated with 50 nM topotecan, a camptothecin derivative, for 24 h and subsequently topotecan was withdrawn from the cultured medium. Western blot analysis showed that Chk1 phosphorylation was expressed at similar levels in all four cell lines regardless of *SLFN11* status after topotecan treatment. Notably, Chk1 phosphorylation diminished in DMS273 and H526 cells, as early as 12 h upon topotecan withdrawal. In contrast, Chk1 phosphorylation exhibited a marked decline at 36 h post-withdrawal in H82 and H69 cells (Fig. 3). Thus, we concluded that *SLFN11* plays a vital role in maintaining Chk1 phosphorylation in SCLC cells. Moreover, p-RPA2, a well-recognized marker for ssDNA from DNA end resection, was induced in all cell lines tested, suggesting that the formation of ssDNA, a prerequisite for efficient HR repair, was

not changed. Following the results of RPA2 phosphorylation, Rad51 expression was induced in all four cell lines, indicating that *SLFN11* is not involved in the initiation of HR repair. However, the expression level of Rad51 was quickly declined only in SCLC cells with high *SLFN11* expression (Fig. 3). These results suggested that *SLFN11* is required for maintenance rather than the initiation of the RSR and HR repair pathways.

Knocking-down/overexpression of *SLFN11* could decrease/increase the sensitivity of topotecan in SCLC

We hypothesized that *SLFN11* is a key determinant for an efficient DDR and the response to topotecan. To test this hypothesis, the knockdown and overexpression approaches were employed to address the role of *SLFN11* in topotecan-treated SCLC cells. First, we generated two *SLFN11* stable-knockdown DMS273 cells showing significant down-regulated in both mRNA and protein expression levels (Fig. 4a). Furthermore, the depletion of *SLFN11* suppressed apoptosis in DMS273 cells while *SLFN11* overexpression induced programmed cell death (Supplementary Fig. S1). We next examined the effect of *SLFN11* knockdown on DDR using time-course analyses. As shown in Fig. 4b, Knockdown of *SLFN11* recovered robust Chk1 phosphorylation and Rad51 with the prolongation of topotecan treatment time compared with the scramble control. We also assessed the effects of exogenous overexpression of *SLFN11* in SCLC cells showing minimal expression of *SLFN11*. As depicted in Fig. 4a, the expression of wild-type *SLFN11* was strongly induced by transient transfection. Interestingly, H82 cell overexpressing wild-type *SLFN11* caused apparent defects in Chk1 phosphorylation and Rad51 induction upon topotecan treatment (Fig. 4c). Importantly, *SLFN11* overexpression caused accumulation of γ -H2AX while *SLFN11* knockdown suppressed H2AX phosphorylation (Fig. 4b, 4c). Taken together, our data indicate that *SLFN11* exerts a suppressive role in the maintenance of DNA damage checkpoint signals.

Given that *SLFN11* is a central player in the DDR network, we evaluated the effect of *SLFN11* knockdown or overexpression on cell viability. As seen in Fig. 4d, depletion of *SLFN11* in DMS273 cells resulted in a less sensitive phenotype to topotecan (IC₅₀ of shCtrl: 0.04 μ M, IC₅₀ of shSLFN11-2: 0.12 μ M, IC₅₀ of shSLFN11-3: 0.21 μ M). On the contrary, ectopic expression of *SLFN11* in H82 cells remarkably re-sensitized the cells to topotecan (IC₅₀ of vehicle: 0.90 μ M, IC₅₀ of *SLFN11* OE: 0.03 μ M). At the same time, we examined the effect of *SLFN11* expression on the cell cycle. It was found that the cell cycle did not change significantly upon *SLFN11* knockdown (Supplementary Fig. S2a), while *SLFN11* overexpression in H82 cells led to G₀-G₁ phase arrest (Supplementary Fig. S2b).

HDAC inhibitor FK228 sensitizes SCLC cells to topotecan via epigenetic induction of *SLFN11* in SCLC

FK228, an HDAC inhibitor, has been reported to induce *SLFN11* expression in human fibrosarcoma and erythroleukemia cells [15]. We sought to determine whether HDAC inhibitors induced *SLFN11* in SCLC cells. *SLFN11*-deficient H82, H69 cells and *SLFN11*-proficient H526, DMS273 cells were incubated with 1 nM and 2 nM FK228 for 24 and 48 h, and the cells were then harvested for protein detection. Western blot analysis showed that *SLFN11* expression was markedly increased in the FK228-treated cells versus DMSO-treated cells at 24 and 48 h post-treatment (Fig. 5a). Strikingly, an apparent increase in *SLFN11* expression was also observed in *SLFN11*-proficient cells at 24 and 48 h post-treatment. Notably, the induction of *SLFN11* was stable and dose-dependent in the presence of FK228 (Fig. 5a). To rule out the possibility of PI3K inhibitor's contribution to *SLFN11* induction since FK228 is a dual HDAC and PI3K inhibitor, pan-HDAC inhibitor SAHA was also employed to treat SCLC cells [22, 23]. As shown in Fig. 5b, SAHA treatment also substantially increased *SLFN11* in H82 and H69 cells 24 h after treatment. Consistent with the results in

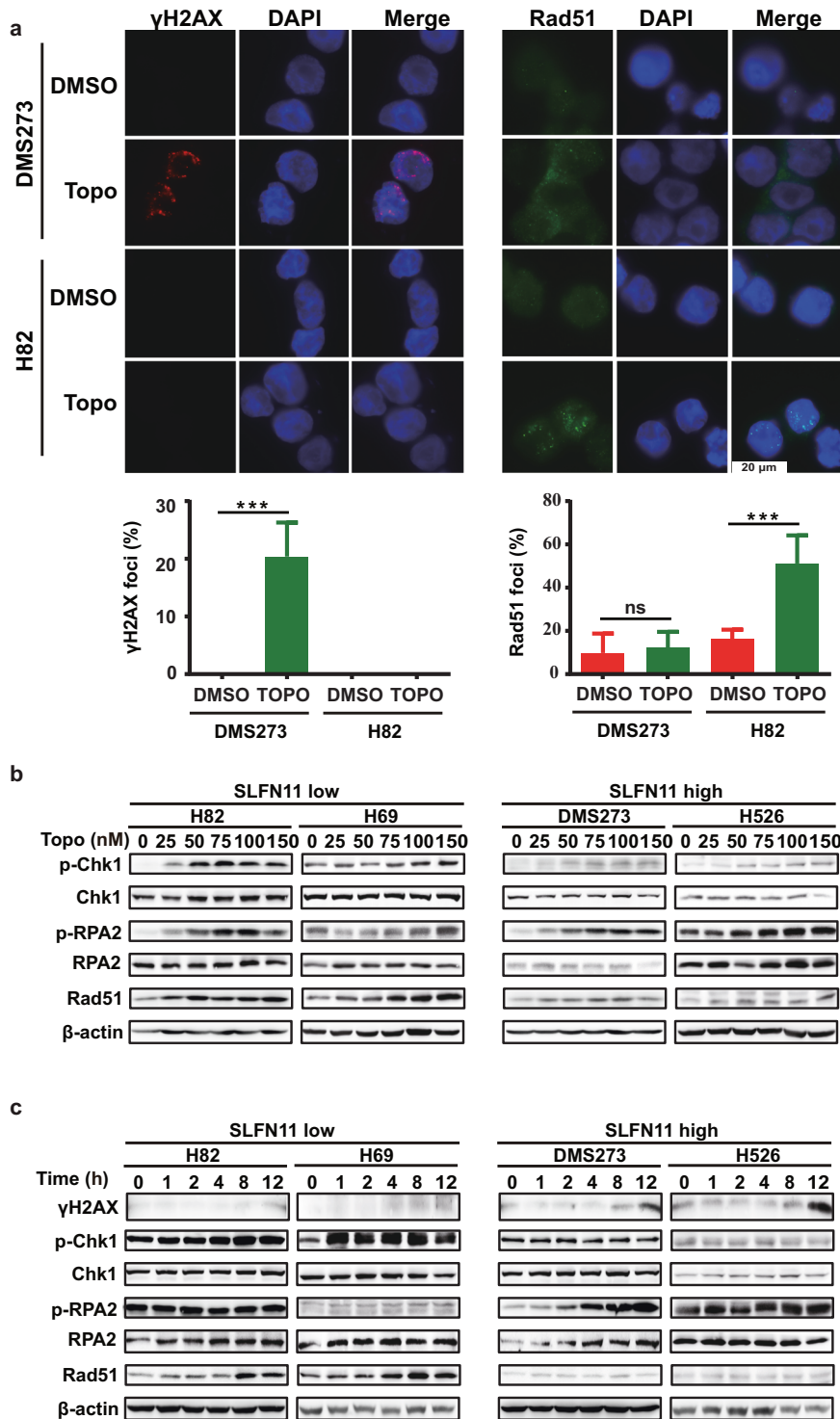


Fig. 2 Topotecan induces different activation strength of DNA damage response in SCLC. **a** Representative images of immunofluorescence staining for γ -H2AX and Rad51 and quantification of γ -H2AX and Rad51 signals. The cells were treated with 50 nM topotecan for 12 h and subjected to immunofluorescence staining. *** $P < 0.001$; ns, no significance. **b** Western blotting analysis of Chk1 and RPA2 phosphorylation and Rad51 in SCLC cells treated with different doses of topotecan. **c** Western blotting analysis of Chk1 and RPA2 phosphorylation, and Rad51 expression in SCLC cells treated with 50 nM topotecan at indicated time points. β -Actin was used as a loading control.

FK228-treated experiments, the level of SLFN11 was markedly increased in *SLFN11*-proficient DMS273, H526 cells following SAHA treatment (Fig. 5b). Concomitantly, a dose-dependent induction of *SLFN11* at the mRNA level was observed upon FK228 (Fig. 5c) or SAHA (Supplementary Fig. S3a) treatment. These data suggested that HDACi transcriptionally regulates SLFN11 expression in SCLC

cells. Next, we sought to investigate whether HDACi-induced SLFN11 could enhance the activity of topotecan to SCLC cells. Different concentrations of FK228 were incubated with H82, H69, H526, and DMS273 cells to induce SLFN11 expression and subsequently withdrawn from the medium 16 h (for H69) or 24 h (for H82 and H526) post-incubation. The cells were then

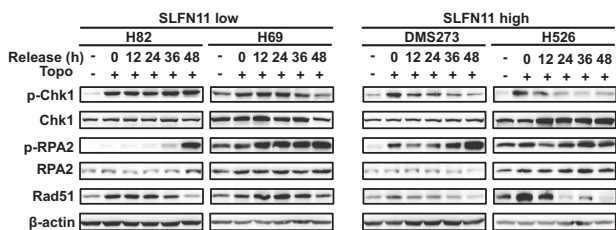


Fig. 3 Western blotting analysis of the kinetics of p-Chk1, p-RPA2 and Rad51 in SCLC cell lines expressing *SLFN11* at high (DMS273 and H526) and undetectable levels (H82 and H69), respectively. The indicated cells were treated with 50 nM topotecan for 24 h. β-Actin was used as a loading control.

treated with a range of concentrations of topotecan for 48 h. Cell viability assays demonstrated that FK228 pre-incubation augmented the sensitivity to topotecan in SCLC cells (Fig. 5d). Importantly, pretreatment with FK228 for 16 or 24 h had minimal effect on cell viability in four cell lines examined (Supplementary Fig. S3b). These results confirmed that the reactivation of SLFN11 by HDAC inhibitor is responsible for the increased activity of topotecan in SCLC cells.

Epigenetic modulation is responsible for FK228-induced SLFN11 and *SLFN11* promoter methylation is prevalent in clinical SCLC samples

To explore the molecular mechanism of FK228-induced re-expression of SLFN11, we assessed the possible epigenetic regulation of SLFN11 by FK228. *SLFN11* expression varies across human cancer cell lines. We first retrieved *SLFN11* expression data from the CCLE database and compared *SLFN11* expression in SCLC cells to lung adenocarcinoma (LUAD) cells. The results showed that *SLFN11* expression was much higher than that in LUAD (Fig. 6a). We next evaluated the methylation status in the promoter of *SLFN11* using the methylation data from CCLE. The majority (34/49) of SCLC cell lines demonstrated a high methylation level at the promoter of *SLFN11*, even though some SCLC cells show high *SLFN11* expression. We wondered whether *SLFN11* expression was associated with the methylation level at the promoter of *SLFN11*. Pearson correlation analysis showed a negative correlation between these two factors (Fig. 6b, $n = 49$), suggesting that *SLFN11* expression is suppressed by promoter methylation in SCLC. Interestingly, we noticed a strong positive correlation between *SLFN11* promoter methylation and the IC_{50} values of topotecan from the JNCI dataset (Fig. 6c, $n = 36$). Similar results were observed in the GDSC2 dataset (Fig. 6c, $n = 27$). We then hypothesized that FK228-induced SLFN11 might involve epigenetic modulation. First, MSP analysis confirmed that methylation at the promoter of *SLFN11* existed in all cell lines tested. FK228 treatment completely abrogated the methylation signals (Fig. 6d). Similar results were also observed in the cells treated with SAHA (Supplementary Fig. S4a). To further elucidate the mechanism leading to SLFN11 re-expression by FK228, ChIP-PCR was employed to examine the changes of histone acetylation at the promoter of *SLFN11* upon FK228 treatment. The results demonstrated that histone acetylation was significantly increased (Fig. 6e, 6f). But the status of histone methylation was not influenced by FK228 (Supplementary Fig. S4b). These data indicated that a FK228 induced-increase of histone acetylation causes a striking reduction of DNA methylation at the promoter of *SLFN11*, thereby leading to the reactivation of SLFN11. Given that FK228 facilitates the sensitivity of topotecan in SCLC cellular models and the methylation status in the promoter region of *SLFN11* might be informative for using FK228 to potentiate the therapeutic efficacy of topotecan, we extended our investigation to SCLC patient samples. The methylation status in the promoter region of *SLFN11* in 27 human primary SCLC was examined by

MSP. Among 27 cases of SCLC, complete methylation was observed in 2 cases of samples; 22 cases exhibit partial methylation; unmethylation was only found in 3 cases of SCLC samples. Together the promoter of *SLFN11* was methylated in 88.89% (24/27) of primary SCLC specimens (Fig. 6g). These results suggest that the promoter of *SLFN11* is frequently methylated, and FK228 might have a much broader application to overcome topotecan's resistance in SCLC.

DISCUSSION

The second-line therapeutic options for small cell lung cancer are limited after relapse. Topotecan has long been considered the only second-line drug for refractory SCLC until the recent approval of lurbinectedin by the FDA. Unfortunately, the topotecan's response rate is so low that less than one-quarter of SCLC patients can benefit from this treatment [24]. Thus, the development of small molecules to broaden the therapeutic potential of topotecan is urgently needed. This study used cellular models to investigate the signal pathways leading to differentiated topotecan sensitivity in SCLC cells. Our data demonstrated that topotecan-insensitive cells unleashed the DNA damage checkpoint and induced Rad51 expression in response to topotecan in the absence of *SLFN11*, whereas *SLFN11* expression in topotecan-sensitive cells blocked the maintenance of DNA damage checkpoint and HR repair, leading to striking DSBs and subsequent cell death. Furthermore, *SLFN11* knockdown and ectopic overexpression in SCLC cells could reverse the sensitivity to topotecan. We further confirmed that *SLFN11* expression was a critical factor in determining the differentiated phenotypes. Importantly, *SLFN11* expression was epigenetically controlled, and HDAC inhibitors FK228 and SAHA epigenetically induced *SLFN11* expression, rendering the cells sensitive to topotecan. Our investigation unveils a strategy to render SCLC cells more sensitive to topotecan.

Previous studies have shown that expression of *SLFN11* sensitized the cells to many DNA damage agents, including camptothecin, topotecan, hydroxyurea, and cisplatin [5, 12, 25]. Furthermore, *SLFN11* has been shown to preferentially associate with euchromatin and be recruited to stalled replication fork by several replication factors upon DNA damage. *SLFN11* acts as a reader of ATR-CHK1 activation, thereby persistently blocking DNA replication and DNA repair [5, 6, 21]. Our study showed that Chk1 phosphorylation was rapidly and robustly induced in the absence of *SLFN11*, but not in the presence of *SLFN11* in SCLC cells. Concomitantly, Rad51, a key HR factor, was also strongly induced in *SLFN11*-proficient cells compared with *SLFN11*-deficient cells. Our data suggest that *SLFN11* works as an executioner to maintain the DDR signals, amplify the DNA damage checkpoint signals, and stimulate HR repair.

The re-expression of *SLFN11* has been reported to be induced by several types of small epigenetic molecules. Although a recent investigation demonstrated that SLFN11 expression could be induced by FK228 and other HDAC inhibitors in several cancer cells, no SCLC cells have ever been tested. In this study, we demonstrated that FK228 removed the DNA methylation mark via the promotion of histone acetylation. These data confirmed coordinated crosstalk between DNA methylation and histone acetylation [26, 27]. Notably, DNA methylation inhibitor 5-Aza did not induce SLFN11 expression in partial SCLC cell lines, which could be induced by EZH2 inhibitor [16], suggesting that other epigenetic events besides DNA methylation might be involved in the suppression of SLFN11. Our study confirmed that FK228 could trigger SLFN11 expression as long as CpG island methylation exists in the promoter of *SLFN11*, no matter whether the cells are *SLFN11*-deficient or *SLFN11*-proficient. The increased histone H3 acetylation by FK228 treatment in the *SLFN11* promoter region might reverse SLFN11 methylation in the CpG island, thereby

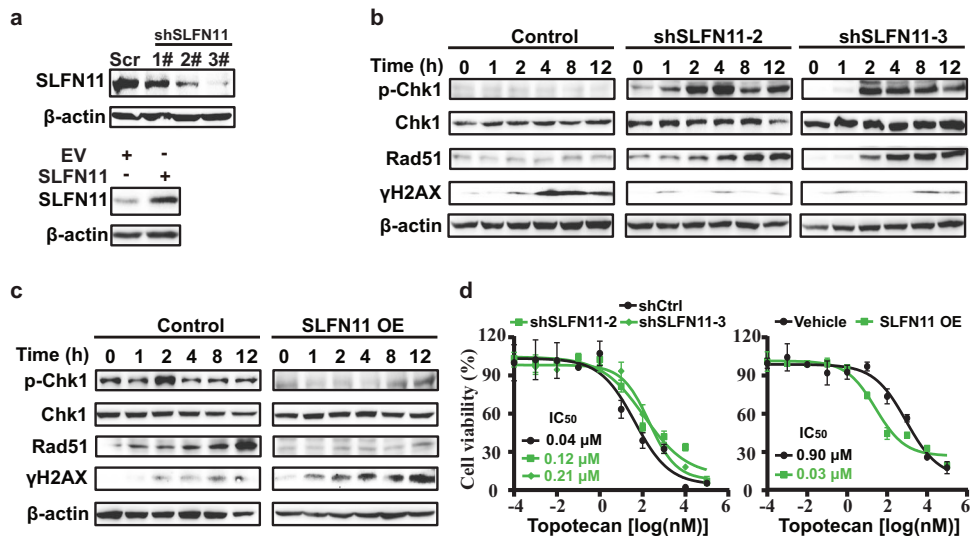


Fig. 4 SLFN11 expression determines the sensitivity of topotecan in SCLC cells. **a** Detection of SLFN11 protein expression in DMS273 cells with *SLFN11* knockdown and H82 cells with *SLFN11* overexpression by Western blotting. **b, c** Western blotting analysis of p-Chk1, Rad51, and γ -H2AX expression in DMS273 cells after knockdown of *SLFN11* (**b**) or in H82 cells after overexpression of *SLFN11* (**c**). **d** Growth inhibition curves of topotecan in DMS273 cells with *SLFN11* knockdown or H82 cells with *SLFN11* overexpression. The cells were treated with different concentrations (10^{-4} nM–100 μ M) of topotecan for 72 h. The cell viability was assessed by the CellTiter-Glo assay. The IC_{50} values were determined from the sigmoidal dose-response curves using PRISM6 software.

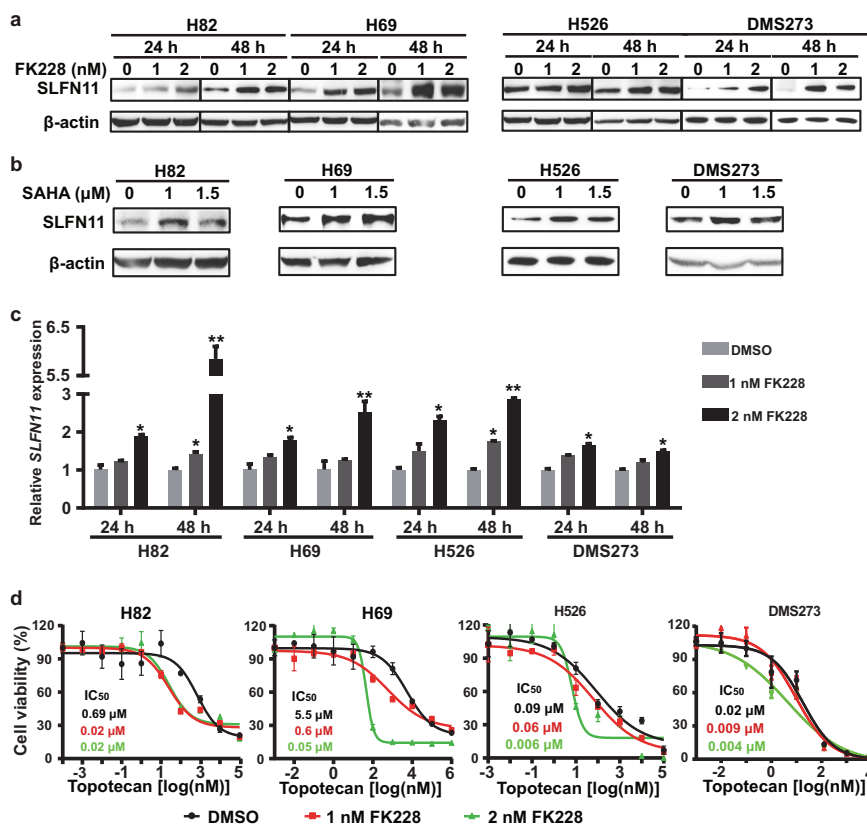


Fig. 5 FK228 increases the sensitivity of SCLC cells to topotecan via the up-regulation of SLFN11. **a** Western blotting analysis of SLFN11 expression in a panel of SCLC cell lines treated with various concentrations of FK228 at indicated times. **b** Western blotting analysis of SLFN11 expression in a panel of SCLC cell lines treated with various concentrations of SAHA for 24 h. **c** RT-qPCR analysis of *SLFN11* expression upon FK228 treatment. **d** Growth inhibition curves of topotecan in a panel of SCLC cell lines (H82, H69 and H526) pretreated with FK228 for 16 h for H69 cells and 24 h for H82 and H526. The cells were then treated with different concentrations (10^{-4} nM–100 μ M) of topotecan for 48 h. The cell viability was assessed by CellTiter-Glo assay.

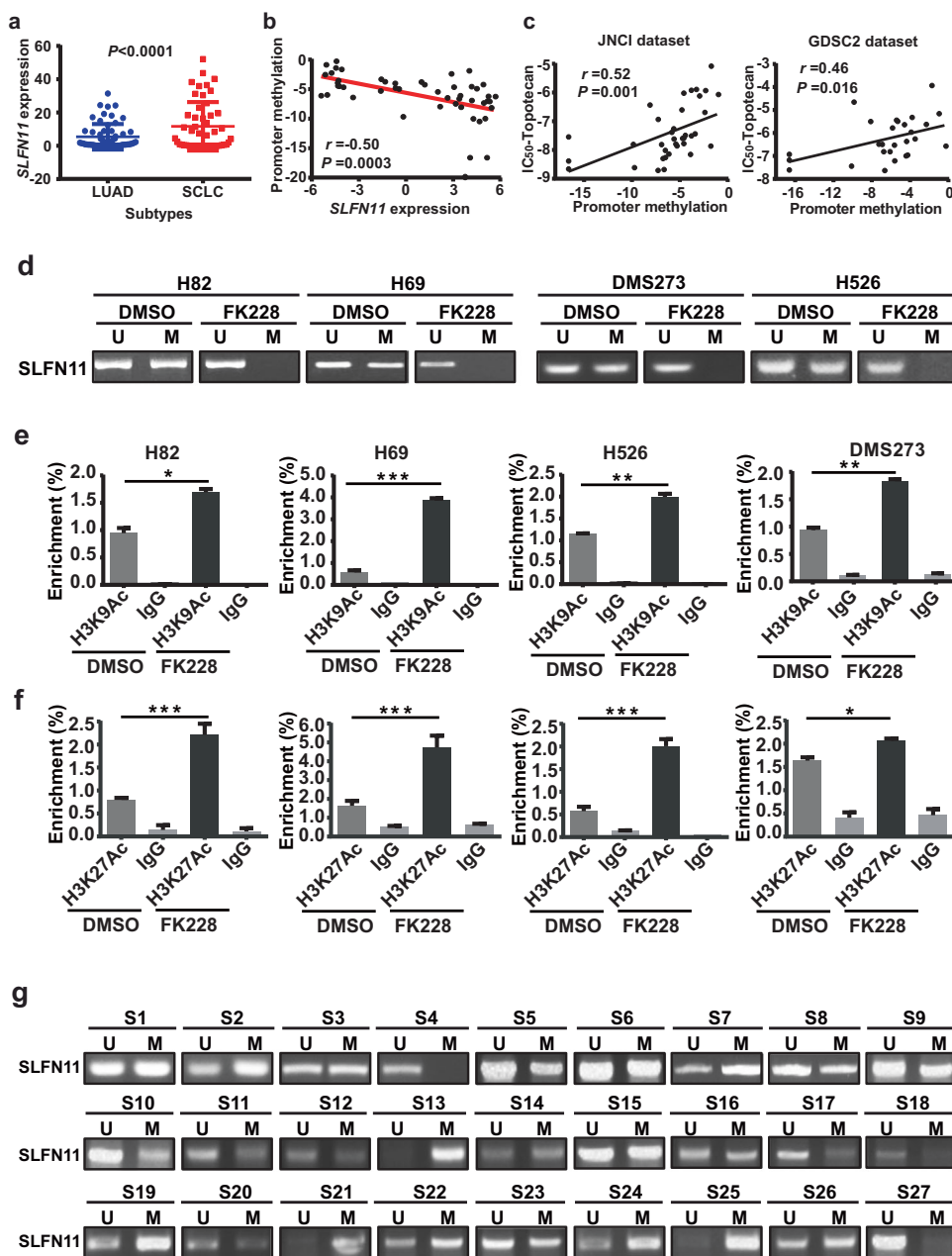


Fig. 6 FK228 induces SLFN11 by decreasing the methylation of the *SLFN11* promoter. **a** The comparison of *SLFN11* expression between lung adenocarcinoma cell lines (LUAD, $n = 59$) and SCLC cells ($n = 49$). **b** The expression level of *SLFN11* correlates to the methylation level of *SLFN11* in 49 SCLC cell lines. **c** IC₅₀ to topotecan is positively correlated with *SLFN11* promoter methylation. Pearson correlation analysis of *SLFN11* promoter methylation and IC₅₀ values of topotecan from the JNCI dataset (left panel) and GDSC2 dataset (right panel). **d** Methylation status of *SLFN11* detected by MSP in SCLC cell lines after FK228 treatment for 24 h. M: methylated alleles, U: unmethylated alleles. Small cell lung cancer cell lines H82, H69, DMS273, and H526 were treated with DMSO and FK228 for 4 h, and then semi-quantitative PCR was performed. **e, f** ChIP-PCR assays to detect the association of H3K9Ac (**e**) and H3K27Ac (**f**) to the promoter of *SLFN11* upon FK228 treatment. The relative activity of H3K9Ac to the *SLFN11* promoter was detected by qPCR. Anti-normal mouse IgG was used as a negative control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **g** Methylation status of *SLFN11* detected by MSP in 27 SCLC clinical samples. M: methylated alleles, U: unmethylated alleles.

reactivating SLFN11 expression. Based on these observations, we speculate that histone modifications might play a more critical role than DNA methylation in the regulation of *SLFN11*. Overall, these lines of evidence prompted us to speculate that the status of *SLFN11* promoter CpG island methylation could be used as a predictive biomarker for choosing FK228 to sensitize SCLC tumors to topotecan.

SLFN11 has been reported to be inactivated in a significant fraction of tumors [10]. Detection of *SLFN11* promoter methylation by MSP has shown that more than half of colorectal cancer

samples display *SLFN11* methylation. Our investigation also showed that more than 80% of SCLC specimens had either complete or partial methylation. These studies suggest that *SLFN11* promoter methylation might be more prevalent than initially estimated. More efforts might be needed to investigate the *SLFN11* promoter methylation pattern across cancer types. On the other hand, re-expression of SLFN11 by epigenetic inhibitors or other small molecules might have more clinical significance. Furthermore, given that SLFN11 expression is critical for multiple DNA damage agents, it is worth further illustrating whether

re-expression of SLFN11 by epigenetic inhibitors such as FK228 augments the sensitivity/overcomes the resistance of other DNA damage agents besides topotecan [16].

In conclusion, SLFN11 could determine the activation strength of the DDR network and repair efficacy in SCLC. *SLFN11* promoter is frequently methylated, and induction of SLFN11 by epigenetic inhibitor FK228 could enhance the sensitivity of SCLC cell lines to topotecan. A clinical trial to test the sensitizing role of FK228 to topotecan in SCLC might be an important future consideration to translate these promising findings into a clinical setting.

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AUTHOR CONTRIBUTIONS

WCL conceived and designed the study. YPY, LYM performed experiments and acquired the data. XTL performed flow cytometry analysis. GZC performed statistical analyses. JHH collected clinical information and performed correlation analyses; YPY wrote the original draft. WCL wrote and reviewed the paper. All authors reviewed the paper and approved the conclusions.

ADDITIONAL INFORMATION

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