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T790M mutation sensitizes non-small cell lung cancer cells to radiation via suppressing SPOCK1

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ABSTRACT

Background: Approximately 50% of patients harbor the T790M mutation after developing first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) resistance. Evidence has showed the major treatment failure is local relapses and limited metastases. Several studies have demonstrated the value of radiotherapy in metastatic non-small cell lung cancer (NSCLC) with the EGFR T790M mutation after the development of TKI resistance. The aim of this study was to explore the role of radiation in T790M-mutant NSCLC and the value of early radiotherapy for NSCLC with T790M-mediated EGFR-TKI resistance.

Methods: Gefitinib-resistant NSCLC cell lines were established via stepwise exposure to increasing concentrations of gefitinib (PC-9-GR). Droplet digital PCR was used to determine the relative T790M subclone abundance. *In vitro* and *in vivo* models were established using different mixtures of PC-9-GR and PC-9 cells. Differentially expressed genes were identified using RNA sequencing. Two research models were constructed (salvage and prophylactic radiotherapy) to determine the effects of early radiotherapy on gefitinib-resistant cells.

Results: PC-9-GR cells exhibited higher radiosensitivity than PC-9 cells (sensitivity enhancement ratio = 1.5). Salvage radiation reduced the number of T790M-mutant subclones, and the relative T790M abundance was significantly lower than that without radiation at 90 days (10.94% vs. 21.54%). Prophylactic radiation prevented the development of T790M subclones. These results were also confirmed *in vivo*. qRT-PCR revealed threefold elevation of miR-1243 in PC-9-GR cells, and the increased radiosensitivity of PC-9-GR cells was inhibited when miR-1243 was knocked down. RNA sequencing revealed that SPOCK1 was downregulated in PC-9-GR cells. Interestingly, bioinformatic analysis showed that SPOCK1 was a target gene of miR-1243. SPOCK1 knockdown markedly increased the radiosensitivity of PC-9 cells.

Conclusion: Gefitinib-resistant NSCLC with the T790M mutation had higher radiosensitivity than that without the mutation, possibly mediated by SPOCK1. Early radiotherapy can eliminate T790M subclones, providing evidence for the benefit of early local treatment in patients with TKI-resistant NSCLC.

1. Introduction

For several decades, lung cancer has remained the most prevalent cancer and the primary cause of cancer-related mortality worldwide [1]. Approximately 40–50% of Chinese patients with non-small cell lung cancer (NSCLC) harbor epidermal growth factor receptor (EGFR) mutations. EGFR-tyrosine kinase inhibitors (EGFR-TKIs) are highly effective clinical therapies for NSCLC patients with EGFR mutations, with an objective response rate of almost 70% and a prolonged progression-free survival (PFS) of 8–13 months [2–7]. However, the development of drug resistance remains an important challenge for successful EGFR-TKI therapy in terms of disease control. In two clinical

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trials, one from the Memorial Sloan Kettering Cancer Center and one from Yonsei University, 60% of patients experienced local relapse in the thoracic region, and, in another study, treatment failed in 62.5% of patients in the thoracic area [8]. Thus, thoracic radiation may benefit such patients.

In 2011, an exploratory prospective study was conducted to evaluate the efficacy of a combination of thoracic radiation and EGFR-TKIs [9]. The median PFS was 10.2 months and median overall survival was 21.8 months in those patients. Early integration of radiotherapy (RT) in patients with non-squamous NSCLC who responded to EGFR-TKIs was effective in another study, yielding a median PFS of 16 months [10]. In patients with local relapse, RT remains valuable despite the development of third-generation EGFR-TKIs.

The T790M mutation in EGFR is a major mechanism of resistance to first-generation EGFR-TKIs. Several clinical studies have demonstrated the value of early RT in patients with EGFR-mutant NSCLC. However, little is known about the value of RT specifically for patients with NSCLC with the T790M mutation. In this study, we aimed to explore the value of early RT in NSCLC cell lines exhibiting T790M-mediated EGFR-TKI resistance.

2. Methods and materials

2.1. Cell culture

Human NSCLC cell line PC-9 was supplied by the National Collection of Authenticated Cell Cultures (Shanghai, China). Gefitinib-resistant PC-9 cells (PC-9-GR) were successfully established by stepwise exposure to increasing concentrations of gefitinib, as described in a previous study [11]. These cell lines were maintained in RPMI 1640 media with 10% Gibco fetal bovine serum (New York, USA). All experiments were approved by the Ethics Committee.

2.2. MiRNA array and RNA sequencing

Total RNA was extracted from PC-9 and PC-9-GR cells by using TRIzol Reagent (Invitrogen, Waltham, MA USA) and reverse transcribed into cDNA by using PrimeScript RT Master Mix (Takara, Kusatsu, Japan). We used the TaqMan Array Human MicroRNA Card v2.0 (Applied Biosystems, Waltham, MA, USA) to determine miRNA expression profiles. MiRNAs with $\geq$2 fold changes were selected for further analysis. RNA sequencing was performed using Illumina mRNA deep sequencing by LC Sciences (Houston, TX, USA). Sequencing results were obtained as fragments per kilobase of exon per million reads (FPKMs) for each transcript. Gene expression level was quantified according to FPKMs. We used edgeR v3.30.3 [12] to define differentially expressed genes with the following criteria: $p < 0.05$ and fold change $\geq$2.

2.3. MiRNA target prediction

TargetScanHuman (Release 8.0, https://www.targetscan.org) and miRDB (http://mirdb.org/mirdb/index.html) were used for miRNA target prediction.

2.4. Transfection and viral infection

MiRNA inhibitor negative controls (NCs) were obtained from GenePharma (Shanghai, China). Cells were transfected with 30 nM miRNA NC or miRNA inhibitor by using Lipofectamine 3000 according to the manufacturer's instructions.

The pSLenti-U6-shRNA (SPOCK1)-CMV-EGFP-F2A-Puro-WPRE and pSLenti-U6-shRNA (NC)-CMV-EGFP-F2A-Puro-WPRE plasmids were purchased from Obio Technology (Shanghai, China). Cells were transfected with 2.5 μg of either plasmid with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Silencing effectiveness was verified by western blotting.

2.5. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA from tumor cells was isolate by using TRIzol Reagent. cDNA Reverse transcribed from total RNA (PrimeScript RT Master Mix, Takara) was used to determine miRNA and mRNA (Mir-X miRNA First-Strand Synthesis Kit, Clontech Laboratories, Mountain View, CA, USA). RNA expression was subsequently determined by qRT-PCR according to the manufacturer's protocol (SYBR Kit, Clontech Laboratories). U6 expression was used to normalize the miRNA expression. The primers used in this study were as follows: U6-Forward (5′-GGAACGATACAGAGAAGATTAGC-3′), U6-Reverse (5′-TGGAACGCTTCACGAATTTGCG-3′), hsa-miR-100–5p (AACCCGTAGATCCGAACTTGTG), hsa-miR-1233 (TGAGCCCTGTCCTCCCGCAG), hsa-miR-1243 (AACTGGATCAATTATAGGAGTG), hsa-miR-1275 (GTGGGGGAGAGGCTGTC), hsa-miR-139–5p (TCTACAGTGCACGTGTCTCCAG), and hsa-miR-505–5p (GGGAGCCAGGAAGTATTGATGT).

2.6. Clonogenic formation assay

One thousand to 6000 tumor cells were plated in 6-well plates. The cells were irradiated using a Precision X-RAD 225 machine (source-to-cell distance: 36 cm; dose rate: 1.3 Gy/min). Methanol was used to fix the colonies 10–14 days after irradiation. The colonies were stained with 0.1 % crystal violet for 30 min. A linear quadratic model was used to fit the survival curves.

2.7. Western blotting (WB)

WB was performed as previously described [13]. Rabbit *anti*-SPOCK1 polyclonal antibody (1:2000; Abcam, Cambridge, UK) and *anti*-GAPDH polyclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) were used to detect antigens. We used anti-rabbit IgG and anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, USA) as secondary antibodies.

2.8. In vivo tumor experiments

Male, 6-to-8-week-old BALB/c nude mice were used for *in vivo* experimentation (Experimental Animal Center of Hangzhou Medical College, Hangzhou, China). All animal experiments complied with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978). PC-9 and PC-9-GR cells were subcutaneously administered to the right axilla of the mice. A single, 12 Gy dose of radiation was administered when the average tumor volume reached 200 mm^3. Tumor volume was assessed every second day with a caliper and computed using the following formula: volume (mm^3) = (length × $width^2$)/2.

2.9. Statistical analysis

Data analysis and figure presentation were performed using GraphPad Prism, Version 9.50 (GraphPad Software, San Diego, CA, USA). Each experiment was performed in triplicate.

3. Results

3.1. Radiosensitivity of PC-9-GR cells

Subclones of NSCLC cells with a stable resistance to gefitinib and persistent EGFR T790M mutation were selected for further research (PC-9-GR cells). We determined the IC_{50} for gefitinib in PC-9-GR cells as 8.6 μM. To explore the radiosensitivity of gefitinib-resistant NSCLC cells, we performed colony formation assays using PC-9-GR and PC-9 cells. PC-9-GR cells had higher radiosensitivity than PC-9 cells (sensitivity enhancement ratio = 1.5; Fig. 1).

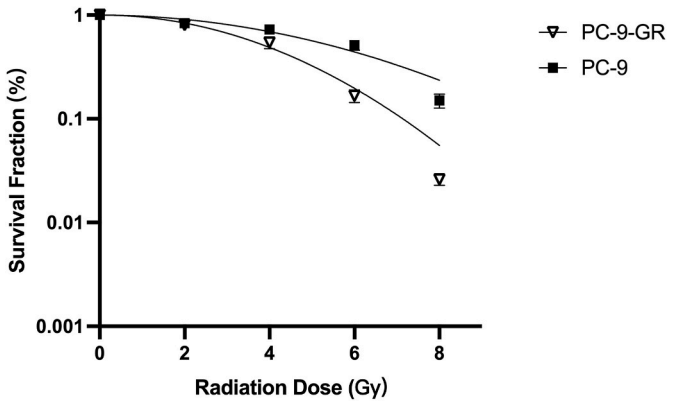

Fig. 1. Colony formation assays of PC-9 and PC-9-GR cells. N = 3 wells/group.

3.2. In vitro study

Two research models were constructed (salvage and prophylactic RT) to determine the effects of early RT on gefitinib-resistant cells. Fig. 2A illustrates the pipeline of the research models.

In the salvage RT model (Fig. 2B), PC-9 cells were initially exposed to gefitinib at IC_{30}. When T790M subclones were detected at an abundance of 0.5% by digital droplet PCR, the cells were exposed to an escalated concentration of gefitinib (IC_{50}) and also treated with 4 Gy or 0 Gy irradiation. After 5 days of culturing, the proportions of T790M subclones were 0.18% and 0.42% in the salvage RT and control groups, respectively. After 15 days of culturing, those proportions had increased to 0.47% and 1.05%, respectively. At that point, the concentration of gefitinib was increased to IC_{70}. After 30 days, the proportions of T790M subclones had increased to 5.52% and 18.39% in the salvage RT and control groups, respectively, and the concentration of gefitinib was increased to IC_{90}. After 90 days, the proportions of T790M subclones were 10.94% and 21.54%, respectively. These results indicate that salvage RT can reduce the proportion of T790M-mutant subclones of PC-9 cells.

In the prophylactic RT model (Fig. 2C), PC-9 cells were exposed to increasing concentrations of gefitinib, followed by 4 Gy or 0 Gy radiation after 24 h. At IC_{30} and IC_{50}, no T790M mutant subclones were detected in either group. When gefitinib was increased to IC_{70}, the proportions of T790M subclones were 0% and 0.06% in the prophylactic RT and control groups, respectively. At IC_{90}, the proportions of T790M subclones were 0% and 1.5% in those two groups, respectively. When cells were cultured with gefitinib at IC_{90} for 1 year, T790M mutations were still not detected in the prophylactic RT group. These results indicated that prophylactic RT can delay T790M-induced resistance to EGFR-TKIs.

3.3. In vivo study

TKI resistance is gradually acquired. To mimic the different stages, different proportions of PC-9-GR cells were mixed with PC-9 cells: 0%, 1%, 10%, and 50% PC-9-GR cells; each of these were separately implanted into mice. When the tumors grew to 200 mm^3, radiation was administered with or without gefitinib. All tumors were excised when they grew to 1000 mm^3. The proportion of T790M mutant cells was significantly lower in the RT and RT + TKI groups than in the TKI and control groups, indicating that radiation reduced the proportion of T790M subclones (Fig. 3A). Moreover, tumor growth was markedly inhibited after radiation in all groups (Fig. 3B).

3.4. Preliminary determination of mechanism of increased radiosensitivity in T790M subclones

To explore the mechanism of high radiosensitivity in T790M-mutant lung cancer cells, miRNA chip analysis was performed on PC-9 and PC-9-GR cells. Six miRNAs (miR-100–5p, miR-1243, miR-505–5p, miR-1233, miR-1275, and miR-139–5p) were selected, as they had fold changes >1.5 between the cell types. qRT-PCR revealed a threefold elevation in miR-1243 expression in PC-9-GR cells compared to PC-9 cells (Fig. 4A). A colony formation assay revealed that the increased radiosensitivity of PC-9-GR cells was inhibited when miR-1243 was knocked down (Fig. 4B).

We performed RNA sequencing on PC-9 and PC-9-GR cells. The results revealed 442 up-regulated and 326 down-regulated genes in PC-9-GR cells. SPOCK1 was down-regulated in PC-9-GR cells (Fig. 4C). We

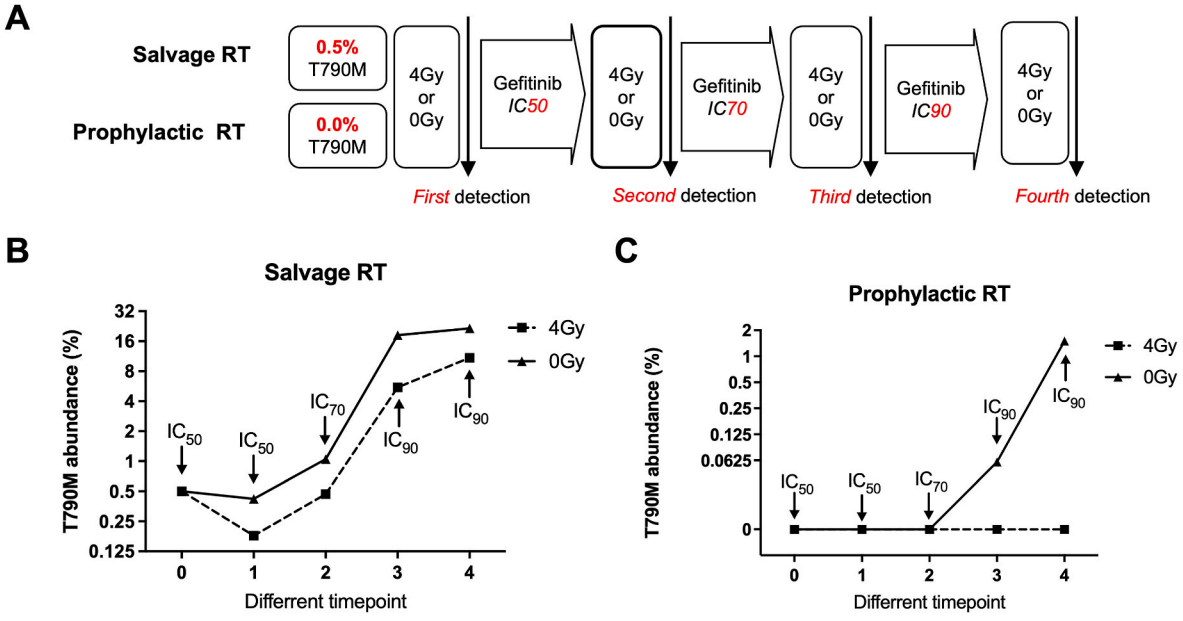

Fig. 2. Impact of radiation on relative T790M abundance. (A) Pipeline of salvage and prophylactic radiotherapy (RT) in *vitro* research models. (B) Administration of gefitinib in lung cancer cells with a 0.5% initial proportion of T790M subclones, with or without 4 Gy irradiation. (C) Administration of gefitinib in lung cancer cells with a 0% initial proportion of T790M subclones, with or without 4 Gy irradiation.

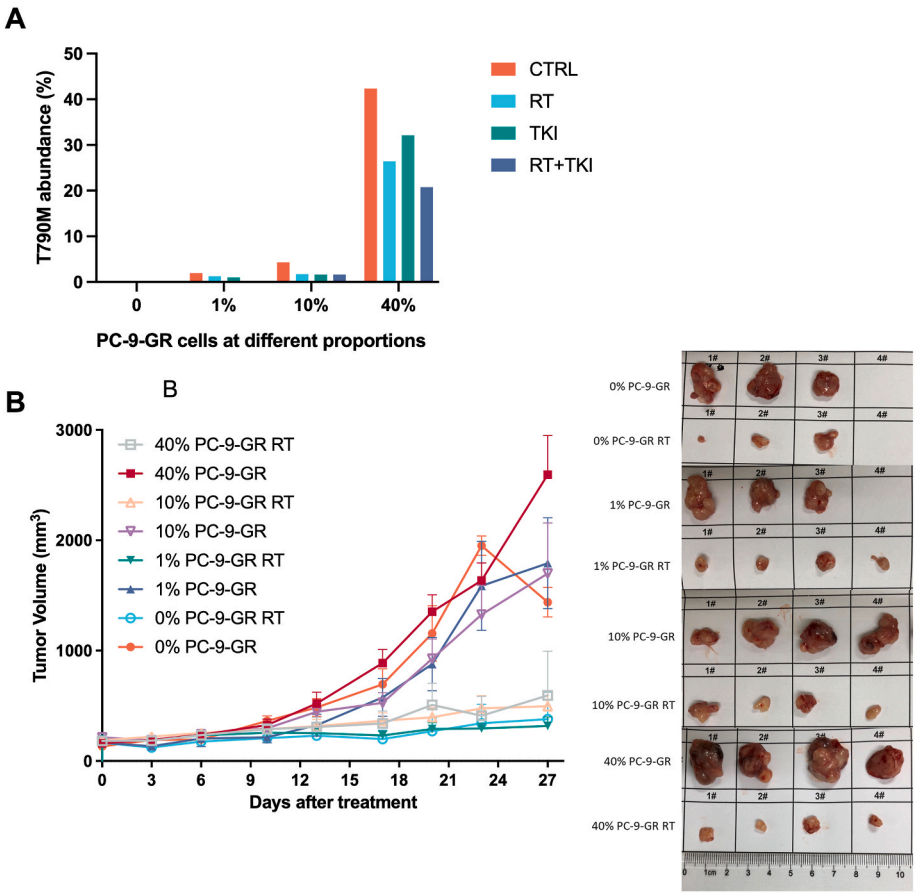

Fig. 3. Radiation inhibits T790M subclones and tumor growth *in vivo*. (A) Relative T790M abundance after radiotherapy (RT) of PC-9-GR cells at different proportions (0%, 1%, 10%, and 50%) among PC-9 cells. These mixed cells were implanted into mice. A single 10 Gy irradiation was performed when the tumor size reached 200 mm^3. (B) Tumor growth curve with or without radiation in different proportions of PC-9-GR cells (0%, 1%, 10%, and 50%) mixed with PC-9 cells. These mixed cells were implanted into mice. A single 10 Gy irradiation was performed when the tumor grew to 200 mm^3. CTRL, control; TKI, tyrosine kinase inhibitor.

used the miRBase database to predict the target genes of miR-1243. SPOCK1 was a target gene of miR-1243. To validate the role of SPOCK1 in radiosensitivity, three small hairpin RNAs (shRNAs) were used to knock down SPOCK1. The knockdown of SPOCK1 in PC-9 cells was confirmed at the protein level (Fig. 4D). SPOCK1 knockdown markedly increased the radiosensitivity of PC-9 cells (Fig. 4E).

4. Discussion

EGFR-TKIs are the standard of care for patients with EGFR-sensitive NSCLC. Although several studies have demonstrated the superiority of third-generation TKIs to first-generation TKIs in nationwide population databases [14–16], gefitinib is still widely used in many regions with less inclusive insurance policies or poorer economic situations. Additionally, osimertinib reportedly does not improve the overall survival of patients with EGFR exon 21 mutations [14]. Therefore, first-generations TKI still warrant further research. As tumor cells inevitable acquire resistance to EGFR-TKIs, these treatments yield a median PFS of 8–12 months. Most cases of disease progression involve local relapses or limited metastases [17]. RT offers additional survival benefits for such patients [18]. Lim et al. analyzed 49 patients who developed gefitinib resistance and discovered that local treatment, including RT, prolonged PFS by 5.1 months [19]. EGFR-TKIs may increase the radiosensitivity of lung cancer cells [11,20,21]. This may partially explain the superiority of the combination of RT and EGFR-TKIs demonstrated in our study. Our study revealed that RT can effectively eliminate T790M subclones. Notably, innate radiosensitivity increased in gefitinib-resistant cells harboring the T790M mutation in our study.

In patients without disease progression, preemptive RT to residual sites reportedly yields a survival benefit [22]. Zheng et al. revealed that concurrent administration of EGFR-TKIs and thoracic RT prolonged PFS to 13 months and the time to local failure to 20.5 months [23]. Yen et al. analyzed 1475 patients receiving first-line EGFR-TKIs and 295 patients who received thoracic RT for lung tumors during the EGFR-TKI response [24]. They demonstrated that early RT was associated with better overall survival. Another study confirmed the benefit of early RT in EGFR-TKI responders [25]. Several studies have indicated that early RT to residual sites can significantly improve tumor control and prolong survival. However, the underlying mechanisms have not yet been fully elucidated. In our study, preemptive RT inhibited the development of T790M subclones. In our *in vitro* study, constant exposure to gefitinib stimulated the development of T790M mutants, whereas early RT, before T790M development, extended the time of susceptibility to gefitinib.

SPOCK1 is a heparan sulfate/chondroitin sulfate proteoglycan belonging to the multidomain testicular proteoglycan family. SPOCK1 is reportedly associated with cell proliferation and metastasis [26,27]. Váncza et al. revealed that SPOCK1 overexpression is correlated with a poor prognosis in patients with ovarian cancer [28]. In our study, SPOCK1 was downregulated in gefitinib-resistant lung cancer cells. In a study by Gao et al. SPOCK1 was upregulated in osimertinib-resistant lung cancer cells, and SPOCK1 knockdown inhibited osimertinib-resistant cell growth, overcoming its resistance [29]. These results are not contradictory, because gefitinib-resistant lung cancer cells harboring the T790M mutation are sensitive to osimertinib. Several studies have demonstrated that SPOCK1 promotes tumor growth and

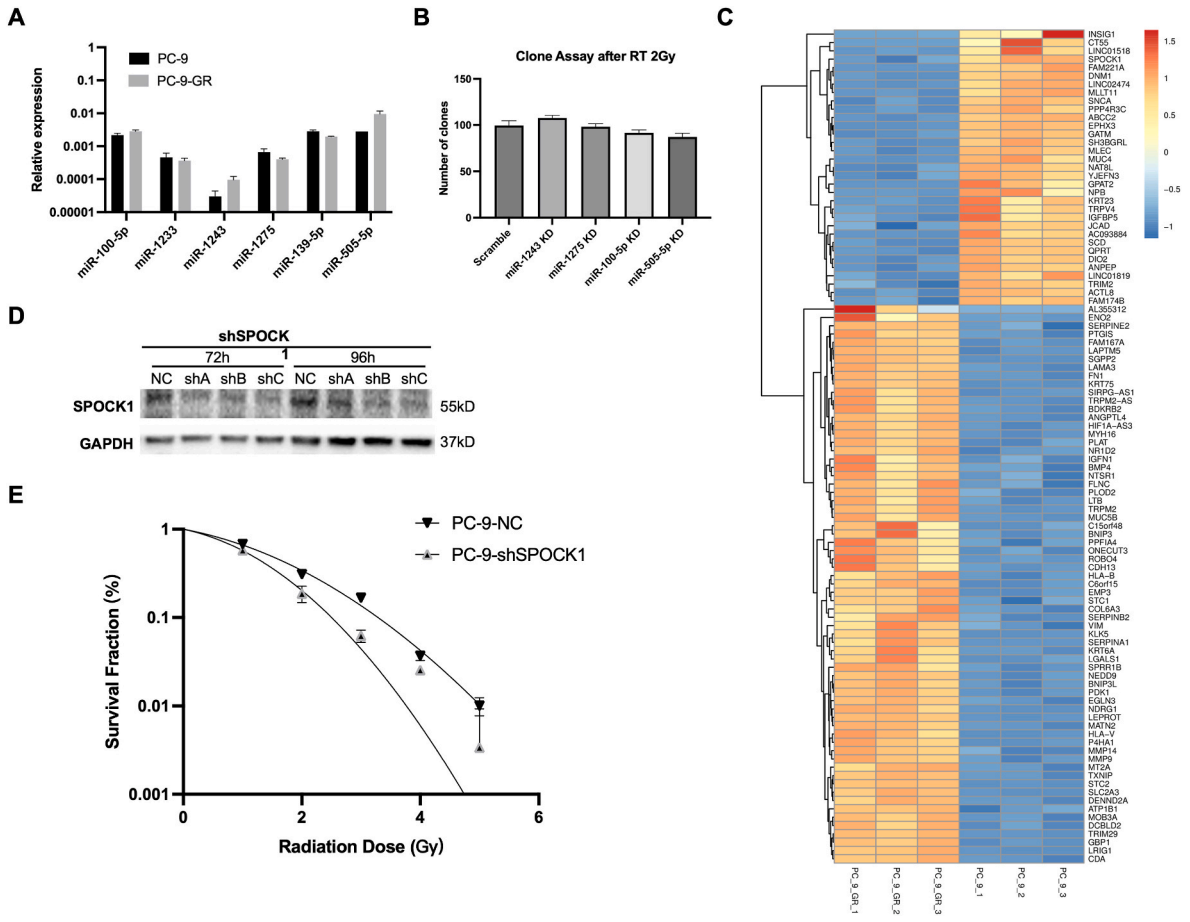

Fig. 4. Reduced SPOCK1 expression in T790M mutant non-small cell lung cancer contributes to increased radiosensitivity. (A) qPCR of six miRNAs (miR-100–5p, miR-1243, miR-505–5p, miR-1233, miR-1275, and miR-139–5p) in PC-9 and PC-9-GR cells. (B) Colony formation assays in PC-9 cells after 2-Gy irradiation with miRNA knockdown (KD). (C) RNA sequencing result of PC-9 and PC-9-GR cells. (D) Western blot of PC-9 transduced with a small hairpin RNA (shRNA) negative control, shSPOCK1-A, shSPOCK1-B, or shSPOCK1-C. (E) Colony formation assays in PC-9 cells and in PC-9 cells with SPOCK1 KD. RT, radiotherapy.

metastases [30–32]. However, research on SPOCK1 and tumor radiosensitivity is limited. In our study, SPOCK1 was downregulated in PC-9-GR cells, and the radiosensitivity of PC-9-GR cells was higher than that of PC-9 cells. When SPOCK1 was knocked down, the radiosensitivity of PC-9 cells increased.

To the best of our knowledge, this is the first study to explore the mechanism of early RT on EGFR-TKIs, *in vivo* and *in vitro*. SPOCK1 may be the key to the increased radiosensitivity of gefitinib-resistant lung cancer. However, the downstream targets of SPOCK1 were not identified in our study. The key genes affecting radiosensitivity were also not explored. These aspects warrant exploration in future studies.

In conclusion, our study revealed that gefitinib-resistant NSCLC with the T790M mutation exhibited higher radiosensitivity than NSCLC without the mutation. Early RT eliminated T790M subclones, providing evidence for the benefit of early local RT in patients with TKI-resistant NSCLC. SPOCK1 and its downstream pathways might be hot topics for future radiation research.

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Data availability statement

The original contributions presented in the study are included in the article material. Further inquiries can be directed to the corresponding authors.

CRediT authorship contribution statement

Yasi Xu: Writing – original draft, Writing – review & editing. **Pengjun Zhao:** Conceptualization, Formal analysis. **Xiao Xu:** Methodology. **Shirong Zhang:** Resources. **Bing Xia:** Funding acquisition, Writing – review & editing. **Lucheng Zhu:** Data curation, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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