Hypo-fractionation radiotherapy normalizes tumor vasculature in non-small cell lung cancer xenografts through the p-STAT3/HIF-1 alpha signaling pathway

Fan Tong*, Chun-jin Xiong*, Chun-hua Wei, Ye Wang, Zhi-wen Liang, Hui Lu, Hui-jiao Pan, Ji-hua Dong, Xue-feng Zheng, Gang Wu and Xiao-rong Dong

Abstract

Background: Hypo-fractionation radiotherapy (HFRT) was considered to be an important treatment for non-small cell lung cancer (NSCLC), but the radiobiological effects of HFRT on NSCLC remain unclear. The aim of this study was to investigate specific biological effect of HFRT on tumor angiogenesis, compared with conventional radiotherapy (CRT).

Methods: The subcutaneous xenograft models and the dorsal skinfold window chamber (DSWC) models of nude mice bearing H460 and HCC827 NSCLC cells were irradiated with doses of 0 Gy (sham group), 22 Gy delivered into 11 fractions (CRT group) or 12 Gy delivered into 1 fraction (HFRT group). At certain time-points after irradiation, the volumes, hypoxic area, coverage rate of pericyte and micro-vessel density (MVD) of the subcutaneous xenograft models were detected, and the tumor vasculature was visualized in the DSMC model. The expressions of phosphorylated signal transducer and activator of transcription (p-STAT3), hypoxia-inducible factor 1-α (HIF-1α), CXCL12 and VEGFA were detected.

Results: Compared with the CRT groups, HFRT showed more-efficient tumor growth-suppression, accompanied by a HFRT-induced window-period, during which vasculature was normalized, tumor hypoxia was improved and MVD was decreased. Moreover, during the window-period, the signal levels of p-STAT3/HIF-1α pathway and the expressions of its downstream angiogenic factors (VEGFA and CXCL12) were inhibited by HFRT.

Conclusion: Compared with CRT, HFRT induced tumor vasculature normalization by rendering the remaining vessels less tortuous and increasing pericyte coverage of tumor blood vessels, thereby ameliorating tumor hypoxia and enhancing the tumor-killing effect. Moreover, HFRT might exert the aforementioned effects through p-STAT3/HIF-1α signaling pathway.

Keywords: HIF-1α, hypo-fractionation radiotherapy, non-small cell lung cancer, STAT3, vascular normalization

Introduction

Radiotherapy plays a pivotal part in cancer treatment, especially for non-small cell lung cancer (NSCLC). Ablation hypo-fractionation radiotherapy (SBRT) and stereotactic radiosurgery, refers to high-precision radiotherapy techniques designed to safely deliver effective radiation doses in fewer (commonly between one and eight) fractions. This is achieved through the use of advanced treatment planning and delivery techniques, including onboard imaging for image-guided radiation therapy delivery. Several clinical trials have demonstrated that ablative HFRT is an effective and well-tolerated therapy for early-stage NSCLC in medically-inoperable patients. Compared with conventional
Radiation therapy (CRT), HFRT increased the biologically equivalent dose to the tumor volume with superior local control of the primary disease in inoperable NSCLC patients. In addition, Jin et al. demonstrated that for sufficiently high prescription doses, HFRT was associated with less relative damage-volume than with that of CRT, as predicted by a local threshold dose model. However, the radiobiological mechanisms of ablative HFRT remain poorly understood.

According to the 5 'R model, the killing effect of HFRT on tumor cells seems to be weaker than that of CRT; however, this is inconsistent with current clinical findings. Over the past few years, this narrow radiobiological view has shifted towards understanding the central role of the tumor microenvironment. The oxygenation status has been reported to be a key factor that might affect the efficacy of radiotherapy. As the previous study showed, compared with CRT, HFRT exhibited the advantages of remodeling the normalization of tumor vasculature and increasing re-oxygenation in tumor. Tumor blood vessels are composed of an endothelial lining surrounded by a supportive layer(s) of pericytes, which are essential for vascular maturation and physiological perfusion, therefore, the pericyte coverage was widely used to assess the normalization of tumor blood vessels. So far researchers have not yet reached a consensus about effects of radiation on the angiogenesis, which depend on the total dose, fraction size and the type of radiotherapies, as well as the location and stage of tumors.

Hypoxic condition results in many solid tumors when micro-vessels are structurally and functionally abnormal, and hypoxic tumor cells are resistant to radiotherapy. In normoxia, hypoxia-inducible factor 1-α (HIF-1α) is known to be hydroxylated on specific proline residues by prolyl hydroxylases, thereby becoming a substrate for Von Hippel–Lindau tumor suppressor protein (pVHL), a E3 ligase for proteasome degradation. Under hypoxic condition, the degradation of HIF-1α is inhibited, and stabilized HIF-1α activates the transcription of genes related to in cell survival, proliferation, apoptosis, glucose metabolism and angiogenesis. Tumor vasculatures are induced by hypoxia principally via two major pathways: (1) the colonization of circulating endothelial progenitor cells to the hypoxic region through CXCL12 and its receptor CXCR4 (vasculogenesis); (2) sprouting from and endothelial proliferation of local vessels through the VEGF pathway (angiogenesis). Therefore, hypoxic condition is one of the most important environmental factors that induce tumor angiogenesis. And, based on current clinical findings, exploring the differences of the post-radiation changes between HFRT and CRT in tumor vasculature and oxygenation might help understand the advantage of HFRT over CRT shown by current clinical findings.

In this study, we established a mouse model bearing NSCLC H460 and HCC827 xenografts, and dynamically observed the structural and functional changes in tumor micro-vessels after HFRT or CRT, to explore the differences between HFRT and CRT in ameliorating hypoxic conditions. In addition, we investigated the mechanism by which HFRT improves hypoxia in the tumor microenvironment.

**Methods and materials**

**Cell cultures and animals**

H460 and HCC827 cells were maintained in the Laboratory Center of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The in vitro experiments were approved by the committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and the approval number was C3256. The cells were cultured in the Roswell Park Memorial Institute (RPMI)-1640 culture medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂-humidified incubator. The cells grown until the logarithmic phase were used in the experiment. Six-week-old Babl/c nude female mice were procured from the Beijing HFK Bioscience Company, China. The mice, weighing about 20 g on average, were housed, with four to five animals per cage, in laminar flow hoods and pathogen-free rooms to minimize the risk of infection. Animal models were established by subcutaneous injection of 5 × 10⁶ cells into the right proximal hind legs of the mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Wuhan, China. And the IACUC number was S2323 provided by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Adequate efforts were taken to reduce the number of animals required for a set of experiments and mitigate their suffering.
Tumor irradiation and measurement
When the xenograft volumes reached approximately 70 mm$^3$, the transplanted mice were randomly divided into three groups (n = 5 mice in each group): sham group (0 Gy), CRT group (22 Gy/11F) and HFRT group (12 Gy/1F). Irradiation was delivered using a Varian Clinac 600C X-ray unit at 250 cGy/min (80 cm source-to-skin distance). Before irradiation, each mouse was anesthetized by giving 5% chloral hydrate and was shielded by a lead cover, with only the tumor exposed to the field. The mice in CRT group received five daily fractions of 2 Gy per week (exclusive of weekends). Tumor diameters were measured every other day by using a caliper. The tumor volume was calculated by the formula: volume $V = \text{length} \times \text{width} \times \text{width} \times 0.5$.

Cells irradiation
When H460 and HCC827 cells grew to 80–90% confluence, the cells were irradiated with different doses in these corresponding groups: sham group (0 Gy), CRT group (22 Gy/11F) and HFRT group (12 Gy/1F). S3I-201 (STAT3 inhibitor) was purchased from Merck (Rockland, MA, USA) and was dissolved in dimethyl sulfoxide (DMSO). Twenty-four hours before irradiation, the cells were treated by DMSO-dissolved S3I-201 at a final concentration of 100 μM. The same volume of DMSO was used as negative control. After irradiation, the cells were returned to the CO$_2$ incubator and maintained at 37°C in 5% CO$_2$/95% air for 3, 6, 24 and 48 h post-irradiation.

Immunofluorescence staining
The tumor sections (6 μm) were fixed in 4% paraformaldehyde for 3–4 h at room temperature, and rinsed with phosphate-buffered saline (PBS). After washing with PBS, the non-specific binding sites were blocked with 5% non-fat skimmed milk (GTX27481, GeneTex) for 1 h at room temperature. The samples were incubated with one or two primary antibodies, that is, mouse monoclonal anti-CD34 (1:50, Abcam, USA) and rabbit polyclonal anti-α-SMA (1:50, Abcam, USA), simultaneously in 1% goat serum overnight. After washing, the sections were incubated with the secondary antibodies: Alexa Flour® 568 goat anti-rabbit IgG (H+L) (1:200, A11011, Invitrogen) and Alexa Flour® 488 goat anti-mouse IgG (H+L) (1:200, A1106, Invitrogen). After washing three times with PBS for 5 min, the nuclei were stained with DAPI (S36939, Invitrogen) for 15 min, and the sections were then examined under a confocal scanning microscope (BX41F, Olympus, Tokyo, Japan). The ratio of α-SMA/CD34 was calculated by dividing the positive area of α-SMA adjacent to CD34-positive vessels by the total area of CD34-positive tumor vasculature under five 200× high-powered randomly chosen fields per slide.

Immunohistochemistry
Immunohistochemical staining with CD34 or pimonidazole was performed on formalin-fixed and paraffin-embedded tissue sections. Briefly, after antigen retrieval, tissue sections were incubated with anti-CD34 antibody (Abcam, USA) overnight at 4°C, and then incubated with biotinylated secondary antibody, and then with avidin–biotin–peroxidase complex (DAKO, Glostrup, Denmark). Finally, the tissue sections were incubated with 3′, 3′-diaminobenzidine (Sigma, St Louis, MO, USA) and counterstained with hematoxylin. In negative controls, primary antibodies were not applied. Pimonidazole hydrochloride (Chemicon International, Temecula, CA, USA) was used to detect the tumor hypoxia as previously described. Briefly, tumor-bearing mice were intraperitoneally injected with pimonidazole hydrochloride (0.1 mg/g body weight) dissolved in 10 mg/ml in 0.9% saline 1 h before sacrifice. The ratio of pimonidazole-positive area (%) was defined as the pimonidazole-positive area divided by the visible tumor area under 100-fold magnifications. The tumor micro-vessel density (MVD) was expressed as the ratio of CD34 positive stained area per total tumor area in a 200× high-power field.

Western blotting
Proteins were extracted from the cells and tissues by using a protein extraction kit (Pierce Biotechnology Inc., IL, USA) in accordance with the manufacturer’s protocol. In addition, cytoplasmic and nuclear proteins were also extracted subsequently. Protein extracts were first separated on 15% sodium dodecyl sulfate polyacrylamide gels and then transferred onto a nitrocellulose membrane at 150 mA. After blocking with 5% non-fat skimmed milk, the membrane containing the protein extracts was incubated overnight with primary antibody diluted with 2% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 at 4°C. The primary antibodies were as follows: signal transducer and activator of transcription3 (STAT3) (1:1000, Cell Signaling...
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Technology, USA), p-STAT3 (1:1000, Cell Signaling Technology, USA), HIF-1α (1:1000, Cell Signaling technology, USA), VEGFA (1:1000, Cell Signaling technology, USA), CXCL12 (1:1000, Cell Signaling technology, USA) and GAPDH (1:10000, Bioworld, USA). On the next day, the proteins were visualized using the enhanced chemiluminescence detection system (Pierce, USA) after incubation with respective horseradish peroxidase-conjugated secondary antibodies (1:1000), and then exposed to medical X-ray film. The intensity of the blots was quantified by employing a gel image analyzer (JS380; Peiqing Science and Technology, Shanghai, China).

Isolation of RNA and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from tumor tissues of different groups using RNase Mini Kit (Qiagen, Valencia, CA, USA) and following kit instructions. Primer sequences were designed by using Beacon Designer software package (Bio-Rad). The sequences of primers were as follows: CXCL12 sense 5’-GCT ACA GAT GCC CAT GCC GAT-3’ and anti-sense 5’-AGC TTC GGG TCA ATG CAC ACT-3’, GAPDH sense 5’-TCA CCACCA TGG AGA AGG-3’ and anti-sense 5’-GCT AAG CAG TTG GTG GTG CA-3’, VEGFA sense 5’-CTG TGC AGG CTG CTG TAA CG-3’ and anti-sense 5’-GTT CCC GAA ACC CTG AGG AG-3’. All primers were synthesized by Invitrogen (Groningen, Netherlands). Real-time quantitative polymerase chain reaction (qPCR) for cDNA analysis was conducted at 60–95°C for 45 cycles on a Sequence Detection System (ABI Prism 7000, Applied Biosystems, Darmstadt, Germany) by following the instructions given with the kit and using SYBR Green Reaction Master Mix (TaKaRa Biotechnology Co. Ltd., Dalian, China). For each sample, GAPDH served as the housekeeping gene. Fold-expression was calculated from the threshold cycle values. For the calculation of relative changes, the gene expression measured in sham-irradiated tissues was taken as baseline value.

Dorsal skinfold window chamber model

The dorsal skin window chamber (DSWC) in the mouse was prepared as previously described.24 Briefly, female BALB/c-nu mice (20–22 g body weight) were anesthetized (with inhalant isoflurane at 3% maintenance) and placed on a heating pad. Two symmetrical titanium frames were implanted into a dorsal skinfold, to sandwich the extended double layer of the skin. An approximately 15 × 15 mm² layer was excised. The surgical site was monitored for 48 h and then 1 × 10⁶ tumor cells were injected into the window chamber. Radiation treatments were initiated 10 days after the cell implantation when substantial vascularization was observed within the growing tumor mass.

Intravital microscopy and the quantitative analysis of tumor microvascular networks

The mice with window chambers were fixed to the microscope stage during the intravital microscopy, and the body core temperature was kept at 37–38°C by using a hot-air generator. Imaging was performed by using an inverted fluorescence microscope (IX-71; Olympus, Munich, Germany) and a black and white CCD camera (C9300-024; Hamamatsu Photonics, Hamamatsu, Japan). The tumor vasculature was visualized by transillumination using a 4× objective lens and a filter passing green light, resulting in images with a pixel size of 3.7 × 3.7 μm². To study the function of tumor vasculature, first-pass imaging movies were recorded after a 0.2-ml bolus of 50 mg/ml of tetramethylrhodamine isothiocyanate-labeled dextran (Sigma-Aldrich) with a molecular weight of 155 kDa, which was injected into the lateral tail vein. First-pass imaging movies were recorded at a frame rate of 22.3 frames per second by using a 2× objective lens, resulting in a time resolution of 44.8 ms and a pixel size of 7.5 × 7.5 μm². The algorithms used for identification of microvascular networks were implemented in MATLAB software (The MathWorks, Natick, MA, USA). Background heterogeneities were removed by using a white top hat transformation. The images were eroded by linear structure elements, and local thresholding was carried out. Finally, the images were cleaned up by applying scrapping and hole filling procedures. Vascular masks established from high-resolution images (i.e. images recorded by a 4× objective lens) were used to compute morphological parameters. The following morphological parameters were computed: total vessel length per μm² tumor area, length of large vessels (i.e. vessels with diameter ≥23.8 μm, corresponding to 7 pixels) per μm² tumor area, vascular area fraction [i.e. #pixels (vascular mask)#pixels (tumor)] and interstitial distance (i.e. the median of the distance from a tumor pixel outside the vascular mask to the nearest pixel within the vascular mask).
Statistical analysis
All the data were obtained from at least three independent experiments and were expressed as the mean ± standard error of the mean. The one-way analysis of variance was used to assess the significant differences among the groups, followed by Student’s t tests by using SPSS software package (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered to be statistically significant.

Results
HFRT inhibited tumor growth more than did CRT
To investigate the effect of irradiation on tumor bearing mice which were transplanted with H460 and HCC827 cells, tumors with size approximately 70 mm³ were irradiated at indicated doses. Tumor growth was significantly delayed in both HFRT and CRT groups [Figure 1(a) and (b)] as compared with their corresponding sham group. Moreover, HFRT exposure delayed tumor growth more effectively than did CRT exposure.

HFRT ameliorated tumor hypoxia, decreased tumor MVD and increased pericyte-coverage in tumor vessels compared with CRT
On the seventh and 14th days post irradiation, tumor hypoxia was evaluated by immune-histochemical staining combined with pimonidazole hydrochloride [Figure 2(a) and (b)]. On the seventh day, the hypoxia in HFRT and CRT groups was decreased when compared with sham groups, with the reduction being more obvious in the HFRT group. On the 14th day, the tumor hypoxia stayed at a much lower level compared with the sham groups. However, in the CRT groups, the tumor hypoxia, to some extent, restored virtually to the level of sham groups in H460 cell lines [p < 0.01, Figure 2(a), (b), (e) and (g)].

Immunohistochemical staining of the endothelial surface biomarker CD34 revealed that MVD of the tumors was decreased in both CRT and HFRT groups. The MVD of the tumors in the CRT and HFRT groups began to decrease on the seventh day post irradiation, and increased on the 14th day after irradiation. Moreover, MVD was significantly lower in the HFRT groups during the entire observation period compared with the sham and CRT groups [p < 0.01, Figure 2(c), (d), (f) and (h)], while the difference was smaller between CRT group and sham group.

We further investigated the vascular structure of tumors after irradiation. The tumor sections were immune-fluorescently stained with CD34 and α-SMA (a pericyte marker) antibodies to identify the morphology of the tumor vessels, and the ratio of α-SMA/CD34 was used to detect and calculate pericyte cell coverage. The result showed that vessels in the HFRT group were more likely to be covered by pericytes [Figure 3(a) and (b)]. Nonetheless, plenty of tumor vessels in the CRT and sham group were rarely covered by pericytes [Figure 3(a) and (b)]. The ratio of α-SMA/CD34 showed significantly more pericyte-covered vessels in the HFRT groups than in the other two groups (p < 0.01; Figure 3(c) and (d)).

Figure 1. HFRT inhibited tumor growth more than CRT. (a) Tumor growth curve in H460 xenograft mice models. (b) Tumor growth curve in HCC827 xenograft mice models. CRT, conventional radiation therapy; HFRT, hypo-fractionation radiotherapy.
HFRT reduced vascular density and increased vessel segment length in tumor vessels more than CRT.

Dorsal window chambers were used to morphologically assess tumor vascular networks on high-resolution trans-illumination images. Tumor-bearing mice were irradiated 10 days after cell implantation. The tumors were subjected to intravital microscopy once at the end of the irradiation (day 0) and twice post irradiation (days 7 and 14). The vasculature under an intravital microscope and the quantitative analysis confirmed that the vessel density was significantly lower and interstitial distance was significantly greater in HFRT-treated tumors than in the other two groups \( p < 0.05; \) \((a)\) and \((b))\),

The HFRT-induced decrease in vessel density was 53% with H460 tumors and 26% with HCC827 tumors. In the other two groups, the diameter of individual vessels increased during tumor growth. Collectively, these findings suggest that HFRT treatment could reduce growth-induced diameter increase in the surviving vessels compared with CRT treatment.

Activation of STAT3 mediated HFRT-inhibited tumor neovasculature through HIF-1α/CXCL12 and HIF-1α/VEGFA signaling pathway

Hypoxia partially results from leaky and disorganized tumor vasculature. Moreover, HIF-1α is
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Figure 3. HFRT increased pericyte-coverage in tumor vessels more than CRT. (a) and (b) Fluorescence microscopy images of CD34-positive endothelial cells and α-SMA-positive pericytes [red and green, respectively, 400×] of each group on day 7 and day 14. (c) and (d) The ratio of α-SMA/CD34 was measured by calculating the number of CD34-positive spots overlapping α-SMA-positive spots to the total number of CD34-positive spots in five randomly selected sets of 40 high-magnification [400×] fields per slide, of five slides per tumor (*p < 0.05, one-way analysis of variance test).

α-SMA, anti-α smooth muscle actin; CRT, conventional radiation therapy; d, day; HFRT, hypo-fractionation radiotherapy.

stabilized under hypoxic conditions and acts as transcription factor for genes implicated in tumor neovascularature. Based on the results in this study, we surmised that different radiotherapy regimens affected tumor angiogenesis and vasculogenesis via HIF-1α/CXCL12 or HIF-1α/VEGFA signaling pathway. Thus, we detected the base-line expression of HIF-1α/CXCL12 and HIF-1α/VEGFA on day 7 and day 14 in the sham group by using real time qPCR [Figure 5(a)] and western blotting [Figure 5(b)] and found that HIF-1α was increased gradually with tumor growth during the observation period [Figure 5(b)]. On day 7 post irradiation, we found that the expression of HIF-1α was down-regulated in the CRT and HFRT groups compared with sham group, while increased thereafter on day 14 after irradiation [Figure 5(b)]. Moreover, the down-regulation of HIF-1α expression in HFRT group was much more conspicuous than that in CRT group [Figure 5(b)]. Furthermore, real time qPCR [Figure 5(a)] and western blotting [Figure 5(b)] results showed that the expressions of VEGFA and CXCL12, the downstream genes of HIF-1α, presented similar changes with HIF-1α.

Hypoxia-induced active STAT3 accelerates the accumulation of HIF-1α protein and prolongs its half-life in solid tumor cells.25,26 We further analyzed the level of phosphorylated STAT3 (p-STAT3) by western blotting. Our results showed that the phosphorylation level of STAT3 at Tyr705 was also decreased 7 days post irradiation, and then increased 14 days after irradiation [Figure 5(b)]. Moreover, the down-regulation of p-STAT3 expression in HFRT group was much more conspicuous than that in CRT group [Figure 5(b)].

To further elucidate the role of the p-STAT3/HIF-1α signaling pathway in our models, we examined the effect of STAT3 inhibitor, S3I-201, on downstream signaling molecules in vitro.
After irradiation, the expression of p-STAT3 was markedly down-regulated in both H460 and HCC827 cells after irradiation, with the expressions of HIF-1α, VEGFA and CXCL12 being concomitantly down-regulated [Figure 5(c) and (d)], and the difference between sham group and HFRT group was more obvious. Between the two irradiation groups, expression of p-STAT3 was gradually increased 24 h after irradiation in CRT group while no re-increase in p-STAT3 expression was found in HFRT group at different time-points during the observation period [Figure 5(c) and (d)]. When STAT3 phosphorylation was inhibited by S3I-201 in H460 and HCC827 cells, the expressions of HIF-1α, VEGFA, CXCL12 and CXCR4 were significantly down-regulated both in sham and in HFRT groups [Figure 5(c) and (d)]. However, S3I-201 exerted no significant effect on the expressions of HIF-1α/VEGFA or HIF-1α/CXCL12 in CRT group.

Discussion
Lung cancer represents the most common malignancy world-wide and is associated with a high mortality.1 Approximately 76% of lung cancer patients reportedly could benefit from radiation therapy.2 Several clinical trials have demonstrated that ablative HFRT is an effective and well-tolerated therapy for early-stage NSCLC in medically-inoperable patients,3,7 and can help the patients achieve superior local control compared with CRT.8–10 Currently, the radiobiological mechanism of HFRT remains elusive. According to the 5 ‘R model, the killing effect of HFRT on tumor cells seems to be weaker than that of CRT,12 but this is not consistent with current clinical findings. The in vitro findings of Zhang H et al. have indicated that HFRT has advantages over CRT, because early-passage NSCLC cells line that received CRT exposure have more aggressive phenotypes than cells that received HFRT exposure.27 Moreover, the tumor growth curve of the present study (Figure 1) showed that HFRT inhibited tumor growth more than CRT. Some studies suggested that this inconsistency might be due to a secondary tumor-killing effect as a result of vascular injury caused by HFRT-induced endothelial cell apoptosis and the specific normalizing effect of HFRT on structural and functional
Figure 5. Activation of STAT3 mediated irradiation-inhibited tumor neovasculature through HIF-1α/CXCL12 and HIF-1α/VEGFA signaling pathway. (a) VEGFA and CXCL12 mRNA expression levels in H460 and HCC827 xenograft mice models were assessed by quantitative reverse transcription polymerase chain reaction; GAPDH is used as an internal control. Each bar represents mean ± SD of triplicate samples from a representative experiment (*p < 0.05, p-value was calculated by one-way analysis of variance). (b) p-STAT3, HIF-1α, VEGFA and CXCL12 protein expression levels in H460 and HCC827 xenograft mice models were assessed by western blotting; GAPDH is used as an internal control. S3I-201, a STAT3 inhibitor, was used to examine the effect on downstream signaling in vitro. P-STAT3, HIF-1α, VEGFA and CXCL12 protein expression levels in H460 (c) and HCC827 (d) cells were assessed by western blotting; GAPDH is used as an internal control. CRT, conventional radiation therapy; d, day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFRT, hypo-fractionation radiotherapy; HIF-1, hypoxia inducible factor-1; STAT3, signal transducers and activators of transcription 3; VEGF, vascular endothelial growth factor.
disturbances of the tumor vasculature. The potential favorable impact of HFRT on tumor control, partly contributed to by the advantage in re-oxygenation effect of HFRT, has been demonstrated by Figlia et al. and Onishi et al. Previous studies exhibited that HIF-1α is recognized by the pVHL protein and rapidly degraded by the ubiquitin-proteasome pathway under normoxia. The stabilized expression of HIF-1α was maintained by the hypoxic environment-induced phosphorylation of tyrosine residue (Y705) at the C-terminus of STAT3, forming p-STAT3, which binds to the C-terminus of HIF-1α. Accumulated HIF-1α translocates from the cytoplasm into the nucleus, where it dimerizes with HIF-1β, thereby forming the functional HIF-1 complex, which regulates cellular responses to hypoxia, including formations of tumor vasculature. The two major processes that mediate tumor vasculatures are: (1) the migration of circulating endothelial progenitor cells to the hypoxic area, as mediated by CXCL12 binding to its receptor CXCR4 (vasculogenesis), (2) sprouting and proliferation of endothelial cells from existing vessels, as mediated by the VEGF pathway (angiogenesis). In this study, we found that the hypoxic tumor microenvironment formed and the baseline expression of p-STAT3 and HIF-1α was induced during the growth of NSCLC (Figure 2 and Figure 5). After HFRT, the baseline levels of p-STAT3 and HIF-1α were significantly down-regulated, while after CRT, p-STAT3 and HIF-1α expression was hardly down-regulated. Coincident with the decreased HIF-1α expression after HFRT, the expression of VEGFA and CXCL12 was reduced. This reduced expression ultimately inhibited formation of new disordered vessels and promoted normalization of tumor vessels in vivo. We also found that 2 weeks after HFRT, the expression of p-STAT3 and HIF-1α was up-regulated, leading to increased expression of VEGFA and CXCL12, thereby promoting the renewed formation of disordered vessels and de-normalization of tumor vessels. This transient or temporary short-time down-regulation might contribute to the window period of HFRT-elicited normalization of tumor vasculature.

Furthermore, our in vitro study found that expression of p-STAT3 was significantly down-regulated in both H460 and HCC827 cells after irradiation, and after HFRT, this down-regulation was more conspicuous and duration of decrease was longer, with expression of HIF-1α, VEGFA and CXCL12 being more strongly inhibited, as compared with CRT. When the STAT3-phosphorylation in H460 and HCC827 cells was inhibited by S3I-201, a selective inhibitor of
STAT3-phosphorylation (Figure 5), the expression of HIF-1α/VEGFA and HIF-1α/CXCL12 was significantly down-regulated. Moreover, the down-regulating effect of HFRT on HIF-1α/VEGFA and HIF-1α/CXCL12 was further enhanced by S3I-201. But, interestingly, after CRT, S3I-201 could not inhibit STAT3-phosphorylation and its down-stream genes, demonstrating that p-STAT3/HIF-1α play a more important role in tumor microenvironment post-HFRT than that post-CRT, and HFRT might promote normalization of tumor vasculature via p-STAT3/HIF-1α signaling pathway.

In summary, this study showed that, compared with CRT, HFRT induced tumor vasculature normalization by rendering the remaining vessels less tortuous and more uniformly covered by pericytes, thereby ameliorating tumor hypoxia and enhancing the tumor-killing effect. Moreover, the p-STAT3/HIF-1α signaling pathway might be a key molecular mechanism by which HFRT exerts the aforementioned effects.

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Conflict of interest statement
The authors declare that there is no conflict of interest.

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ORCID ID
Fan Tong https://orcid.org/0000-0002-4588-8520

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