Alteration in subclinical lesions of epidermal growth factor receptor-mutated non-small cell lung cancer transplanted tumors in nude mice suggest the delineation of clinical targets volume Changes of subclinical lesions after EGFR-TKI treatment

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Research Article

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Abstract

Objective

To investigate the alteration of subclinical lesions of subcutaneous xenograft tumors in nude mice after EGFR-TKI treatment.

Methods

Human lung adenocarcinoma cell lines were subcutaneously transplanted on the right hind legs of the nude mice in control and treatment groups. Three-dimensional pathological section was taken to observe the subclinical lesion areas when the tumors' long diameters in the control group grew to specified length. In the Gefitinib treatment group, the subclinical lesions were observed likewise after tumor shrinkage. The results were compared.

Results

The long diameters of transplanted tumors in group C were 5, 10, 15, and 20mm, the smallest infiltration range of subclinical lesions was 0.23, 0.78, 1.24, and 2.98mm. The edge of transplanted tumor was extended 5.14mm, to include 95% of subclinical lesions. The long diameter of the transplanted tumor in the T group shrank to 15, 10, 5, and 0mm, the smallest infiltration range was 5.74, 2.13, 2.13, and 0.11mm, the edge of the transplanted tumor was extended 8.99mm to include 95% of subclinical lesions. The extended range in T group was significantly larger than group C (P=0.000).

Conclusion

The subclinical lesion receded after gefitinib treatment, but was wider than that of tumor of subclinical lesion without treatment. Even if the complete imaging response was achieved, there were still small infiltrating lesions.

Introduction

Recent studies show that therapeutic benefits have been observed after treating non-small cell lung cancer(NSCLC) with lung Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors(EGFR-TKI) for 3 months. At this stage, Stereotactic Body Radiation Therapy(SBRT), combined with EGFR-TKI, can significantly prolong the progression-free survival(PFS) and overall survival(OS)[1–2]. Likewise, the meta-analysis of 16 prospective studies on radiotherapy combined with EFGR-TKI for primary tumor shows both OS and PFS are prolonged significantly [3], confirming the prolonging effect of three-dimensional therapy on survival for stage IV NSCLC, especially in the case of oligometastatic [4]. These studies, however, failed to explain the key issue of target delineation and the interrelationship between recurrence and target volume during the process of three-dimensional therapy, or simply say the radioactive therapy is decided in accordance with the radiologist's own plan. International Committee on Radiation Units and Measurements (ICRU) reports 50 and 62 [5–6], the highest and lowest clinical target volume (CTV) are

similar to those of planning target volume (PTV). A 5% error may result in practical radioactive impairment and change of tumor control rate, and is closely related with local recurrence. In the case of treating primary tumor with the combination of three-dimensional therapy and radioactive therapy, the clinical target delineation of prolonged survival can be achieved by 6–8 mm outward extension from the edge of known gross target volume (GTV) [7–8]. When it comes to first-line treatment for stage IV NSCLC with EFGR-TKI [9–14], whose effective rate is higher than 70%, problems arise. First, EGFR-TKI hasn't been officially recommended as the therapy for NSCLC. Second, the subclinical infiltration range of the tumor, which the pathological examination, cannot be acquired even if EGFR-TKI treatment show benefits after surgery for stage IV NSCLC, due to the requirements of the ethical protocol. Human sensitive mutant cells fail to form tumors when transplanted into normal mice. Lung cancer cells derived from LLC mice have no sensitive mutation. Furthermore, imaging technology to measure small lesions has not been available for visualized measurement [15–16]. It has not been reported whether there is a small lesion surrounding the edge of the primary lesion after tumor shrinkage from the EFGR-TKI treatment, or CTV delineation after radioactive therapy can be made based on the tumor shrinkage resulting from chemotherapy for stage IV NSCLC or small cell lung cancer [17–18]. Considering the mechanism of EGFR-TKI treatment is inhibiting tumor, different from chemotherapy, the alteration range of post-treatment subclinical lesion may be different too. In this study, the EGFR-(19Del) human lung adenocarcinoma cell line was used to establish an animal model of subcutaneously transplanted tumor in nude mice to explore the distribution characteristics and alteration mechanism of the edge of the transplanted tumor before and after EGFR-TKI treatment, providing reference for EGFR-(M+) NSCLC Delineation of the clinical target area.

Materials And Methods

Cell culture. Human lung adenocarcinoma cell line HCC827 (STR identification, E746-A750 deletion, Wuhan Prose Life Technology Co., Ltd.), 10% fetal bovine serum RPMI1640 medium, were cultured and reproduced at 37 Celsius degrees in 5% CO2 incubator. 0.5ml-1ml of 0.25% trypsin solution was kept at 37°C for 1 min and centrifuged at 1000 r/min for 3 min to prepare cell suspension.

Grouping of tumor-bearing mice and establishment of EGFR-M + xenograft animal model. Sixty SPF BALB/c-nu nude mice [SPF (Beijing) Biotechnology Co. Ltd. SCXK (jing) 2019-0010], six weeks old, weight (21.49 ± 1.63)g, half male and half male, were randomly divided into control group (Group C) and Treatment Group (Group T), 30 rats in each. The cells in the logarithmic growth phase were digested with trypsin to prepare a cell suspension with a concentration of 1.0×10^7 cells/mL, and 0.2 mL/ was inoculated into the right hind limb of each of the nude mice subcutaneously to establish animal models of lung adenocarcinoma transplantation tumor. Observation of the growth of subcutaneous transplanted tumors was performed on a daily basis. When the long diameters of the transplanted tumors grew to 5 mm, 10 mm, 15 mm, and 20 mm in group C, and 20 mm in group T, the intervention of Gefitinib by gastric infusion was introduced. After the transplanted tumor retreated to 15 mm, 10 mm, 5 mm, and 0 mm in long diameter, the same method was used for sub-clinical lesion measurement on the nude mice with transplanted tumors in their hind limbs. The nude mice were fed in the SPF animal room of the Animal

Experiment Center by the principles of animal experiment 3R (Reduction, Replacement, Refinement), observing the ethic requirements and sterilized conditions.

EGFR-TKI treatment. After sugar-coating removal, Gefitinib Tablets (AstraZeneca) was ground, and dissolved in sterilized ultrapure water to prepare a 7.5 mg/mL solution and stored in a constant temperature refrigerator at 4°C. Nude mice in group T were given the prepared solution at 0.5mL/100g by gastric perfusion, once a day, to observe the changes in the long diameter of the transplanted tumor in nude mice [19]. When the long diameters of the transplanted tumors decreased to 1.5cm, 1.0cm, 0.5cm, At 0.0cm (complete response, CR), the hind limbs of nude mice were obtained to measure the subclinical lesion of the transplanted tumor.

Definition and detection methods of subclinical lesions. The subclinical lesion area is defined as the distance between the small lesion in the three-dimensional direction and the edge of the transplanted tumor by HE staining and 200x to 400x microscope examination [20–21]. In group C, when the longest diameters of the transplanted tumors grew to 5mm, 10mm, 15mm, and 20mm, the hind limbs with transplanted tumors were taken from three nude mice and fixed with formalin solution respectively, altogether 12 nude mice were sacrificed. The transplanted tumors were sectioned on the largest diameter plane, HE stained, and observed under a microscope. The extent of subclinical lesions is recorded. In group T, Gefitinib was injected into the stomach when the long diameter of the transplanted tumor grew to 20mm, and the changes in the long diameter of the transplanted tumor were observed daily. When the cancer was reduced to 15mm, 10mm, 5mm, and 0mm, the same method as group C was followed for pathological observation of clinical lesions.

Observation indicators. Observed under the microscope, the subclinical lesion range and change the rule of the transplanted tumor in group C under different long diameters, the longest diameter of the transplanted tumor was reduced with the treatment of gefitinib in group T, the subclinical lesion range and change rule.

Statistical analysis. SPSS26.0 data statistics software was used for analysis. T-testing was adopted for the inter-group comparison. There is a statistical difference between group C and T($p < 0.05$). The range included in 95% of subclinical lesions was calculated as a cumulative percentage.

Results

EGFR-(19Del) human lung cancer transplanted tumor model in nude mice. During the establishment of the 60 nude mice transplanted tumor models, 1, 3, 2, 4, and 2 deaths (40%) occurred on the 2nd, 3rd, 5th, 7th, and 8th day in group C, respectively. In group T, 2, 2, 5, and 1 deaths (33.3%) occurred on days 2, 4, 7, and 12. No tumor metastasis was found in the anatomy of dead nude mice. The average body weight of nude mice in groups C and T were (21.61 ± 1.63) g and (21.37 ± 1.64) g ($t=-0.315$, $P = 0.754$), and the completion time of the transplanted tumor model was (24.57 ± 5.79) days and (24.10 ± 5.69) days ($t=-0.568$, $P = 0.572$), the number of models established was 18 and 20 ($t = 0.528$, $P = 0.599$), there was no significant difference.

Measurement of subclinical lesions of transplanted tumor in group C nude mice.

When the transplanted tumor of nude mice grew to 5mm, 10mm, 15mm, and 20mm in length, the range of subclinical lesions was 0.23-2.13mm (0.99 ± 0.44mm), 0.78-2.53mm (1.41 ± 0.46mm), 1.24-4.99mm (3.06 ± 0.82mm), and 2.98-5.79mm (4.63 ± 0.84mm), respectively. Ninety-five percent of the subclinical lesions was included after an outward extension of 5.14mm from the edge of the solid tumors. Table 1 and Fig. 1.

Table 1
Group C includes the scope of 95% subclinical lesions

ME(mm)	Number	Cumulative (%)
0.00–1.00	24	20.0
1.01-2.00	37	50.8
2.01-3.00	15	63.3
3.01-4.00	19	79.2
4.01-5.00	14	90.8
5.01–5.14	**5**	**95.0**
5.15–5.59	3	97.5
5.60-6.00	3	100.0
Total	120	100.0

Subclinical Infiltration of Transplanted Tumor in Group T Nude Mice. After the intervention of gefitinib in group T, the long diameters of the transplanted tumors were reduced to 15mm, 10mm, 5mm, and 0mm, and the subclinical lesions ranged from 5.74-9.00mm (7.66 ± 0.87), 2.13-10.78mm (7.35 ± 2.09), 2.13-9.89mm (7.49 ± 1.82), 0.11-3.88mm (1.74 ± 1.24) according, an outward extension of 8.99mm from the edge of the transplanted tumor covered 95% of the subclinical lesions. Table 2 and Fig. 2.

Table 2
The range required for the T group, including 95% of subclinical lesions

ME(mm)	Number	Cumulative (%)
0.00–1.00	6	5.6
1.01-2.00	3	8.3
2.01-3.00	8	15.7
3.01-4.00	5	20.4
4.01-5.00	3	23.1
5.01-6.00	5	27.8
6.01-7.00	13	39.8
7.01-8.00	29	66.7
8.01-9.00	**31**	**95.4**
9.01-10.00	4	99.1
≥ 10.00	1	100.0
Total	108	100.0

Comparison of subclinical lesions in groups C and T. When transplanted tumors in group C were 5mm, 10mm, 15mm, and 20mm, the subclinical lesions were (2.52 ± 1.59) mm, with the smallest being 0.23mm and the largest 5.79mm. When the transplanted tumors in group T receded to 15mm, 10mm, 5mm, 0mm, the subclinical lesions were (6.54 ± 2.68) mm with the smallest being 0.11mm and the largest 10.78mm, including 95% of the subclinical lesions, there was a statistical difference in the marginal extrapolation range of the transplanted tumor (χ^2 = 126.21 P = 0.000). While the transplanted tumors of the C and T groups were of the same size, and the subclinical lesion infiltration range of the T group was more extensive than that of the C group (P = 0.000). Table 3 and Fig. 3.

Table 3
Comparison of subclinical lesions between transplanted tumors of the same size in groups C and T.

Group	The long diameter of transplanted tumor (mm)			t	*P*
	5mm	10mm	15mm		
group C	0.99 ± 0.44	1.41 ± 0.46	3.06 ± 0.82	13.92	0.000
group T	7.49 ± 1.82	7.35 ± 2.09	7.66 ± 0.87		

Discussion

The NSCLC target area is closely related to local control and radiation damage [22].

According to ICRU reports No. 50 and No. 62, the periphery of GTV in the concept of target volume has subclinical involvement. CTV includes the tumor focus and the surrounding subclinical focus range. The control over the tumor depends on the dose and its changes in the CTV. A dosing error higher than 5% may lead to real radiation damage and increased risk of tumor recurrence [5–6]. NSCLC subclinical lesions need a minimum irradiation of 50Gy to achieve the purpose of local control [23]. Analysis by Senan et al [24] pointed out that early clinical studies did not agree on the role of CTV. They believed that the CTV of NSCLC three-dimensional conformal radiotherapy was simply an extension of 7-10mm or 5mm of GTV [25–26], even CTV delineation was unnecessary[27–28]. Current conventional segmented three-dimensional radiotherapy for NSCLC is based on the study results of 70 surgical specimens. The CTV formed by 8mm and 6mm outward extension of GTV in the cases of lung adenocarcinoma and squamous cell carcinoma can cover more than 95% of subclinical lesions [8]. After stage III NSCLC chemoradiation, IMRT technology was applied, (omitting CTV can obtain) a 3-year local control rate of 67.3% cann be achieved [17]. However, no comparative study report has been available on the range of CTV in patients with sensitive mutations and harmful NSCLC-driven genes. There is no report on peripheral subclinical lesions after EGFR-TKI intervention in receded tumor lesions, either. This study was conducted through pathological studies of animal models and surgical specimens. Pathological analysis of surgical samples of 2 cases of EGFR-19Del and 1 case of L858R sensitive mutant NSCLC showed that the smallest subclinical lesion of three-dimensional direction (X, Y, Z) of was 3.25 mm, and the largest 10.23mm, with similar mean values of 6.69mm, 5.24mm, 7.14mm and 6.97mm, 5.19mm, 6.27mm, respectively ($P > 0.05$). The coverage of 95% of small lesions needed a outward extension of 7.35mm from the edge of solid tumors. It may indicate that the range of subclinical lesions in human lung adenocarcinoma is similar to that of EGFR sensitive mutations and negative cases. The CTV of three-dimensional radiotherapy requires an external extension of GTV by 8mm in the three-dimensional direction [3]. The tumor-forming time of the transplanted tumor in group C was around 24 days [29]. Infiltration was observed in pathological when the long diameters of the transplanted tumors were 5mm, 10mm, 15mm, and 20mm (Fig. 1), indicating that in EGFR mutation (19Del) human lung adenocarcinoma, the shortest distance of clinical lesion infiltration was 0.23mm and the longest 5.79mm. The mean value varies from 0.99mm to 4.63mm with the change of the long diameter of the transplanted tumor. The CTV coverage of 95% of the subclinical lesions needs an outward expansion of 5.14mm from the edge of the transplanted tumor. It is suggested that there are subclinical lesions outside the transplanted tumor of EGFR mutation (19Del) human lung adenocarcinoma in nude mice, and the subclinical range expands with the increase of the tumor's long diameter. If three-dimensional radiotherapy is to be performed, an expansion of 5-6mm from the margin of the gross tumor.to ensure CTV is safe and reliable.

However, the tumor-effect mechanism of chemotherapy and EGFR-TKI are different. Cytotoxic drugs can make small lesions reach biological CR. As the tumor shrinks after chemotherapy or concurrent chemotherapy and radiotheapy, CTV and other irradiation targets shrink accordingly without increasing the risks of local tumor recurrence, the radiation damage to normal tissue is decreased consequently.

[17–18]. However, EGFR-TKI can inhibit the growth, metastasis, and angiogenesis of tumors by competing for the ATP binding site in the catalytic region of the epidermal growth factor receptor tyrosine kinase, blocking its downward signal transduction pathway and inhibiting tumor growth [30]; it suggests that the subclinical range of EGFR-(M+) NSCLC after EGFR-TKI treatment may be different from that of chemotherapy. This study showed that in animal experiments, subcutaneously transplanted tumors in nude mice in group T shrank after the intervention of Gefitinib; the minimal distance of subclinical lesions was 0.11mm, the maximal was 10.78mm. The long diameter was reduced to 15mm, 10mm, and 5mm; the farthest distance of the subclinical lesion was 9.0-10.78mm. More importantly, after the intervention of Gefitinib in nude mice, observations of the tumores by naked eyes, palpatation, and animal CT scans showed that CR had been reached, a small lesion of 2mm was still seen under pathological examination. The CTV covering 95% of the of subclinical lesions need an outward expansion of 8.99mm from the edge of the transplanted tumor. Further comparison was made of the subclinical lesion distance of the transplanted tumor in nude mice before and after the intervention of EGFR-TKI when the long diameter of the transplanted tumor was the same, the results showed that the subclinical lesion in group T was significantly more extensive than that of the C group ($P = 0.000$). To include 95% of the subclinical infiltrating lesions, CTV needed to be increased by about 2/3 before EGFR-TKI intervention. Even if the CT scan of the transplanted tumor was CR, small lesions of 2mm still was observed in the range, suggesting that if radiotherapy is recommended after the EGFR-TKI treatment of the primary tumor, the delineation range of the CTV in the target area may not follow that for targeted drugs, an expansion is needed. Even if CR has been obtained in imaging, local radiotherapy is required in the original tumor bed area based on the residual traces of the image after treatment so that local control or biological CR may be achieved. Then it may lower the risk of the clinical progression in the chest after Gefitinib treatment [31]. In particular, attention should be paid to patients with stage IV NSCLC with oligometastatic EGFR-sensitive mutations through three-dimensional radiotherapy.

Conclusions

The subclinical lesion receded after gefitinib treatment, but was wider than that of tumor of subclinical lesion without treatment. CT scans show there are still small lesions during CR.

Declarations

Ethical Approval.

The nude mice were fed in the SPF animal room of the Animal Experiment Center of Guizhou Medical University [SYXK (Qian) 2018-0001] by the principles of animal experiment 3R. Ethical lot number: 2101039.

Consent for publication

All authors Agree to published.

Availability of supporting data.

Supplementary material for this study available from the corresponding author on reasonable request.

Conflict of interest: none.

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Authors' contributions.

Jie Liu prepared the manuscript, conducted the literature search, and reviewed and edited the manuscript. Wei Zhang, Shengfa Su, Qingsong Li, Yichao Geng, Wengang Yang, Xiaxia Chen, Weiwei Ouyang, Zu Ma, and Bing Lu reviewed the manuscript. All authors have read and approved the final manuscript.

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Figures

Figure 1

The manifestations of subclinical lesions with the long diameters of the transplanted tumosr in group C being 5mm, 10mm, 15mm, and 20mm, respectively.(a: solid tumor,×200;b: subclinical lesions,×400)

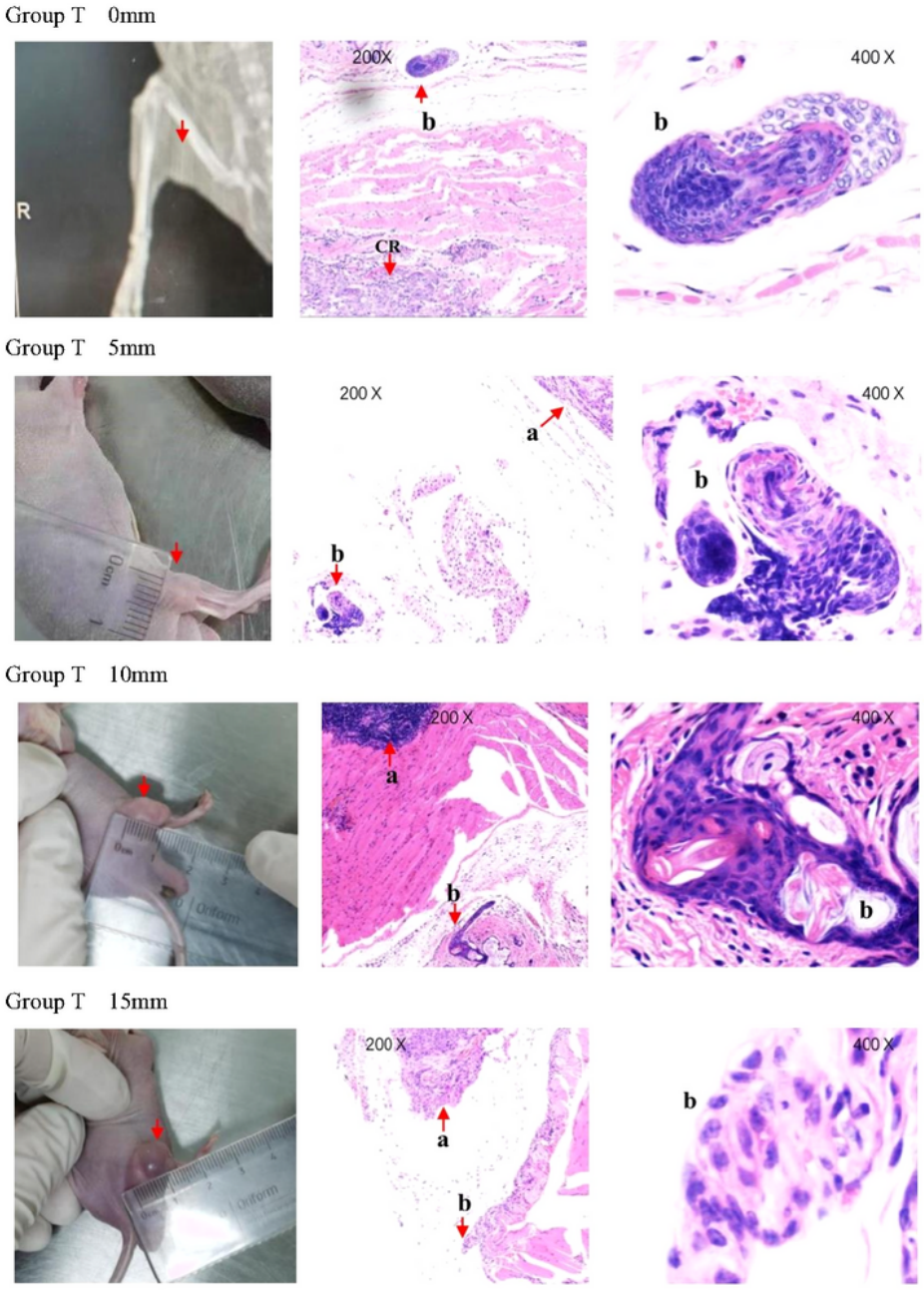

Figure 2

The manifestations of subclinical lesions in group C when the transplanted tumor shrinks to 15mm, 10mm, 5mm, and 0mm after targeted therapy.(a: solid tumor,×200;b: subclinical lesions, ×400;CR: complete response).

Figure 3

Cumulative distribution of subclinical lesions in groups C and T.(Group C included 95% of subclinical lesions with a cumulative extension of 5.14 mm; Group T included 95% of subclinical lesions with a cumulative extension of 8.99 mm)