Peer Review File

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Reviewer A

General Comments:

This is an interesting paper on a clinically-relevant topic. The unpredictability of radiation-induced lung injury is an important problem to be addressed. Some elements of the paper are unclear; additional information is needed to provide the proper context and interpretation.

Specific Comments:

Two major concerns:
Comment 1: Radiation doses should be clarified:
Lines 92-94: Please provide type of radiation, source, fractions, frequency of treatment, total duration of treatment.

Reply 1: Thank you for your suggestion. We have added the treatment details to the text and table 1.

Changes in the text: The following was added to Page 7, lines 102–114.
Of the 21 patients, four received stereotactic radiation therapy (SRT) and 17 received intensity-modulated radiation therapy (IMRT). Of the four patients who received stereotactic irradiation, one received it in combination with chemotherapy and three received radiation therapy alone; all of which with a total dose, number of fractions, and duration of treatment of 48-Gy, 4fr, and one week, respectively. Of the 17 patients who received IMRT, five received hyper-fractionated intensity modulated radiation therapy (HF-IMRT), and 12 received standard fractionated intensity-modulated radiation therapy (SF-IMRT). One patient in the SF-IMRT group had to be stopped immediately after irradiation due to an adverse event. Excluding this patient, the mean total dose, number of fractions, and duration of treatment were 55.76-Gy, 35.6fr, and 3.6 weeks (45–64-Gy, 30–40fr, 3–4 weeks) for the HF-IMRT group and 57.1-Gy, 28.1fr, and 5.8 weeks for the SF-IMRT group, respectively (40–64-Gy, 19–40fr, 4–8 weeks).

Comment 2: Lines 159-165: I understand that patient treatment schedules may have differed, but It is important to describe the treatment parameters in some way. Large-dose-per-fraction therapy would produce a different response from hyperfractionation.

Reply 2: Thank you. We have added the information to the text and table 1.

Changes in the text: The following was added to Pages 9–10, lines 162–168.
Of the 20 patients, 10 were treated with RT alone (six with SRT, three with SF-IMRT, and one with HF-IMRT) and 14 were treated with chemotherapy (one with SBRT, nine with SF-IMRT, and four with HF-IMRT). Of the patients in the combined chemotherapy group, two were treated

with CDDP combined and 12 with CBDCA combined. One patient received one course of platinum combination therapy, one received two courses, one received three courses, and 12 received four courses.

Comment 3: Interpretation of the results:
In the results generally and Figure 3 especially: Why was the dose of 30-48 Gy chosen as the relevant "time point"? Radiation pneumonitis and fibrosis occur with a 50% incidence after around 10 Gy. Why is the data going in the opposite direction for 15-29 Gy not considered important? How do the authors forsee applying TGFbeta as a predictor, if it goes down up to 29 Gy, and up above 30 Gy? Would it be fair to say that "TGFbeta elevation in the second half of a radiotherapy course" is the relevant measurement?

Reply 3: Thank you for your suggestion. The onset of radiation pneumonitis (RP) is characteristic, and most cases occur between one and three months after the end of irradiation, although it may occur immediately after irradiation or after about 5–6 months (1,2). Classic RP, which commonly appears within the irradiated volume, has been proposed to have three onset phases: early (latent) phase, intermediate (acute pneumonitis) phase, and late (pulmonary fibrosis) phase (3,4). The early phase refers to the period immediately after the start of irradiation to a few weeks, when no clinical changes are seen but intracellular changes have begun. In particular, cytokines released within the first two weeks include IL-1, TNF-α, IL-6, high molecular weight mucin, and PDGF-β (5). In the intermediate phase, a few weeks to a few months after irradiation (acute noninfectious pneumonia), the patient presents with clinical acute to subacute pulmonary inflammatory findings due to infiltration of inflammatory cells, such as lymphocytes and plasma cells, that obstruct the capillaries due to vascular endothelial cell damage. During this process, inflammatory cells that contribute to tissue remodeling produce TGF-beta as a proinflammatory cytokine, leading to fibroblast activation. TGF-beta is also produced by lung cells and fibroblasts (6,7). This, in turn, causes myofibroblasts to secrete excess collagen, fibronectin, and proteoglycans (7), which are thought to enter the late phase (8,9).
The period of 30–48 Gy irradiation observed in the present study falls right in the middle of this phase (acute non-infectious pneumonia; the period when inflammatory cytokines, such as TGF-beta are induced from the initial intracellular changes, leading to fibroblast activation), especially in the RP group, where an increase in TGF-beta We consider it consistent with the observed increase in the pre-irradiation ratio in the group wherein RP occurred.

References:
1. Kayoko Tsuji, et al. Japanese Journal of Lung Cancer. 2019;59:333-341
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3. Hanania AN, et al. Chest. 2019;156:150-162. 4.
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6. Larici AR, et al. Radiographics. 2011;31(3):771–89.
7. Kendall RT, Feghali-Bostwick CA. Front Pharmacol. (2014) 5:123.
8. Denham JW, Hauer-Jensen M. Radiother Oncol. (2002) 63:129–45.
9. Toussaint O, et al. Mech Ageing Dev. (2002) 123:937–46.

Comment 4: What do the authors see as the chance of a type I error, given their statistical approach?

Reply 4: As you indicated, the possibility of a type 1 error cannot be ruled out due to the small number of cases. We believe that additional analysis with a larger number of cases is needed in the future.

Other points requiring clarification:
Comment 5: Line 46: Suggest "to the time of 30-40 Gy irradiation"

Reply 5: Thank you for your suggestions. We corrected as follows in Page 3, line 45.

Changes in the text: Page 3, line 45.
irradiation to the time of 30–48-Gy irradiation ($p = 0.011$).

Comment 6: Line 52: Did both the "before" and "after" TGFbeta measurements have predictive value? Or rather, was the value found in the "change from baseline"?

Reply 6: Thank you for pointing this out. What is important is the relative ratio of TGFβ at the time of 30–48 Gy irradiation to baseline. The actual pre-irradiation TGFβ values varied from case to case, and it was difficult to evaluate the actual TGFβ values at the time of 30–48 Gy irradiation only. However, since TGFβ tended to increase rapidly in cases of RP, we examined the ratio of TGFβ from baseline (rate of increase) for each case. To examine the rate of increase, it is necessary to measure both baseline and the time point of 30–48 Gy irradiation.

Comment 7: Line 67: Pulmonary fibrosis is a continuous process beginning at the time of irradiation; it becomes evident as a distinct entity after radiation pneumonitis wanes. Perhaps the authors are referring to a clinically-recognized entity? This could be made more clear.

Reply 7: Thank you for pointing this out. As you say, radiation pulmonary fibrosis is a condition secondary to radiation pneumonitis, and that is diagnosed from clinical symptoms and CT findings. I have added the information to the text.

Changes in the text: The text was changed as follows in Page 5, lines 63–75.
In the initial phase, interstitial shadows, such as ground-glass attenuation and reticular shadows, appear in the lungs within the irradiation field. In this period, the symptoms are often still asymptomatic to mild. During the next organizing phase, patchy consolidation occurs in the same area as the irradiated area, regardless of the anatomical boundaries, such as lung areas and lobes. When the degree of patchy consolidation is severe, various respiratory symptoms appear. After this phase, the fibrotic phase occurs, and linear scarring, increased density consolidation, and decreased lung volume are observed in line with the irradiated area. During this phase, the symptoms of the acute phase disappear and stabilize; however, respiratory function may be impaired if the volume loss is marked. The clinical course of the disease is usually stable with follow-up, but in cases of severe symptoms, treatment with steroids is required, and the disease may spread to the irradiated area, becoming severe and rarely fatal.

Comment 8: Line 106: Please define "standard steroid treatment" including name of the drug used, dose, and duration of treatment/taper

Reply 8: Thank you. I have added.

Changes in the text: The text was added as follow in Page 8, lines 124–126 and Page 10, lines 170–174.
Page 8, lines 124–126: Patients diagnosed with RILI Grade 2 or higher were started on prednisolone at 0.5~0.8 mg/kg/day, titrated down by 5 mg/day over 1–2 weeks according to symptoms.
Page 10, lines 170–174: Treatment with prednisolone was initiated at 0.5–0.8 mg/kg/day depending on symptoms with a mean duration of treatment of 19.1 weeks (4~31 weeks); three patients continued maintenance therapy at 5–10 mg/day due to relapse of RP during steroid

reduction.

Comment 9: Line 109: What type of diagnostic imaging was used? CT?

Reply 9: CT was used. We added the information about this.

Changes in the text: The text was added as follows in Page 8, lines 139–142.
Patients with new infiltration or cord-like shadows on CT and "Grade 2: Symptomatic and requiring therapeutic intervention" or higher in CTCAE v5.0 "Pneumonia" were classified as RP+, and those with "Grade 1: No therapeutic intervention required" or lower were classified as RP-.

Comment 10: Line 128: typo, should be "compare"

Reply 10: Thank you. I have corrected the word.

Change in the text: The word was changed as follows.
Page 9, line 151: “compare”

Comment 11: Line 146-147: What is meant by "Additionally, we included treatment cases"?

Reply 11: Thank you. I meant "both groups include those who received radiotherapy only.”. The text has been corrected.

Changes in the text:
Page 10, lines 178–179: Additionally, both groups include those who received radiotherapy only.

Comment 12: Line 180: Does this mean "change from baseline?"; if so, please state.

Reply 12: Thank you. It is "relative to baseline", not "change from baseline". Corrected for clarity.

Changes in the text: We have changed as follows.
Page 12, lines 210–212: **Between-group comparison of the relative to baseline of serum TGF-β levels**
We performed between-group comparisons of the transition of the relative to baseline of the pre-irradiation serum TGF-β levels (figure 3).

Comment 13: Line 205 and 206: "early onset" is described by weeks, and "late onset" by dose delivered. The same units should be used for these categories, either "weeks" or "Gy" but not a mix.
Reply 13: Thank you. The description was incorrect.
Changes in the text: We have corrected as follows.
Page 13, lines 236–237: Specifically, seven patients developed early-onset disease by 14 weeks after irradiation, while two patients developed late-onset disease more than six months after irradiation.

Comment 14: Lines 345-356: The relevance of ICI therapy to this paper is tangential; is this section necessary?

Reply 14: Thank you for pointing this out. We have omitted it.

Changes in the text: The following text has been omitted.

Line 345-356 of the first draft: There has been extensive research on ICIs for NSCLC (20,21). The Phase 3 PACIFIC trial showed that patients who underwent CCRT and durvalumab combination therapy had longer progression-free survival and overall survival than patients who received CCRT alone (22,23). Moreover, irradiation of cancer cells in the primary lesion has a shrinking effect on non-irradiated metastatic tumors (24,25). This phenomenon, known as the abscopal effect, can be effective in combination with ICIs (26). In patients with chemorefractory metastatic NSCLC, where anti-CTLA antibodies failed to demonstrate significant efficacy alone or in combination with chemotherapy (27), radiation therapy and ipilimumab combination therapy were found to activate systemic antitumor T cells. Additionally, objective responses and disease control were observed in 18% and 31% of the enrolled patients, respectively (28).

Reviewer B

Comment 15: What is considered “standard steroid treatment” at your clinic? Figure 5 shows that most RP+ RILI occurs early. Early RP+ RILI patients are graded between day 0 and day 14 and steroid treatment is initiated. It is well published that steroids regulate TGF-beta levels. Without better data classification, it is not clear if the observations in relative changes in TGF-beta is a direct consequence of treatment or an independent measure of RILI. Late onset of RP+ RILI could be more interesting because patients are not receiving steroid treatment for at least 30 days post treatment. However, with just 2 patients with broad differences in relative concentration, no conclusions can be made.

Reply 15: Thank you for your suggestion. There have been no prospective clinical trials on "standard steroid therapy" to date, and empirical treatment has been used. In general, oral prednisolone is started at 0.5–1 mg/kg/day, depending on the condition, and is often withdrawn over 6~12 weeks. In this study, all patients were started at 0.5–0.8 mg/kg/day and tapered down by 5 mg/day over 1–2 weeks according to symptoms. The description was inadequate, so we have added it to the text.
The onset of TGF-β modification by steroids is undeniable, as you pointed out, and the time of onset shown in Figure 5 is based on the number of weeks after the end of irradiation. Since there was no modification by steroids at the time of 30–48 Gy irradiation, we believe that these results are very significant in predicting the onset of RILI.

Changes in the text: The text was added as follows.
Page 8, lines 124-126: Patients diagnosed with RILI Grade 2 or higher were started on prednisolone at 0.5~0.8 mg/kg/day, titrated down by 5 mg/day over 1–2 weeks according to symptoms.
Page 10, lines 170–174: Treatment with prednisolone was initiated at 0.5–0.8 mg/kg/day depending on symptoms, with a mean duration of treatment of 19.1 weeks (4~31 weeks); three patients continued maintenance therapy at 5–10 mg/day due to relapse of RP during steroid

reduction.

Reviewer C

Comment 16: This is a limited small study of a group of lung cancer patients with clinical stage ranging from stage 1 to stage 3, and determining whether the use of serum TGF-β levels can be predictive of what the authors call radiation-induced lung injury. The results are interesting and confirm much of the information published in the 1970s and 1980s by pioneer clinical radiation biologists, Philip Rubin, Paul O'Kunieff, Michael Anscher, and others showing that TGF-β levels could not be predictive of irradiation induced acute injury (pneumonitis) or late radiation injury (pulmonary fibrosis). With a complex method of analysis, the authors compare TGF-β levels with that of G-CSF and another cytokine KC-6, and determine that in the mid-dose range of around 30 – 40 Gy irradiation TGF-β levels can be predictive of lung injury. The results are interesting in view of the relative lack of data published on TGF-β in the past 40 years. However, you can conclude from careful analysis of the data that TGF-β levels are, in fact, not predictive. The data are presented in total, so the reviewer and reader are able to see the spread of data. The complex statistical analysis is used to determine the relative level of pre-treatment TGF-β with that at the mid-dose point of around 30 – 40 Gy, and the authors interpret this change as predictive of radiation-induced lung injury in a subset of patients.
The authors should be encouraged to continue their studies, but include more cytokines as controls, and other predictive measures, such as miRNA levels, oxidative lipid levels, and potentially confounding variables. A sample size of over 100 patients would be necessary to make the conclusions that the authors suggest from this limited sample of 21 patients.

Reply 16: Thank you for your suggestion. Indeed, the sample size in this study was limited, and we believe that further large-scale studies are needed in the future to corroborate the results of this study.

Comment 17: The most important information that is required is the volume of lung in the initial treatment planning volume, any reduced field (cone-down) volumes, and how treatment planning was done. Were the patients treated with 3-dimensional treatment planning or intensity modulated radiotherapy (IMRT)? The designation of chemotherapy, the tables, is important, but one must know the drugs, whether the patients completed a full course of chemotherapy, and which drugs were used. With this additional information and the larger sample size, the results could be potentially useful and alert clinicians to use TGF-β levels as a predictor. The most important piece of information, which is lacking, is how the patients, who had indication of RIPF were treated? Having a predictive marker of fibrosis would be important if it leads clinicians to either stop

treatment, reduce the field size, or intervene with a countermeasure.

Reply 17: Thank you for pointing this out. We have added the detailed information on radiotherapy to the text and Table, as it was missing. We have also added the details of chemotherapy.

Changes in the text: The following was added to Pages 9-10, lines 162–168.
Of the 20 patients, 10 were treated with RT alone (six with SRT, three with SF-IMRT, and one with HF-IMRT) and 14 were treated with chemotherapy (one with SBRT, nine with SF-IMRT, and four with HF-IMRT). Of the patients in the combined chemotherapy group, two were treated with CDDP combined and 12 with CBDCA combined. One patient received one course of platinum combination therapy, one received two courses, one received three courses, and 12 received four courses.