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Prospective Exploratory Study of the Relationship Between Radiation Pneumonitis and TGF-β1 in Exhaled Breath Condensate

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Abstract. *Background/Aim: We conducted a prospective exploratory study to investigate the relationship between radiation pneumonitis (RP) and transforming growth factor-β1 (TGF-β1) in exhaled breath condensate (EBC). Patients and Methods: The inclusion criteria were: patients who 1) received thoracic radiotherapy (RT) for lung cancer, 2) were aged ≥20 years, and 3) provided written informed consent. EBC was collected before and 1 month after RT. TGF-β1 levels in EBC were measured using an enzyme-linked immunosorbent assay. We evaluated RP using the Common Terminology Criteria for Adverse Events v4 and analyzed the relationship between grade (G) 2 RP and TGF-β1 levels in EBC. Results: Ten patients were enrolled [median age, 75 years (range=60-81 years)], and none of them had interstitial lung disease. Conventional fractionation, accelerated hyperfractionation, hypofractionation, and stereotactic ablative fractionation were used in four, one, two, and three patients, respectively. G1 and G2 RP were observed in five patients each; no G3-G5 RP occurred. The median TGF-β1 levels in EBC before and 1 month after RT were 79.1 pg/ml (0.1-563.7 pg/ml) and 286.9 pg/ml (33.7-661.3 pg/ml), respectively. Of the seven patients with increased TGF-β1 levels in EBC 1 month after RT than before RT, five (71%) experienced G2 RP, whereas the remaining three patients with decreased TGF-β1 levels had G1 RP (p=0.083, one-sided Fisher's exact test). Conclusion: Increased TGF-β1 levels in EBC 1 month after RT might be promising for the detection of G2 RP.*

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Radiation pneumonitis (RP) is a major complication of thoracic radiotherapy (RT) for lung cancer. Transforming growth factor-β (TGF-β) plays a dominant role in RP (1). A study reported that TGF-β1 levels in bronchoalveolar lavage fluid (BALF) 1 month after RT tended to be greater in patients with ≥grade (G) 2 RP than in those with <G1 RP (2). Although BALF contains inflammatory cytokines in the lungs, invasive procedures are required to obtain it. Common complications include transient hypoxemia, post-BAL fever, bronchospasm, and, rarely, pneumothorax (3). In contrast, exhaled breath condensate (EBC), which also contains inflammatory cytokines in the respiratory tract, can be obtained non-invasively (4). Exhaled breath comes into contact with a cooled EBC collecting apparatus and is condensed into liquid. The obtained EBC consists of mainly water and a small amount of airway lining fluid (4). EBC contains a large number of mediators including adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, nitrogen oxides, peptides, and cytokines (5). Several systematic reviews have reported the potential usefulness of EBC to assess the activation of biomarkers in pediatric and adult asthma (6, 7). As for TGF-β1 in EBC, a study reported that in patients with sarcoidosis, TGF-β1 levels in EBC were comparable to those in BALF (8). To detect RP early, we conceptualized the substitution of EBC for BALF in patients treated with thoracic RT. Therefore, we conducted this prospective exploratory study to investigate the relationship between RP and TGF-β1 in EBC.

Patients and Methods

Patients. This prospective study (clinical trial registration number: UMIN000040894) was approved by the Institutional Ethics Committee (approval number: H30-094). The inclusion criteria were as follows: patients who 1) received thoracic RT for lung cancer, 2)

were aged ≥20 years, and 3) provided written informed consent. The exclusion criteria were as follows: patients who 1) felt a physical or mental burden by enrolling in this study, and 2) considered unsuitable for this study by physicians.

RT planning. Planning computed tomography (CT) scan under free breathing without breath coaching was performed using four-dimensional computed tomography (4D-CT) or a long-time scan (3 seconds/scan). The CT simulation was performed using a 3-dimensional radiation treatment planning system. RT was delivered using a 6-MV photon from a linear accelerator. RT policies, fractionations, and radiation doses per fraction at the isocenter were as follows: conventional fractionation (2 Gy/fraction) in definitive chemoradiotherapy or preoperative chemoradiotherapy for non-small cell lung cancer; accelerated hyperfractionation (1.5 Gy/fraction by twice a day) in definitive chemoradiotherapy for small cell lung cancer; hypofractionation (2.5-3 Gy/fraction) in a centrally located solitary lung tumor without lymph node metastasis; and stereotactic ablative fractionation (12 Gy/fraction) in a peripheral solitary lung tumor without lymph node metastasis.

EBC collection and measurement. Based on a previous study that reported TGF-β1 levels in BALF (2), we collected EBC before and 1 month after RT using a collection device (RTube; Respiratory Research, Austin, TX, USA). EBC was collected according to the recommendations of the American Thoracic Society/European Respiratory Society Task Force (5). The EBC collection method is illustrated in Figure 1. Regarding the collection time, 10 min is recommended as it provides an adequate sample for assay and is well tolerated by patients (5). The use tidal breathing for EBC sampling is also recommended (5). The collection device has a mouth piece with separated inlet avoiding salivary contamination. TGF-β1 levels in EBC were measured using an enzyme-linked immunosorbent assay using Human Transforming Growth Factor-Beta Detection Assay Kit (Chondrex, Woodinville, WA, USA) according to the manufacturers' instructions.

Evaluation and statistics. In this study, patients were followed up 1, 3, and 6 months after RT to observe the treatment effect and adverse events. Based on the clinical symptoms, chest X-ray, and CT images, we evaluated RP using the Common Terminology Criteria for Adverse Events v4. Main criteria of the RP grading were as follows: Grade 1 was asymptomatic, clinical or diagnostic observations only, or intervention not indicated; Grade 2 was symptomatic, medical intervention indicated, or limiting instrumental activity of daily living. Using the worst grade of RP for each patient, we analyzed the relationship between G2 RP and TGF-β1 levels in EBC. Statistical significance was defined as a *p*-value <0.05. JMP Pro ver. 15 (SAS Institute, Cary, NC, USA) was used for statistical analyses. The sample size was not calculated to prioritize the feasibility of the exploratory study.

Results

A total of 10 patients were enrolled between November 2018 and October 2019. Patient characteristics are listed in Table I. Histopathology of lung cancer at the time of RT was as follows: two patients each with adenocarcinoma, squamous cell carcinoma, and relapsed adenocarcinoma; one patient each with small cell carcinoma, relapsed squamous cell carcinoma, relapsed adenosquamous cell carcinoma, and relapsed adenoid cystic carcinoma. Clinical stage according to the 8th edition of the Union for International Cancer Control at the time of RT was as follows: three patients with stage I, two with stage II, four with stage III, and one with stage IV with an isolated pulmonary metastasis relapsed after surgery.

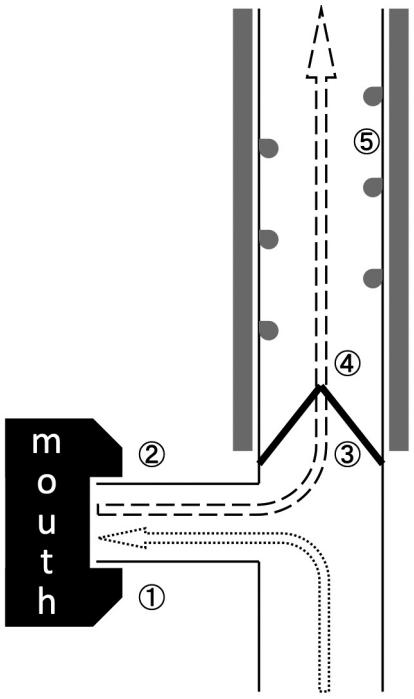

Figure 1. *A method of the exhaled breath condensate (EBC) collection. 1) After inserting a collection device in the patients' mouth, they were instructed to inhale naturally (dotted line). 2) Patients were then instructed to exhale naturally (broken line). 3) The exhaled breath passes through a one-way valve (bold line). 4) The exhaled breath is then chilled by a cooling sleeve, which covers a collection device (gray bar). 5) The EBC attaches to an inner wall of a collection device (gray drop). Patients were instructed to breathe naturally to a collection device for 10 min. After that, we collected the EBC within the collection device.*

All patients experienced RP within 6 months after RT. G1 and G2 RP were observed in five patients each; no G3-G5 RP occurred. The median incidence timing of G2 RP was 9 weeks (range=5-17 weeks) after the completion of RT; all G2 RP occurred after EBC collection 1 month after RT.

During the 10 min for every EBC collection, no remarkable changes were observed in the patients' blood pressure, pulse rate, percutaneous oxygen saturation, and general condition. Changes in TGF-β1 levels from before RT to 1 month after RT are shown in Table II. The median difference in TGF-β1 levels (1 month after RT minus before

Table I. *Patient characteristics*.

		Number	%	Median (Range)
Age (years)				75 (60-81)
Sex	Male	9		
	Female	1		
ECOG-PS	0	3	30	
	1	7	70	
History of lung cancer at the time of RT	Newly diagnosed	4	40	
	Relapsed after surgery	6	60	
With smoking history	Yes	8	80	
	No	2	20	
With COPD	Yes	3	30	
	No	7	70	
With ILD	Yes	0	0	
	No	10	100	
With DM	Yes	4	40	
	No	6	60	
RT policy	Definitive	9	90	
	Preoperative	1	10	
CTx with RT[a]	Concurrent CTx with RT	4	40	
	Induction CTx followed by RT	1	10	
	None	5	50	
Treatment after RT	Durvalumab	2	20	
	Carboplatin and etoposide	1	10	
	Surgery	1	10	
	None	6	60	
RT fractionation[b]	Conventional fractionation	4	40	
	Accelerated hyperfractionation	1	10	
	Hypofractionation	2	20	
	Stereotactic ablative fractionation	3	30	
V_{30Gy}[c] (%)[d]				15.3 (1.3-35.8)
V_{20Gy}[c] (%)[d]				19.8 (2.3-39.8)
V_{13Gy}[c] (%)[d]				23.6 (3.7-43.5)
V_{5Gy}[c] (%)[d]				38.9 (10.5-61.2)
VS_{5Gy}[e] (cc)[d]				1,562 (999-2,921)
MLD (Gy)[d]				2.1 (11.2-22.0)

[a]Regimens of CTx for each patient are listed in Table II. [b]Radiation doses at the isocenter for each patient are listed in Table II. [c]V_{nGy} was the percent of the volume of the lungs at least irradiated n Gy. [d]These raw dose-volume histogram parameters without adjustments are just for exploratory reference purposes as various fractionations were used in this study. [e]VS_{5Gy} indicates lung volumes spared from a 5 Gy dose. ECOG-PS: Eastern Cooperative Oncology Group Performance Status; RT: radiotherapy; COPD: chronic obstructive pulmonary disease; ILD: interstitial lung disease; DM: diabetes mellitus; CTx: chemotherapy; MLD: mean lung dose.

RT) was 109.1 pg/ml (range=–182.0 to 541.4 pg/ml). The median ratio of TGF-β1 levels (1 month after RT/before RT) was 5.9 (range=0.3-4520.0).

In seven patients, TGF-β1 levels increased from before RT to 1 month after RT. Of the seven patients with an increase in TGF-β1 levels in EBC 1 month after RT, five (71%) experienced G2 RP, whereas the remaining three patients with decreased TGF-β1 levels had G1 RP (p=0.083, one-sided Fisher's exact test) (Figure 2).

One of the two patients with G1 RP experienced early tumor progression and had an increase in TGF-β1 levels, and this was brain metastasis 17 weeks after the completion of RT; this metastasis occurred after EBC collection 1 month after RT.

As for the RT fractionations, all three patients treated with stereotactic ablative fractionation had a decrease in TGF-β1 levels (p=0.008, one-sided Fisher's exact test).

Discussion

To the best of our knowledge, this is the first study to investigate the relationship between RP and TGF-β1 levels in EBC. TGF-β plays a dominant role in RP through tissue remodeling and inflammation (1). TGF-β1 is considered as a master switch for the fibrotic program (9). To predict RP, TGF-β1 levels were studied in plasma and BALF (2, 10). In such background, we conceptualized the substitution of EBC for BALF, and conducted this prospective exploratory study.

Table II. *Changes in TGF-β1 levels from before RT to 1 month after RT.*

Increased (n=7)	RP	CTx with RT	RT dose at the isocenter	V_{20Gy}[a] (%)[b]	MLD (Gy)[b]	TGF-β1 before RT (pg/ml)	TGF-β1 1 month after RT (pg/ml)
Pt 1	G2	None	60 Gy/24 fr	15.1	9.5	295.1	481.7
Pt 4	G1	Concurrent CTx with RT[c]	48 Gy/24 fr[d]	26.4	14.3	0.1	452.0[e]
Pt 6	G2	Concurrent CTx with RT[c]	60 Gy/30 fr	34.0	20.3	119.9	661.3
Pt 7	G2	Induction CTx followed by RT[f]	60 Gy/30 fr	22.1	12.6	20.1	331.2
Pt 8	G2	Concurrent CTx with RT[f]	60 Gy/30 fr	39.8	22.0	38.2	242.6
Pt 9	G1	None	60 Gy/20 fr	17.5	9.7	2.2	33.7
Pt 10	G2	Concurrent CTx with RT[g]	45 Gy/30 fr[h]	27.6	12.8	2.6	34.0
Decreased (n=3)							
Pt 2	G1	None	48 Gy/4 fr	2.3	2.6	254.1	72.1
Pt 3	G1	None	48 Gy/4 fr	10.7	6.4	141.6	94.1
Pt 5	G1	None	48 Gy/4 fr	2.4	2.1	563.7	472.8

[a]V_{nGy} was the percent of the volume of the lungs at least irradiated n Gy. [b]These raw dose-volume histogram parameters without adjustments are just for exploratory reference purposes as various fractionations were used in this study. [c]Cisplatin and docetaxel. [d]This patient was treated with preoperative chemoradiotherapy followed by surgery. [e]Exhaled breath condensate 1 month after RT was collected before surgery. [f]Carboplatin and nab-paclitaxel. [g]Carboplatin and etoposide. [h]Twice a day. TGF-β1: Transforming growth factor-β1; RT: radiotherapy; RP: radiation pneumonitis; CTx: chemotherapy; MLD: mean lung dose; Pt: patient; G: grade; fr: fraction.

Based on a previous study that reported TGF-β1 levels in BALF (2), we collected EBC before and 1 month after RT. Before RT was selected as a baseline. To detect G2 RP early on, 1 month after RT was also chosen as the most promising timing based on the above-mentioned study (2). As a result, marginal significance was observed; of the patients with an increase in TGF-β1 levels in EBC 1 month after RT, 71% experienced G2 RP. This was consistent with the results of the above-mentioned study that TGF-β1 levels in BALF 1 month after RT tended to be greater in patients with ≥G2 RP than in those with <G1 RP (2). In the study that used BALF (2) and in our study where we used EBC, we think that the main cause of the marginal significance was the small sample size (*n*=11 and 10, respectively) and small event size (≥G2 RP occurred in six and five patients, respectively). Further larger studies are needed to confirm the significance of the results.

TGF-β1 affects not only fibrosis, but also tumor resistance or metastasis through epithelial-to-mesenchymal transition (11). A meta-analysis indicated that TGF-β expression significantly predicted poor prognosis in patients with lung cancer (12). Another study showed that an increase in the plasma TGF-β1 level during RT was correlated with poor prognosis in locally advanced non-small cell lung cancer (13). In our study, one of the two patients with G1 RP experienced early tumor progression and had an increase in TGF-β1 levels, and this was brain metastasis 17 weeks after the completion of RT. We anticipate that our study might provide an insight into the TGF-β1 influence on not only fibrosis, but also poor prognosis.

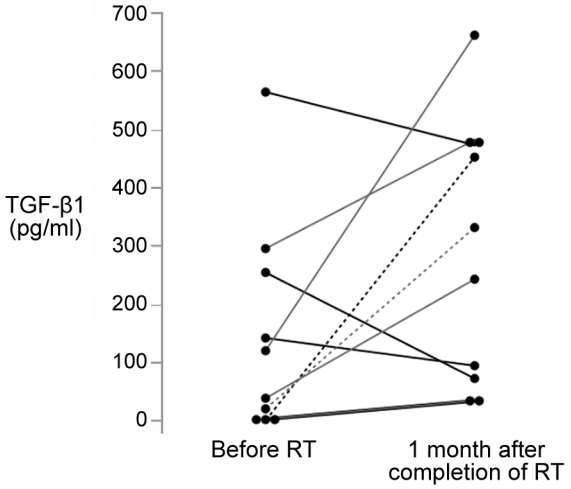

Figure 2. *Changes in transforming growth factor-β1 (TGF-β1) levels in exhaled breath condensate from before radiotherapy (RT) to 1 month after RT. Gray solid lines indicate patients with grade (G) 2 radiation pneumonitis (RP) alone. Gray broken lines indicate patients with both G2 RP and early tumor progression. Black broken lines indicate patients with early tumor progression alone. Black solid lines indicate patients without these events.*

As for the RT fractionations, all three patients treated with stereotactic ablative fractionation had a decrease in TGF-β1

levels with a statistical significance. As shown in Table II, these three patients had low V_{20Gy} and mean lung dose, although these raw dose-volume histogram parameters without adjustments are just for exploratory reference purposes as various fractionations were used in this study. Relatively low dose to the lung might affect not increasing TGF-β1 levels through the limited lung fibrosis. Moreover, stereotactic ablative fractionation is well-known to have better prognosis of lung cancer than conventional fractionation (14, 15). This good prognosis might also affect not increasing TGF-β1 levels after stereotactic ablative fractionation.

As mentioned previously, we collected EBC at the two timepoints and analyzed TGF-β1 based on a previous study using BALF (2). However, EBC can be obtained relatively easily without invasiveness. Therefore, it might be possible to measure TGF-β1 levels at more frequent timepoints to observe the changes in TGF-β1 levels related to thoracic RT for lung cancer in detail.

Our study has other limitations, such as the single institutional design, various histories of lung cancer, different RT policies, various RT fractionations, and several treatment modalities. These factors may have influenced the occurrence of RP. Therefore, further controlled studies are needed to obtain more solid data from a more homogeneous group than that of our exploratory study.

In conclusion, increased TGF-β1 levels in EBC 1 month after RT might be promising for the detection of G2 RP. Further controlled studies with larger sample sizes are required.

Conflicts of Interest

The Authors have no conflicts of interest regarding this study.

Authors' Contributions

This study was coordinated by ST and TS. Data were collected by ST, MA, TK, and TN. Collected data were analyzed by ST. This article was drafted by ST. Data interpretation and article revision were done by all Authors: ST, MA, TK, TN, and TS. All Authors approved this submitted article.

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