Supplementary information

Analysis of volatile organic compounds in exhaled breath after radiotherapy

Dianlong GE[1,2], Xue ZOU[1]✉, Yajing CHU[1,2], Jijuan ZHOU[1,2], Wei XU[1,2], Yue LIU[1], Qiangling ZHANG[1], Yan LU[1,3], Lei XIA[1], Aiyue LI[1], Chaoqun HUANG[1], Pei WANG[3]✉, Chengyin SHEN[1,3], Yannan CHU[1]

[1]*Anhui Province Key Laboratory of Medical Physics and Technology, Institute of Health and Medical Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China*

[2]*University of Science and Technology of China, Hefei 230026, China*

[3]*Department of Laboratory Medicine, Hefei Cancer Hospital, Chinese Academy of Sciences, Hefei 230031, China*

✉Corresponding author:

Xue ZOU, xzou@cmpt.ac.cn;

https://orcid.org/0000-0001-9540-6764

Pei WANG, 61775618@qq.com

https://orcid.org/0000-0002-8613-6480

Materials and methods

1 Breath sample collection

All patient-subjects were diagnosed with lung cancer and were hospitalized at Hefei Cancer Hospital, Chinese Academy of Sciences. The exhaled breaths of the subjects were obtained 0.5 hours before and 0.5, 2.0, 4.0, and 6.0 hours after radiotherapy. For each subject, breath samples 0.5 hours before and after radiotherapy were repeatedly collected four times at one-week intervals. Meanwhile, 600-mL home-made glass bottles were used for storing the samples. During the

sampling process, the valves at both ends of each bottle were opened. Each subject took a deep breath and deeply exhaled into one of the valves. The blowing process was made to repeat thrice to ensure that the bottle was filled with exhaled gases. Then, both valves were closed immediately. Finally, the bottles were placed inside a light-proof box to avoid photolysis of VOCs.

2 SPME-GC/MS conditions

Exhaled VOC detection was performed on a mass spectrometer (TSQ QUANTUM XLS, Thermo, San Jose, USA) coupled with gas chromatography (TRACE GC ULTRA, Thermo, Milan, Italy). A 30 m×0.32 mm×1.80 μM capillary column (TG-624SILMS, Thermo, Bellefonte, USA) was utilized for VOC separation. Before GC/MS analysis, the SPME fiber was inserted into the GC injection port for 30 min to remove residual VOCs. Then, it was exposed to the breath samples for 40 min at 37 °C before reinserting into the GC injection port for desorption for 30 s. The temperature of the GC injection port was set to 200 °C, while the flow rate of helium carrier gas was set to 1.5 mL/min. The splitless mode was applied in this experiment. Both the temperatures of the transfer line and ion source were set to 200 °C. MS detection was undertaken in a full scan from 45 to 300 amu. Electron impact energy was 70 eV. The temperature of the column was maintained at 40 °C for 1 min, before raising it to 180 °C at a speed of 5 °C/min, holding it afterward for 2 min.

Considering the performance fluctuation of GC/MS, ethanol of standard concentration was added to the breath samples as an internal standard. To prepare standard ethanol gas, 1.14 mL of analytically pure liquid ethanol was injected into a 500-mL volumetric flask with 498.86-mL ultrapure water. Then, 20 mL of the standard solution was injected into a 100-mL headspace flask. After vapor-liquid equilibrium, ethanol concentration in the headspace was sustained at 200 ppm. Finally, 5-mL headspace gas was inserted into a 600-mL breath sampling bottle as the internal standard.

3 Statistical analysis

A hot spot map was drawn using the Multi-Experiment Viewer (MeV) to depict the changing trends of exhaled VOCs after radiotherapy. In addition, the *Mann-Whitney* U test was applied to

compare the significant differences of exhaled VOCs 0.5 hours before and after radiotherapy and identify the biomarkers of radiotherapy. A $p<0.05$ condition was considered to indicate statistical difference. VOCs with $p<0.05$ were then selected for orthogonal partial least squares discriminant analysis (OPLS-DA) with Pareto (Par) scaling. A sevenfold cross-validation method was applied to estimate robustness and predictive ability of the OPLS-DA model. Variable importance in projection (VIP) scores were used to select VOCs that had significantly contributed to discrimination. Finally, permutation testing (200 permutations) was employed for further validation.

Results

Quantitative analysis of biomarker VOCs

Propionic acid, triacetin, and indole were quantitatively analyzed. The calibration gases of propionic acid at varying levels were prepared by adding 0.002-, 0.004-, 0.006-, 0.008-, and 0.012-mL gas samples into 600-mL glass bottles filled with pure nitrogen gas. The calibration gases of triacetin at varying levels were made by adding 0.3-, 0.6-, 1.5-, 3-, and 4.5-mL gas samples into 600-mL glass bottles filled with pure nitrogen gas. Meanwhile, the calibration gases of indole at varying levels were made by adding 0.1-, 0.5-, 0.8-, 1.2-, and 1.8-mL gas samples into 600-mL glass bottles filled with pure nitrogen gas. Fig. S1 presents the calibration curves. The linear regression equation of propionic acid is $y=6.8112\times10^{7}x-7.0790\times10^{5}$, $r^2=0.9861$ (Fig. S1A); the linear regression equation of triacetin is $y=2.1206\times10^{7}x+5.5856\times10^{4}$, $r^2=0.9941$ (Fig. S1B); the linear regression equation of indole is $y=8.1812\times10^{7}x-9.8591\times10^{3}$, $r^2=0.9987$ (Fig. S1C).

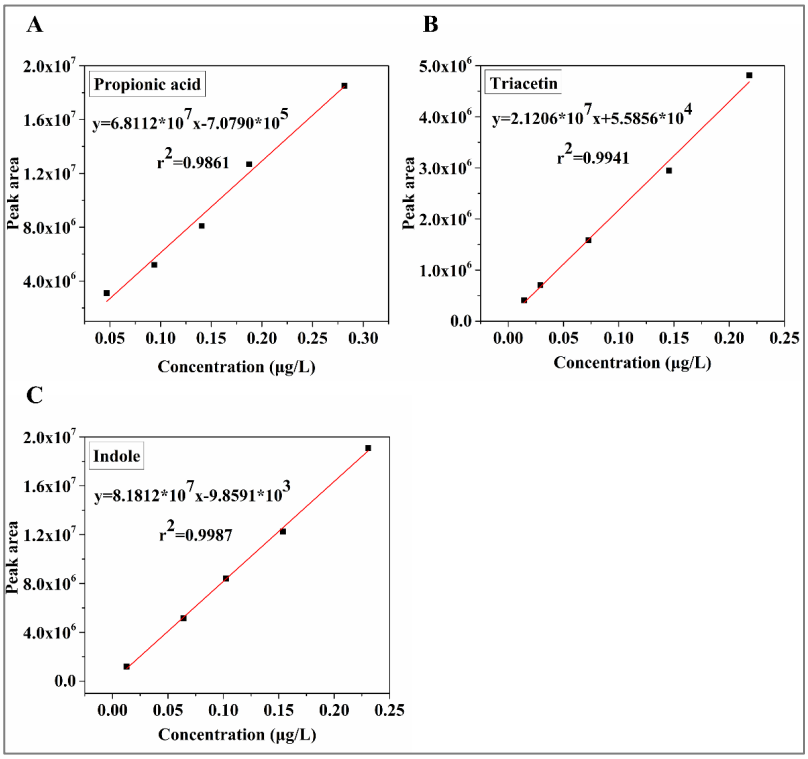

Fig. S1 Quantitative analysis of propionic acid, triacetin, and indole. (A) Calibration curve for the quantification of propionic acid; (B) calibration curve for the quantification of triacetin; (C) calibration curve for the quantification of indole.