

In-air microparticle induced X-ray emission analysis of particles in interstitial pneumonia lung tissue obtained by transbronchial biopsy

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Interstitial pneumonia develops in association with inhaled particles. In-air microparticle induced X-ray emission (in-air micro) analysis was previously employed to assess the spatial distribution and content of particles in surgical lung biopsy specimens. The aim of this study was to assess the efficacy of in-air micro-analysis for transbronchial lung biopsy specimens in patients with or without occupational exposure. The elements composing lung particles and their locations could be identified by in-air micro-analysis. Silicon was the major component of particles. Quantitative analysis revealed that the elements composing lung particles varied between patients. In a patient with suspected nickel exposure, aluminium, vanadium, and calcium were detected, but was not detected. In a patient without a work history (housewife), various elements were detected. In-air micro-analysis was useful for assessing the spatial distribution and content of particles in specimens from patients. Occupational exposure was not necessarily associated with deposition of particles in the lungs. Therefore, in the diagnosis of, elemental analysis of specimens by in-air micro-analysis could be useful for assessing exposure to particles objectively.

Key Words: transbronchial biopsy, in-air micro-PIXE analysis, elemental analysis, particles, interstitial pneumonia

Occupational and environmental exposure to particles lead to various respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), interstitial pneumonia (IP), pulmonary hypertension, and lung cancer.^(1,2) In patients with IP, inhaled particles cause inflammation and fibrosis of the lung parenchyma as well as DNA damage resulting from biochemical and immunological responses.^(3–5) Analysis of particles in lung tissue by X-ray methods has been done on bronchoalveolar lavage (BAL) fluid in patients with silicosis, which is a type of IP, using electron microscopy with semiquantitative X-ray microanalysis (SXM) or energy dispersive X-ray microanalysis (EDXA).^(6,7) EDXA analysis of BAL cells or transbronchial biopsy (TBLB) specimens has proven useful for identifying silica in patients with silicosis (Si).⁽⁸⁾

An in-air microparticle induced X-ray emission (in-air micro-PIXE) system was developed at the TIARA facility of the Japan Atomic Energy Agency.⁽⁹⁾ This system performs elemental analysis of materials by irradiation with a proton microbeam, and allows visualization of the spatial distribution and quantitation of various elements with very low background noise compared to electron beam methods.^(10,11) When samples for in-air micro-PIXE are prepared, tissue specimens do not need any chemical treatment.

Application of this method of analysis had been done for asbestosis and idiopathic interstitial pneumonia (IIP) lung specimens, and the distribution and content of asbestos particles or other particles has been determined in association with the expression of disease-related proteins.^(12–14) Those studies were performed on lung tissue specimens obtained by video-assisted thoracoscopic surgery (VATS) or open lung surgery, so application of in-air micro-PIXE to TBLB specimens has not yet been done. TBLB specimens are smaller (about 2 mm³) than specimens obtained by VATS or open lung biopsy (>2–3 cm³). However, TBLB specimens can be obtained by bronchofiberscopy, which is less invasive for patients compared with lung surgery.

The aims of the present study were to investigate the detection efficacy of in-air micro-PIXE for TBLB specimens, and to determine the particle content of specimens from patients with or without a history of occupational exposure.

Methods

Subjects. Patients with evidence of IP on computed tomography were enrolled in this study. Bacterial pneumonia was excluded by various examinations, including blood, sputum, and urine tests, before bronchofiberscopy. Lung specimens were obtained by TBLB at bronchofiberscopy using a biptome (FB-19c or FB-20c-1, Olympus, Tokyo, Japan). Serum levels of KL-6, surfactant protein D (SP-D), and anti-nuclear antigen (ANA) were examined, and then a diagnosis was made according to the disease criteria reported previously.^(15,16) Patient no. 1 (pt1) worked in the battery making industry, and exposure to Ni was suspected (Table 1). Exposure to automobile exhaust fumes (diesel or oil) was suspected for pt4, while pt2 and pt3 had no history of work-related exposure to particles. All patients showed IP on lung images, and activity was confirmed by high serum levels of KL-6 (>500 U/ml) or SP-D (>110 ng/ml). The clinical diagnosis of pt1 and pt4 was collagen vascular disease-related IP, while the diagnosis for pt2 and pt3 was idiopathic IP. The characteristics of the patients enrolled and their diagnoses are shown in Table 1. This study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the Human Research Committee of Jobu Hospital for Respiratory Disease.

Elemental analysis. Specimens obtained by TBLB were prepared for in-air micro-PIXE analysis as reported previously.⁽¹²⁾ Specimens about 2 mm³ obtained with a biptome at bronchofiber-

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Table 1. Characteristics of interstitial pneumonia patients

Pt number	Age	M/F	Smoking history (pack-years)	Job	Locality of residence area	KL-6 (U/ml)	SP-D (ng/ml)	ANA (X)	Clinical diagnosis
Pt1	58	M	38	battery making	Shibukawa city, Gunma (mountain area)	1292	69.9	320	CVD-IP
Pt2	60	M	never	cook	Maebashi city, Gunma (suburban)	4024	355	40	IIP
Pt3	75	F	never	housewife	Kiryu city, Gunma (mountain area)	3888	554	40	IIP
Pt4	64	M	ex-smoker	management of car parking	Maebashi city, Gunma (suburban)	2235	913	320	Systemic sclerosis, CVD-IP

Normal range of serum KL-6, surfactant protein-D (SP-D) and anti-nuclear antigen were <500 U/ml, <110 ng/ml and <X40, respectively. Clinical diagnosis of lung diseases were collagen vascular related-interstitial pneumonia (CVD-IP) and idiopathic interstitial pneumonia (IIP).

scopy were embedded in paraffin, and cut into sections 5 μm thick. Each section was dried, placed onto 5 μm polyethylene terephthalate film, and fixed in the sample holder for in-air micro-PIXE analysis. This is a method of elemental analysis in which the sample is irradiated with a proton ion microbeam. A 3.0 MeV proton ion-micro-beam was accelerated by a single-ended machine at the Takasaki Advanced radiation research institute, and irradiated samples yields specific X-ray peaks for various elements. Based on these peaks and their strength, the in-air micro-PIXE system can visualize the spatial distribution and content of various substances. Identification of particles was done as previously.⁽¹²⁾ Briefly, elemental maps which indicated spatial distribution of particles were obtained as large scale of 980 μm \times 980 μm . Since Si forms the core of particles, and was good marks of particles at large scale analysis, the microbeam was focused on areas of Si particles. Then small scale analysis focused area of 50 μm \times 50 μm was done, and the particles from the analysis area were gated. Visualization and calculation of the content were done for magnesium (Mg), aluminium (Al), silicon (Si), phosphorus (P), sulphur (S), chlorine (Cl), potassium (K), calcium (Ca), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn). The X-ray intensity of Si in each sample was set as maximum intensity in gray scale in an elemental map of a sample, and the intensity of other metals were demonstrated according to the scale. Since Si forms the core of particles, quantitation of the elements in particles was expressed as a ratio relative to Si (element/Si). Haematoxylin-eosin stain was performed as previously described,⁽¹²⁾ and section were examined under microscope (BX50F4, Olympus, Tokyo, Japan).

Statistics. The quantities of element A and element B were compared as follows:

$$\frac{m_B}{m_A} = \frac{Y_B M_B \text{eff}_A \sigma_A}{Y_A M_A \text{eff}_B \sigma_B}$$

where Y is the net count of the elements, M is the atomic weight of the element (amu), m is the quantity of the element in grams (g), eff is the detection efficiency for the element, and σ is the cross-sectional area of the specific X-ray peak for the element.⁽¹²⁾ Then the content of each element was calculated as a ratio to that of Si.

Results

Elemental analysis was done on TBLB specimens. All particles identified in the specimens had Si as the core. Peaks of the gated areas were obtained as shown in Fig. 1a (peak line 2), and the gated areas on elemental map were as shown in Fig. 1b. Elemental

analysis of the TBLB specimen from pt1 showed a high Si peak (Fig. 1a), while the elemental map revealed that Al was markedly co-localized with Si, while V was weakly co-localized. Ca was diffusely distributed in the tissue, but co-localization with Si was weak (Fig. 1b). The peaks of the gated area for pt2 are shown in Fig. 2a. Ca was markedly co-localized with Si in the gated area, while Mg, Al, and Zn were weakly co-localized (Fig. 2b). The peaks obtained in pt3 are shown in Fig. 3a. Fe, Cr, Ni and Co were obviously co-localized with Si, while Mg, Mn, Ni, and Zn were weakly co-localized (Fig. 3b). Ca was diffusely distributed in the tissue, but its co-localization with Si was only weak. The peaks obtained in pt4 are shown in Fig. 4a. Fe, Al, Ti, and Ca were obviously co-localized with Si, while Mg, Co, Cr, and V were weakly co-localized (Fig. 4b). Quantitative analysis of the content of various elements relative to Si showed that mAl/mSi was high (1.223) in pt1, while mV/mSi and mCa/mSi were low at 0.011 and 0.046, respectively (Fig. 5a). In pt2, mMg/mSi, mAl/mSi and mCa/mSi were high at 0.179, 0.131, and 0.169, respectively, while mZn/mSi was a low 0.033 (Fig. 5b). In pt3, mFe/mSi was high (3.725), while mMg/mSi, mCr/mSi, mMn/mSi, mNi/mSi, mCo/mSi, mZn/mSi, and mCa/mSi were 1.031, 0.656, 0.094, 0.037, 0.658, 0.044, and 0.337, respectively (Fig. 5c). In pt4, mFe/mSi was low (0.047), but mMg/mSi and mAl/mSi were high at 0.229 and 0.284, respectively. However, the values of mTi/mSi, mCo/mSi, mCr/mSi, mV/mSi, and mCa/mSi were all low at 0.0149, 0.005, 0.004, 0.007 and 0.031, respectively (Fig. 5d).

Discussion

This study showed that in-air micro-PIXE could detect various elements in TBLB specimens. Previous studies have demonstrated that the elements composing asbestos or particles can be analyzed in VATS or open lung biopsy specimens from patients,⁽¹²⁻¹⁴⁾ but application of in-air micro-PIXE to TBLB specimens has not been reported. Although TBLB specimens are smaller than specimens obtained by surgery, bronchofiberscopy is less invasive for patients. In the present study, pt1 had suspected occupational exposure to Ni, but in-air micro-PIXE analysis could not detect co-localization of Ni and Si deposits in the TBLB specimen, and also Ni without co-localization to Si was not detected in focused area. From this analysis, the IP of pt1 was unlikely to be Ni-induced, and airway exposure was considered to be well-controlled in the workplace. In pt4, inhalation of automobile exhaust fumes was suspected. The specimen obtained from pt4 contained Fe, Mg, Al, Ti, Co, Cr, V, and Ca. A previous study showed that the particles in air obtained along a highway included Na, Mg, Al, Si, S, Cl, K, Ca, and Fe on PIXE analysis.⁽¹⁷⁾ Diesel exhaust fumes contain Na, Mg, Si, Cl, Ca, Fe, and Zn as major components by PIXE analysis.⁽¹⁸⁾ These findings indicate that automobile exhaust fumes examined *ex vivo* contained similar

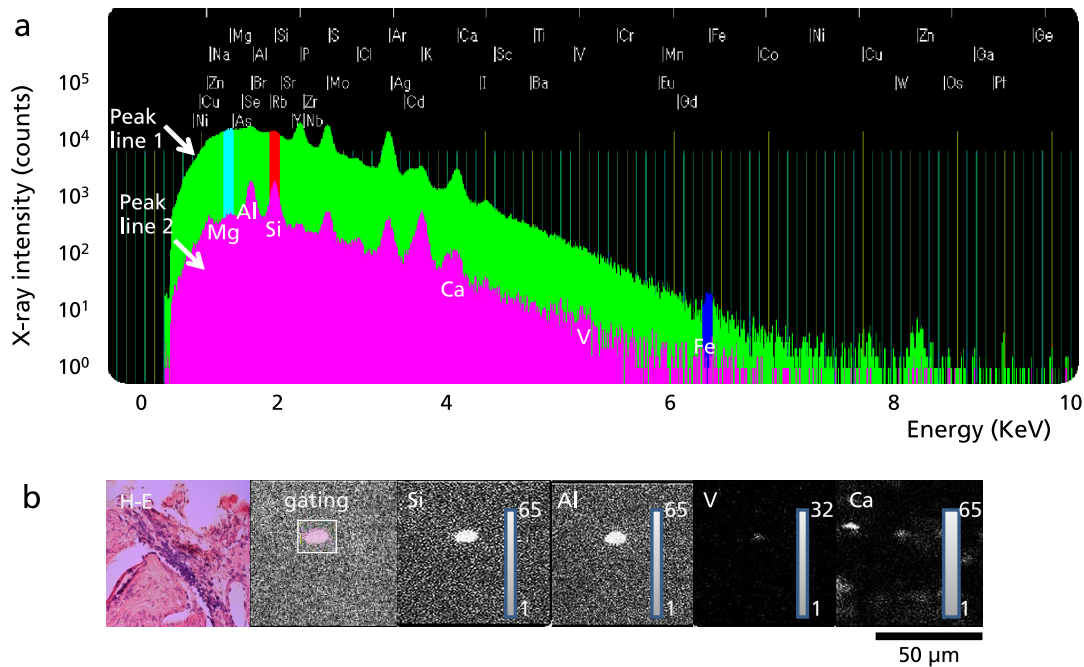


Fig. 1. Peaks of each element and elemental map for pt1. Peak line 1 indicates all elements in the beamed area and peak line 2 indicates the spectrum of the gated area (a) shown as white square (b). A serial section of irradiated area showed tiny black particles with accumulation of lymphocytes and dense fibrosis (Hematoxylin-eosine stain (H-E), $\times 400$). The intensity of metals in lung tissue is shown by gray to white bars. Si is marked on the elemental map. White dots indicate the location of elements in the TBLB specimen; Al and V are seen to co-localize with Si. Some dots of Ca are co-localized with Si, Al, and V (b).

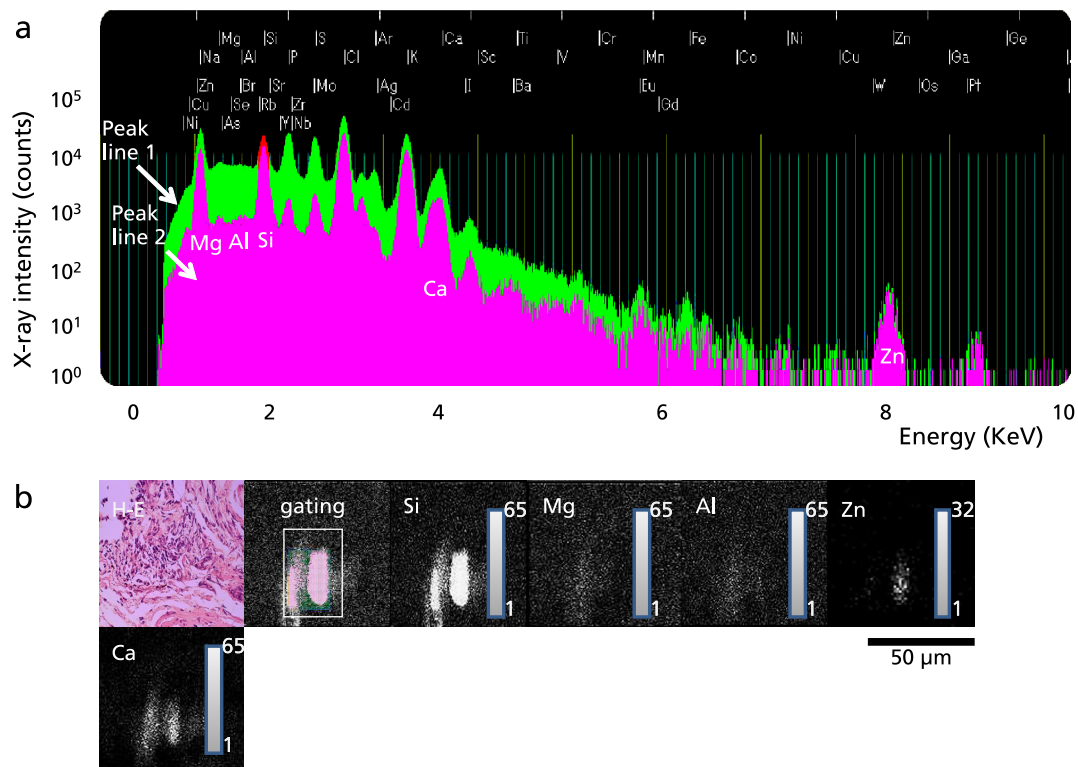


Fig. 2. Peaks of each element and elemental map for pt2. Peak line 1 indicates the spectrum for the entire beamed area and peak line 2 indicates the spectrum of the gated area (a) shown as white square (b). A serial section of irradiated area showed accumulation of lymphocytes and destructed small airways (Hematoxylin-eosine stain (H-E), $\times 400$). The intensity of metals in lung tissue is shown by gray to white bars. Si is marked on the elemental map. White dots indicate the locations of elements in the TBLB specimen; Mg, Al, Zn, and Ca are co-localized with Si (b).

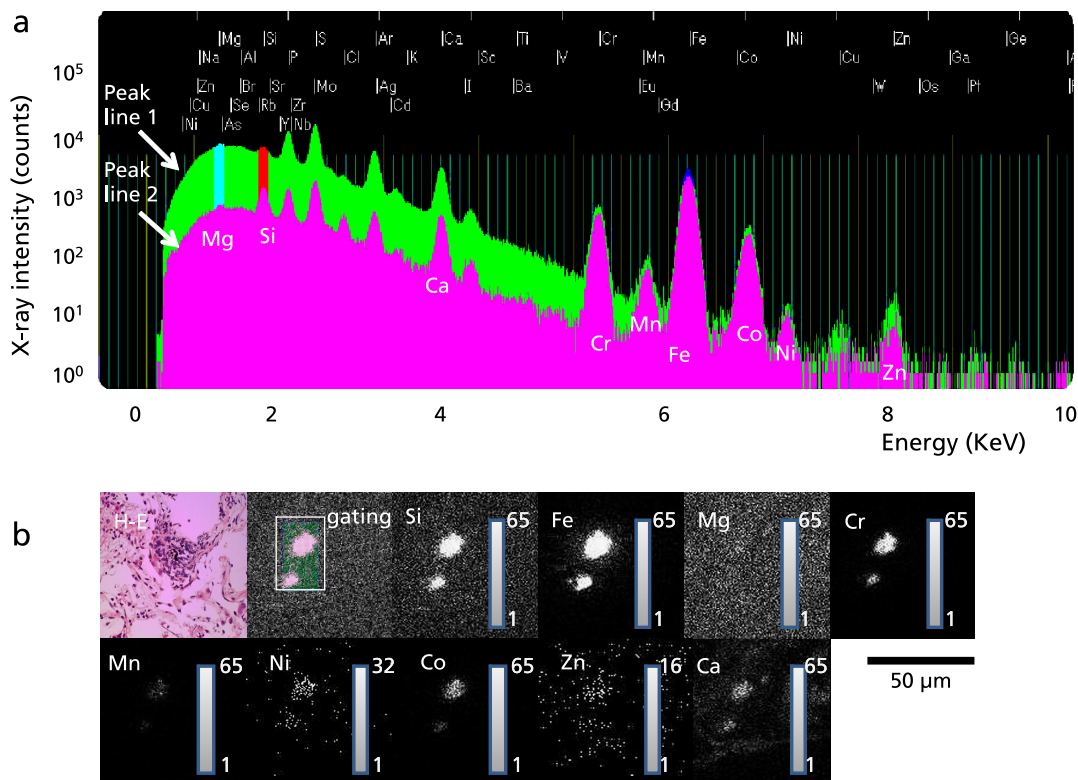


Fig. 3. Peaks of each element and elemental map for pt3. Peak line 1 indicates the spectrum for the entire beamed area and peak line 2 indicates the spectrum of the gated area (a) shown as white square (b). A serial section of irradiated area showed dense black particles with accumulation of lymphocytes and macrophages (Hematoxylin-eosine stain (H-E), $\times 400$). The intensity of metals in lung tissue is shown by gray to white bars. Si is marked on the elemental map. White dots indicate the locations of elements in the TBLB specimen; Fe, Mg, Cr, Mn, Zn, and Co are co-localized with Si. Some dots of Zn and Ca are co-localized with Si, Fe, Mg, Cr, Mn, and Co (b).

particles to the TBLB specimen of pt4, and that inhalation of these particles may have been related to IP. However, to determine occupational exposure of pt4, it would be necessary to examine airborne particles in his workplace. Both pt2 and pt3 had less risk of occupational or environmental exposure to particles. However, pt2 was a cook, and cooks can inhale particles from fuel that affect pulmonary function.⁽¹⁹⁾ Solid fuel stoves or open fires are sources of pollutants, but patient 2 did not use these methods of cooking. Patient 3 was a housewife and she had no history of occupational exposure to particles or apparent environmental exposure, but many elements were still detected. Resident area of patient 2 was Kiryu city of Gunma prefecture which is located in northeast from Tokyo, Japan about 100 km distance. Previous study revealed the existence of airborne particles originated from local and continental soils, coal combustion and diesel exhaust in Gunma prefecture.⁽²⁰⁾ Environmental exposure might have been occurred in pt3 without her awareness. Particles that can be inhaled exist in the air, and environmental exposure can lead to pulmonary disease without the subject being aware of exposure. Patient 4 was a manager of car parking, and had the risk of particle exposure. Diesel particles (DEP) synergistically promote lung tissue injury by enhancing bacterial endotoxin production.⁽²¹⁾ Therefore, it could be useful to identify the origin of particles when environmental exposure (especially DEP) is suspected in patients with respiratory diseases, such as IP.

Quantitative analysis of particle composition showed various findings in our patients. Previous analysis of BAL by electron microscopy and EDXA has shown that inorganic particles in the lungs include silica, fibrous silicates, non-fibrous aluminium silicates, or metals.⁽⁷⁾ Thus, the high ratio of Al to Si in pt1 was

possibly due to aluminium silicate. In pt 3, the particles of Fe were detected. In general, asbestos in lung tissue contain Fe,⁽¹²⁾ but there were no history of asbestos exposure in pt3. Although in-air micro-PIXE analysis was not done for BAL samples in present study, BAL analysis by microscopic examination did not show asbestos fibers in pt3. Iron exposure is a risk factor for IP, and high levels of Fe are related to apoptosis via Fas overexpression in the lungs of patients with IIP.^(14,22) Therefore, Fe was possibly considered to be associated with IP in pt3.

Tobacco smoking influences the pathogenesis of IP.⁽²³⁾ Among our patients, two were smokers. A previous study demonstrated there were patients whose exhaled breath condensate (EBC) contained high levels of cadmium (Cd) and Lead (Pb) analysed by electrothermal atomic absorption spectroscopy (ETAAS) combination with inductively coupled plasma-mass spectrometry (ICP-MS).⁽²⁴⁾ The surface of the lower airway is covered with airway lining fluid (ELF), and exhaled breath includes various substances including metals as an ELF aerosol. The substance of ELF is obtained by cooling breath at -20°C .⁽²⁵⁾ In the present study, the influence of particles related to smoking could not be determined in the smokers (pt1 and pt4), because Cd and Pb were not detected in their TBLB specimens. Although analysis using ETAAS combination with ICP-MS on liquid sample was considered having high detection efficiency than in-air micro-PIXE, in-air micro-PIXE can be applied both on liquid and solid state samples with two-dimensional analysis. Therefore, existence of particles can be visually identified with spatial distribution. The concentrations of Cd and Pb were not always high in smoker⁽²⁵⁾ and the determination of concentrations of those metals in EBC has not yet been established.⁽²⁶⁾ Further analysis is needed to

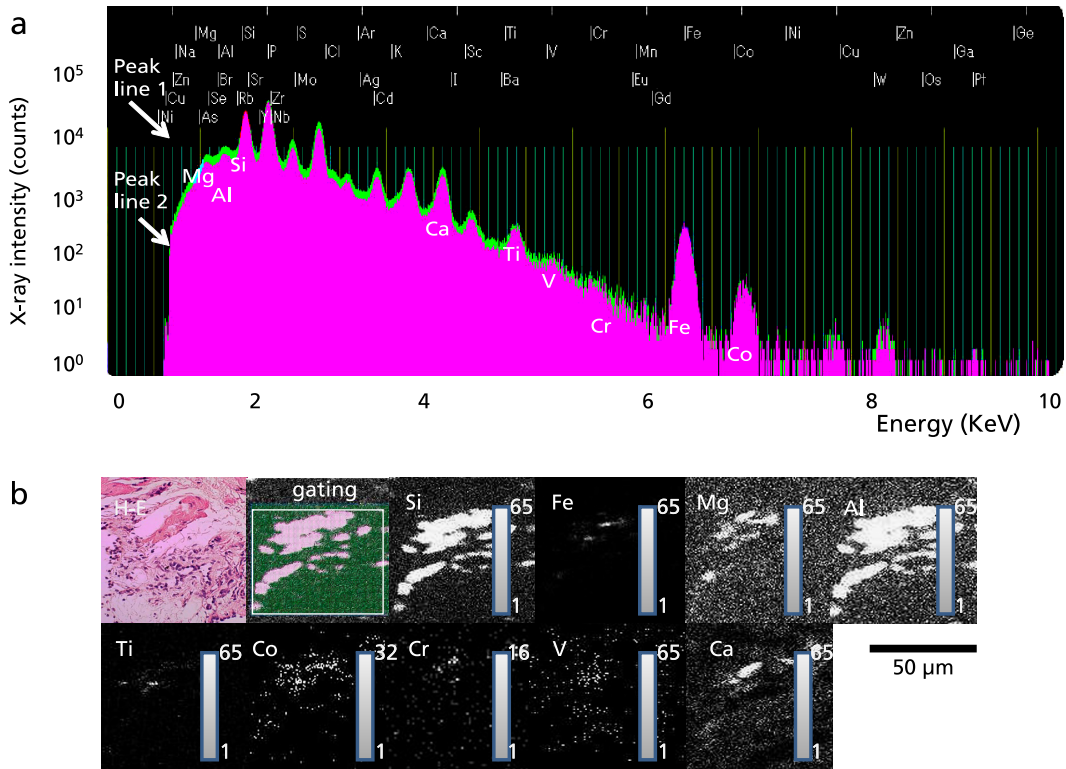


Fig. 4. Peaks of each element and elemental map for pt4. Peak line 1 indicates the spectrum for the entire beamed area and peak line 2 indicates the spectrum of the gated area (a) shown as white square (b). A serial section of irradiated area showed accumulation of lymphocytes and destroyed small airways (Hematoxylin-eosine stain (H-E), x400). The intensity of metals in lung tissue is shown by gray to white bars. Si is marked on the elemental map. White dots indicate the locations of elements in the TBLB specimen; Fe, Mg, Al, Ti, Co, Cr, V, and Ca are co-localized with Si (b).

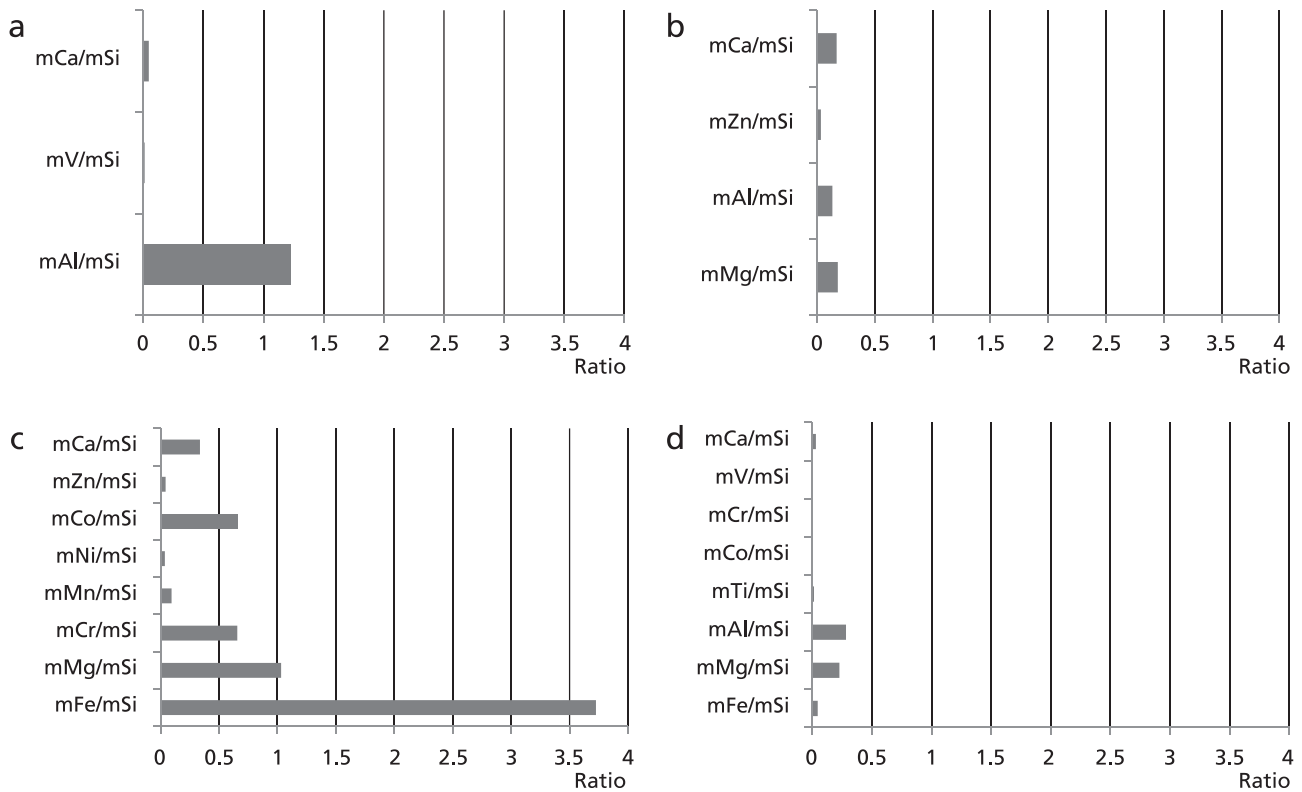


Fig. 5. Quantitative analysis of lung tissue specimens from IP patients obtained by TBLB. In-air micro-PIXE analysis shows the ratio (m) of each element to Si for pt1 (a), pt2 (b), pt3(c), and pt4 (d).

determine the concentration of these metals in relation to smoking. The chief limitation of this study is the small number of subjects. We revealed environmental exposure to particles in patients without a history of occupational exposure, but the present study could not demonstrate the origin of these particles. How subclinical particle occurs in IP patients, or how particle deposition associated with the workplace can be proven by in-air micro-PIXE analysis were not examined. Analysis of airborne particles in residential areas and workplace is needed in the future. The effect of elemental leakage on specimen by paraffin-embedd procedure was not completely excluded. However, detected elements were differences from each patient in present study, and previous study revealed that co-expression of the disease-related proteins and the distribution of metals in lung by using paraffin-embedd VATS specimens.⁽¹⁴⁾ Influence of chemical leakage by paraffin-embedd procedure appeared to be a few, when in-air micro-

PIXE analysis was applied to deposited exogenous dust in lung tissue.

Finally, present study showed that application of in-air micro-PIXE to TBLB specimens can be useful in IP patients. Their history of occupational or environmental exposure did not necessarily correspond with the particles deposited in lung tissue. Accordingly, elemental analysis of TBLB specimens by in-air micro-PIXE can provide useful information when examining occupational and environmental factors related to IP.

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