Talc and Amosite/Crocidolite Preferentially Deposited in the Lungs of Nonoccupational Female Lung Cancer Cases in Urban Areas of Japan

Hiroyuki Yamada, Hisako Hashimoto, Masako Akiyama, Yoshinori Kawabata, and Kazuro Iwai

Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Kiyose, Tokyo, Japan

To analyze the correlation between asbestos lung burden and lung cancer, lungs of 211 female cases with and without lung cancer were examined. Phase-contrast microscopic (PCM) counting of ferruginous bodies (FBs) and uncoated fibers (UFs), which had length longer than 5 µm and aspect ratios greater than 3:1, revealed a significantly higher level of FBs plus UFs in urban lung cancer cases than urban non-lung cancer cases (1380.5 vs. 550.3; $p<0.001$). No difference was noted between rural lung cancer and non-lung cancer cases. Analytical electron microscopic studies identified various kinds of mineral fibers with an aspect ratio greater than 3:1 in the lung tissue including chrysotile, actinolite/tremolite, amosite/crocidolite, fibrous talc, mica, silica, iron, wollastonite, chlorite, kaoline, and others. The most frequently detected fibers were thin, short chrysotile fibers, most of which could not be found by PCM, followed by relatively thick, long actinolite/tremolite fibers, fibrous talc, and in a smaller number, amosite/crocidolite of intermediate length and width. Amosite/crocidolite and fibrous talc counts in urban lung cancer cases were greater than those of urban non-lung cancer cases, rural lung cancer, and rural non-lung cancer cases; these findings were consistent with PCM analysis. Therefore, it is suggested that fibers detected in PCM observation may be mainly amosite/crocidolite with some parts fibrous talc and that fibrous talc in urban environments may be another candidate for carcinogenic or cocarcinogenic factors of female lung cancer. *Key words:* asbestos, female, ferruginous bodies, fibrous talc, lung cancer, mineral fibers, nonoccupational exposure. *Environ Health Perspect* 105:504–508 (1997)

Lung cancer mortality has been increasing worldwide. Factors including smoking, occupational exposure to particulate dusts, diesel exhaust particles, asbestos fibers suspended in the air, and radon gas absorbed in dust particles have been implicated as lung carcinogens (*1*). Among them, long mineral fibers were considered to be carcinogens. Persons occupationally exposed to asbestos, such as asbestos factory workers, have been frequently associated with lung cancer, pleural plaque, and mesothelioma, with or without interstitial lung fibrosis (*2,3*). Numerous asbestos fibers or ferruginous bodies (FBs) were found in digested lung tissue samples using phase-contrast microscopical (PCM) or electron microscopical observations, indicating that the heavy asbestos lung burden may cause the fibrotic and neoplastic changes.

The nonoccupational asbestos lung burden in the general population has been studied by many investigators (*4–7*). Urban inhabitants usually show a larger number of uncoated fibers than rural inhabitants, but the comparison of asbestos fiber counts in the lung tissues of lung and non-lung cancer cases has revealed controversial results. In some studies, FB counts estimated by light microscopy revealed a significantly larger magnitude in lung cancer cases and adenocarcinoma cases (especially in males) than non-lung cancer cases (*8–10*), but other studies do not show this (*11,12*).

Although some investigators using the PCM methods revealed no difference in the number of asbestos fibers between the two groups (*13,14*), recent studies counting uncoated fibers (UFs) by electron microscope have demonstrated higher uncoated fiber counts in the lungs of lung cancer cases than in those of non-lung cancer cases (*15*). To elucidate whether asbestos fibers in the air are responsible for lung cancer in the general population, it is important to analyze subjects with a defined residential and smoking history (*7*).

In order to compare the number of FBs or UFs between urban and rural inhabitants and between the lung cancer and non-lung cancer cases in the present study, female lung cancer and non-lung cancer cases without occupational asbestos exposure were chosen and a quantitative analysis of FBs and UFs in the lungs was performed using a phase-contrast microscope. In addition, we performed analytical transmission electron microscope (ATEM) examinations with an X-ray microanalyzer to detect fibers that cannot be observed by PCM and identify as well as measure the length and width of the fibers.

Materials and Methods

Source of subjects. Subjects included 103 female lung cancer cases over 40 years of age and 108 female non-lung cancer (control) cases that were matched by age (± 5 years) to the cancer cases were selected for the study. No asbestos-related occupational history was noted in any cases. Histological types of 103 primary lung cancers consisted of 83 adenocarcinomas, 3 squamous cell carcinomas, 8 small cell carcinomas, 1 each of atypical carcinoid and carcinosarcoma, and 7 of unclassifiable histological type. Paired case and control subjects were obtained from urban (Tokyo and Osaka) and rural (Iwate and Nagano Prefecture) areas in Japan. Information on residence and smoking history was obtained for all subjects. Age distribution of the cases examined (Table 1) showed no significant difference among the four groups.

Lung tissue sampling. A lung tissue sample of about 1 cm^3 was taken from apparently normal lung tissue of these cases. The lung tissues of all lung cancer cases and most non-lung cancer cases were obtained from surgical materials; in some non-lung cancer cases, samples were obtained from autopsied lungs. Lung specimens were sent to our institute in Tokyo and stored frozen until use. Wet and dry weights of the tissue samples were measured before tissue digestion.

Digestion procedure. Procedures for digestion and sample preparation for ATEM analysis were performed using a modified Kohyama method (*16*). Briefly, the dried tissue was digested with 30 ml of Clean 99 K-200 (Clean Chemical Co. Ltd., Osaka, Japan) bleach solution for clinical laboratory use, consisting of 30% sodium hypochlorite, 4% potassium hydroxide, and

Address correspondence to Hiroyuki Yamada, Division of Pathology, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3-1-24, Matsuyama, Kiyose, Tokyo, 204, Japan.

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Table 1. Age distribution of cancer cases[a]

	Urban		Rural		
Age	Lung cancer	Non-lung cancer	Lung cancer	Non-lung cancer	Total
40s	6 (1)	11 (4)	6 (2)	7 (5)	30 (12)
50s	22 (14)	22 (10)	7 (5)	8 (3)	59 (32)
60s	27 (17)	21 (7)	7 (5)	9 (1)	64 (30)
70s	20 (10)	22 (14)	8 (6)	8 (5)	58 (35)
Total	75 (42)	76 (35)	28 (18)	32 (14)	211 (109)

[a]Number of cases, with cases of nonsmokers indicated in parentheses.

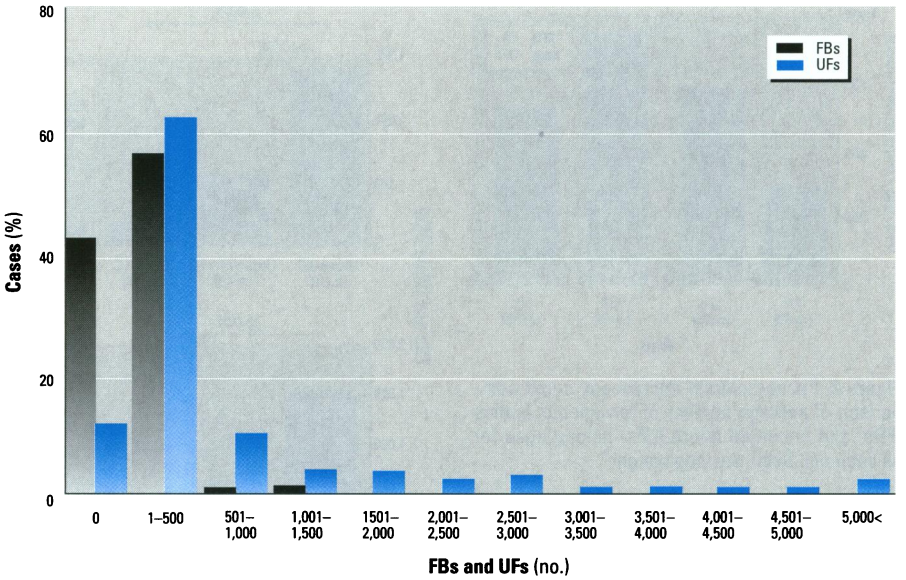

Figure 1. Frequency distribution of ferruginous bodies (FBs) and uncoated fibers (UFs) for all subjects as determined by phase-contrast microscopy.

detergent. The bleach solution was previously filtered through an FG 0.2 µm Millipore fluoropore filter. After overnight digestion of the lung tissue at 80°C in an oven, the digested fluid was centrifuged at 10,000 rpm for 30 min. Five milliliters of 30% H_2O_2 was added to the sediment for complete digestion. Finally, the sediments were sonicated for 1 min and resuspended in ion-exchanged water that had been passed through a UF 0.2 µm Millipore membrane filter.

Preparations for PCM observations. The resuspended fluid, 25 or 50 ml, was filtered with a UF 5 µm Millipore membrane filter. The dried filter was mounted on a glass slide and exposed to acetone vapor in a petri dish for 5 min to make the filter transparent. A cover slip was mounted with a drop of triacetin solution. FBs and UFs were counted using a Nikon OPTIPHOT with a phase-contrast device Ph at 400× over the entire filter area. Very straight, thin, and semi-translucent fibers with an aspect ratio greater than 3:1 that showed a distinct dark phase contrast in a bright field were counted as uncoated fibers.

Preparation for ATEM sections. Seven nonsmoking age-matched cases with detailed information for residential history were chosen from each of four groups including urban or rural lung and non-lung cancer cases. Lung tissues of these 28 cases were digested as described above, and 5 or 10 ml of the digested fluid was filtered through 0.2 µm pore size Nuclepore polycarbonate filter with a filtration area of 1.2 cm^2 (ADVANTEC, Tokyo, Japan). The filter was dried, subjected to carbon coating, cut into 3 × 3 mm pieces, and mounted on a nickel grid. The polycarbonate filter was dissolved by exposure to chloroform vapor.

All UFs or FBs of aspect ratio greater than 3:1 were counted in all the grid openings under 3,000× magnification, and 5 to 10 grid openings in the same grid were randomly selected and examined at 20,000× magnification to examine shorter uncoated fibers. Length and width were estimated and UF and FB core fibers were identified under an electron microscope (JEOL JEM-1200EX) with an energy dispersive X-ray (EDX) microanalyzer (KEVEX-7000). Electron diffraction was also performed to identify fiber types that had similar EDX patterns (*16*). Fiber contamination from reagents or laboratory air during PCM and ATEM sample preparation was assessed by the same procedure without lung tissue and revealed no contaminated fibers. Several sets of Millipore and Nuclepore filters were also examined to detect asbestos fiber contamination.

The numbers of FBs + UFs were calculated according to the following formulas and expressed per gram dry lung weight (DLW).

For PCM counts,

$$n = F \times (V / V') \times 1/W$$

where F = number of FBs and UFs counted, V = total volume of digested tissue solution (ml), V' = volume of used tissue digested solution for filtration (ml), and W = dry digested lung weight (g).

For ATEM counts,

$$n = F \times (V / V') \times (1 / W) \times (1.2^{*} / (H \times 10^{-4\,**})$$

where F = number of FBs and UFs counted, V = total volume of digested tissue solution (ml), V' = volume of digested tissue solution used for filtration (ml), W = dry digested lung weight (g), H = number of observed grid openings, an asterisk indicates the available area of membrane filter for filtration (cm^2), and two asterisks indicate the area of a grid opening (cm^2).

Statistical analysis. Calculated average numbers of UFs and FBs of each group in PCM examination were compared by Student's *t*-test.

Results

PCM analysis. FBs and UFs were found in 123 (58.3%) and 186 (88.2%) of the total 211 cases, respectively, and in 93% of all cases, either FBs or UFs were found. FB and UF frequency distribution is shown in Figure 1. About 98% of the cases had less than 500 FBs, 78% less than 50 UFs/g of dry lung tissue, but a higher number of fibers, up to over 5,000 UFs, was found in a small number of cases even though no asbestos workers were included in this series.

FB + UF counts in each age group are shown in Figure 2. Mean FB + UF count increases with age, with the highest count shown in the 60s but a slightly lower count in the 70s, indicating peak accumulation of uncoated fibers in the 60s with a gradual disappearance and/or less inhalation in later life.

Average FB + UF counts in lung cancer and non-lung cancer cases are shown in Figure 3A. A significantly higher FB + UF count was found in urban lung cancer cases in comparison with urban non-lung cancer cases (1380.5 vs. 550.3 FBs + UFs/g DLW, respectively, p = 0.001), whereas no difference was seen between rural lung cancer and rural non-lung cancer cases (151.4 vs. 125.6 FBs + UFs/g DLW, respectively, p>0.5). To exclude effects of smoking, subjects were limited to nonsmoking cases. A significantly larger number of FBs + UFs was again demonstrated in the urban lung cancer cases, whereas no significant difference was

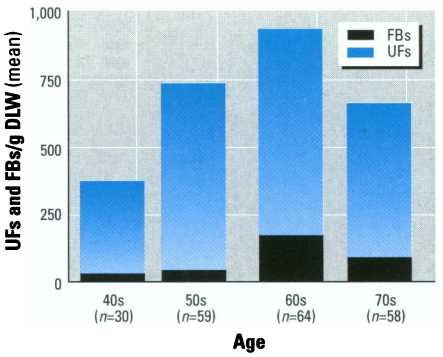

Figure 2. Phase-contrast microscopy count comparison of average number of ferruginous bodies (FBs) and uncoated fibers (UFs) among ages for all subjects. DLW, dry lung weight.

found among the other three groups. An average number of FBs + UFs showed 1525.8, 599.2, 184.8, and 233.2/g DLW for nonsmokers in urban lung cancer, urban non-lung cancer, rural lung cancer, and rural non-lung cancer cases, respectively. Urban groups of both lung cancer and non-lung cancer cases showed higher counts than rural groups.

ATEM analysis. Electron microscopical observations with an energy dispersive X-ray microanalyzer allowed identification of the observed fibers. At first, we examined the presence of fibrous materials in unused Nuclepore filters, which has been reported by Rodgers (*17*). We found that there were numerous short chrysotile fibers in the filter; however, most of them were shorter than 0.1 µm in length (data not shown) and undetectable in PCM observation.

The identified fibers included actinolite/tremolite, amosite/crocidolite, chlorite, chrysotile, fibrous talc, iron, mica, silica, and wollastonite. Average counts for some fiber types are shown in Figure 4A for all lengths, and in Figure 4B for fibers longer than 5 µm. Total average counts of the all length fibers (Fig. 4A) showed greater counts in actinolite/tremolite, mica, and chrysotile than other types of fibers; however, no definite tendency of fiber counts among urban, rural, lung cancer, and non-lung cancer cases, as seen in PCM observation, was found.

Figure 4B illustrates a comparison of fiber counts where lengths were 5 µm or longer. For actinolite/tremolite counts, the urban lung cancer group showed higher counts than the urban non-lung cancer group, but the rural non-lung cancer group had the highest counts. Chrysotile counts for the rural non-lung cancer group were much greater than for the other three groups. On the other hand, profiles for amosite/crocidolite and fibrous talc counts were very similar, whereas the fibrous talc counts were much higher than those of the amosite/crocidolite.

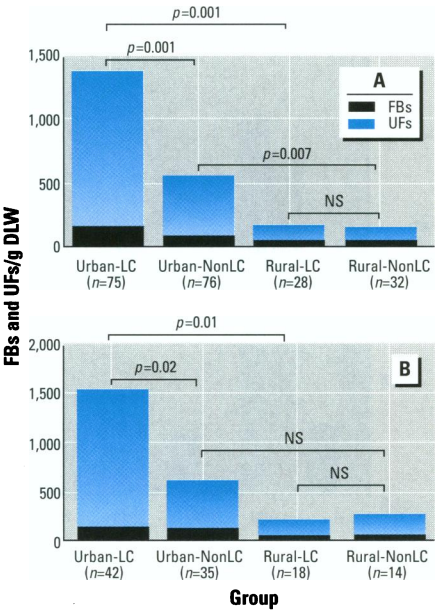

Figure 3. Phase-contrast microscopy count comparison of average ferruginous bodies (FBs) and uncoated fibers (UFs) for lung cancer (LC) and non-lung cancer (NonLC) cases in urban and rural areas for (A) all subjects and (B) nonsmokers. DLW, dry lung weight; NS, not significant.

With respect to these two fiber types, urban lung cancer groups showed the highest counts and the pattern of the profiles was similar to that of PCM analysis (Fig. 3B).

Length and diameter distributions of asbestos fibers, including actinolite/tremolite, amosite/crocidolite, chrysotile, mica, and fibrous talc are shown in Figure 5 as three-dimensional diagrams. Most of the chrysotile fibers were shown to have shorter lengths and thinner diameters than other types of fibers; only 15.8% of chrysotile fibers were longer than 5 µm in length, whereas amosite/crocidolite fibers of comparable length were found in 63.5%, actinolite/tremolite in 82.3%, and fibrous talc in 75.6%, respectively. Furthermore, the percentages of actinolite/tremolite, amosite/crocidolite, and fibrous talc fibers longer than 10 µm were 26.4, 30.2, and 23.8%, respectively, whereas those of chrysotile and mica were 5.7 and 3.5%, respectively. On the other hand, chrysotile fibers thinner than 0.5 µm in diameter were seen in 99.4%, whereas amosite/crocidolite fibers with such diameters were seen in 72.9%, actinolite/tremolite fibers in 40.3%, and fibrous talc in 23.3%, respectively. Mean fiber lengths and diameters are shown in Table 2.

Discussion

The present study quantitatively examined FBs and UFs in digested lung tissues by means of the phase-contrast microscope.

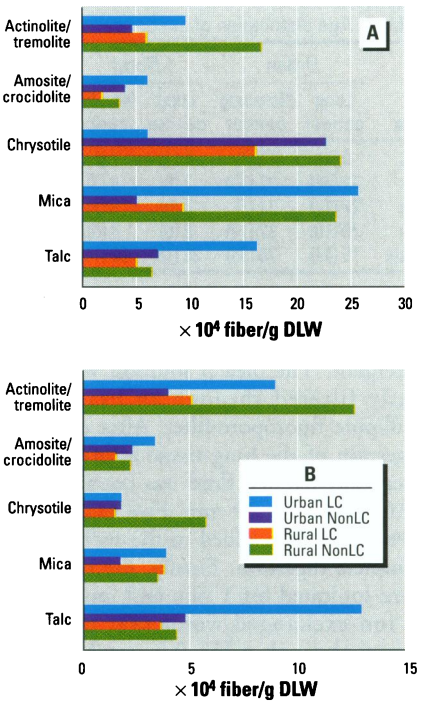

Figure 4. Analytical transmission electron microscope examination count results of (A) fibers of all lengths and (B) fibers longer than 5 µm. Abbreviations: LC, lung cancer; NonLC, non-lung cancer; DLW, dry lung weight. For each lung cancer and non-lung cancer group from both urban and rural areas, $n = 7$ nonsmoking cases.

ATEM examinations were performed both to detect fibers that cannot be observed by PCM analysis and to identify and measure fiber length and width. This PCM study revealed a significantly larger UF + FB count in the lungs of urban lung cancer cases compared to those of urban non-lung cancer cases. This result differs from other studies revealing no correlation between phase-contrast positive ferruginous bodies and lung cancer incidence. Because this discrepancy may partly be related to sex and histological lung cancer types, we compiled only female lung cancer cases, most of which were adenocarcinomas, for this study. Females generally have less occupational exposure to asbestos and a lower smoking rate than males and thus are more appropriate subjects for the study of environmental factors. Smoking has been believed to be another important pathogenic factor for lung cancer, especially when coexposed with asbestos; even when subjects were limited to nonsmokers, almost identical counts were obtained (Fig. 3B).

Electron microscopic observations are superior for detecting short or thin fibers that cannot be detected by PCM analysis or EDX identification of these uncoated fibers. Of all chrysotile fibers detected in this ATEM study, 84% of the fibers were shorter

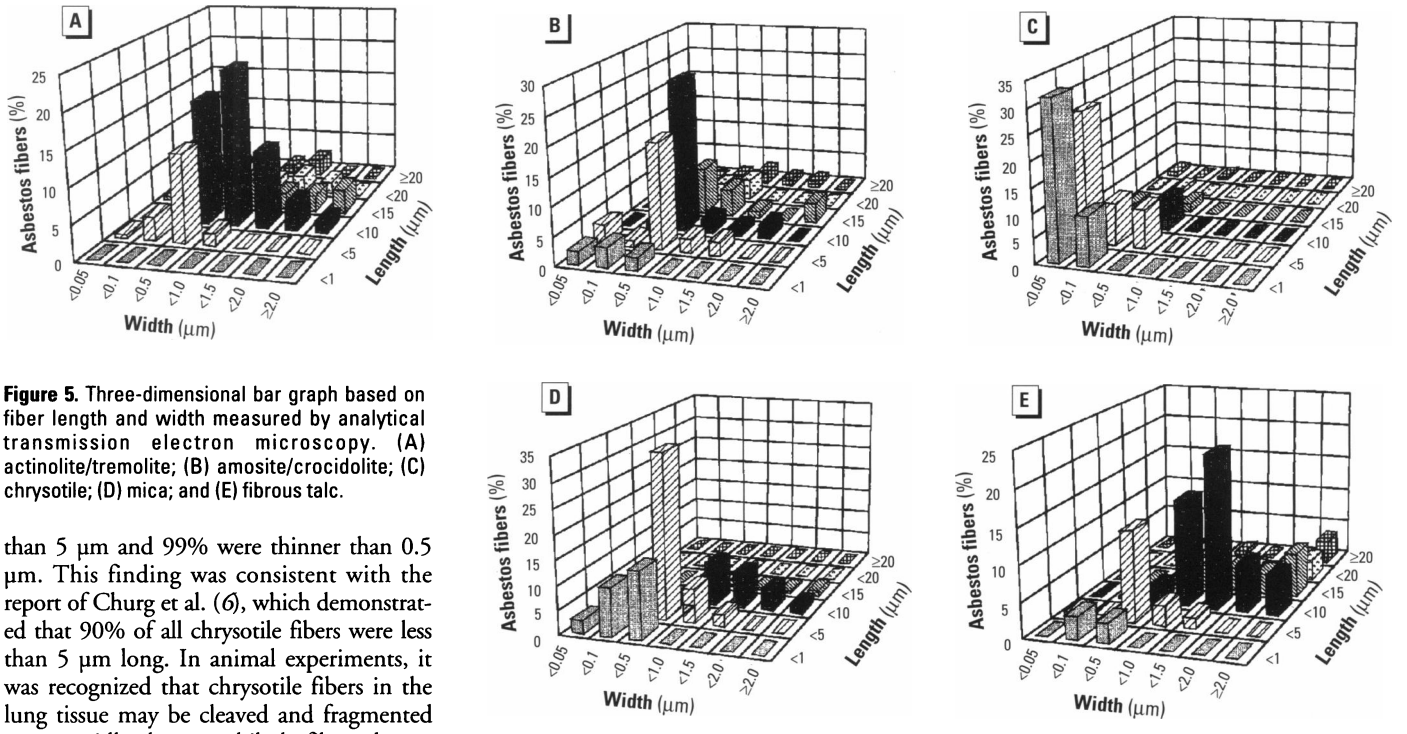

Figure 5. Three-dimensional bar graph based on fiber length and width measured by analytical transmission electron microscopy. (A) actinolite/tremolite; (B) amosite/crocidolite; (C) chrysotile; (D) mica; and (E) fibrous talc.

than 5 μm and 99% were thinner than 0.5 μm. This finding was consistent with the report of Churg et al. (*6*), which demonstrated that 90% of all chrysotile fibers were less than 5 μm long. In animal experiments, it was recognized that chrysotile fibers in the lung tissue may be cleaved and fragmented more rapidly than amphibole fibers due to higher fragility and leaching of magnesium from chrysotile fibers. Similar changes may occur in human lung tissue (*18*). These shorter and/or thinner fibers cannot be detected by the 0.5 μm resolving power of PCM. However, because PCM examination can estimate larger amounts of digested lung tissue, it provides less sampling bias and less variability in counting fibers than ATEM.

In the present electron microscopic study, chrysotile counts were greater than for other types of fiber as a whole. Total chrysotile counts amounted to two times those of actinolite/tremolite and four times of amosite/crocidolite (Fig. 4A). However, comparison including all fiber lengths shows greater chrysotile counts in non-lung cancer groups in both urban and rural areas in comparison to lung cancer groups. Furthermore, for fibers longer than 5 μm, average counts of chrysotile and actinolite/tremolite fibers in rural non-lung cancer cases had the highest value of all groups (Fig. 4B). Therefore, since counts of these two types of fibers are in inverse proportion to counts using PCM analysis, it is difficult to relate these fiber burdens with lung cancer incidence. On the contrary, both amosite/crocidolite and fibrous talc showed the highest counts in the urban lung cancer group, and the results correlated very well to those of PCM analyses, particularly when limited to nonsmokers. Fiber length seems to be required for carcinogenesis, and the findings that the fibers longer than 10 μm were found in a relatively greater percentage in actinolite/tremolite, amosite/crocidolite, and talc (but not in chrysotile) may support this view. We did not expect to find significant amounts of fibrous talc deposited in the lung tissue. Also, the average fiber counts of urban lung cancer cases was definitely greater than non-lung cancer and rural cases. It should be noted that all the examined subjects in this study were female.

Talc is one of the silicates and is manufactured for various uses in modern society, especially in cosmetics (face powder). Some epidemiological studies on talc industry workers have demonstrated a high incidence of lung cancer among the workers; other studies have not. Recent animal talc inhalation studies have revealed a significant occurrence of lung tumors in female rats, whereas no tumorigenicity was shown in mice (*19*). However, some authors have argued that the tumorigenicity in these studies was due only to an unusual overload of talc powder in the animals, and the actual effect of fibrous talc on human lung carcinogenesis still remains to be explained (*20*).

We have concluded that in this PCM analysis, urban counts were significantly greater than rural ones (particularly the urban lung cancer group, which had the highest counts) and in ATEM analysis, various types of fibers were detected in the lungs under nonoccupational exposure conditions. Although chrysotile fibers were the most frequently detected because their lengths and widths were shorter and thinner than the resolving power of PCM, counted fibers in the PCM examination may be amphibole (especially amosite/crocidolite) and fibrous talc that may have been inhaled with particulate powder. The reason large amounts of fibrous talc were detected in this study may be because subjects of the study were restricted to female cases. Although there is a possibility that talc powder may be contaminated from surgical gloves during an operation or autopsy, a higher count of talc was noted in only the urban lung cancer group. Fibrous talc may persist longer in the bronchiolo–alveolar area than particulate talc by resisting the mucociliary clearance system of the lung. We also concluded that although some fibrous materials (including chrysotile and actinolite/tremolite) may be inhaled in large amounts, these fibrous materials may not affect lung carcinogenesis, particularly in rural areas. In addition, some of the fibers may be partly derived from soil. All types of fibrous materials are not always linked to lung cancer incidence.

Finally, it remains to be clarified whether asbestos and fibrous talc in an urban environment could promote lung cancer by themselves at ordinary nonoccu-

Table 2. Average thickness and length of fibers (μm)[a]

Type of fiber	Thickness	Length
Actinolite/tremolite	0.75 ± 0.57	8.48 ± 7.63
Amosite/crocidolite	0.47 ± 0.64	8.31 ± 10.1
Chrysotile	0.06 ± 0.09	2.73 ± 4.63
Talc	1.11 ± 0.80	7.32 ± 4.97
Others	0.48 ± 0.74	3.11 ± 3.76

[a]Expressed as mean ± standard deviation.

pational exposure levels or whether these fibrous materials foster lung cancer incidence with carcinogenic pollutants common in urban areas. Further studies are still required to establish the role of fibrous talc other than asbestos fibers in female lung cancer incidence.

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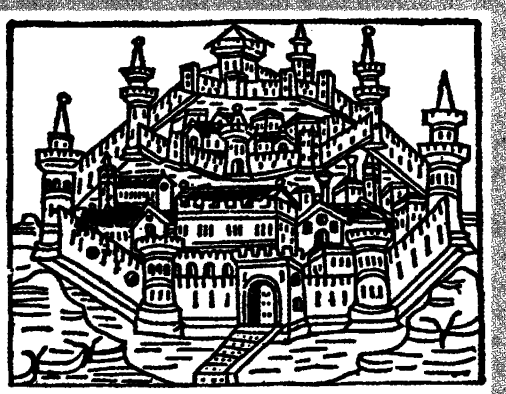

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