The Detection of Increased Amounts of the Extracellular Domain of the Epidermal Growth Factor Receptor in Serum During Carcinogenesis in Asbestosis Patients

Riitta Partanen, BS,
Kari Hemminki, MD,
Heikki Koskinen, MD,
Jiin-Chyuan Luo, MD, MPH,
Walter P. Carney, PhD,
Paul W. Brandt-Rauf, MD, ScD, DrPH

Overexpression of the epidermal growth factor receptor (EGFr) has been implicated in the pathogenesis of a wide variety of human malignancies and may be related to asbestos-induced carcinogenesis. Overexpression of the EGFr can be detected immunologically by quantitation of the extracellular domain (ECD) in the extracellular fluid in vitro *and in serum* in vivo. *An enzyme-linked immunosorbent assay (ELISA) for the EGFr ECD was used to examine banked serum samples of 38 asbestosis patients who subsequently developed cancer, 72 age-sex-race-smoking-asbestos exposure matched asbestosis controls without cancer, and 20 age-sex-race-smoking matched nonasbestosis noncancer controls. The mean serum level for the EGFr ECD in the cancer cases (636 ± 299 fmol/ml) was statistically significantly elevated ($P < 0.05$) in comparison to the mean level in the asbestosis controls (546 ± 147 fmol/ml) or the nonasbestosis controls (336 ± 228 fmol/ml). Defining a positive elevation of the serum EGFr ECD as any value more than 2 standard deviations above the nonasbestosis control mean, 7 (18%) of the cancer cases were positive compared to 4 (6%) of the asbestosis controls and one (5%) of the nonasbestosis controls. In addition, all of these cancer cases had positive*

Overexpression of the epidermal growth factor receptor (EGFr; also termed the p170 c-*erbB*-1 oncogene protein) has been implicated in experimental cellular transformation and has been identified in a wide range of human cancers including brain, lung, bladder, stomach, breast, head and neck, esophagus, ovary, prostate, pancreas, kidney, thyroid, and sarcomas.[1] Such EGFr overexpression can be immunologically quantitated in human tumor tissue lysate using an enzyme-linked immunosorbent assay (ELISA).[2] In addition, it has been suggested that asbestos-induced carcinogenesis may involve alterations in EGFr expression, since frequent nonspecific chromosomal alterations have been observed in asbestos-transformed cells, including alterations in chromosome 7, the site of the EGFr gene.[3] For example, polysomy of chromosome 7 has been observed frequently in human mesothelioma cells, indicating that increased dosage and concomitant overexpression of a gene on chromosome 7 may play a role in the asbestos-induced transformation of these cells.[3]

We have previously demonstrated that overexpression of the related transmembrane growth factor receptor, p185 c-*erbB*-2, can be detected *in vivo* in patients with asbestosis who develop subsequent malignancies by using an ELISA to quantitate the extracellular domain (ECD) of the receptor in serum, since cells that

From the Center for Nutrition and Toxicology, Karolinska Institute, Huddinge, Sweden (Riitta Partanen, Dr Hemminki); the Institute of Occupational Health, Helsinki, Finland (Dr Koskinen); the Division of Environmental Sciences, Columbia University, New York, New York (Dr Luo, Dr Brandt-Rauf); and Oncogene Science, Cambridge, Massachusetts (Dr Carney).

Address correspondence to: Paul W. Brandt-Rauf, MD, Division of Environmental Sciences, Columbia University School of Public Health, 60 Haven Avenue, B-1, New York, NY 10032.

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serum samples prior to the time of disease diagnosis (average = 5.1 years). These results suggest that serum EGFr ECD may be elevated at an early stage of carcinogenesis in some asbestosis patients and that further prospective study of the utility of this biomarker is warranted.

overexpress p185 c-*erbB*-2 release increased quantities of its ECD into the extracellular environment.[4,5] Similarly, it is known that tumor cells that overexpress EGFr release increased quantities of its ECD into the extracellular environment, and increased amounts of the EGFr ECD can be detected immunologically via an ELISA in the sera of mice bearing tumors that overexpress EGFr.[6,7] Thus, in this study, we have used an ELISA for the EGFr ECD to quantitate this oncoprotein in the multiple banked sera samples of asbestosis patients who have developed cancer as well as age-sex-race-smoking-asbestos exposure matched asbestosis controls without cancer and age-sex-race-smoking matched nonasbestosis noncancer controls to evaluate the potential role of EGFr overexpression in asbestos-mediated carcinogenesis and the use of the EGFr ECD as a potential biomarker for this process.

Materials and Methods

A previously described cohort of 110 patients with asbestosis was followed at the Institute of Occupational Health in Finland from 1978 to 1987.[8] The patients included in this cohort were all cases with compensated pneumoconiosis who fulfilled the usual diagnostic criteria for their disease. These patients received regular periodic follow-up examinations every 1 to 3 years during this period, and from 1981 to 1987 this included the collection of a venous blood sample as well. Cancer incidence within this cohort was determined for cases until the end of June 1993 from the Finnish Cancer Registry, a national registry with a complete coverage of diagnosed cancers. Thirty-eight of the 110 patients developed malignant tumors during this follow-up period. These included 27 lung cancers (6 adenocarcinomas, 5 squamous cell carcinomas, 4 small cell carcinomas, and 12 lung cancers that were not further specified), 3 malignant pleural mesotheliomas, and one each of the following: prostate cancer, brain cancer, non-Hodgkins lymphoma, laryngeal cancer, gallbladder cancer, rectal cancer, kidney cancer, and pancreatic cancer.

Information on age, sex, race, smoking habits, and occupational exposure history was available on the 38 asbestosis cancer cases as well as the 72 asbestosis noncancer controls in this cohort. The characteristics of these two groups are presented in Table 1 and demonstrate that the cancer cases and the asbestosis controls were virtually identical in terms of age, sex, race, smoking status, and asbestos exposure. In both groups, all were Caucasian, most were male, and most were current or ex-smokers with an average of 20 years of asbestos exposure, and the average age and the range of ages were almost the same.

Blood samples had been collected from these individuals by routine venipuncture techniques at various time intervals, as noted. The serum had been separated and stored frozen at −70°C until the time of analysis. For most of these patients (81), multiple serum samples (2 to 7) were available for analysis from various points in time between 1981 and 1987, whereas for the remaining 29 patients only one serum sample was available for analysis. A total of 105 serum samples were available from the 38 cancer cases, and a total of 260 serum samples were available from the asbestosis controls.

In addition, a group of 20 non-asbestos-exposed noncancer control patients was selected from a previously described cohort of 190 patients admitted to the surgical services of Columbia-Presbyterian Medical Center with noncancer diagnoses in 1987.[9] These patients were group-matched to the asbestosis cancer cases and controls on the basis of age, sex, race, and smoking habits. The characteristics of this group are also presented in Table 1 and demonstrate that they are virtually identical to the other groups, except for asbestos exposure. The patients in this group had approximately the same average age and age range, and were Caucasian, most were male, and most were current or ex-smokers. Individual serum samples were available from these patients from the time of admission to the hospital; these had been collected in the same fashion and stored frozen at −70°C until the time of analysis.

Serum samples were assayed blind to diagnosis for levels of the EGFr ECD via a sandwich ELISA that utilizes a mouse monoclonal capture antibody and a rabbit polyclonal detector antibody that specifically recognize protein domains on the ECD of EGFr.[7,10] For the assay, microtiter wells are precoated with the mouse monoclonal antibody at a concentration of 3 μg/well. Diluted standards (final concentrations 0 to 80 fmol EGFr ECD/ml) and serum samples (1:50 in PBS, pH 7.4, containing 0.1% sodium azide, 1% BSA, and 10% normal mouse serum) are added as 100-μl aliquots to the microtiter wells for incubation overnight at room temperature. After exhaustive washing, 100 μl of the rabbit antiserum is added to each well and incubated for 30 min at room temperature. Then, 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well and incubated for 30 min at room temperature. After exhaustive washing, the wells are incubated with 100 μl of *o*-phenylenediamine substrate solution in the dark at room temperature for 1 hour, and the color is measured at 490 nm using a microplate reader. A standard curve is generated from the absorbance of serial dilutions (80, 60, 40, 20, 10, 5 and 0 fmol EGFr ECD/ml; in duplicate) containing EGFr obtained from A431 tumor cell lysates.[6] This assay will detect 5 fmol EGFr ECD/ml at a signal level that is approximately three times background and generates a linear standard curve up to 80 fmol EGFr ECD/ml, and the assay does not cross-react with similar antigens such as the c-*erbB*-2 oncoprotein. With serum samples spiked with known quantities of EGFr, this assay gives an average

TABLE 1
Serum EGFr ECD in Cancer Cases, Asbestosis Controls, and Non-Asbestosis Controls

	Age (yrs)	Sex	Race	Smoking Status	Asbestos Exposure (yrs)	Diagnosis	Serum EGFr ECD, (fm/ml) (+ Positive)*
Cancer cases ($n = 38$)	64.8 (40–84)	95%M 5%F	100%W	47%E 34%C 16%N 3%NA	20 (2–44)	71% Lung Cancer 8% Mesothelioma 21% Other	636 ± 299 (18%+)
Asbestosis Controls ($n = 72$)	67.8 (47–89)	92%M 8%F	100%W	42%E 26%C 19%N 13%NA	20 (5–43)		546 ± 147 (6%+)
Non-Asbestosis Controls ($n = 20$)	63.0 (39–81)	91%M 9%F	100%W	50%E 20%C 25%N 5%NA			336 ± 228 (5%+)

* Positivity defined as value greater than two standard deviations above the mean of the non-asbestosis controls, i.e., greater than 792 fm/ml.

M, male; F, female; W, white; E, ex-smoker; C, current smoker; N, non-smoker; NA, not available.

recovery of 71.0–94.6%. Intra-assay variability ranges from 5.8 to 8.1%, and inter-assay variability ranges from 6.0 to 14.6%. The concentration of EGFr ECD in samples (assayed in duplicate) is determined by interpolation of sample absorbance from the standard curve.

Means and standard deviations in terms of fmol EGFr ECD/ml were calculated for the samples from the asbestosis cancer cases, the asbestosis controls, and the nonasbestosis controls. Means were compared using the two-tailed Student's *t* test for the comparison of independent samples, and a *P* value of 0.05 or less was considered statistically significant. Using the mean and standard deviation of non-asbestosis controls as comparison, an *a priori* cut-off for establishing an individual positive elevation of serum EGFr ECD was chosen as any value greater than two standard deviations above the control mean.

Results

The aggregate results for serum levels of the EGFr ECD in the asbestosis cancer cases, the asbestosis controls, and the nonasbestosis controls are presented in Table 1. The average serum EGFr ECD in the cancer cases was 636 ± 299 fmol/ml, in the asbestosis controls was 546 ± 147 fmol/ml, and in the nonasbestosis controls was 336 ± 228 fmol/ml. The difference in the means between the two control groups was statistically significant ($P < 0.001$), and the differences in the means between the cancer group and either control group were also statistically significant ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, using the definition of a positive serum elevation of EGFr ECD as any value greater than two standard deviations above the nonasbestosis control mean, 5% of the nonasbestosis controls and 6% of the asbestosis controls were positive, but 18% of the cancer cases were positive. As shown in Table 2, cancer cases with positive serum samples included 3 lung cancers (one adenocarcinoma, one squamous cell carcinoma, one unspecified), one mesothelioma, and the cases of non-Hodgkins lymphoma, kidney cancer, and pancreas cancer. The individual sample values for serum EGFr ECD, their positivity or negativity, and their date of collection in relation to the date of cancer diagnosis for these patients are presented in Table 2. As shown, these cancer cases had positive serum samples for elevated EGFr ECD before the time of disease diagnosis. The average time between initial serum positivity and diagnosis in these seven cases was 5.1 years (range = 0–10). It should be noted that for five of these cases, the earliest available samples were positive, so that the first significant elevation of the EGFr ECD may have occurred earlier, suggesting that the average lead time to diagnosis might be even longer.

Discussion

The results on serum EGFr ECD in this study are consistent with tissue studies of EGFr expression in human cancers. As noted, increased expression of EGFr has been identified in a number of different human tumors, including most of those associated with elevated serum EGFr in this study.[1] For example, increased tissue EGFr has been identified in human lung cancers to varying degrees depending on histologic type, including in 45–100% of squamous cell carcinomas, 23–75% of adenocarcinomas, and 17–100% of other cell types, except for small cell carcinomas in which none (0%) have been found to overexpress EGFr.[11–18] In the present study, increased serum EGFr ECD was detected in one of 5 (20%) cases of squamous cell lung cancer, one of 6 (17%) cases of adenocarcinoma of the lung, one of 12 (8%) other lung cancers, and 0 of 4 (0%) cases of small cell lung cancer. The somewhat lower percentages of positives in serum compared to tissue for lung cancer

TABLE 2
Timing of EGFr ECD Positive Samples in Relation to Date of Cancer Diagnosis

Cancer	Serum EGFr ECD, fm/ml (Positivity)* Date of Sample Collection							Date of Cancer Diagnosis
	1981	1982	1983	1984	1985	1986	1987	
Lung, A	1384(+)		1208(+)					1985
Lung, S	1221(+)							1981
Lung, U	1017(+)					1088(+)	1067(+)	1988
Mesothelioma	1438(+)	471(−)			1258(+)	1257(+)		1991
Lymphoma	375(−)	463(−)	400(−)	694(−)	4583(+)	4038(+)		1987
Kidney	925(+)							1988
Pancreas	694(−)		563(−)	663(−)	969(+)		888(+)	1991

* Positivity defined as value greater than two standard deviations above the mean of the non-asbestosis controls, i.e. greater than 792 fm/ml.

A, adenocarcinoma; S, squamous cell carcinoma; U, unspecified.

types may reflect differences in methods for determining cut-offs in serum compared to tissue (which is usually by visual assessment of immunohistochemical staining) or the fact that all tumors that overexpress EGFr may not necessarily release ECD into the circulation; also, the number of positive serum cases is too small as yet to allow strict comparisons. Further study of larger cohorts with matched tissue and serum samples will be necessary to resolve these and other issues. It should also be noted that increased serum EGFr ECD was found in one case each of cancer of the kidney, cancer of the pancreas, mesothelioma, and lymphoma. With the exception of lymphomas, overexpression of EGFr has been reported in a large proportion of tumor tissues for all of these cancers.[19–23]

In addition, in at least one study of tissue expression of EGFr in lung cancer, overexpression was identified in both the tumor tissue and in histologically normal-appearing adjacent bronchial epithelium in several cases.[18] The authors concluded that EGFr overexpression thus represented an early alteration of growth control, allowing cells to proliferate continually and escape terminal differentiation.[18] This would be consistent with the findings in this study of increased serum EGFr ECD in several cases years before the development of clinically detectable disease.

As noted, it is possible that asbestos plays a specific role in the induction of EGFr in carcinogenesis by the production of chromosome 7 abnormalities.[3] If this is the case, one would expect some individuals with asbestos exposure but without cancer as yet to also have elevated serum EGFr ECD, as is evident in this study by the significantly elevated mean level in the asbestosis controls compared to the nonasbestosis controls. Further follow-up of this cohort will be necessary to confirm that it is those individuals with consistently elevated serum EGFr ECD among the asbestosis controls who will go on to develop malignancy. Based on the results on banked serum samples from individuals with asbestosis who have already developed cancer, one would anticipate that this will be the case. Thus, overall these results suggest that overexpression of EGFr may play a role in some asbestos-induced carcinogenesis and that this process may be detectable via identification of increased amounts of the EGFr ECD in the serum of exposed individuals before the detection of clinical disease. Since several of the other EGFr ECD-negative cancer cases in this cohort have been previously found to have elevated serum c-*erbB*-2 ECD or *ras* p21 oncoprotein prior to clinical disease,[4,8] ultimately it may be possible to use a battery of such serum tests for early detection of most individuals at risk for such cancers.

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The Caspian Also Rises

An inexplicable rise in the level of the Caspian Sea is alarming Russia, Iran, and the other three states along its shores. In the past 15 years this salty landlocked sea has risen by some 2.1 meters (nearly 7 feet), flooding villages, factories, and farmland. It is continuing to rise by 15 centimeters (6 in) per year. Scientists at the Caspian Research Centre in Baku, the capital of Azerbaijan, believe the sea could rise by over 5 meters by 2010.

What is happening? Derek Scott, a consultant with the International Waterfowl and Wetlands Research Bureau who has studied Caspian wetlands, says that some Russians put the rise down to natural fluctuations, but the Iranians blame the Russians. Russia's blocking of the Gulf of Kara-bogaz, which previously acted as a vast natural evaporation pan, accounts, in Iran's view, for 40 centimeters of the rise in the Caspian's level.

The sea has gone up and down inexplicably in the past. It fell consistently between the late 1920s and the 1970s. By 1977 it had dropped to almost 30 meters below sea level—its lowest point for five centuries. In the 1940s Iran and the USSR began to cover the drying sea bed with factories, houses, and tourist playgrounds. Now the rising sea is reclaiming this land. Russian and Iranian environmental organizations reckon that the current rise has so far inundated 32,000 square kilometers (12,360 square miles) of land. The loss has been exacerbated by the shallowness of the water—in some places 30 km (19 miles) from land, the Caspian is only two meters deep—and by the flatness of the shore. ...

... [T]he rise in the sea's level is not just a threat to the region's economy; it has serious environmental implications, too. After years of indiscriminate industrial dumping, the Caspian is already heavily polluted with oil, phenol, and heavy metals. In Baku harbor ... the average concentration of oil is 12 times the permitted maximum. Pollution, together with the damming of rivers, already threatens the sea's valuable stocks of fish, including sturgeon. The rising sea may make pollution worse. Much of the Caspian shore is rich in oil, and western oil companies are busily hunting for ways to exploit it. Some existing wells, drilled on the previously exposed sea bed, have already been flooded. Many more are surrounded by dikes. So far the dikes have generally kept the sea at bay, but occasionally storms have washed water over the walls and swept oil out to sea ....

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