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SHORT REPORT

p53 autoantibodies predict subsequent development of cancer

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Because *TP53* mutations can induce an immune response and can occur early in the carcinogenic process for some tumors, p53 autoantibodies may be useful biomarkers for risk of development of cancer. Using banked serum samples from an asbestosis cohort at high risk for cancer, we demonstrate for the first time a statistically significant relationship between p53 autoantibodies and the subsequent development of malignancy (hazard ratio [HR] = 5.5, 95% confidence interval [CI] = 2.8–10.9) with a positive predictive value of 0.76 and an average lead time to diagnosis of 3.5 years. p53 autoantibodies were also significantly associated with p53 alterations in the resultant tumors ($\kappa = 0.78$, $p = 0.01$).

Key words: p53 mutation; biomarker; cancer risk; predictive value

The *TP53* tumor suppressor gene is the site identified most frequently for mutations in human cancers.[1] In many cases, mutations in *TP53* cause an increase in the stability of the mutant p53 protein leading to its accumulation in cells.[1,2] Thus, mutations in the *TP53* gene or accumulations of the mutant p53 protein have been identified frequently in many different types of human tumors. In some cases, they have even been identified in common pre-malignant lesions and in histologically normal tissue adjacent to tumors suggesting that these can be early events in the carcinogenic process.[3–5] It has also been found that individuals with tumors that contain accumulations of mutant p53 can mount an antibody response against the protein, due presumably to the conformational alterations produced by the mutations that cause it to be identified as foreign by the body's immune system.[2] Such p53 autoantibodies have been detected in the sera of patients with most types of cancer with a good general correlation between the presence of the antibodies and the occurrence of *TP53* mutations or accumulations of mutant p53 protein in the tumor tissue.[2,6] p53 autoantibodies have also been found in individuals with pre-malignant conditions such as oral leukoplakia and Barrett's esophagus and may even be detectable before the clinical diagnosis of malignant or pre-malignant disease.[6] For example, p53 autoantibodies have been found in isolated cases of heavy smokers before the development of lung and other tobacco-related cancers and of workers exposed to workplace carcinogens before the development of occupational cancers.[7–10] These reports include 13 cases with an average lead time to diagnosis of approximately 2 years. This suggests that p53 autoantibodies may have predictive value for the subsequent development of cancer, but this has not been formally investigated. The purpose of our current study was to test this hypothesis.

Material and methods

In 1978–79 a cohort of 115 cases of compensable asbestosis was assembled at the Finnish Institute of Occupational Health in Helsinki.[11] On return visits from 1980–88, serum samples were collected on 103 of these cases, aliquoted and stored frozen at −70°C for a total of 268 serum samples (1–5 per case). This group consisted of 94 males and 9 females with an average age of 66.8 years at the end of sample collection in 1988. They had an average of 20 years of employment in asbestos-related industries in job categories with high likelihood of asbestos exposure (*i.e.*, asbestos mining, insulation, spraying, cement work). All patients had asbestosis and relatively high cumulative exposures to asbestos with an average estimated cumulative exposure of 538 fiber-years/ml (range = 13.5–1750 fiber-years/ml), which for purposes of statistical analysis was divided into tertiles of 33 cases ≤200 fiber-years/ml, 37 cases from 201–500 fiber-years/ml and 33 cases >500 fiber-years/ml. They included 19 never-smokers and 84 current or ex-smokers. They were obviously a high-risk cohort for subsequent development of cancer. Cancer incidence in this group was followed up through December 31, 2001, from the Finnish Cancer Registry, a national registry with complete coverage of diagnosed cancers in the country.[12] At that time, there had been 49 cancers (31 lung cancers, 4 mesotheliomas, 14 others of various types including cancers of the prostate, bladder, pancreas, colon, brain, esophagus, gallbladder, kidney, melanoma of the skin and non-Hodgkin's lymphoma).

Serum samples from all cohort members were analyzed for p53 autoantibodies by enzyme-linked immunosorbent assay, as described previously.[13] Briefly, the p53 autoantibodies were detected by a sandwich-type enzyme-linked immunosorbent assay based on matching microtiter plates coated with either glutathione-S-transferase (GST) conjugated p53 fusion protein or GST protein alone. For the assay, 100 μl serum samples (in duplicate) diluted 1:50 were added to the microtiter wells on separate plates that were pre-coated with the GST-conjugated p53 protein or the GST protein alone and incubated overnight at 4°C. After washing, 100 μl of a conjugate solution of horseradish peroxidase-conjugated goat anti-human IgG was added to each well and incubated for 12 hr at 37°C. After washing again, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution was added to each well and incubated for 5 min at room temperature followed by the addition of 100 μl sulfuric acid stop solution. The absorbance of each well was read on a spectrophotometric plate reader at 450 nm. For each sample, the ratio of optical density on the mean of the GST-p53 plate to the mean of the GST alone plate was calculated. Known antibody-positive and antibody-negative controls were included on each plate. This assay has been shown to be highly reproducible and to give results in good agreement with those obtained by immunoblotting on the same samples.[13] These prior studies were used to establish an *a priori* cut-off for sample positivity that best distinguished between cancer cases and controls, which was used in our

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study. Furthermore, p53 autoantibodies have been found to be very stable over time, allowing for reliable retrospective analyses on frozen serum samples,[2,6] as carried out in our study. Our own prior studies have demonstrated the stability of p53 autoantibodies in frozen serum samples for more than a decade.[14]

Results for p53 autoantibodies were compared to the subsequent development of cancer in univariate (Fisher's exact test) and multivariate (Cox proportional hazards model for time-dependent repeated measurements; Kaplan-Meier survival analysis) statistical analyses. In addition, tumor samples were available from 10 of the resultant tumors and had been analyzed previously for *TP53* mutations and mutant p53 accumulations[15] for comparison with the serum results (Cohen's κ test).

Results

p53 autoantibodies were found in 31 serum samples: in at least one serum sample in 13 of 49 (26.5%) individuals who subsequently developed cancer (11 lung, 1 mesothelioma, 1 lymphoma) compared to 4 of 54 (7.4%) individuals who did not develop cancer. The pattern of p53 autoantibody results in these 17 individuals is shown in Table I.

In univariate analysis, p53 autoantibodies were significantly associated statistically with subsequent cancer ($p = 0.015$), representing a negative predictive value of 0.58 and a positive predictive value of 0.76 with an average lead time to clinical diagnosis (time from first positive sample to diagnosis) of 3.5 years (range = <1–12 years). p53 autoantibodies were not significantly associated statistically with smoking, which is consistent with our prior studies of p53 autoantibodies in lung cancer cases[13] and is possibly due to the small numbers involved. p53 autoantibodies were associated with borderline statistical significance with cumulative asbestos exposure (low *vs*. moderate-high tertiles, $p = 0.05$) consistent with prior studies demonstrating increased p53 tissue aberrations and serum p53 autoantibodies in lung cancer cases with asbestos exposure.[16] In multivariate analysis, p53 autoantibodies were highly statistically significantly associated with subsequent cancer (hazard ratio [HR] = 5.5, 95% confidence interval [CI] = 2.8–10.9) controlling for age, gender, smoking and cumulative asbestos exposure. Another way of looking at this is by Kaplan-Meier analysis as shown in Figure 1. This demonstrates the difference in time to cancer diagnosis for p53 autoantibody-positive *vs*. p53 autoantibody-negative cases, where the mean time to diagnosis is 3.9 years for the former compared to 6.8 years for the latter ($p = 0.0009$).

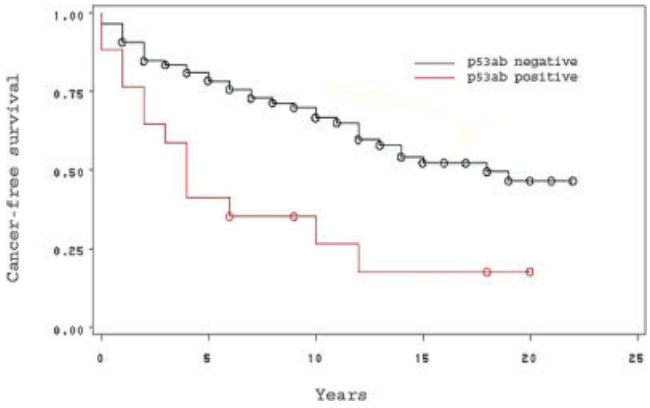

FIGURE 1 – Kaplan-Meier analysis of probability of cancer-free survival vs follow-up time in years from first blood sample for p53 autoantibody-positive and p53 autoantibody-negative cancer cases.

Among the 10 individuals with tumor tissue available for analysis, p53 autoantibodies were found in all 3 of 3 individuals whose subsequent tumors had *TP53* mutations or mutant p53 accumulations but in only 1 of 7 individuals whose tumors were negative for p53 alterations. This represents good agreement between serum and tissue results ($\kappa = 0.78$, $p = 0.01$). Figure 2 shows an example of p53 immunohistochemical positivity in a tissue sample from one of the p53 autoantibody-positive cases.

Discussion

It should be noted that many of the cancer cases in this cohort developed well after 1988, the date of the last serum samples. It is quite possible that some of these cases developed p53 autoantibodies after 1988 but before their diagnosis of cancer but could not contribute to the relative risk estimate between autoantibodies and cancer. Furthermore, among the false–positive individuals (*i.e.*, those antibody-positive who did not develop cancer), all 4 had only one positive sample that was always followed by a negative sample (as opposed to the true positives whose serum samples always remained positive), and the antibody titers for these samples were just marginally positive by the *a priori* cut-off criteria. Thus, use of a higher cut-off for positivity, reliance on multiple

TABLE I – p53 AUTOANTIBODY-POSITIVE ASBESTOSIS CASES IN RELATION TO DIAGNOSIS OF CANCER[1]

Case	Date of serum sample								Date of cancer diagnosis	Cancer type
	1981	1982	1983	1984	1985	1986	1987	1988		
1				+	+	+	+		1988	L
2				+	+	+	+		1987	L
3	+			+	+	+			1987	Ly
4	+		+						1985	L
5	+	+							1983	M
6					−	−	+		1989	L
7				−	+	+			1986	L
8	+								1991	L
9	+		+						1983	L
10	+								1981	L
11		+							1982	L
12	+	+							1982	L
13							+		1999	L
14				+	−	−	−	−	NA	
15				−	−	+	−		NA	
16					+		−		NA	
17				−	−	+	−		NA	

[1]L, lung; M, mesothelioma; Ly, lymphoma; NA, not applicable since two of these cases (14 and 15) were cancer-free and died of other causes in 1990 and 1993, respectively, and two of these cases (16 and 17) were alive and cancer-free as of 12/31/01.

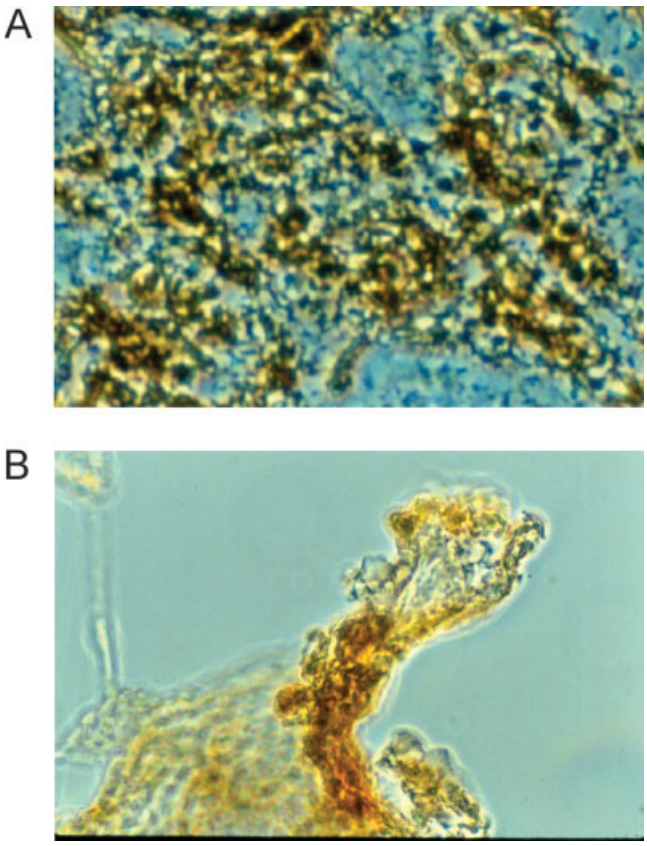

FIGURE 2 – Example of p53 immunohistochemical positivity in both tumor tissue (*a*) and histologically normal adjacent tissue (*b*) from a p53 autoantibody-positive individual (immunoperoxidase staining, 400×).

positive samples to define a positive individual or access to additional serum samples after 1988 could improve the relationship between p53 autoantibodies and subsequent development of cancer even further.

For the serum-tissue comparison, the lack of agreement in one case may be attributable to sampling error in the DNA and protein analyses of the tumor, subsequent loss of the mutant *TP53* allele with tumor progression, or development of an autoantibody response due to a second undiagnosed mutational site in the patient. Nevertheless, the agreement overall between p53 autoantibodies and p53 alterations in the subsequent tumors was reasonably good and statistically significant. Furthermore, as shown in Figure 2, it was possible to identify instances where both tumor tissue and histologically normal tissue adjacent to the tumor contained areas of p53 positivity, providing an explanation for the p53 autoantibody-positivity before clinical diagnosis of malignancy. Similarly, because of the noted association in our study and others[16] between p53 autoantibodies and asbestos exposure and p53 autoantibodies and p53 tissue alterations, this finding of p53 positivity in histologically normal tissue also suggests that alterations in p53 produced by asbestos can be a relatively early event in the process of asbestos-induced carcinogenesis.

Most importantly, these results demonstrate for the first time a statistically significant relationship between the presence of p53 autoantibodies and the subsequent development of cancer. This relationship was independent of other known risk factors for cancer in this cohort, including smoking and asbestos exposure. Although the specificity of p53 autoantibodies for cancer was high (0.93) in this cohort, sensitivity was lower (0.27), but this is consistent with the fact that not all cancers would be expected to contain mutant p53 and not all mutant p53-positive cancers will necessarily generate an autoantibody response. Furthermore, several other cancer-related protein biomarkers have been detected in the samples from this cohort before the diagnosis of cancer,[17,18] so combinations of these biomarkers along with p53 autoantibodies could be used to significantly increase the sensitivity for cancer detection. For example, by combining the results from all biomarkers tested for far in this cohort, the positive predictive value (0.76), the negative predictive value (0.66) and the specificity (0.85) remain high and the sensitivity increases considerably to 0.51. As additional biomarkers are developed, this potentially could be improved even further.

The results from our study can have practical significance because they clearly demonstrate that in high-risk cohorts such as this one, p53 autoantibodies can have high predictive value for cancer thereby identifying individuals who could benefit from more aggressive preventive interventions. Because several new interventions directed against mutant p53 are under investigation currently,[19–21] p53 autoantibodies could be used to target individuals not only for p53-specific chemotherapy but also for p53-specific chemoprophylaxis, as well as being used to monitor the effectiveness of the intervention in these individuals.

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