Survivin Autoantibodies Are Not Elevated in Lung Cancer When Assayed Controlling for Specificity and Smoking Status

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Abstract

The high mortality rate in lung cancer is largely attributable to late diagnosis. Case–control studies suggest that autoantibodies to the survivin protein are potential biomarkers for early diagnosis. We tested the hypothesis that sandwich ELISA can detect autoantibodies to survivin before radiologic diagnosis in patients with early-stage nonsmall cell lung cancer (NSCLC). Because previous studies assayed survivin autoantibodies with the direct antigen-coating ELISA (DAC-ELISA), we first compared that assay with the sandwich ELISA. Based on the more robust results from the sandwich ELISA, we used it to measure survivin autoantibodies in the serum of 100 individuals from a well-controlled population study [the Dutch–Belgian Lung Cancer Screening Trial (NELSON) trial] composed of current and former smokers (50 patients with NSCLC, both before and after diagnosis, and 50 matched, smoking-habit control subjects), and another 50 healthy nonsmoking control subjects. We found no difference in specific autoantibodies to survivin in NSCLC patients, although nonspecific median optical densities were 24% higher \( P < 0.001 \) in both NSCLC patients and smokers, than in healthy nonsmokers. Finally, we confirmed the ELISA results with Western blot analysis of recombinant and endogenous survivin (HER-23), which showed no anti-survivin reactivity in patient sera. We conclude that specific anti-survivin autoantibody reactivity is most likely not present in sera before or after diagnosis. Autoantibody studies benefit from a comparison to a well-controlled population, stratified for smoking habit.

Introduction

Long-term survival in lung cancer depends mainly on early detection and immediate start of treatment of the tumor (1). During tumor development, tumor-antigen expression elicits cellular and humoral immune responses (2–4). Identifying autoantibodies to tumor-associated antigens (TAA) is thought to be a promising method of early lung cancer diagnosis (5–8), especially because these autoantibodies have been found up to 5 years before CT detection (9, 10).

Antibodies to survivin are one of the autoantibodies described most frequently in lung cancer (5, 11–18). Survivin, also known as baculoviral IAP repeat-containing protein 5 (BIRC5), is a member of the inhibitor apoptosis proteins (IAP) family. It promotes cell proliferation and inhibits apoptosis, thereby favoring the growth and progression of transformed cells and tumors. Although survivin is abundantly expressed in fetal cells, transformed cell lines, and various tumors, it is undetectable in most normal, differentiated adult tissues (19). If overexpressed in lung cancer, it may lead to antibody responses to this protein. Various studies have compared amounts of survivin autoantibodies in lung cancer patients and healthy blood-donor control subjects; the presence of antibodies against survivin in lung cancer sera collected after diagnosis was reported to range between 8% (16) and 58% (15).

In our institute, a well-controlled multicenter population study was conducted aiming at early detection of lung cancer with specific emphasis on smoking habit, the NELSON trial (20, 21). We wanted to test the hypothesis that, in this population, anti-survivin autoantibodies are detectable before the radiologic diagnosis of non–small cell lung cancer (NSCLC) and establish how these autoantibodies may emerge over time in the disease. For that purpose, we collected, NSCLC cases from the NELSON trial with well-matched controls to the NSCLC cases, subdivided into individuals who were currently smoking or were former smokers. In addition, we included late-stage NSCLC patients from the Dutch Association of Pulmonologists for Lung Diseases and Tuberculosis (NVALT)-12 study (22) and a control group of generally healthy blood donors, similar to that used in the publications on survivin autoantibodies (5, 11–18).

All published studies on survivin autoantibodies in lung cancer have used the direct antigen-coating (DAC) form of an ELISA with a recombinant survivin (5, 11–18). However, the DAC-ELISA assay can give false-positive results if antibodies bind to...
impurities in the antigen preparation. In our study we therefore chose to assess the presence of autoantibodies to survivin with the sandwich ELISA. In this assay, a highly specific capture antibody is absorbed to the solid phase and incubated with an antigen solution. Only autoantibodies to survivin are then detected in the serum samples. At the cost of less signal, this greatly increases the specificity of the assay.

**Materials and Methods**

**Subjects**

We obtained serum from 50 NSCLC cases (82% adenocarcinoma; 90% stage I/II) before and after diagnosis and from 50 smoking-habit controls drawn from current and former smokers in the NEILSON trial, as described previously (20, 21). Smoking-habit controls were matched to the NSCLC cases for age, gender, smoking status, smoking duration, number of cigarettes smoked per day, chronic obstructive pulmonary disease (COPD) status, asbestos exposure, and blood collection center (Supplementary Table S1). For the reference population, serum was derived from 50 healthy nonsmoking blood donors of the Sanquin Blood Supply Rotterdam. These nonsmoking controls were matched to cases and smoking-habit controls for age and gender (Supplementary Table S1). From the NVALT-12 study, we obtained baseline serum from 20 patients (10 men and 10 women; 6 current smokers, 12 former smokers, 2 nonsmokers; mean age, 63.2 ± 9.1 years) with stage IV NSCLC (80% adenocarcinoma; 20% large cell carcinoma). From Sanquin Blood Supply Rotterdam we obtained 7 independent serum samples from healthy nonsmoking controls with a normal serum IgG (mean 10.5 ± 1.78 g/L). Professor Anastasios E. Germenis (16) made available for our study 9 serum samples labeled positive for anti-survivin antibodies by the DAC assay from NSCLC patients (8 men and 1 woman; mean age, 57.6 ± 8.0 years) in his laboratory. Upon diagnosis, these patients—part of a cohort of 117 NSCLC patients (108 males and 9 females; mean age, 64.2 ± 9.3 years)—had been shown by DAC-ELISA to have higher anti-survivin antibody levels than 100 healthy, age- and gender-matched individuals (99 men and 1 woman; mean age, 68.2 ± 5.8 years).

For all human samples, a medical ethical statement according to the Declaration of Helsinki was given by the METC Erasmus MC Rotterdam. Written informed consent was obtained from all participants for the use of their samples.

**Serum-collection protocol**

During the participants’ visits to the center, one serum gel tube was collected per participant. The venous blood was allowed to clot, and was centrifuged for 10 minutes at 1400 × g and 4°C within 2 hours after collection. After centrifugation, the serum was stored immediately in aliquots at −80°C. All samples were blinded and analyzed in random order.

**Detection of anti-survivin antibodies by ELISA**

Antibody reactivity against recombinant full-length human survivin fused to calmodulin (Cam-tag) (BIRC5, Abcam) was measured by DAC-ELISA as described previously (16) and by sandwich ELISA. To validate that both assays detected survivin autoantibodies and to assess their dynamic ranges, we used a rabbit monoclonal antibody (mAb) to survivin (0.06–10.00 ng/mL; Abcam) to establish standard curves (Supplementary Fig. S1). The standard curve of rabbit anti-survivin showed a dynamic range from 0.06 to 40 ng/mL in the DAC-ELISA and from 0.06 to 1000 ng/mL in the sandwich ELISA (Supplementary Fig. S1). To determine if these assays could distinguish human auto-antibody–positive from –negative sera, we analyzed serum from 7 NSCLC patients that had been previously reported (16) to be positive for survivin autoantibodies and from 7 healthy nonsmoking control subjects, the type of control group used by Karanikas and colleagues (16). The replicability of our sandwich ELISA was established by repeatedly testing a single sample for intra-assay variation, as well as testing inter-assay variation. The intra-assay coefficient of variation (CV) for the optical densities (OD) with variation, as well as testing inter-assay variation. The intra-assay coefficient of variation (CV) for the optical densities (OD) with survival of 20 replicates of 1 serum sample from a nonsmoking control was 6.9%, the interassay (plate-to-plate) CV for 6 serum samples from nonsmoking controls ranged from 5.3% to 11.0%.

**DAC-ELISA for anti-survivin antibodies.** One half of a microtiter plate (Nunc-Immuno Maxisorp flat bottom, Thermo Scientific) was incubated with 100 μL of the 2 μg/mL recombinant Cam-tagged survivin in 0.05 mol/L carbonate buffer (pH 9.6) and the other half only with 0.05 mol/L carbonate buffer for 20 hours at 4°C. After washing 5 times with phosphate-buffered saline containing 0.05% Tween-20 (PBST), plates were blocked with 200 μL 5% bovine serum albumin (BSA; Sigma) in PBST for 20 hours at 4°C. Plates were washed 5 times with PBST, and wells with and without survivin on the same plate were incubated for 1 hour at room temperature with 100 μL of serum sample either diluted 1:40, as described previously (16), diluted 1:100, or with serial dilutions of rabbit anti-survivin in PBST. After washing as before, plates were incubated for 1 hour at room temperature with 100 μL of horseradish peroxidase (HRP)–conjugated goat anti-human IgG (H+L; 100 ng/mL; Vector) or HRP-conjugated goat antirabbit IgG (Fc) (80 ng/mL; Jackson) in 1% BSA-PBST. Plates were washed 6 times, and enzyme activity was visualized by adding 100 μL of 3,3’,5,5’-tetramethylbenzidine substrate (TMB; Sigma-Aldrich). Absorbance at 450 nm was measured after 10 minutes. Absorbance values were corrected by blank subtraction.

**Sandwich ELISA for anti-survivin antibodies.** Microtiter plates were coated overnight at 4°C with 100 μL of 0.50 μg/mL Cam-tag specific mAb (Santa Cruz Biotechnology) in 0.05 mol/L carbonate buffer. Plates were washed 5 times with PBST and blocked with 200 μL 5% BSA-PBST for 1.5 hours at room temperature. After washing 5 times with PBST, one half of the plate was incubated with 100 μL of 400 ng/mL recombinant Cam-tagged survivin in 5% BSA-PBST, and the other half with only 5% BSA-PBST for 3.5 hours at room temperature. Plates were washed as before, and 100 μL of serum sample diluted 1:100 or serial dilutions of rabbit anti-survivin in PBST were added to wells with and without survivin on the same plate. After 1-hour incubation at room temperature followed by washing, wells were incubated with 100 μL of (HRP)-conjugated goat anti-human IgG (H+L; 100 ng/mL) or (HRP)-conjugated goat anti-rabbit IgG (Fc) (80 ng/mL) in 1% BSA-PBST for 1 hour at room temperature. After washing 6 times, enzyme activity was visualized by adding 100 μL of TMB. After 10-minute incubation, absorbance at 450 nm was measured. Absorbance values were corrected by blank subtraction.

**Sensitivity and specificity of reagents.** The linearity of the sandwich ELISA was determined by diluting the rabbit anti-survivin positive control into 6 nonsmoking control serum samples and 10 NSCLC patient serum samples, to create a standard curve. ODs of serial
dilutions of rabbit anti-survivin in human sera were identical to those spiked in diluent. To validate the utility and specificity of our secondary antibody (goat anti-human IgG (H+L)), we designed a similar sandwich ELISA using as a positive control a different human protein and sera that were known to contain autoantibodies to it. Patients with paraneoplastic neurologic syndromes (PNS) can generate autoantibodies against the onconeural antigen HuD (23). Recombinant HuD antigen (ELAV4) was applied to serial dilutions of patient serum samples, known to be positive for anti-HuD antibodies. For this test, one healthy donor sample was used as negative control.

To determine nonspecific reactions, samples were analyzed in wells with and without survivin on the same plate. After blank subtraction, the net absorbance at 450 nm for each sample was calculated using the following equation: Net OD = OD with survivin – OD without survivin.

Western blot analysis

With CaM-survivin antigen. To determine the specificity of the DAC-ELISA and sandwich ELISA, selected samples diluted 1:200 were analyzed by Western blotting of CaM-tagged survivin (50 ng/lane). Rabbit anti-survivin serially diluted (1.6–200 ng/mL) was used to check the sensitivity of the Western blot. Blots were incubated with a 1:10,000 dilution of IRDye 800CW goat anti-human IgG (H+L) or IRDye 680RD donkey anti-rabbit IgG (H+L), and scanned with an Odyssey Infrared Imager (LI-COR) to simultaneously visualize proteins at anti-human and anti-rabbit dual wavelengths.

With HEK-293 cell lysate. Blots were performed using HEK-293 cell lysate as a source of endogenous survivin. For equal loading of proteins, 100 µL HEK-293 cell lysate was loaded into a single-well comb slot for Western blot analysis. After protein transfer, the blot was cut into strips containing equal amounts of protein per centimeter of strip. Antibody response to HEK-293 antigens was analyzed with either serum samples diluted at 1:200 or rabbit anti-survivin diluted at 1:2,500. Proteins were detected by fluorescence as described previously or by chemiluminescence using the ECL Western blotting substrate (Thermo Scientific) according to the protocol provided.

Preparation of HEK-293 cell lysate as source of endogenous survivin

Potentially, posttranslational modification of survivin may be necessary for immune recognition and may be a reason that a positive survivin antibody serum is missed. We prepared human embryonic kidney (HEK)-293 cell lysate that endogenously produces survivin (24). HEK-293 cells were cultured in DMEM with GlutaMax (Life Technologies) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37°C in 5% CO₂. The identity and homogeneity of the HEK-293 culture were verified by light-microscopic inspection of cell morphology and growth patterns. No additional authentication was performed. Cells were washed with PBS and lysed in 1 mL SDS sample buffer (±10⁶ cells/mL). The cell lysate was centrifuged and stored in aliquots at –20°C until analysis.

NanoLC Orbitrap mass spectrometry measurement of endogenous survivin in HEK-293 cell lysate

Proteins in the HEK-293 cell lysate were separated by SDS-PAGE. Protein bands (band 1–5) in the 15- to 16-kDa range were tryptic digested for LC/MS measurement as described previously (25). Tryptic peptides were measured by LC/MS on an Ultimate 3000 nano-RSLC system (Dionex) coupled online to a hybrid linear ion trap/Orbitrap MS (Orbitrap Fusion, Thermo Fisher Scientific). The trap column was then switched online with the analytical column (PepMap C18, 75 µm ID × 500 mm, 3-µm particle and 100 Å pore size; Dionex), and peptides were eluted by a 90-minute gradient from 4% to 38% acetonitrile at a flow rate of 250 nL/minute. For electro-spray ionization (ESI), metal-coated nano ESI emitters (New Objective) were used at a spray voltage of 1.7 kV. Two different methods for MS detection were used. First, all samples were run according to a data-dependent shotgun method whereby MS1 survey scans of 400 to 1,600 m/z were acquired in the Orbitrap at 120,000 resolution. Subsequently, CID MS/MS spectra were acquired in the linear iontrap using the top speed mode of the instrument. Second, in a further measurement of protein band 2, the method described above was adjusted to exclusively trigger MS/MS spectra of precursor masses matched with the predicted tryptic peptides of survivin within a 12-ppm m/z window.

Statistical analysis

Before samples were included in this study, the clinical characteristics of nonsmoking controls, NSCLC cases, and smoking-habit controls were statistically analyzed. To detect statistically significant differences in absorbances (OD) among the data sets, the Mann–Whitney U test (two-tailed) was performed. Pearson correlation and linear regression analysis were performed to assess the relationship between the OD of samples measured in wells with survivin and those measured without survivin. All data were analyzed by SPSS (IBM SPSS Statistics 21). A P value of <0.05 was considered statistically significant.

Results

Before we started our studies on the development of survivin autoantibodies in NSCLC, we tested both the DAC-ELISA and the sandwich ELISA for robustness and specificity.

DAC-ELISA for survivin antibodies

The antibody response to human survivin was measured in 7 NSCLC serum samples previously found positive for survivin antibodies in the provider’s laboratory (16) and in 7 serum samples from healthy nonsmokers. Tests were carried out at a 1:40 (Fig. 1A) and a 1:100 dilution (Supplementary Fig. S2). Comparisons between patients and healthy nonsmokers were made. The Mann–Whitney U test showed no significant difference between the NSCLC cases and the healthy nonsmoking controls at two different dilutions (1:40 dilution, P = 1.000; 1:100 dilution, P = 0.620). In addition, no significant differences were found in specific binding, between OD readings in assays that contained the antigen, survivin, and those without antigen, either at a 1:40 (P = 0.535) or at 1:100 (P = 0.535) dilution.

Sandwich ELISA for survivin antibodies

We first determined the replicability of our sandwich ELISA by repeatedly testing a single sample for intra-assay variation, as well as testing inter-assay variation (see Materials and Methods) and found no sample matrix interference in the sandwich ELISA (Supplementary Figs. S3 and S4).
To determine whether our sandwich ELISA technique can detect known human autoantibodies, we tested the assay using the onconeural antigen HuD (ELAV-like protein 4) and serial dilutions of an anti-HuD–positive serum from a patient with PNS. Serum from a healthy donor was used as a negative control. The patient serum gave a linear standard curve at serum dilutions of 1:10 to 1:640 and a relatively high OD (2.378) at the 1:100 dilution that is commonly used for immunohistology (Supplementary Fig. S5). The negative control serum had a low OD (0.147) at a dilution of 1:100. When antigen was omitted from the sandwich ELISA and serum used at a 1:100 dilution, the patient serum had a much lower OD (0.102), but the OD of the negative donor serum was virtually unchanged (0.146). Thus, the anti-human secondary antibody reaction works correctly in demonstrating the presence of immobilized antigen-bound human auto-antibody in our sandwich assay.

Comparison of DAC-ELISA to sandwich ELISA

Having validated the sandwich ELISA as capable of detecting human autoantibodies, we retested the same 7 NSCLC patient and healthy control samples that we had assayed with the DAC-ELISA, at a serum dilution of 1:100. We observed that ODs with and without antigen were significantly higher for patients [median, 0.108; interquartile range (IQR), 0.095–0.121] than for the nonsmoking control subjects [median, 0.075; IQR, 0.065–0.092; Mann–Whitney U test (MW), P = 0.011] and were strongly correlated. Pearson correlation and linear regression between OD with survivin and OD without survivin of these samples resulted in an $R^2$ value of 0.96 ($P < 0.001$) with an intercept of 0.014 (95% CI, 0.003–0.025) and a slope of 0.896 (95% CI, 0.781–1.011). Specific binding was essentially zero, median net ODs (ODs with survivin − ODs without survivin) ranged from 0.000 to 0.008.

Survivin sandwich ELISAs of NELSON samples

We tested sera for the presence of autoantibodies to survivin from 50 NSCLC cases, before and after diagnosis, 50 matched, smoking-habit controls from the NELSON trial, and another 50 nonsmoking controls (n = 200). All samples were randomized before measurement. Specific reactions (subtracting background values obtained in the absence of survivin in the sandwich ELISA) were almost zero, ranging from −0.004 to −0.001 (Fig. 2). However, as noted with the sera from the study by Karanikas and colleagues (16), the background median OD was significantly higher in NSCLC patients ($P < 0.001$) and smoking-habit controls than in nonsmoking controls (Fig. 2 and Table 1). Thus, the choice of control may be critical for these studies if background is not removed before reporting data.

We also tested for autoantibodies in sera of 20 NSCLC patients from the late-stage NVALT-12 study (median, 0.051; IQR, 0.027), 13 smoking-habit controls [median, 0.059; IQR 0.045; P = 0.253], and 13 nonsmoking controls [median, 0.062; IQR,
None of these late-stage NSCLC cases was positive for antibody binding to survivin. Sandwich ELISA results confirmed by Western blot analysis

CaM-tagged survivin was used in Western blots for further testing of serum samples from 9 NSCLC patients previously found positive by DAC-ELISA (16), 5 patients before diagnosis of NSCLC, 5 patients after diagnosis of NSCLC, 2 smoking-habit controls, and 2 nonsmoking controls (Supplementary Fig. S6). To check the sensitivity of the Western blot, we analyzed rabbit anti-survivin serially diluted from 1:5,000 to 1:625,000 (1.6–200 ng/mL), finding a sensitivity of at least 1.6 ng/mL for rabbit anti-survivin antibodies. The 20 NVALT-12 study samples were also analyzed as described above. Sandwich ELISA and Western blot analysis both showed all selected human serum samples to be negative.

Western blot analysis with HEK-293 cell lysate

To rule out the possibility that autoantibodies are not reactive to recombinant survivin but only to endogenous survivin, blots were also performed with HEK-293 cell lysate as the source of endogenous survivin. Proteins in the HEK-293 cell lysate were separated by SDS-PAGE, and bands of interest (bands 1–5) were excised from the gel for MS.

**Table 1.** Survivin antibody absorbances of NSCLC cases, smoking-habit controls, and nonsmoking controls

<table>
<thead>
<tr>
<th>Sample set</th>
<th>N</th>
<th>OD at 450 nm Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmoking controls</td>
<td>50</td>
<td>0.054 (0.023) vs.</td>
</tr>
<tr>
<td>Smoking-habit controls</td>
<td></td>
<td></td>
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<tr>
<td>NSCLC cases before dx</td>
<td></td>
<td>0.068 (0.029)</td>
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<tr>
<td>NSCLC cases after dx</td>
<td></td>
<td>0.067 (0.037)</td>
</tr>
<tr>
<td>NSCLC cases after dx</td>
<td></td>
<td>0.066 (0.028)</td>
</tr>
<tr>
<td>Smoking-habit controls and NSCLC cases before dx</td>
<td>150</td>
<td>0.067 (0.017)</td>
</tr>
</tbody>
</table>

Abbreviations: dx, diagnosis of lung cancer; IQR, interquartile range; N, number of sandwich ELISA measurements; OD, absorbance.

* Differences in absorbance values were statistically determined by the Mann-Whitney U test (two-tailed).

**Figure 2.** Results of sandwich ELISA for antibody response to recombinant human survivin in sera from 50 smoking-habit controls, 50 cases measured before and after diagnosis (dx) of NSCLC, and 50 healthy nonsmoking controls. Data, OD at 450 nm (A) and net OD at 450 nm (net OD = OD with survivin – OD without survivin) (B) with bars representing median OD (IQR) for each group. A, no difference in specific autoantibodies to survivin was found in NSCLC patients, although nonspecific median ODs were 24% higher (P < 0.001) in both NSCLC patients and smokers, than in healthy nonsmokers. B, specific median ODs (subtracting background values obtained in the absence of survivin in the sandwich ELISA) were almost zero, ranging from −0.004 to −0.001. For the 200 samples, ODs with survivin and ODs without survivin were strongly correlated. Pearson correlation and linear regression analysis of log-transformed OD values resulted in an R² value of 0.83 (P < 0.001) with an intercept of −0.117 (95% CI, −0.288–0.053) and a slope of 0.971 (95% CI, 0.781–1.011).

0.035; P = 0.113).
MS/MS database search identified survivin (predicted: 16 kDa) in 16-kDa protein bands 2 and 3 (Fig. 3). The presence of survivin was verified by a targeted MS analysis on protein band 2, which identified four peptides of BIRC5: KKEEETAK, HSSGCAFLSVK, AIEQLAAM, and RAEQLAAMD. Western blot analysis showed that rabbit anti-survivin recognized a band of 15- to 16-kDa protein in the HEK-293 lysate (Fig. 3) that was in agreement with band 3 of the mass spectrometry (MS) analysis. Thus, Western blot and MS both confirmed the presence of survivin in HEK-293 cells.

Antibody response to antigens in HEK-293 cell lysate was analyzed in serum of 7 randomly selected nonsmoking controls and 9 longitudinally collected sample sets, each set consisting of one case analyzed before diagnosis (b, dark blue box) and after diagnosis of NSCLC (a, red box) and one smoking-habit control matched to this case (c, green box). Immunodetected proteins at 16 kDa were observed for all samples, including smoking-habit control subjects and nonsmoking control subjects. No anti-survivin reactivity was detected in any of the patient sera.

Discussion

No survivin-specific autoantibodies were detected by sandwich ELISA in sera from NSCLC cases, smoking-habit controls, or healthy nonsmoking controls, even though background nonspecific binding was 24% higher (P < 0.001) in NSCLC cases and smoking controls than in healthy nonsmoking controls. Thus, if background is not subtracted, significantly higher OD values for cases and smokers can be misleading when compared only with nonsmoking healthy controls. A study of autoantibodies to a panel of 10 TAAs reported that smokers—lung cancer patients and non–lung cancer patients alike—consistently had significantly higher background binding when assaying for autoantibodies than did healthy non-smoking controls (18).

Specific antibody reactivity for survivin was no higher in any of the 50 NELSON NSCLC patients than in smoking-habit controls. Positive rabbit control samples gave a strong response in the assay. Thus, the assay was functioning correctly, and survivin in vivo had not induced significant antibody responses before or after diagnosis of NSCLC. This observation is supported by the absence of cytolytic responses against survivin peptides (26). Western blot analysis revealed no antibody reactivity with the recombinant survivin, consistent with these ELISA results.

To our knowledge, the population we used is the best well-controlled population related to smoking habit in lung cancer case–control studies on survivin autoantibodies to date. Our inability to find survivin autoantibodies in NSCLC patients is not in agreement with the results reported by others (11–18), who found survivin autoantibodies with an 8% to 52% prevalence. Although these inconsistent findings may have been due to differences in the type of tumor, the stage of lung cancer, or the source of antigen, the most likely explanation is the difference in assay methodology.

Forty-one (82%) of the 50 NSCLC patients in our study were diagnosed with adenocarcinoma, and 45 (90%) were classified as early stage (I and II). This uneven distribution of pathology might explain the absence of survivin autoantibodies. Antibodies to survivin have nevertheless been found irrespective of tumor type and clinical stage of NSCLC (15, 16). To exclude the possibility that our selection of patients had been unbalanced, we also investigated late-stage (stage IV) NSCLC.
serum samples. In these, too, sandwich ELISA and Western blot analysis revealed no positive samples.

To date, the method used most widely to detect survivin autoantibodies involved direct adsorption assays with immobilized recombinant survivin. If the antigen solution is not absolutely pure, contaminating antigens may be coimmobilized in much the same way as Escherichia coli proteins, which are very often copurified with the recombinant protein (27). Due to the high prevalence of E. coli infections in humans, background responses to the E. coli bacteria used in producing recombinant proteins have been found to be a major problem (28, 29). We also detected these antibody responses to E. coli proteins in a Western blot of E. coli extract with sera of lung cancer patients (data not shown). Although the specificity of the survivin recognition in the DAC-ELISAs was confirmed by decreased reactivity after preabsorption of sera with soluble survivin, reactivity is also likely to be reduced by nonspecific reactions with impurities in the survivin solution.

In solid-phase immunoassays, nonspecific binding for auto-antibodies has been found to correlate with increased concentrations of IgG and other inflammatory mediators (30). Despite the fact that the DAC-ELISA showed higher apparent anti-survivin levels than the sandwich ELISA, in our hands this assay showed no significant difference between lung cancer patients previously reported (16) to be positive for anti-survivin and healthy nonsmoking control subjects—in addition, any differences disappear when background is subtracted. In general, DAC-ELISA may incorrectly assess antibodies to survivin and, presumably, to other tumor antigens, because it does not compensate for nonspecific binding. Much more than DAC-ELISA, sandwich ELISA makes it possible to significantly reduce—and also monitor—nonspecific binding. Due to better specificity, absorbance in the sandwich ELISA was an order of magnitude lower than that in the DAC-ELISA.

To test if any differences in posttranslational modifications of endogenous survivin, compared with recombinant survivin, could be the cause of a lack of anti-survivin reactivity to recombinant survivin, we also used HEK-293 cell lysate as the source of endogenous survivin antigen. Although Western blot analysis with HEK-293 cell lysate showed no autoantibody reactivity to endogenous survivin (16 kDa), it showed that the individual patterns of antibodies against HEK proteins before and after the diagnosis of lung cancer were almost identical in each NSCLC cancer patient.

In conclusion, we demonstrated that survivin autoantibody reactivity is not present in sera from NSCLC cases, smoking-habit matched controls, and healthy nonsmoking controls. Higher apparent survivin autoantibody reactivity in the serum samples of smokers than in nonsmokers that has been previously reported is likely the result of nonspecific binding in smokers. In general, autoantibodies to lung tumor antigens should be investigated using sandwich ELISA or another well-characterized technology in a well-controlled population, stratified for at least smoking habit.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: I. Broodman, J.M. Dekker, R.J. van Klaveren, T.M. Luider
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Writing, review, and/or revision of the manuscript: I. Broodman, M.M. VanDuijn, C. Stingl, L.J.M. Dekker, H.J. de Koning, R.J. van Klaveren, J.G. Aerts, J. Lindemans, T.M. Luider
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References


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