

Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection

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Abstract

Survivin, an inhibitor of apoptotic protein, is over-expressed in many cancers but not in normal differentiated adult tissues. Recently, antibodies to survivin have been demonstrated in patients with lung and colorectal cancer. Whether antibodies to survivin can be used as a marker for the diagnosis of cancer, and how antibody to survivin is related to antibodies against tumor suppressor protein p53 and oncoprotein c-myc remains to be evaluated. In the present study, the full-length recombinant proteins survivin, p53 and c-myc, were expressed and used as antigens in enzyme-linked immunosorbent assay (ELISA) and Western blot for the detection of antibodies to these three proteins. Sera from 1137 patients with 11 different types of cancer were analyzed. Antibodies to survivin were detected in 8.4% (96/1137), with a significant difference from the control groups consisting of normal individuals and autoimmune disease patients ($p < 0.05$). Of 1137 cancer sera, 546 were also tested for the presence of antibodies to p53 and c-myc. Frequencies of antibodies to p53 and c-myc were 11.5 and 12.3%, respectively. Although antibodies to either one of three antigens do not reach levels of sensitivity, which could become routinely useful in diagnosis, it appears that there are different patterns of antibody frequency in individual cancer type. The results also indicated that when the presence of antibody to any one of these three antigens was considered, the cumulative frequency was increased to 27.3% (149/546) for the total group of cancer patients. It became apparent from our data that the combination of antibodies might acquire higher sensitivity.

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Keywords: Autoantibody; Survivin; p53; c-myc; Cancer

1. Introduction

Studies in autoimmune diseases have provided abundant evidence suggesting that the autoantibodies are antigen-driven responses and that autoantibodies can be viewed as reporters from the immune system revealing the identity of antigens, which might be playing roles in the pathophysio-

logy of the disease process [1,2]. How the intracellular proteins participate in the malignant transformation process and whether a similar mechanism in autoimmune diseases might be involved in humoral immune responses in cancer remains to be established, but appears to be a possibility. Cancer has long been recognized as a multi-step process which involves not only genetic changes conferring growth advantage but also factors which disrupt regulation of growth and differentiation [3]. It is possible that some of these factors could be identified and their functions evaluated with the aid of autoantibodies arising during

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tumorigenesis. The multi-factorial and multi-step nature in the molecular pathogenesis of human cancers must be taken into account in both the design and interpretation of studies to identify biomarkers, which will be useful for early detection of cancer.

Recent studies demonstrated that cancer sera contain antibodies, which react with a unique group of autologous intracellular antigens known as tumor-associated antigens (TAAs) [4–6]. The production of autoantibodies can be induced by a variety of proteins associated with malignant transformation processes of cells such as tumor suppressor protein p53 [7], oncoprotein c-myc [8] and IGF-II mRNA binding protein p62 [9,10]. The inhibition of apoptosis is an important mechanism in cancer progression [11], and apoptosis pathways can be blocked by the inhibitor of apoptosis protein (IAP) family proteins [12–14]. Survivin is a structurally unique IAP molecule, characterized by a developmentally regulated expression during human and mouse differentiation. More importantly, survivin was undetectable in normal adult tissues but abundantly expressed in transformed cell types and a variety of human cancers in vivo [15]. In neuroblastoma, the presence of survivin identified patients with negative prognostic factors for disease progression, whereas expression of survivin in gastric cancer was significantly associated with bcl-2 and p53 abnormalities [16]. Recently, antibodies to survivin have been demonstrated to be present in patients with lung and colorectal cancer [17]. Whether antibodies to survivin can be used as a marker for the diagnosis and prediction of cancer, and how the immune responses to survivin are associated with antibodies to p53 and c-myc in cancer remains to be investigated and evaluated. In the present study, we determined the frequency of antibodies to survivin, p53 and c-myc in patients with 11 types of cancer, and evaluate the usefulness of anti-survivin antibody as additional marker for the diagnosis of cancer.

2. Materials and methods

2.1. Sera and antibodies

In this study, sera from two groups of patients (Group 1, 546; Group 2, 591; total, 1137) with different types of cancer, and 82 sera from normal individuals were obtained from the serum bank of the Tumor Cell Engineering Laboratory of Xiamen University (Fujian Province, PR China). These two groups of cancer sera were collected from the same hospitals in Fujian Province of China, and basically there was no genetic background difference between these two groups. Based on the clinical information, all cancer sera were collected at the first time of diagnosis, and patients did not receive treatment with any chemotherapy or radiotherapy. Normal control sera were collected during annual health examination and who had no obvious evidence of malignancy. One hundred and three human autoimmune

disease sera (62 patients with systemic lupus erythematosus, 41 patients with Sjögren's syndrome) from the Autoimmune Disease Center of the Scripps Research Institute (La Jolla, CA) were also available for this study. Human prototype sera containing autoantibodies to previously identified cellular antigens [1,2] were obtained from the reference controls of the Autoimmune Disease Center of the Scripps Research Institute and were used to monitor the sensitivity of Western blotting immunoassays. Rabbit polyclonal anti-survivin antibody raised against the C-terminal peptide was obtained from ProSci Inc. (Poway, CA). p53 and c-myc monoclonal antibodies were purchased from Oncogene Research Products (Boston, MA). This study was approved by the Institutional Review Boards of the respective academic institutions.

2.2. Expression and purification of recombinant proteins

cDNAs from p53, c-myc and survivin were, respectively, subcloned into pET28 vector (Novagen, Madison, WI), which provides the NH₂-terminal fusion protein with 6× histidine and T7 epitope tags. p53 clone (p53SN3) was kindly provided by Dr. Yuxin Yin of Columbia University, New York. cDNA from c-myc was amplified by polymerase chain reaction from human fetal liver tissue and survivin cDNA was made from human survivin EST clone (BG258433). For increased expression and purification, the recombinant polypeptides were expressed in *Escherichia coli* BL21 (DE3) and purified using nickel column chromatography. The protocol used for high-level expression and purification of 6× His-tagged proteins was performed as described (QIAGEN Inc., Valencia, CA). Elution buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 4.5) was used to elute the recombinant protein.

2.3. Western blotting analysis

Western blotting was performed essentially as described by Chan and Pollard [18]. Purified recombinant proteins were electrophoresed on SDS-PAGE and transferred to nitrocellulose paper. After preblocking with phosphate-buffered saline containing 0.05% Tween-20 (PBST) and 5% nonfat milk for 30 min at room temperature, the nitrocellulose strips were incubated for 60 min at room temperature with a 1:100 dilution of serum. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) was applied as secondary antibody using 1:2000 dilution. Immunoreactive bands were detected using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

2.4. In vitro translated products and immunoprecipitation

The survivin cDNA was transcribed and translated in vitro using TnT coupled reticulocyte lysate system (Promega Biotec, Madison, WI) in the presence of [³⁵S]

methionine (ICN, Irvine, CA), as described [9]. Labeled products were used as substrates for immunoprecipitation analysis. The standard protocol for immunoprecipitation assay was described in a previous study [9].

2.5. Enzyme-linked immunosorbent assay

Purified recombinant proteins were diluted in PBS to a final concentration of 0.5 µg/ml for coating immulon two microtiter plates (Dynatech Laboratories, Alexandria, VA). Human sera diluted 1:200 were incubated in the antigen-coated wells. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) and the substrate 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, FRG) were used as detecting reagents. Each serum sample was tested in duplicate, and the average OD value at 405 nm was used for data analysis. The cut-off value designating positive reaction was the mean OD of 82 normal human sera (NHS) + 3S.D. As described in previous study [19], due to the reason that several hundred sera were analyzed at different time periods, each run of ELISA included 10 NHS samples and 2 positive control samples. The 10 NHS samples represented a range above and below the mean of the 82 normal human sera, and the average value of the 10 normal sera was used in each run to normalize all absorbance values to the standard mean of 82 normal sera. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. The chi-square (χ^2) test with Yates' correction, and two significance levels (0.05 and 0.01) were used to determine whether the frequencies of positive sera in each type of cancer patients were significantly higher than in the normal individuals.

2.6. Absorption of autoantibodies with recombinant survivin

Sera (2.5 µl) were diluted in 500 µl PBS, and incubated for 2 h at room temperature with 15 µg survivin recombinant protein. This mixture was centrifuged at 10,000 × g for 10 min, and the supernatant was then used for the analysis by ELISA with survivin recombinant protein as coating antigen.

3. Results

3.1. Frequency of antibodies to survivin in diverse malignancies

In this study, the full-length recombinant survivin protein was used as antigen, and sera from patients with different types of cancer, autoimmune diseases, and normal individuals were examined for the presence of antibodies to survivin. Table 1 shows the frequency of autoantibodies to survivin using enzyme immunoassay. The sera that were tested included the 1137 patients from China with different

Table 1
Frequency of autoantibodies to survivin in cancer

Cancer	No. tested	Autoantibody positive (%) ^a
Breast	105	12 (11.4)*
Colorectal	151	10 (6.6)
Esophageal	148	9 (6.1)
Gastric	205	16 (7.8)
HCC	160	15 (9.4)*
Lung	189	20 (10.6)*
Lymphoma	44	6 (13.6)*
Pharyngeal	46	1 (2.2)
Ovarian	39	1 (2.6)
Thyroid	23	2 (8.7)
Uterine	27	4 (14.8)*
Total	1137	96 (8.4)*
NHS	82	2 (2.4)
SLE	62	1 (1.6)
SS	41	0 (0)

NHS, normal human sera; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.

^a *p*-Value relative to NHS: **p* < 0.05.

types of cancer including breast, colorectal, esophageal, gastric, lung, ovarian, uterine and so on, 82 normal human sera from the same region in China as the cancer patients, and as further controls representing noncancer autoimmune diseases, 62 patients with systemic lupus erythematosus (SLE), and 41 with Sjögren's syndrome (SS). A positive test for antibody to survivin was taken as an absorbance reading above the mean + 3S.D. of the 82 normal human sera. Of a total of 1137 cancer sera analyzed, 8.4% (96/1137) were shown to have antibodies to survivin by ELISA. Higher frequency was observed in sera from patients with uterine cancer (14.8%), lymphoma (13.6%), breast cancer (11.4%) and lung cancer (10.6%). Of the 96 ELISA positive sera, 91 were available for further studies, and 78 (85.7%) had positive reactions in Western blotting. The more reactive ELISA positive sera were also positive by immunoprecipitation using *in vitro* translation products. Several representative positive sera by Western blotting and immunoprecipitation assay were shown in Fig. 1.

The specificity of recombinant survivin as antigen for ELISA was confirmed by absorption studies. As shown in Fig. 2, five cancer sera (BC50, LC40, LC 52, GC 4 and HCC 12), which had positive reactivity with survivin were preincubated with recombinant survivin protein and used subsequently for ELISA. One normal human serum (NHS71) was also tested as control. Reactivity of cancer sera decreased substantially after preincubation with survivin protein, but not in control serum.

3.2. Anti-survivin antibodies and its association with antibodies to p53 and c-myc

One of the most extensively studied tumor-associated antigens is the tumor suppressor protein p53. Autoantibodies to p53 in cancer were first reported in 1982 [20] and since

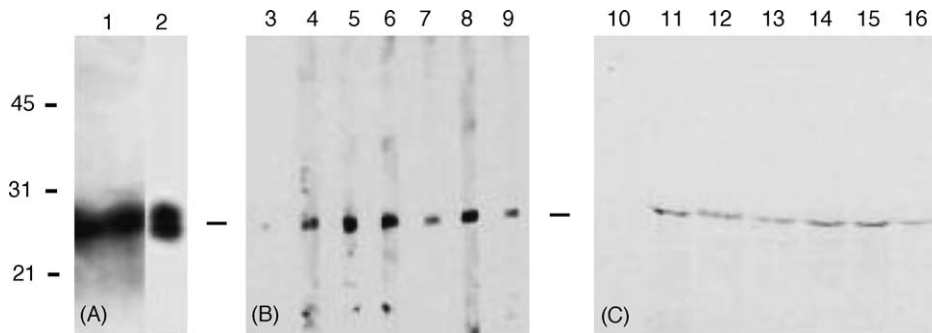


Fig. 1. Western blot and immunoprecipitation analysis of autoantibodies to survivin. Survivin recombinant protein expressed as a $6 \times$ histidine tag protein in *E. coli* was purified using nickel column chromatography. (A) A 28 kDa peptide that corresponded to the size of ORF of survivin was detected in SDS-PAGE with Coomassie blue staining (lane 1). Rabbit polyclonal anti-survivin antibody was reactive with survivin recombinant protein in Western blot analysis (lane 2). (B) Reactivity of six cancer sera in Western blotting against survivin recombinant protein. Lane 3, normal human serum; lanes 4–9, six positive sera with anti-survivin in ELISA also showed strong reactivity with a 28 kDa survivin recombinant protein in Western blotting analysis. (C) Immunoprecipitation of in vitro translated survivin cDNA. Lane 10, normal human serum; lanes 11–16, a 28 kDa protein was immunoprecipitated by antibodies from the six positive sera which were the same sera used in Western blotting analysis in panel B.

then there have been numerous reports confirming and extending this finding [21]. The oncoprotein c-myc is another tumor-associated antigen with antibodies reported in 13.2% of patients with lung cancer [22]. In order to explore the relationship between antibodies to survivin, p53 and c-myc, the first group of 546 cancer sera was further analyzed for the presence of antibodies to p53 and c-myc (Table 2), and the second group of 591 cancer sera was not analyzed in this study. Frequencies of antibodies to survivin, p53 and c-myc were 9.3, 11.5 and 12.3%, respectively. Basically, there was no significant difference for frequency of anti-survivin antibodies in most type of cancer sera between Table 2 and Table 1 except for breast and esophageal cancer. The reason for the difference of anti-survivin frequency in certain type of cancer sera between these two tables may relate to the clinical backgrounds of cancer patients such as clinical

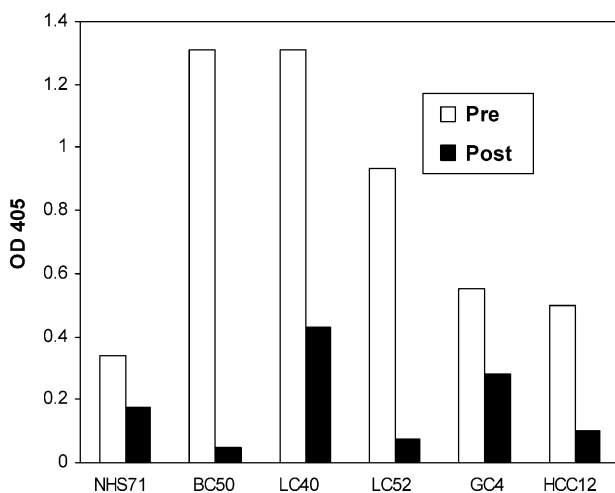


Fig. 2. Absorption of autoantibodies with recombinant survivin protein. Five cancer sera (BC50, LC40, LC52, GC4 and HCC12), which had positive reactivity with survivin were preincubated with recombinant survivin protein and used subsequently for ELISA. One normal human serum (NHS71) was also assessed as control. The experiment was repeated three times, and the average OD values were used to draw this figure.

stages and so on. Because the detailed clinical information for these serum samples was not available we could not give further explanation at this point. It is of interest to note that antibody responses to these three antigens in ovarian, thyroid and uterine cancer were very weak compared to other types of cancer. The small sample size could be one of the reasons for this result. The data also showed that when the presence of antibody to any one of these three antigens was considered, the cumulative frequency was raised to 27.3% (149/546) for the total group of cancer patients, which was significantly higher than that in normal human sera (4.9%) and autoimmune disease sera (4.8% in SLE and 0 in SS). In particular, antibodies to these three antigens in several types of cancer were shown to have higher frequency, for example in HCC (38.5%), lung cancer (32.1%) and gastric cancer (30.8%). The range of antibody titers to survivin, p53 and c-myc are shown in Fig. 3. The high titer reactivity of many cancer sera and the distinct difference between cancer and normal and autoimmune sera were also demonstrated in this figure. Many cancer sera showed OD value several fold above the cut-off (mean + 3S.D. of normal sera), indicating that antibody responses to survivin, p53 and c-myc in some cancer patients were quite robust and not just mildly elevated.

Table 3 shows the analysis of different combinations of antibodies to the three antigens (p53, c-myc and survivin). Of 149 cancer sera with any one positive of the three antibodies, the frequency of antibodies to individual antigen p53, c-myc and survivin was 24.2, 28.2 and 28.9%, respectively, which was much higher compared to the frequency of either two or three antibody combinations (2.7–8.7%).

4. Discussion

Survivin, a member of IAP family proteins, is over-expressed in many cancers but not in normal differentiated

Table 2
Frequency of autoantibodies to p53, c-myc and survivin in cancer

Cancer	No. tested	Anti-p53 no. (%) ^a	Anti-c-myc no. (%)	Anti-survivin no. (%)	Any of three Ags no. (%)
Breast	64	5 (7.8)	12 (18.8)**	5 (7.8)	17 (26.6)**
Colorectal	45	8 (17.8)**	2 (4.4)	2 (4.4)	11 (24.4)**
Esophageal	77	11 (14.3)**	9 (11.7)**	8 (10.4)*	23 (29.9)**
Gastric	91	12 (13.2)*	14 (15.4)**	9 (9.9)*	28 (30.8)**
HCC	65	7 (10.8)*	16 (24.6)**	7 (10.8)*	25 (38.5)**
Lung	56	9 (16.1)**	6 (10.7)**	6 (10.7)*	18 (32.1)**
Lymphoma	44	4 (9.1)	3 (6.8)*	6 (13.6)*	10 (22.7)**
Pharyngeal	32	3 (9.4)	5 (15.6)**	1 (3.1)	9 (28.1)**
Ovarian	22	1 (4.5)	0	1 (4.5)	2 (9.1)
Thyroid	23	2 (8.7)	0	2 (8.7)	3 (13.0)
Uterine	27	1 (3.7)	0	4 (14.8)*	5 (18.5)*
Total	546	63 (11.5)*	67 (12.3)**	51 (9.3)*	149 (27.3)**
NHS	82	2 (2.4)	0	2 (2.4)	4 (4.9)
SLE	62	0	3 (4.8)	1 (1.6)	3 (4.8)
SS	41	0	0	0	0

NHS, normal human sera; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.

^a *p*-Value relative to NHS: **p* < 0.05; ***p* < 0.01.

adult tissues [16,23–28]. Overexpression of survivin by cancer cells may lead to anti-survivin antibody responses and cytotoxic T-lymphocyte responses against the cancer [17,27,28]. One recent study also demonstrated that the expression of survivin is the essential event in the early stage of colorectal carcinogenesis and may play an important role in the malignant transformation pathway, suggesting that survivin may be a new diagnostic and therapeutic target in colorectal cancer [29].

It is a well-established observation that autoimmunity can be associated with cancer and one of the forms of its expression is the development of antibodies to autologous cellular antigens. Studies on p53 tumor suppressor protein could be used to understand how cellular proteins become immunogenic and drive an immune response in cancer. p53 is a phosphoprotein barely detectable in the nucleus of normal cells [30]. Upon cellular stress, particularly that induced by DNA damage, p53 can arrest cell cycle progression, thus allowing the DNA to be repaired [31] or it can lead to apoptosis [32]. The most common changes of p53 in human cancers are missense point mutations within the coding sequences of gene [33,34]. For example, the abnormalities of the p53 gene have been shown to be present in approximately 50% of patients with breast cancer and in this subgroup, 20–30% have been found to have antibodies to p53 [35]. Patients with point mutations had single amino acid substitutions, which resulted in production of proteins with altered functions. These altered proteins had increased

stability in contrast to wild type p53 which is rapidly degraded. The majority of patients with lung cancer who had autoantibodies to p53 had such point mutations resulting in altered proteins with increased stability. Antibodies to p53 have now been found in many other types of human cancer, including esophageal, oral, colon, gastric, hepatic, prostate, thyroid and bladder cancers [21]. Many studies have been done for evaluating the clinical value of p53 antibodies. There is generally a correlation between the presence of p53 antibodies and tumors with poor differentiation as well as short survival [21]. The myc oncogene was originally discovered as the cellular sequence contained within the acute transforming virus MC29 which was isolated from a chicken myelocytoma. MC29 also induced carcinoma of the liver and kidney, sarcomas and mesotheliomas. In addition to c-myc, there are two other highly related family members, L-myc and N-myc. The myc family proteins are comprised of several motifs that are commonly associated with transcription factors. Myc has been rigorously studied due to its involvement in a number of neoplastic conditions as well as its apparent contribution to cell growth regulation [8]. Antibody to c-myc was found in 13.2% of patients with lung cancer [22], and was not reported in other types of cancer.

In this study, we used recombinant survivin protein as coating antigen in ELISA for the detection of anti-survivin antibody in sera from 1137 patients with 11 different types of cancer. Autoantibodies to survivin were detected in 8.4%, with a significant difference from the control populations consisting of normal subjects and autoimmune disease patients. Of 1137 cancer sera, 546 were also analyzed for the presence of antibodies to p53 and c-myc. Frequencies of antibodies to p53 and c-myc were 11.5 and 12.3%, respectively. The analysis of different antibody combinations showed that one fourth of cancer sera contained one of these three antibodies, and less than 10% of sera had either two or three of these antibodies. The observation in this study suggests that in certain number of cancer cases only

Table 3
Analysis of different antibody combinations in cancer

Antibodies								Total
p53	+	+	+	+	–	–	–	
c-myc	–	+	+	–	+	+	–	
Survivin	–	–	+	+	–	+	+	
Numbers	42	13	4	4	43	7	36	149
%	28.2	8.7	2.7	2.7	28.9	4.7	24.2	100.0

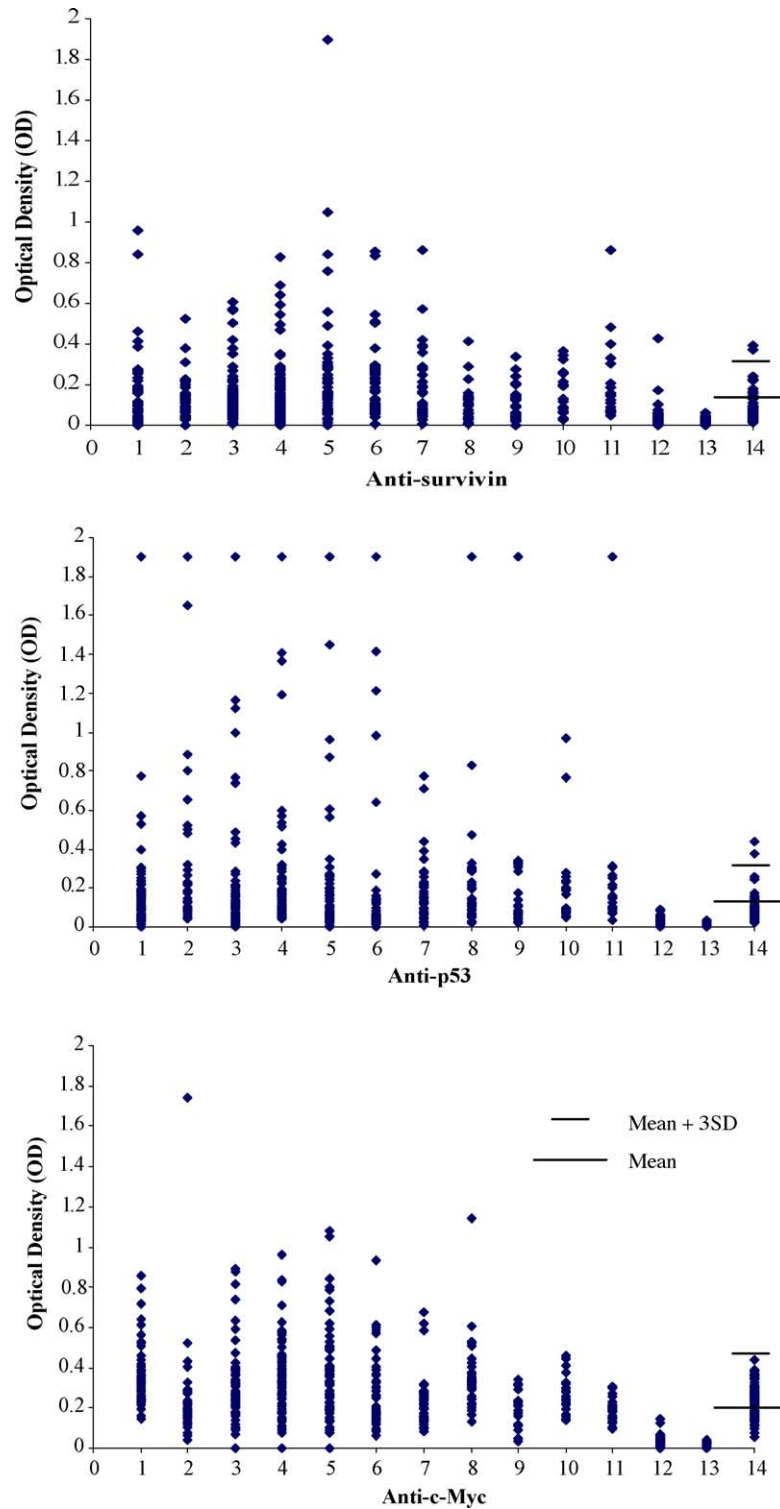


Fig. 3. Autoantibodies to survivin, p53 and c-myc in different cancers. The range of antibody titers to survivin, p53 and c-myc is expressed as optical density (OD) obtained from enzyme-linked immunosorbent assay (ELISA). The mean and 3S.D. of 82 normal human sera are shown in relationship to cancer sera and autoimmune disease sera from SLE and SS. The high titer reactivity of many cancer sera and the distinct difference between cancer and normal and autoimmune sera are demonstrated in this figure. The X-axis represents the type of serum samples. 1, breast cancer; 2, colorectal cancer; 3, esophageal cancer; 4, gastric cancer; 5, HCC; 6, lung cancer; 7, lymphoma; 8, pharyngeal cancer; 9, ovarian cancer; 10, thyroid cancer; 11, uterine cancer; 12, SLE; 13, SS; 14, NHS.

one of these three antigens may be involved in the malignant transformation process, and a few of cases could be related to either two or three of these antigens. The data also showed that when the presence of antibody to any one of these three antigens was considered, the cumulative frequency was raised to 27.3% for the total group of cancer patients. Although antibodies to either one of three antigens do not reach levels of sensitivity, which could become routinely useful in diagnosis, it appears that there are different patterns of antibody frequency in individual cancer type. For example, in breast cancer, c-myc appears to be more reactive antigen than p53 and survivin; in esophageal and lung cancer, both p53 and c-myc appear more reactive antigens, whereas in colorectal cancer it is only p53 (see Table 2). It also became apparent from our data that the combination of antibodies might acquire higher sensitivity. As demonstrated in many other studies, cancer has long been recognized as a multi-step process, which involves not only genetic changes conferring growth advantage but also factors which disrupt regulation of growth and differentiation. Gene alterations or overexpression tend to occur in combinations that vary from tissue to tissue [36]. One recent study demonstrated that the tissue expression of survivin and p53 might play important role in the tumorigenesis of intraductal papillary-mucinous tumor [37], and another study showed that c-myc and p53 gene alterations are important for tumor metastasis, and also important for genetical and pathological staging [38]. If autoimmune responses represent immune system reactions to abnormal gene expression [2], one might expect differences in autoantibody profiles from one type of tissue cancer to another. It is conceivable that autoantibody profiles involving different panels or arrays of “cancer antigens” might be developed in the future and the approach could be useful for cancer diagnosis. Further studies should be directed at selecting other tumor-specific antigens, be attempted to design a unique antigen panels for different types of cancer, and determine whether a mini-array of multiple TAAs would further enhance antibody detection and be a useful approach to early detection and diagnosis.

Acknowledgements

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