Humoral immune response to p16, a cyclin-dependent kinase inhibitor in human malignancies

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Abstract. The p16 protein is a cyclin-dependent kinase (CDK) inhibitor, which plays an important role in the regulation of the cell cycle by inactivating the cyclin-dependent kinase (CDK) that phosphorylates the retinoblastoma (Rb) protein. Overexpression of p16 protein has been found in many types of human malignancy. Autoantibody response to p16 in cancer has not been reported. This study determined the extent and frequency of autoantibodies to p16 in diverse malignancies. p16 recombinant protein was expressed in *E. Coli* BL21 (DE3) cells, and purified using GST fusion protein purification system. In further studies, p16 recombinant proteins were used as antigens in enzyme-linked immunoassay (ELISA) and Western blotting. Sera from 479 cancer patients and 82 normal individuals were analyzed. Autoantibodies to p16 were found in 11.7% in cancer, with significant difference from the normal individuals ($p<0.05$). The results in this study also showed that the frequency of antibodies to p16 is relatively higher in nasopharyngeal cancer (28.6%), breast cancer (17.1%) and hepatocellular carcinoma (HCC, 21.4%). Of the 56 ELISA positive sera with the anti-p16 antibodies, 85.7% (48/56) had positive reactions in Western blotting. The antigen-antibody absorption experiment was also performed to confirm the specificity of the anti-p16 antibody. In order to increase the frequency of antibody detection in cancer, a combination of three tumor-associated antigens (TAAs) p16, p53 and c-myc were used. Increased frequencies at $p<0.01$ were found for antibodies to p16 in breast, esophageal, and nasopharyngeal cancer as well as HCC. For antibodies to c-myc, increased frequencies at $p<0.01$ were found in breast, cervical, colorectal and lung cancer. For antibodies to p53, increased frequencies at $p<0.01$ were only found in breast cancer. With the successive addition of three TAAs, there was a stepwise increase of positive antibody reaction up to 44% in breast cancer and 43% in nasopharyngeal cancer. In summary, the results in this study suggest that the combination of antibodies might acquire higher sensitivity for early cancer diagnosis. It is conceivable that autoantibody profiles involving different panels or arrays of TAAs might be developed in the future and the results could be useful for cancer diagnosis.

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Introduction

The highly specific autoantibody response in systemic autoimmune diseases generally predicts the biologic phenotype of the diseases, making autoantibodies clinically valuable and diagnostically useful (1). Whether a similar mechanism might be involved in humoral immune response in cancer remains to be established, but appears to be a possibility (2). Previous studies demonstrated that cancer sera contain antibodies which react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs) (3-5). 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 (6). It is possible that some of these factors could be identified and their functions evaluated with the aid of autoantibodies arising during 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. One of our previous studies indicated that detection of autoantibodies in cancer can be enhanced by using mini-array of a panel of TAAs as target antigens (7). It is conceivable that autoantibody profiles involving different panels or arrays of TAAs might be developed and the results could be useful for cancer diagnosis.

The tumor suppressor protein p16 is a cyclin-dependent kinase inhibitor, and also a negative regulator of the mammalian cell cycle (8,9). Normal proliferating cells do not express significant levels of p16 prior to extensive rounds of cell division, suggesting a late-stage, anti-proliferative role as in replicative cell senescence. Missense mutations in p16 gene are strongly linked to several types of human cancer (10).

Previous studies demonstrated that proteins associated with malignant transformation of cells such as p53 (11), c-myc (12), p62 (4,13,14) and survivin (15,16) can induce the production of autoantibodies that could serve as parameters to monitor tumor progression. How the tumor suppressor protein p16 induces immune response is still unknown. Whether antibody to p16, like antibodies to p53, c-myc, p62 and survivin can be also used as a marker for the diagnosis and prediction of cancer remains to be investigated and evaluated. In this study, a recombinant p16 protein was purified and further used as an antigen for detection of antibodies to p16 in human cancers.

Materials and methods

Serum samples and antibodies. Sera from 479 patients with different types of cancer, and 82 sera from normal individuals were obtained from the serum bank of the Tumor Cell Engineering Laboratory of Xia'men University (Fujian Province, P.R. China). Based on the clinical information, all cancer sera were collected at the first time of cancer diagnosis, and patients did not receive treatment with any chemotherapy and radiotherapy. Normal human sera were collected during annual health examinations from persons who had no obvious evidence of malignancy. p16, p53 and c-myc monoclonal antibodies were purchased from Oncogene Research Products (Boston, MA). This study was approved by the Institutional Review Board of the respective academic institutions.

Expression and purification of recombinant p16 protein. For expressing the p16 protein, a primer pair with BamH I and EcoR I was synthesized: 5'-AGCGGATCCATGGGTGCC CCGACGTTG-3', and p16-2, 5'-GGTGAATTCAATCGGG GATGT-3'. p16 cDNA was amplified by reverse-transcriptase polymerase chain reactions (RT-PCR) using HeLa cell mRNA as a template. RT-PCR was performed using the one-tube methods as described by Pfeffer *et al* (17). p16 cDNA derived from RT-PCR was further subcloned into a pGEX vector, which fused the p16 gene with a glutathione-S-transferase (GST) domain. pGEX vector has a tac promoter which can be induced by the lactose analog isopropyl Beta-D thiogalactoside (IPTG). The vector also has an ampicillin resistant gene and a multiple cloning site. For increased expression and purification, p16 recombinant protein was expressed in *E. coli* BL21 (DE3) cells and purified using GST fusion protein purification kit (Glutathione Sepharose 4B, Amersham Biosciences). The protocol used for expression and purification was performed as described (Amersham Biosciences). Elution buffer (50 mM Tris-HCl, pH 8; 150 mM reduced glutathione; 0.5 M DDT, pH 9.2) was used to elute the recombinant protein.

Enzyme-linked immunosorbent assay (ELISA). Purified recombinant p16 proteins were diluted in PBS to a final concentration of 0.5 μg/ml for coating Polystyrene 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). Human serum samples diluted 1:200 were incubated in the antigen-coated wells. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories) and the substrate 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, Germany) were used as detecting reagents. Each sample was tested in duplicate, and the average OD at 405 nm was used for data analysis. In this study, all positive sera were tested at least twice. The cut-off value designating positive reaction was the mean optical density (OD) of 82 normal human sera plus 3 standard deviations (SD). The detailed protocol of ELISA was used as described by Rubin (18).

Western blotting. Western blotting was performed essentially as described by Chan and Pollard (19). In brief, the purified p16 protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, p16 protein was further electrotransferred to a nitrocellulose membrane. Nitrocellulose membrane was cut in strips and preblocked with phosphate-buffered saline containing 0.05% Tween-20 (PBST) with 5% non-fat milk for 30 min at room temperature. As the primary antibody, 1:100 diluted serum samples were incubated with the nitrocellulose strips for 90 min at room temperature. Horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) was applied as secondary antibody using a 1:3000 dilution. Immunorective bands were detected using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions and followed by autoradiography.

Absorption of antibodies with recombinant p16 protein. Sera (2 μl) were added into 100 μl phosphate-buffered saline (PBS) with different amount of p16 recombinant proteins (final concentration: 0.5, 1.0 and 1.2 μg/ml) and incubated overnight at room temperature. This mixture was centrifuged at 10000 g for 15 min, and the supernatant was recovered and then diluted again for use in ELISA and Western blotting.

Statistical analysis. To determine whether the frequency of autoantibodies to p16 in each cohort of cancer patient sera was significantly higher than that in sera from normal individuals, the data were analyzed using the χ^2 test with Yates' correction. Two significant levels (0.05 and 0.01) were used. Methods for calculating the sensitivity/specificity, false positive/false negative and positive predictive value/negative predictive value was based on the methodology provided by Gordis (20).

Results

Detection of antibodies to p16 in serum samples from cancer patient using ELISA. As described in Materials and methods, p16 cDNA was amplified by RT-PCR from human HeLa cells, and the PCR products were subcloned into the pGEX vector. The GST gene fusion system was used for the expression and purification of p16 recombinant protein. In this study, the full length recombinant p16 protein was used as antigen, and sera from patients with different types of cancer and normal individuals were examined for the presence of antibodies to p16. Table I shows the frequency of antibodies to p16 in ELISA. The cancer sera tested included 479 patients from China with different types of cancer including breast, cervical, colon, esophageal, gastric, lung, nasopharyngeal and ovarian cancer as well as hepatocellular

Table I. Frequency of antibodies to three tumor-associated antigens p16, c-myc and p53 in ELISA.

Type of cancer	No. of tested	Antibodies to p16 no. (%)	c-myc no. (%)	p53 no. (%)	Any of three antigens no. (%)
Breast	41	7 (17.1)[b]	9 (22)[b]	5 (12.2)[b]	18 (43.9)[b]
Cervical	16	1 (6.3)	4 (25)[b]	1 (6.3)	5 (31.3)[b]
Colorectal	57	6 (10.5)[a]	8 (14)[b]	5 (8.8)[a]	16 (28.1)[b]
Esophageal	71	10 (14.1)[b]	5 (7)[a]	5 (7)[a]	16 (22.5)[b]
Gastric	74	8 (11)[a]	2 (2.7)	6 (8.1)[a]	16 (21.6)[b]
Hepatocellular	77	15 (21.4)[b]	1 (1.3)	7 (9.1)[a]	23 (29.9)[b]
Lung	112	3 (3)	14 (12.5)[b]	8 (7.1)[a]	20 (17.9)[b]
Nasopharyngeal	14	4 (28.6)[b]	1 (7.1)[a]	1 (7.1)[a]	6 (42.9)[b]
Ovarian	17	2 (11.8)[a]	0	1 (5.9)	2 (11.8)[a]
Total	479	56 (11.7)[a]	44 (9.2)[b]	39 (8.1)[a]	122 (25.5)[b]
NHS	82	1 (1.2)	0 (0)	1 (1.2)	2 (2.4)

Cut-off value, mean + 3SD of NHS; p-values relative to NHS, [a]$p<0.05$; [b]$p<0.01$.

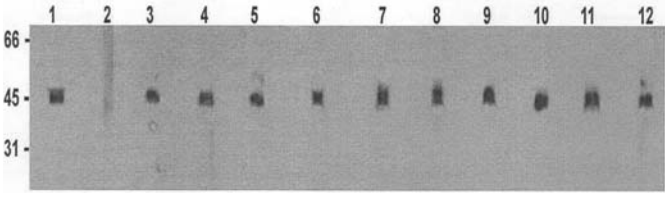

Figure 1. Western blot analysis showing representative cancer sera recognizing p16 recombinant protein. Lane 1, p16 monoclonal antibody was used as positive control; lane 2, a normal human serum was used as negative control; lanes 3-12, nine representative cancer sera which were positive in ELISA also have strong reactivity with p16 recombinant protein in Western blotting.

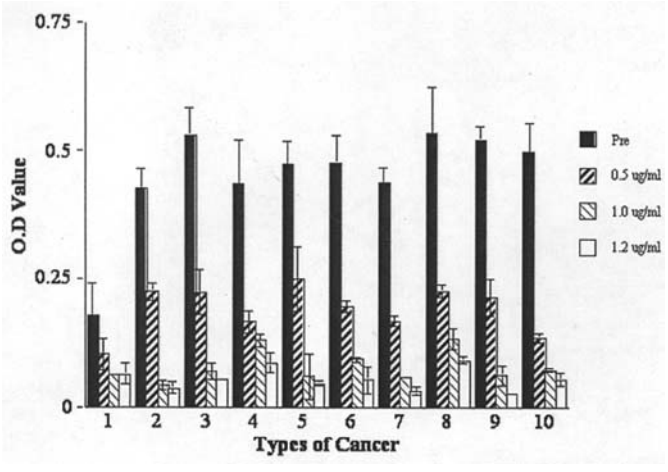

Figure 2. Absorption of antibody with recombinant p16 in ELISA. Sera with anti-p16 positive reactivity were pre-incubated with different concentration of p16 protein (0.5, 1.0, 1.2 μg/ml) and used subsequently for ELISA analysis. The decreasing of OD reading values for each sample indicated the specific absorption of anti-p16 antibody to the recombinant p16 protein. The experiment was repeated three times, and the average OD values were used to draw this figure.

carcinoma (HCC). As control group, 82 normal human sera from the same region in China as the cancer patients were also tested. The cut-off value designating positive reaction was the mean OD of 82 normal human sera (NHS) plus 3 standard deviations (SD). Of the 479 cancer sera analyzed, 11.7% (56/479) were shown to have antibodies to p16 by ELISA, especially in sera from cancer patients with nasopharyngeal (28.6%), breast (17.1%) and esophageal cancer (14.1%) as well as HCC (21.4%). Only one serum sample has a positive reaction with p16 in 82 normal individuals (1.2%). Of the 56 ELISA positive sera, 48 (85.7%) had positive reactions in Western blot. Representative samples in Western blot analysis are shown in Fig. 1.

The specificity of recombinant p16 as antigen for ELISA was confirmed by absorption studies. As shown in Fig. 2, nine cancer sera which had positive reactivity with p16 were pre-incubated by recombinant p16 with different concentrations (0.5, 1.0, 1.2 μg/ml) and used subsequently for ELISA. One normal human serum was also tested as control. Reactivity of cancer sera decreased substantially after pre-incubation with p16 protein. To verify the results from the p16 antigen-antibody absorption experiment in ELISA, five pre-absorbed anti-p16 positive cancer sera were also tested in Western blotting. As shown in Fig. 3, pre-absorbed cancer sera strongly react with p16 recombinant protein, and after treatment with p16 protein, the sera show no reactivity or much less reactivity.

Using a combination of three TAAs such as p16, p53 and c-myc to enhance antibody detection in cancer. Autoantibodies to tumor suppressor protein p53 and oncoprotein c-myc have been extensively detected and evaluated in different types of cancer (11). Antibody to p16 in cancer has not been reported. In order to address whether a combination of TAAs such as

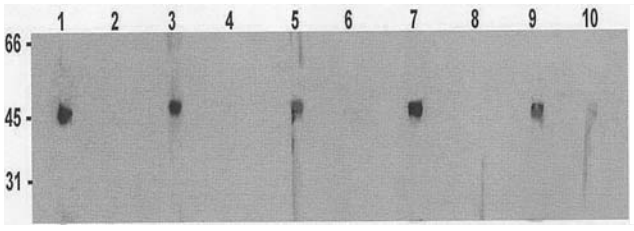

Figure 3. Absorption of antibodies with recombinant p16 in Western blotting. Five cancer sera which had positive reactivity with p16 were pre-incubated with p16 recombinant protein and used subsequently for Western blotting analysis. Lanes 1, 3, 5, 7 and 9 represent that 5 pre-absorbed positive cancer sera strongly react with p16 recombinant protein; lanes 2, 4, 6, 8 and 10 represent these 5 post-absorbed cancer sera have no reactivity or much less reactivity with p16 recombinant protein.

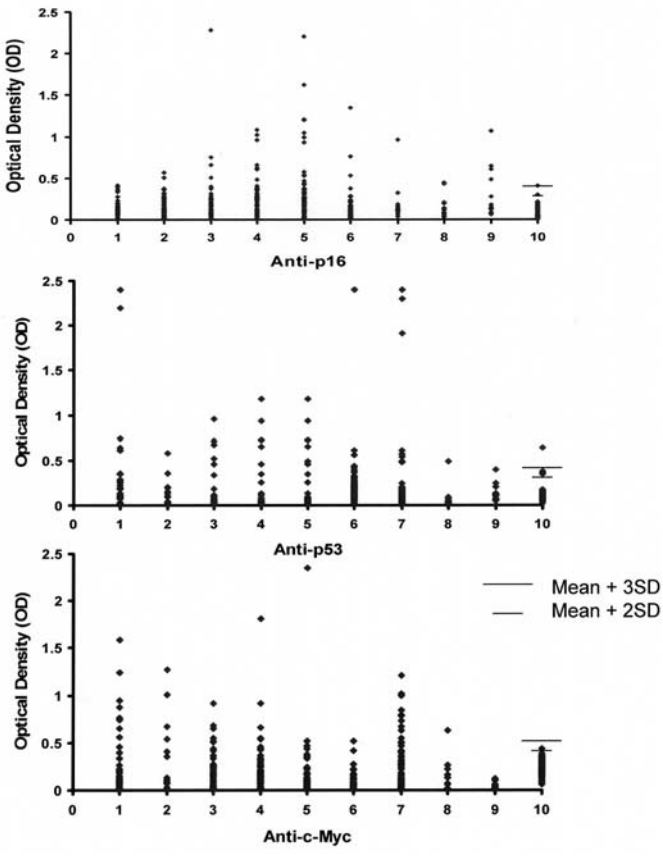

Figure 4. Titers of antoantibody to p16, p53 and c-myc in different cancers. The range of antibody titers to p16, p53 and c-myc is expressed as optical density (OD) obtained from enzyme-linked immunosorbent assay (ELISA). The mean + 3SD and mean + 2SD of 82 normal human sera are shown in relationship to OD value of the cancer sera. X-axis represents the type of cancer sera.1, breast cancer; 2, cervical cancer; 3, colorectal cancer; 4, esophageal cancer; 5, gastric cancer; 6, hepatic cancer (HCC); 7, lung cancer; 8, nasopharyngeal cancer; 9, ovarian cancer; 10, normal human sera (NHS).

p16, p53 and c-myc can enhance the detection of anti-bodies in cancer, the frequencies of antibodies to p53 and c-myc were also analyzed in this study. Table I shows the frequency of antibodies to these three TAAs in ELISA. The range of antibody titers to p16, p53 and c-myc are shown in Fig. 4. The high titer reactivity of many cancer sera and the distinct difference between cancer and normal sera were also demonstrated (Fig. 4). Many cancer sera showed OD value

Table II. Evaluation of antibodies to three TAAs (p16, c-myc and p53) as markers for detection of cancer.

Type of cancer	Sensitivity/ specificity (%)	False negative/ false positive (%)	Positive PV/ negative PV (%)
Breast	43.9/97.6	56.1/2.4	90.0/77.7
Cervical	31.3/97.6	68.7/2.4	71.4/87.9
Colorectal	28.1/97.6	71.9/2.4	88.9/66.1
Esophageal	22.5/97.6	77.5/2.4	88.9/59.3
Gastric	21.6/97.6	78.4/2.4	88.9/58.0
Hepato-cellular	29.9/97.6	70.1/2.4	88.5/59.7
Lung	17.9/97.6	82.1/2.4	50.0/46.5
Nasopha-ryngeal	42.9/97.6	57.1/2.4	75.0/93.0
Ovarian	11.8/97.6	88.2/2.4	50.0/84.2
Total	25.5/97.6	74.5/2.4	98.4/18.3

Calculation was based on the data from Table I; positive PV, positive predictive value; negative PV, negative predictive value.

several fold above the cut-off (mean + 3SD of normal sera), indicating that antibody responses to p16, p53 and c-myc in some cancer patients were quite robust, and not just elevated.

As shown in Table I, increased frequencies at $p<0.01$ were found for anti-p16 antibodies in breast, esophageal, nasopharyngeal cancer and HCC, and at $p<0.05$ in colorectal, gastric and ovarian cancer, but no increased frequencies were found in cervical and lung cancer. For antibodies to c-myc, increased frequencies at $p<0.01$ were found in breast, cervical, colorectal and lung cancer, and at $p<0.05$ in esophageal and nasopharyngeal cancer, but no increased frequencies were found in gastric, ovarian cancer and HCC. For antibodies to p53, increased frequencies at $p<0.01$ were only found in gastric cancer. These results indicate that there are different patterns of antibody frequency in individual cancer type. For example, anti-p16 is more sensitive for esophageal, gastric, nasopharyngeal cancer and HCC compared to antibodies to c-myc and antibodies to p53. It seems that antibody frequency to any individual TAA is variable. For example, the frequency of antibodies to any individual antigen ranges from 12 to 22% in breast cancer. With the successive addition of three TAAs, there was a stepwise increase of positive antibody reaction up to 43.9% in breast cancer. Similar results were also found in other types of cancer. The data suggest that the combination of antibodies might acquire higher sensitivity for cancer detection.

The validity of a test is defined as its ability to distinguish between who has a disease and who does not. In order to address the question of how good is the approach of antibody detection to these three TAAs in separating people with and without cancer in question, the sensitivity/specificity, false negative/false positive and positive predictive value/negative predictive value were calculated and summarized in Table II.

Although the sensitivity of these three TAAs as markers in cancer detection is low (ranging from 11.8 to 43.9), the specificity appears to be very high (97.6%). It is consistent with the results of other two components (false negative and false positive). The positive and negative predictive values were also variable in most types of cancer. In total cancer group, the positive predictive value was quite high (98.4%), and the negative predictive value was only 18.3%.

Discussion

The p16 protein is a cyclin-dependent kinase (CDK) inhibitor, which regulates cell proliferation negatively by inhibiting the kinase activity of Cdk4 and Cdk6, and subsequently promotes phosphorylation and inactivation of the tumor suppressor protein pRb (21). p16 gene mutation was reported in a variety of tumor cell lines and primary tumors (22-25). Overexpression of p16 protein has been found in many types of human malignancy, such as gastric (26), prostate (27) and colorectal cancer (28). In addition, previous studies have also demonstrated that p16 overexpression was associated with tumor progression and poor prognosis in different tumors such as prostate (29), ovarian (30) and breast cancer (31). However, the normal human tissues display low or undetectable levels of p16 protein (27,32-34). Taken together, it could be considered that p16 might be used as a marker for tumor progression and prognosis as well as for tumor diagnosis. Whether or not the p16 protein can induce humoral immune response in cancer, and the antibody to p16 can be used as a marker for detection of cancer remains to be investigated.

In this study, we used recombinant p16 protein as coating antigen in ELISA for the detection of anti-p16 antibody in sera from 479 patients with 9 different types of cancer. Antibodies to p16 were detected in 11.7%, with a significant difference from the normal individuals. Frequencies of anti-p16 antibodies were found in a higher percentage in breast cancer (17.1%) and nasopharyngeal cancer (28.6%) as well as HCC (21.4%). The results from ELISA were also verified by Western blot analysis to confirm the specificity of anti-p16 detection. The ELISA data were in good agreement with the results of Western blotting. Antibodies to p53 and c-myc were also analyzed for their relationship with the anti-p16 antibody. Frequencies of antibodies to p53 and c-myc were 8.1% and 9.2%, respectively. The frequency of antibodies to any individual one of these three antigens was variable but rarely exceeded 15-20%. With the successive addition of three TAAs, there was a stepwise increase of positive antibody reaction up to 25.5% for the total group of cancer patients, but in certain type of cancers the frequencies were over 40%, for example breast cancer (43.9%) and nasopharyngeal cancer (42.9%). Although antibodies to the 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 the individual cancer types. For example, in cervical and colorectal and lung cancer, c-myc appears to be more reactive antigen than p16 and p53; in esophageal, hepatic (HCC) and nasopharyngeal cancer, p16 was more reactive, whereas in breast cancer all the three antigens showed strong reactivity. 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 (35). One recent study demonstrated that the mRNA expression of two tumor suppressor proteins p16 and p14 might play an important role in the pathogenesis of haematological malignancies (36), and another study showed that c-myc and p53 gene alterations are important for tumor metastasis, as well as genetic and pathological staging (37). If autoimmune responses represent immune system reactions to abnormal gene expression (2), one might expect differences in autoantibody profiles from one type of cancer tissue to another. Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence of these autoantibodies in normal individuals and in non-cancer conditions. Enthusiasm for this approach has been tempered by low sensitivity. Data from this study on the one hand indicate that the combination of antibodies might acquire higher sensitivity for diagnosis of cancer. On the other hand, the data also show that in the selection of different antigen-antibody systems, some of the antigens may turn out to be more specific for a certain type of cancer while others not. A comprehensive analysis and evaluation of various combinations of selected antibody-antigen systems will be useful for the development of autoantibody profiles involving different panels or arrays of TAAs, and the results could be useful for the detection and diagnosis of certain types of cancer.

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References

1. Tan EM: Autoantibodies in pathology and cell biology. Cell 67: 841-842, 1991.
2. Tan EM: Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. J Clin Invest 108: 1411-1415, 2001.
3. Old LJ and Chen Y-T: New paths in human cancer serology. J Exp Med 187: 1163-1167, 1998.
4. Zhang JY, Chan EKL, Peng XX, Lu M, Wang X, Mueller F and Tan EM: Autoimmune responses to mRNA binding proteins p62 and Koc in diverse malignancies. Clin Immunol 100: 149-156, 2001.
5. Zhang JY: Tumor-associated antigen arrays to enhance antibody detection for cancer diagnosis. Cancer Detect Prev 28: 114-118, 2004.
6. Vogelstein B and Kinzler KW: The multistep nature of cancer. Trends Genet 9: 138-141, 1993.
7. Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EKL and Tan EM: Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. Cancer Epidemiol Biomarkers Prev 12: 136-143, 2003.
8. Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501-1512, 1999.

9. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D and Barrett JC: Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc Natl Acad Sci USA 93: 13742-13747, 1996.
10. Ruas M and Peters G: The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378: F115-F177, 1998.
11. Soussi T: p53 antibodies in the sera of patients with various types of cancer (Review). Cancer Res 60: 1777-1788, 2000.
12. Yamamoto A, Shimizu E, Takeuchi E, Houchi H, Doi H, Bando H, Ogura T and Sone S: Infrequent presence of anti-c-Myc antibodies and absence of c-Myc oncoprotein in sera from lung cancer patients. Oncology 56: 129-133, 1999.
13. Zhang JY, Chan EKL, Peng XX and Tan EM: A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma. J Exp Med 189: 1101-1110, 1999.
14. Zhang JY and Chan EKL: Autoantibodies to IGF-II mRNA binding protein p62 and overexpression of p62 in human hepatocellular carcinoma. Autoimmun Rev 1: 46-153, 2002.
15. Rohayem J, Diestelkoetter P, Weigle B, Oehmichen A, Schmitz M, Mehlhorn J, Conrad K and Rieber EP: Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. Cancer Res 60: 1815-1817, 2000.
16. Megliorino R, Shi FD, Peng XX, Wang X, Chan EKL, Tan EM and Zhang JY: Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection. Cancer Detect Prev 29: 241-248, 2005.
17. Pfeffer U, Fecarotta E and Vidali G: Efficient one tube RT-PCR amplification of rare transcripts using short sequence-specific reverse transcription primers. Biotechniques 18: 204-206, 1995.
18. Rubin RL: Enzyme-linked immunosorbent assays for antibodies to native DNA, histones, and (H2A-H2B)-DNA. In: Manual of Clinical Laboratory Immunology. Rose NR, Conway de Macario E, Folds JD, Lane HC and Nakamura RM (eds). 5th edition. American Society for Microbiology, Washington DC, pp935-941, 1997.
19. Chan EKL and Pollard KM: Detection of autoantibodies to ribonucleoprotein particles by immunoblotting. In: Manual of Clinical Laboratory Immunology. Rose NR, Conway de Macario E, Folds JD, Lane HC and Nakamura RM (eds). 5th edition. American Society for Microbiology, Washington, pp928-934, 1997.
20. Gordis L: Assessing the validity and reliability of diagnostic and screening tests. In: Epidemiology. Gordis L (ed). 3d edition. Elsevier Saunders, Philadelphia, pp71-94, 2004.
21. Bringold F and Serrano M: Tumor suppressors and oncogenes in cellular senescence. Exp Gerontol 35: 317-329, 2000.
22. Jen J, Harper JW, Bigner SH, *et al*: Vogelstein, deletion of p16 and p15 genes in brain tumors. Cancer Res 54: 6353-6358, 1994.
23. Orlow I, Lacombe L, Hannon GJ, *et al*: Deletion of the p16 and p15 genes in human bladder tumors. J Natl Cancer Inst 87: 1524-1529, 1995.
24. Reed AL, Califano J, Cairns P, *et al*: High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res 56: 3630-3633, 1996.
25. Washimi O, Nagatake M, Osada H, *et al*: *In vivo* occurrence of p16 (MTS1) and p15 (MTS2) alterations preferentially in non-small cell lung cancers. Cancer Res 55: 514-517, 1995.
26. Feakins RM, Nickols CD, Bidd H and Walton SJ: Abnormal expression of pRb, p16 and cyclin D1 in gastric adenocarcinoma and its lymph node metastases: relationship with pathological features and survival. Hum Pathol 34: 1276-1282, 2003.
27. Lee CT, Capodieci P, Osman I, Fazzari M, Ferrara J, Scher HI and Cordon-Cardo C: Overexpression of the cyclin-dependent kinase inhibitor p16 is associated with tumor recurrence in human prostate cancer. Clin Cancer Res 5: 977-983, 1999.
28. Zhao P, Hu YC and Talbot IC: Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma. World J Gastroenterol 9: 2202-2206, 2003.
29. Halvorsen OJ, Hostmark J, Haukaas S, Hoisaeter PA and Akslen LA: Prognostic significance of p16 and CDK4 proteins in localized prostate carcinoma. Cancer 88: 416-424, 2000.
30. Dong Y, Walsh MD, McGuckin MA, *et al*: Increased expression of cyclin-dependent kinase inhibitor 2 (CDKN2A) gene product P16INK4A in ovarian cancer is associated with progression and unfavourable prognosis. Int J Cancer 74: 57-63, 1997.
31. Dublin EA, Patel NK, Gillett CE, Smith P, Peters G and Barnes DM: Retinoblastoma and p16 proteins in mammary carcinoma: their relationship to cyclin D1 and histopathological parameters. Int J Cancer 79: 71-75, 1998.
32. Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, van Tornout JM and Jones PA: Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res 55: 4531-4535, 1995.
33. Jarrard DF, Bova GS, Ewing CM, *et al*: Deletional, mutational and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. Genes Chromosomes Cancer 19: 90-96, 1997.
34. Shapiro GI, Edwards CD, Kobzik L, Godleski J, Richards W, Sugarbaker DJ and Rollins BJ: Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines. Cancer Res 55: 505-509, 1995.
35. Bartek J and Lukas J: Are all cancer genes equal? Nature 411: 1001-1002, 2001.
36. Lee YK, Park JY, Kang HJ and Cho HC: Overexpression of p16INK4A and p14ARF in haematological malignancies. Clin Lab Haematol 25: 233-237, 2003.
37. Yakut T, Egeli U and Gebitekin C: Investigation of c-myc and p53 gene alterations in the tumor and surgical borderline tissues of NSCLC and effects on clinicopathologic behavior: by the FISH technique. Lung 181: 245-258, 2003.