

Detection of autoantibodies to multiple tumor-associated antigens (TAAs) in the immunodiagnosis of breast cancer

Weihong Liu · Ignacio García De La Torre ·
María Cristina Gutiérrez-Rivera · Bo Wang · Yuan Liu ·
Liping Dai · Wei Qian · Jian-Ying Zhang

Received: 22 August 2014 / Accepted: 20 October 2014 / Published online: 30 October 2014
© International Society of Oncology and BioMarkers (ISOBM) 2014

Abstract We aimed to evaluate the immunodiagnostic values of autoantibodies to a panel of six tumor-associated antigens (TAAs) in the detection of patients with breast cancer. This study determines whether a panel of multiple TAAs would enhance antibody detection and be a useful approach in breast cancer detection and diagnosis. The panel of multiple TAAs was composed of six TAAs including Imp1, p16, Koc, survivin, cyclin B1, and c-myc full-length recombinant proteins. Enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies against these six TAAs in 49 sera from patients with breast cancer, 35 sera from patients with benign breast tumor, and 38 sera from normal individuals. Antibody frequency to any individual TAA in breast cancer was variable and ranged from 12.2 to 18.4 %. With the successive addition of TAAs to a final total of six antigens, there was a stepwise increase of positive antibody reactions reaching a sensitivity of 67.3 % and a specificity of 92.2 % in breast cancer. Positive and negative likelihood ratios were 8.52 and 0.36, respectively, which showed that the clinical diagnostic value of a parallel assay of six TAAs was high.

Positive and negative predictive values were, respectively, 91.7 and 68.6 %, indicating that the parallel assay of six TAAs raised the diagnostic accuracy greatly. Agreement rate and kappa value were 78.1 % and 0.57, respectively, which indicated that the observed value of this assay had a middle range of coincidence with the actual value. The data from this study further support our previous hypothesis that the detection of autoantibodies for diagnosis of a certain type of cancer can be enhanced by using a panel of several carefully selected TAAs as target antigens and a panel of multiple TAAs would be a useful approach in the detection and diagnosis of breast cancer.

Keywords Autoantibody · Tumor-associated antigen · Breast cancer · Immunodiagnosis

Introduction

Breast cancer is the most frequent malignant tumor and a leading cause of cancer mortality among women in North America [1]. As with all cancers, early diagnosis and appropriate therapy can reduce the mortality rate of breast cancer and early detection is one of the most significant predictors of survival.

Many studies have demonstrated that cancer sera contain antibodies which react with a unique group of autologous cellular antigens generally known as tumor-associated antigens (TAAs) [2–4]. The generation of these antibodies is the result of the immune response to the dysregulation of the function of certain cellular proteins or the alternation of the molecular structure of these proteins. Antigenic changes in cancer cells can be recognized by the immune system of patients themselves and to respond by producing autoantibodies. These autoantibodies can be used as reporters identifying aberrant cellular mechanisms in tumorigenesis and also

W. Liu (✉) · B. Wang
Department of Clinical Laboratory Technology, Dalian Municipal
Central Hospital, Dalian 116033, Liaoning, China
e-mail: weihongliu1120@hotmail.com

W. Liu · B. Wang · Y. Liu · L. Dai · W. Qian · J.-Y. Zhang (✉)
Department of Biological Sciences, University of Texas at El Paso,
500 West University Avenue, El Paso, TX 79968, USA
e-mail: jzhang@utep.edu

I. G. De La Torre · M. C. Gutiérrez-Rivera
Department of Immunology and Rheumatology, Hospital General de
Occidente and University of Guadalajara, Guadalajara, Jalisco,
Mexico

W. Qian · J.-Y. Zhang
College of Sino-Dutch Biomedical and Information Engineering,
Northeastern University, Shenyang 110189, Liaoning, China

serve as immunodiagnostic markers for cancer detection. Through serological analysis of recombinant complementary DNA (cDNA) expression libraries (SEREX) and proteomic technology, a variety of antigens were identified in recent years. These cellular proteins include mRNA-binding proteins such as p62 [5], Imp1 [6], and Koc [7]; tumor-suppressor protein p53 [8, 9]; cell-cycle nuclear protein cyclin B1 [10]; inhibitor of apoptosis survivin [11]; transcription factor oncoprotein c-myc [12]; and so on. 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 noncancer conditions. Our previous studies observed that if a single TAA was used as target antigen, the sensitivity for cancer immunodiagnosis was usually low, and if a panel of multiple TAAs was used, it can overcome this drawback. The different types of cancer may require different panels of TAAs to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis [13–16]. In the current study, we aimed to evaluate whether a panel of six TAAs would enhance autoantibody detection and be a useful approach in breast cancer diagnosis.

Materials and methods

Serum samples and antibodies

In this study, 49 sera from breast cancer, 35 sera from patients with benign breast tumor, and also 38 normal human sera (NHS) were derived from the sera bank in the Cancer Autoimmunity Research Laboratory at the University of Texas, El Paso (UTEP). These sera were originally provided by our clinical collaborators in Mexico. All cancer sera were collected at the time of cancer diagnosis when the patients had not yet received treatment with any chemotherapy or radiotherapy. All normal human sera were collected from adults during annual health examination in people who had no obvious evidence of malignancy. Due to the regulations concerning studies of human subjects, the patients' names and identification numbers were blinded to investigators. This study was approved by the Institutional Review Board of UTEP and collaborating institutions.

Expression and purification of recombinant TAAs

Six TAAs, including Imp1, p16, Koc, survivin, cyclin B1, and c-myc, were selected for the expression of recombinant proteins. All these six antigens were used for the detection of different types of cancer in our previous study, and the sensitivity and specificity of antibodies against these antigens in cancer sera have been confirmed [13–16]. In brief, Koc cDNA

cloned in the pcDNA3 vector [13] was subcloned to the pET28a vector and the recombinant protein expressed in *Escherichia coli* BL21 (DE3) was purified using nickel column chromatography. Imp1 construct pCMV5-Imp1 was provided by F.C. Nielsen [6] subcloned into pET28a for protein expression. cDNA from c-myc was amplified by polymerase chain reaction from human fetal liver tissue and survivin cDNA from human survivin EST clone (BG258433) before subcloning in the pET28a vector. Recombinant cyclin B1 had been prepared and used previously [10] and was isolated from a pGEX construct expressing cyclin B1 with glutathione *S*-transferase (GST) fusion partner. p16 cDNA was amplified by RT-PCR from human HeLa cells and was subcloned into the pGEX vector. The GST gene fusion system was used for the expression and purification of p16 recombinant protein. The expression of adequate amounts of recombinant protein was examined in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Coomassie blue staining was used to determine that expression products of expected molecular sizes were produced.

Enzyme-linked immunosorbent assay

Purified recombinant proteins (Imp1, p16, Koc, survivin, cyclin B1, and c-myc) were individually diluted in phosphate-buffered saline (PBS) to a final concentration of 0.5 µg/ml, and 100 µl was added into each well to coat microtiter plates (Fisher Scientific, Houston, TX, USA) overnight at 4 °C. Plates were blocked with gelatin post-coating solution for 2 h at room temperature. The human serum samples were diluted at 1:200, incubated with the antigen-coated wells at 37 °C for 90 min followed by washing with PBS containing 0.05 % Tween 20 (PBST), and then incubated with goat antihuman IgG-HRP (Invitrogen, NY) diluted 1:4000 for 90 min followed by washing with PBST. The substrate 2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS, Invitrogen) was used as detecting reagent. The optical density (OD) value of each well was read at 405 nm. Each sample was tested in duplicate. The cutoff value for determining a positive reaction was designated as the mean OD value of the 38 normal human sera plus 2 standard deviations (mean+2 SD). As described in previous studies, eight normal human sera, representing a range of 2 SD above and below the mean of the 38 normal human sera, were always used in each experiment and the average value of the eight normal sera was used in each run to normalize all absorbance values to the standard mean of the entire 38 normal samples. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. The detailed protocol of enzyme-linked immunosorbent assay (ELISA) was used as described in our previous studies [13–16].

Western blotting

Serum samples that were determined to contain autoantibodies using ELISA were further tested by Western blotting to confirm the immunoreactivity to corresponding TAAs. In brief, the purified recombinant proteins (Imp1, Koc, c-myc, cyclin B1, survivin, and p16) were electrophoresed on 10 % SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. After blocking with PBST containing 3 % nonfat dry milk for 1 h at room temperature, the nitrocellulose papers were incubated for 60 min at room temperature with a 1:200 dilution of serum. HRP-conjugated goat antihuman IgG was applied as secondary antibodies at a 1:20,000 dilution. Immunoreactive bands were detected using the ECL kit (Thermo Scientific) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS13.0. The data were analyzed using receiver operating characteristic (ROC) curve and the χ^2 tests with Yates' correction. Two statistically significant levels (0.05 and 0.01) were used, and $P < 0.05$ was considered to indicate statistically significant differences. The comprehensive evaluations of the testing results for each anti-TAA antibody, including the methods for calculating the sensitivity, specificity, Youden's index (YI), positive and negative likelihood ratios, positive (+PV) and negative predictive values (−PV), agreement rate, and kappa value, were based on the methodology provided in the *Epidemiology* textbook [17].

Results

Prevalence of antibodies to a panel of six TAAs in breast cancer

In the present study, a panel of six TAAs was used as coating antigens in ELISA, and sera from 49 patients with breast cancer, 35 patients with benign breast tumor, and 38 normal individuals were examined for the presence of antibodies to the individual TAA and cumulatively to the entire panel of six TAAs. The panel of TAAs used in this study includes Imp1, p16, Koc, survivin, cyclin B1, and c-myc full-length recombinant proteins rather than peptide fragments of these proteins. Table 1 shows the frequency of antibodies to a panel of six TAAs in three groups of sera. When the antibody levels of six TAAs in patients with breast cancer were compared to those of patients with benign breast tumor and to those of normal individuals through the ROC curve, the area under the curve of each TAA is more than 0.5 (Fig. 1). These data indicated

that there was a statistically significant difference of the antibody levels in sera between the breast cancer group and the others. In this study, antibody frequency to individual TAA in breast cancer was variable from 12.2 to 18.4 %. Out of 49 sera from patients with breast cancer analyzed, 67.3 % (33/49) were shown to have antibodies cumulatively to any of these six TAAs, which was significantly higher than the frequency in sera from normal individuals (7.8 %). The ELISA results were also confirmed by immunoblotting analysis. The higher frequency of antibodies to an individual TAA in breast cancer was against cyclin B1 (18.4 %) and Imp1 (18.4 %), Koc (16.3 %), survivin (16.3 %), p16 (14.3 %), and followed by c-myc (12.2 %). The reactivity of benign breast tumor sera and normal human sera was relatively low, ranging from 0 to 8.6 % and from 0 to 2.6 % to any individual TAA, respectively, and the combined frequency of reactivity was 14.2 and 7.8 % against a panel of six TAAs.

Evaluation of diagnostic values of a panel of six TAAs in immunodiagnosis of breast cancer

The validity of a test is defined as its ability to distinguish between who has a disease and who does not. To address the question of how valuable is the approach of antibody detection to a panel of multiple TAAs in separating people with and without cancer in question, a group of parameters, such as the sensitivity/specificity, Youden's index, the +PV/−PV, and so on, were calculated and summarized in Tables 2 and 3. Table 2 shows the comprehensive evaluation of antibodies to a panel of six TAAs. With the successive addition of TAAs to a total of six antigens, there was a stepwise increase of positive antibody reactions up to 67.3 % and there was also a slight decrease of specificity from 97.4 % with one TAA to 92.2 % with a panel of six TAAs. It is consistent with the results of the other two parameters (+PV/−PV). The +PV/−PV were also variable in different combinations of TAAs. In the panel of a total of six TAAs, the +PV was 91.7 % and the −PV was 68.6 %. Youden's index was also increased from 0.16 with one TAA to 0.59 with six TAAs. The positive and negative likelihood ratios were 8.52 and 0.36, respectively, which indicated that the clinical diagnostic value of a parallel assay of six TAAs was high. It also suggests that a parallel assay of six TAAs can raise the diagnostic precision greatly. Agreement rate and kappa value were 78.1 % and 0.57, respectively, which indicated that the observed value of this assay had a middle range of coincidence with the actual value. Taken together, these data show the usefulness of the multiple-antigen array in increasing the clinical diagnostic quality and value for cancer. Positive results of ELISA were also confirmed by Western blot. Western blot analysis of eight representative breast cancer sera is shown in Fig. 2.

Table 1 Frequency of autoantibodies to six TAAs in breast cancer

Sera	No. tested	No. and percentage of autoantibodies to						
		cyclin B1 TAAs	Imp1	Koc	survivin	p16	c-myc	Cumulative to six
BC	49	9 (18.4)*	9 (18.4)*	8 (16.3)**	8 (16.3)**	7 (14.3)**	6 (12.2)**	33 (67.3)*
BBT	35	3 (8.6)	0 (0)	0 (0)	2 (5.7)	1 (2.9)	1 (2.9)	5 (14.2)**
NHS	38	1 (2.6)	0 (0)	1 (2.6)	1 (2.6)	0 (0)	0 (0)	3 (7.8)

BC breast cancer, BBT benign breast tumor, NHS normal human sera

* $p < 0.01$; ** $p < 0.05$ (relative to NHS)

Discussion

It was well demonstrated that breast cancer is one of the most common malignant tumors in women. In recent years, the incidence of breast cancer increased rapidly in some countries, especially in China [18, 19]. Despite tremendous progress, 40 % of patients diagnosed with breast cancer still succumb to the disease. The high mortality and case fatality rate can in part be attributed to a lack of diagnostic methods that allow early detection. Although mammograms are the most effective tool to detect breast cancer, the US Food and Drug Administration (FDA) reports that mammography can find only about 80 % of breast cancers in women [1]. Hence, there is a need for further understanding of tumor biology and host immune response mechanisms so that new diagnostic and therapeutic tools can be developed. Early diagnosis is essential for the optimal management of breast cancer. Thus, extensive studies are being conducted to identify new biomarkers and validate some existing biomarkers that would add to current markers and increase the sensitivity and specificity of breast cancer detection.

Many studies have demonstrated that the immune systems of certain cancer patients can sense some aberrant tumor-associated proteins as unknown antigens and still have the capability to respond by producing autoantibodies [20]. Thus, cancer-associated autoantibodies might be regarded as reporters, identifying aberrant de novo or dysregulated cellular mechanisms in tumorigenesis [2, 3]. In recent years, the potential utility of TAA-autoantibody systems as early cancer biomarker tools to monitor therapeutic outcomes or as indicators of disease prognosis has been explored.

Interest in the use of anti-TAA autoantibodies as serological markers for cancer diagnosis derives from the recognition that these autoantibodies are generally absent or present in very low titers in normal individuals and in noncancer conditions (except autoimmune conditions). Their persistence and stability in the serum of cancer patients is an advantage over other potential markers, including the TAAs themselves, which are released by tumors but are rapidly degraded or cleared after circulating in the serum for a limited time [20]. Furthermore, the widespread availability of methods and reagents to detect serum autoantibodies facilitates their

Fig. 1 ROC analyses for each TAA. Receiver operating characteristic curve for each individual TAA exhibiting different levels between breast cancer patients and benign breast tumor patients (a) or breast cancer patients and normal individuals (b)

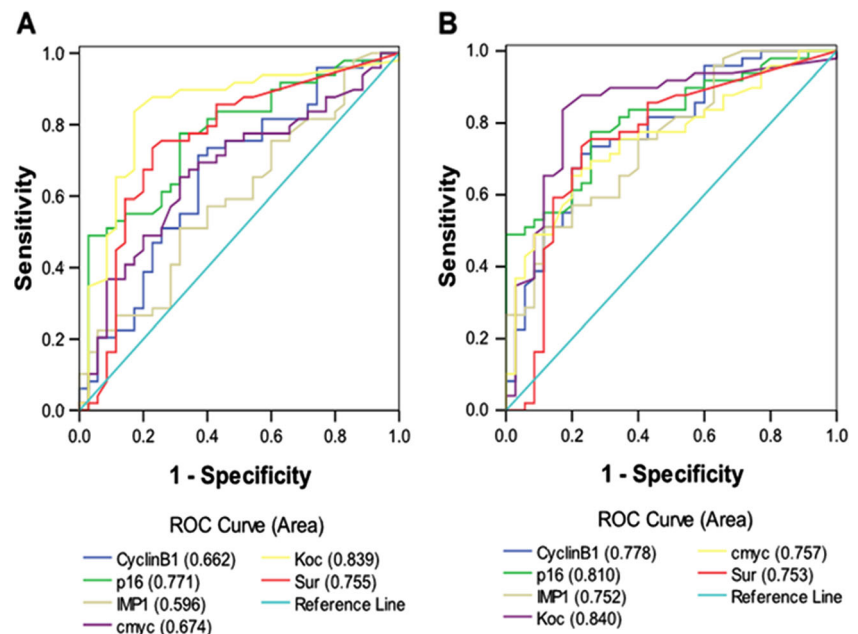


Table 2 Evaluation of diagnostic value of antibodies to different combinations of TAAs in breast cancer

TAAs	BC (%)	BBT (%)	NHS (%)	Sen (%)	Spe (%)	YI	+PV	−PV
Cyclin B1	9 (18.4)	3 (8.6)	1 (2.6)	18.4	97.4	0.16	90.0	48.0
Cyclin B1+Imp1	15 (30.6)	3 (8.6)	1 (2.6)	30.6	97.4	0.28	93.7	52.1
Cyclin B1+Imp1+Koc	21 (42.8)	3 (8.6)	2 (5.2)	42.8	94.8	0.37	91.3	56.2
Cyclin B1+Imp1+Koc+survivin	25 (51.0)	4 (11.4)	3 (7.8)	51.0	92.2	0.43	89.2	59.3
Cyclin B1+Imp1+Koc+survivin+p16	29 (59.1)	5 (14.2)	3 (7.8)	59.1	92.2	0.51	90.6	63.6
Cyclin B1+Imp1+Koc+survivin+p16+c-myc	33 (67.3)	5 (14.2)	3(7.8)	67.3	92.2	0.59	91.7	68.6

BC relative to NHS, Sen sensitivity, Spe specificity, YI Youden’s index, +PV positive predictive value, −PV negative predictive value

characterization in cancer patients and assay development. However, in contrast to autoimmune diseases, where the presence of a particular autoantibody may have diagnostic value, cancer-associated autoantibodies, when evaluated individually, have little diagnostic value primarily because of their low frequency, sensitivity, and specificity [21]. We have observed that this drawback can be circumvented by using mini-arrays of carefully selected TAAs and that different types of cancer may require different TAA arrays to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis.

Our current study has tested breast cancer sera for the presence of autoantibodies to a panel of six selected recombinant TAAs using ELISA and found that the combined autoantibody frequency was 67.3 % (33/49), significantly higher than the frequency in sera from normal individuals (7.8 %; 3/38) and benign breast tumor (14.2 %, 5/35). Antibody frequency to any individual TAA in breast cancer was variable, ranging from 12.2 to 18.4 %. The higher frequencies of antibodies to an individual TAA in breast cancer were against Imp1 (18.4 %) and cyclin B1 (18.4 %), followed by Koc (16.3 %), survivin (16.3 %), p16 (14.3 %), and c-myc

(12.2 %). If we add individual TAA successively to a total of six antigens (cyclin B1, p16, Imp1, Koc, c-myc, and survivin), there was a stepwise increase of sensitivity up to 67.3 % and the specificity remained over 92.2 %. The results indicate that an array of six TAAs might be sufficient to distinguish serologically a breast cancer population from a normal population. However, it needs to be determined whether this TAA combination distinguishes breast cancer from other cancers. Positive results were also confirmed by Western blot. These six TAAs have been used in our previous studies for the detection of other types of cancer, such as liver cancer [13, 14]. The frequency of autoantibodies to these TAAs used in the current study for breast cancer is basically the same as the frequency in other cancers [13, 14]. Actually, this is still the concern of this approach in cancer detection, which may not be suitable to distinguish breast cancer from other types of cancers. As mentioned in the section of “Materials and methods,” most of the serum samples used in the current study were provided by our collaborators. In compliance with the regulations concerning studies of human

Table 3 Summary of diagnostic value of antibodies to a panel of six TAAs in breast cancer

	Any TAA positive	All TAA negative	Total
Breast cancer	33 (A)	16 (C)	49 (R ₁)
NHS	3 (B)	35 (D)	38 (R ₂)
Total	36 (C ₁)	51 (C ₂)	87 (N)

Fourfold table χ^2 test: $\chi^2=31.186, p<0.001$

Sensitivity (%)= $A/(A+C)=33/49=67.3\%$

Specificity (%)= $D/(B+D)=35/38=92.1\%$

Youden’s index= $\text{sensitivity}+\text{specificity}-1=0.673+0.921-1=0.594$

Positive (+) likelihood ratio= $\text{sensitivity}/(1-\text{specificity})=0.673/(1-0.921)=8.52$

Negative (−) likelihood ratio= $(1-\text{sensitivity})/\text{specificity}=(1-0.673)/0.921=0.36$

Percent agreement= $(A+D)/(A+B+C+D)\times 100\%=68/87=78.1\%$

Kappa= $[N(A+D)-(R_1C_1+R_2C_2)]/[N^2-(R_1C_1+R_2C_2)]=0.57$

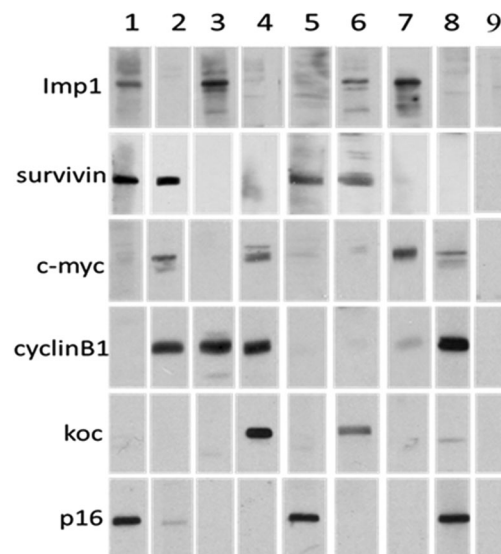


Fig. 2 Western blot analysis of eight representative breast cancer sera. Lanes 1–8 are eight representative breast cancer sera; lane 9 is a normal human serum. It shows different antibody profiles with the six TAAs

subjects, the patients' names and identification numbers were blinded to us. At the present time, we do not have the detailed clinical information from these patients. It is hard for us to further analyze the association between anti-TAA autoantibody biomarkers and some clinical parameters. This may be a limitation of the current study. In conclusion, these preliminary data further support our hypothesis and also suggest that additional breast cancer-specific TAAs will be needed to enhance the sensitivity and specificity of autoantibody detection using an array of multiple TAAs with potential immunodiagnostic value.

Acknowledgments The authors thank Dr. Eng M. Tan (The Scripps Research Institute) for his support. This work was supported by a grant (SC1CA166016) from the National Institutes of Health (NIH). We also thank the Border Biological Research Center (BBRC) Core Facilities at The University of Texas at El Paso (UTEP) for their support, which were funded by an NIH grant (5G12MD007592).

Conflicts of interest None

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics. *CA Cancer J Clin.* 2013;63:11–30.
2. Tan EM. Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. *J Clin Invest.* 2001;108:1411–5.
3. Tan EM, Zhang J. Autoantibodies to tumor-associated antigens: reporters from the immune system. *Immunol Rev.* 2008;222:328–40.
4. Old LJ, Chen YT. New paths in human cancer serology. *J Exp Med.* 1998;187:1163–7.
5. Zhang JY, Chan EKL, Peng XX, Tan EM. A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma. *J Exp Med.* 1999;189:1101–10.
6. Nielsen J, Christiansen J, Lykke-Andersen J, Johnsen AH, Wewer UM, Nielsen FC. A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. *Mol Cell Biol.* 1999;19:1262–70.
7. Muller-Pillasch F, Lacher U, Wallrapp C, Micha A, Zimmerhackl F, Hameister H, et al. Cloning of a gene highly overexpressed in cancer coding for a novel KH-domain containing protein. *Oncogene.* 1997;14:2729–33.
8. Crawford LV, Pim DC, Bulbrook RD. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *Int J Cancer.* 1982;30:403–8.
9. Soussi T. p53 antibodies in the sera of patients with various types of cancer: a review. *Cancer Res.* 2000;60:1777–88.
10. Covini G, Chan EK, Nishioka M, Morshed SA, Reed SI, Tan EM. Immune response to cyclinB1 in hepatocellular carcinoma. *Hepatology.* 1997;25:75–80.
11. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med.* 1997;3:917–21.
12. Yamamoto A, Shimizu E, Takeuchi E, Houchi H, Doi H, Bando H, et al. Infrequent presence of anti-c-Myc antibodies and absence of c-Myc oncoprotein in sera from lung cancer patients. *Oncology.* 1999;56:129–33.
13. Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EKL, Tan EM. Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. *Cancer Epidemiol Biomarkers Prev.* 2003;12:136–43.
14. Zhang JY, Megliorino R, Peng XX, Tan EM, Chen Y, Chan EKL. Antibody detection using tumor-associated antigen mini-array in immunodiagnosing human hepatocellular carcinoma. *J Hepatol.* 2007;46:107–14.
15. Chen Y, Zhou Y, Qiu S, Wang K, Liu S, Peng XX, et al. Autoantibodies to tumor-associated antigens combined with abnormal alpha-fetoprotein enhance immunodiagnosis of hepatocellular carcinoma. *Cancer Lett.* 2010;289:32–9.
16. Dai L, Ren P, Liu M, Imai H, Tan EM, Zhang JY. Using immunomic approach to enhance tumor-associated autoantibody detection in diagnosis of hepatocellular carcinoma. *Clin Immunol.* 2014;152:127–39.
17. Gordis L. Assessing the validity and reliability of diagnostic and screening tests. In: Gordis L, editor. *Epidemiology.* 2nd ed. Philadelphia: Saunders; 2000. p. 63–81.
18. Chen WQ, Zheng RS, Zeng HM, Zhang SW, Li GL, Wu LY, et al. Incidence and mortality of breast cancer in China, 2008. *Thorac Cancer.* 2013;4:59–65.
19. Zeng H, Zheng R, Zhang S, Zou X, Chen W. Incidence and mortality of female breast cancer in China, 2009. *Thorac Cancer.* 2013;4:400–4.
20. Anderson KS, LaBaer J. The sentinel within: exploiting the immune system for cancer biomarkers. *J Proteome Res.* 2005;4:1123–33.
21. Zhang JY, Tan EM. Autoantibodies to tumor-associated antigens as diagnostic biomarkers in hepatocellular carcinoma and other solid tumors. *Expert Rev Mol Diagn.* 2010;10:321–8.