Diseases of the Esophagus (2014) **27**, 790–797
DOI: 10.1111/dote.12145

DISEASES OF THE ESOPHAGUS

Original article

Autoantibody detection to tumor-associated antigens of P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn, and Koc for the screening of high-risk subjects and early detection of esophageal squamous cell carcinoma

S. L. Zhou,[1] W. B. Yue,[1,2] Z. M. Fan,[1] F. Du,[1] B. C. Liu,[1,3] B. Li,[1] X. N. Han,[1] J. W. Ku,[1,4] X. K. Zhao,[1] P. Zhang,[1] J. Cui,[5] F. Y. Zhou,[6] L. Q. Zhang,[1,7] X. P. Fan,[1] Y. F. Zhou,[1,8] L. L. Zhu,[1] H. Y. Liu,[9] L. D. Wang[1]

[1]*Henan Key Laboratory for Esophageal Cancer Research, The First Affiliated Hospital of Zhengzhou University, Zhengzhou and* [2]*Department of Medical Oncology, The General Hospital of Puyang Oil Field, Puyang, Henan and* [3]*Department of Surgery, Shanghai Public Health Clinical Center Affiliated to Fudan University, Shanghai and* [4]*Department of Medical Oncology, The Second Affiliated Hospital of Nanyang Medica College, Nanyang, Henan and* [5]*Cancer Research Center, Xinxiang Medical University, Xinxiang and Departments of* [6]*Thoracic Surgery and* [7]*Gastroenterology, Anyang Tumor Hospital, Anyang and* [8]*Department of Gastroenterology, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou and* [9]*Henan Medical Genetics Institute, People's Hospital of Zhengzhou University (People's Hospital of Henan Province), Zhengzhou, Henan, China*

SUMMARY. **The aim of this study was to evaluate the diagnostic values by detecting sera autoantibodies to eight tumor-associated antigens (TAAs) of P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn and Koc full-length recombinant proteins for the screening of high-risk subjects and early detection of esophageal squamous cell carcinoma (ESCC). Enzyme-linked immunosorbent assay was used to detect autoantibodies against the eight selected TAAs in 567 sera samples from four groups, including 200 individuals with normal esophageal epithelia (NOR), 214 patients with esophageal basal cell hyperplasia (BCH), 65 patients with esophageal dysplasia (DYS), and 88 patients with ESCC. In addition, the expression of the eight antigens in esophageal tissues was analyzed by immunohistochemistry. Statistically significant distribution differences were identified among the four groups for each of the individual autoantibodies to six TAAs (P53, IMP1, P16, cyclin B1, P62, and C-myc); the detection rates of antoantibodies were positively correlated with the progression of ESCC. When autoantibody assay successively accumulated to six TAAs (P53, IMP1, P16, cyclin B1, P62, and C-myc), a stepwise increased detection frequency of autoantibodies was found in the four sera groups (6% in NOR, 18% in BCH, 38% in DYS, and 64% in ESCC, respectively), the risks to BHC, DYS, and ESCC steadily increased about 3-, 9-, and 27-folds. The sensitivity and the specificity for autoantibodies against the six TAAs in diagnosing ESCC reached up to 64% and 94%, respectively. The area under the receiver operating characteristic curve for the six anti-TAA autoantibodies was 0.78 (95% confidence interval 0.74–0.83). No more increasing in sensitivity was found with the addition of new anti-TAA autoantibodies. A combination detection of autoantibodies to TAAs might distinguish ESCC patients from normal individuals and the patients with esophageal precancerous lesions.**

KEY WORDS: **autoantibody, esophageal squamous cell carcinoma, precancerous lesion, tumor-associated antigen (TAA).**

Address correspondence to: Professor Li Dong Wang, MD, PhD, Henan Key Laboratory for Esophageal Cancer Research, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China. Email: ldwang2007@126.com
Competing interests: All authors declare no competing financial interests.
Authors' contributions: LDW conceived and designed this study, LDW and SLZ written the manuscript; SLZ, FD, BCL, ZMF, WBY, and JWK conducted ELISA and immunohistochemical staining and data analysis; BL, XNH, XPF, XKZ, PZ, LLZ, JC, LQZ, FYZ, YFZ, and HYL were responsible for collections of blood samples and clinical data. All the authors have read and approved the final manuscript.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the six most prevalent malignancies in China.[1,2] The overall 5-year survival rate of ESCC is still dismal, only about 14% currently[3] even the diagnosis and treatment have been improved greatly in the past decades. However, after completely surgical removal of the tumor, the 5-year survival rate for early-stage ESCC could be more than 80%.[4] Unfortunately, about 86% of ESCC patients from municipal regions, and more than 95% from rural regions were diagnosed at middle and advanced stage.[5] The main reason for this contradiction is that early-stage ESCC has no obvious symptom, and there is lack of acceptable diagnostic methods for early detection. To date, endoscopy examination and mucosa biopsy are one of the most effective screening tools for early ESCC,[6] which has been widely applied in clinic for early ESCC detection and high-risk population screening on large-scale symptom-free subjects in the high-incidence areas for ESCC in China. However, it is greatly hindered because of economic cost, uncomfortable experience when examined, and lack of experienced endoscopist.[7] Therefore, identification of sera molecular biomarker that is sensitive enough and less invasive is of crucial values for high-risk subjects screening and early detection for ESCC.

Esophageal carcinogenesis has been well recognized as a multistage processes, the early lesions have been characterized as hyperproliferation of esophageal epithelial cells, and morphologically manifested as basal cell hyperplasia (BCH), dysplasia (DYS), and carcinoma in situ, which have been recognized as esophageal precancerous lesions.[8] Accumulated evidences have showed that multiple genetic alterations are involved before or during the morphological progression changes.[9] Malignant cells can activate both the cellular and humoral immune systems during the progression process, which results in the possibility for high-risk subjects screening and early cancer detection by identifying sera autoantibodies that react with autologous cellular antigens from cancer cells called tumor-associated antigens (TAAs).[10,11] Recent studies have indicated that a panel of multiple autoantibodies to TAAs would be used for cancer detection.[12] Our previous study indicated that the frequency of P53 autoantibody was higher in sera from ESCC patients than that from normal people,[13] suggesting that multiple serological biomarkers may emerge in esophageal carcinogenesis. It is noteworthy that most of these studies are focused on single autoantibody detection or on relevant antigen detection in tissue, which results in low detection rate for cancer.[14–18] To evaluate the diagnostic values by detecting sera autoantibodies to TAAs for the screening of high-risk subjects and early detection of ESCC, the present study was thus undertaken to detect antoantibodies to a panel of eight TAAs (P53, P16, P62, C-myc, cyclin B1, Survivn, IMP1, and Koc) in sera from the individuals with normal esophageal epithelia (NOR), and the patients with esophageal precancerous lesions (BCH and DYS) and ESCC with enzyme-linked immunosorbent assay (ELISA) method. Furthermore, to illustrate the correlation of autoantibody alterations in serum and the relevant TAAs protein expression changes in corresponding esophageal tissues with different lesions, immunohistochemical staining of the eight relevant TAAs was performed on esophageal epithelial tissue from the matched subjects and patients for autoantibody assay.

MATERIALS AND METHODS

Study samples

Normal subjects and the patients with esophageal precancerous lesions

The normal subjects and the patients with esophageal precancerous lesions were from the mass surveys for high-risk subjects screening and early detection for ESCC in the high incidence area for ESCC in Linzhou, Henan province, northern China, which was carried out by Henan Key Laboratory for Esophageal Cancer Research of The First Affiliated Hospital, Zhengzhou University from 2009 to 2011. Endoscopic examination and mucosa biopsy, blood sample collection, and questionnaires were performed on each symptom-free subject as described previously.[8] Based on the cellular morphological changes and tissue architecture, the esophageal biopsy tissue was classified as BCH, DYS, and carcinoma in situ.[8] A total of 479 symptom-free subjects were finally enrolled in this study, including 200 individuals with NOR (132 males and 68 females with a mean age of 50 ± 9 and 47 ± 11 years, respectively), 214 patients with BCH (117 males and 97 females with a mean age of 49 ± 12 and 51 ± 10 years, respectively), and 65 patients with DYS (38 males and 27 females with a mean age of 53 ± 9 and 50 ± 8 years, respectively).

Patients with ESCC

Eighty-eight patients with ESCC (49 males and 38 females with a mean age of 58 ± 10 and 56 ± 9 years, respectively) were from Linzhou Esophageal Cancer Hospital. All the patients were performed surgical treatment, and no chemotherapy or radiotherapy was performed before the surgery. All the patients were confirmed as ESCC by histopathologist (LDW).

Blood and tissues samples collection

Five milliliters of peripheral blood was collected before endoscopy or surgery for each participator,

Table 1 The detail information of antibodies involved in the immunochemical analysis

Categories	Properties	Manufacturers
P53	Rat antihuman monoclonal	Oncogene, Inc., Cambridge, MA, USA
IMP1	Goat antihuman polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
P16	Rat antihuman monoclonal	Santa Cruz Biotechnology, Inc.
Cyclin B1	Rabbit antihuman polyclonal	Santa Cruz Biotechnology, Inc.
C-myc	Rat antihuman monoclonal	Vectastain Elite Kit; Vector, Burlingame, CA, USA
Survivn	Goat antihuman polyclonal	Santa Cruz Biotechnology, Inc.
Koc and P62	Rat antihuman monoclonal	Keck Autoimmune Disease Research Center, Los Angeles, CA, USA

centrifuged at 12000 rpm/min, separated sera, and stored at –80°C until autoantibody detection. The surgically resected specimen and biopsy tissues were formalin-fixed, paraffin-embedded, and hematoxylin-eosin staining for pathological diagnosis and immunohistochemistry assay.

This study was reviewed and approved by the Institute Research Ethics Committee of the Zhengzhou University and Linzhou Esophageal Cancer Hospital. Informed consents were obtained from all participants before their blood and tissue samples were used.

ELISA

Eight autoantibodies against the TAAs were selected to determine the serum status in esophageal carcinogenesis. The TAAs consisted of purified full-length recombinant proteins, including P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn, and Koc. All of the eight antigen proteins were kindly donated by Dr Jian Ying Zhang (University of Texas, El Paso, TX, USA). ELISA was performed as previously described.[19] Briefly, The eight kinds purified recombinant proteins were individually diluted in phosphate-buffered saline (PBS) to a final concentration of 0.5 μg/μL, pipetting 100 μL into each wells to coat the microtiter plates overnight at 4°C. After washing with PBS for three times, all human sera (1 : 50) were incubated in the antigen-coated wells at 37°C for 40–60 minutes, followed by washing with PBS containing 0.05% Tween-20 (Sigma., St. Louis, MO, USA) for three times, the horseradish peroxidase-conjugated goat anti-human immunoglobulin G (1 : 5000) (Zhong Shan Golden Bridge Biological Technology, Beijing, China), as a secondary antibody was added 100 μL into each wells, incubating at 37°C for 50 minutes. The substrate 3,3′,5,5′-tetramethylbenzidine (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as the detecting agent. Positive and negative controls were included in the experiment. The positive control was the ESCC patient serum that consistently showed positive staining in previous experiments. In the negative control, only the primary antibody was not added. Each serum sample was tested in duplicate, and the average optical density (OD) at 450 nm was used for data analysis. The cut-off value for positive reactions[19] was designated as above the mean OD of the 200 normal human sera of each subgroup +3 standard deviation (SD). Furthermore, all positive sera and the suspicious negative sera were confirmed with repeated testing.

Immunohistochemical analysis of the eight TAAs

Immunohistochemical analysis was performed using the avidin-biotin-peroxidase complex method, as described previously,[8] to detect the expression of eight proteins, including P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn, and Koc. The detail information of reagents involved in this experiment was stated in Table 1. In each experiment, positive and negative controls were included. The positive control was the esophageal carcinoma tissue, which consistently showed positive staining in previous experiments. In the negative control, only primary antibody was not added. The positive staining (intense dark brown) was mainly located in cytoplasm or nucleus. Five eye-fields under high-power magnification (40×) was examined and when the number of positive cells ≥10% was considered as positive criteria.

Statistical analysis

The χ^2 test, binary logistic regression, and Spearman correlation test were used for the ELISA and immunochistochemistry analysis. Receiver operating characteristic (ROC) curves was used to identify the effectiveness of each outcome. All computations were performed using SPSS (17.0 version; SPSS Inc., Chicago, IL, USA) ($P < 0.05$ of two-sided was considered significant).

RESULTS

Detection frequency of autoantibodies to a panel of eight TAAs in the progression of ESCC

In this study, a panel of eight TAAs was used as coating antigens in ELISA to detect the presence of autoantibodies to each and cumulatively to eight TAAs in the multistage process of ESCC (from NOR to BCH, to DYS, and to ESCC). The absorbance reading cut-off value of ≥mean +3 SD of the 200 normal human sera was considered as positive test for autoantibodies.

The detection frequency of autoantibodies to the eight TAAs in sera from patients with different esophageal lesions was shown in Table 2. The difference of detection rates for the eight autoantibodies between the patients with DYS and ESCC was not dramatic. However, the detection rates both in DYS and ESCC were apparently higher than in the normal individuals. The detection frequency of IMP1-Abs positively correlated with the progression of ESCC (BCH vs. NOR: odds ratio [OR] = 4.85, 95% confidence interval [CI] 1.05–22.43; DYS vs. NOR: OR = 13.90, 95% CI 2.87–67.27; ESCC vs. NOR: OR = 15.63, 95% CI 3.42–71.48). The detection frequencies of autoantibody to C-myc, cyclin B1, P16, P53, and P62 also increased the risk to DYS and ESCC about 3- to 6- and 3- to 15-folds, respectively. Especially, the detection frequency of autoantibody to P53 significantly increased the risk to DYS and ESCC about 7.88-fold and 13.49-fold (DYS vs. NOR: OR = 7.88, 95% CI 2.34–26.53; ESCC vs. NOR: OR 13.49, 95% CI 4.44–41.05). The detection frequency of autoantibody to Koc slightly positively correlated with ESCC (ESCC vs. NOR: OR = 2.73, 95% CI 1.02–7.34). No significant difference of the frequency of autoantibody to Survivn was found during the progression of ESCC. The ROC curve was plotted for the autoantibodies for each TAA. The area under the ROC curve for autoantibody to P53 was 0.74 (95% CI 0.67–0.82), with a sensitivity of 22% and a specificity of 98%, which were much higher than that of other seven autoantibodies (Data not shown).

Table 2 Detection frequency of autoantibodies to eight tumor-associated antigens*

Autoantibodies	NOR ($N = 200$)		BCH ($N = 214$)			DYS ($N = 65$)			ESCC ($N = 88$)		
	n	(%)	*n*	(%)	*P*	OR (95% CI)†	*n*	(%)	*P*	OR (95% CI)	*n*	(%)	*P*	OR (95% CI)
P53	4	(2)	12	(6)	0.07	NA	9	(14)	0.001	7.88 (2.34–26.53)	19	(22)	<0.001	13.49 (4.44–41.05)
IMP1	2	(1)	10	(5)	0.04	4.85 (1.05–22.43)	8	(12)	0.001	13.9 (2.87–67.27)	12	(14)	<0.001	15.63 (3.42–71.48)
P16	5	(3)	7	(3)	0.64	NA	6	(9)	0.03	3.97 (1.17–13.46)	10	(11)	0.004	5.00 (1.66–15.10)
Cyclin B1	6	(3)	7	(3)	0.87	NA	8	(12)	0.007	4.54 (1.51–13.62)	9	(10)	0.02	3.69 (1.27–10.69)
P62	4	(2)	10	(5)	0.14	NA	6	(9)	0.02	4.98 (1.36–18.25)	11	(13)	0.001	7.00 (2.61–22.65)
C-myc	7	(4)	14	(7)	0.17	NA	12	(18)	<0.001	6.24 (2.34–16.64)	16	(18)	<0.001	6.13 (2.42–15.51)
Survivn	7	(4)	11	(5)	0.42	NA	3	(5)	0.69	NA	8	(9)	0.06	NA
Koc	8	(4)	7	(3)	0.69	NA	6	(9)	0.11	NA	9	(10)	0.05	2.73 (1.02–7.34)
Cumulative to eight Ags	15	(8)	40	(19)	0.001	2.84 (1.51–5.32)	25	(38)	<0.001	7.71 (3.73–15.93)	56	(64)	<0.001	21.58 (10.91–42.70)

*P values relative to NOR.
†OR (95% CI) was only shown if the P value ≤0.05.
BCH, basal cell hyperplasia; CI, confidence interval; DYS, dysplasia; ESCC, esophageal squamous cell carcinoma; NA, not applicable; NOR, normal epithelium; OR, odds ratio.

Stepwise increase in detection frequency with successive addition of autoantibodies

The detection frequency of autoantibodies to any individual TAA in patients sera with either ESCC or precancerous lesions was variable from 3% to 22% but rarely exceeded 20% expect P53 in ESCC patients. With a cluster analysis on 88 ESCC sera analyzed, the significant higher frequency ($P < 0.001$) of autoantibodies with individual TAA in ESCC was only found in autoantibody to P53 (22%), C-myc (18%), and IMP1 (14%).

With the successive addition of TAAs to eight antigens, there was a stepwise increase of autoantibody detection frequency in the progression of esophageal carcinogenesis (Table 3). The detection frequency for autoantibodies to TAAs increased up to 64% in ESCC patients when autoantibodies accumulated to a total of six antigens (P53, IMP1, P16, cyclin B1, P62, and C-myc), which was significantly higher than that in DYS (38%), BCH (18%), and normal individuals (6%) (Table 3). The detection frequency of autoantibodies cumulatively to the six TAAs was significantly positively correlated with the progression of ESCC (BCH vs. NOR: OR = 3.38,

Table 3 Stepwise addition of autoantibodies to eight tumor-associated antigens*

Antigen	NOR ($n = 200$)		BCH ($n = 214$)			DYS ($n = 65$)			ESCC ($n = 88$)			
	n	(%)	*n*	(%)	*P*	OR (95% CI)†	*n*	(%)	*P*	OR (95% CI)	*n*	(%)	*P*	OR (95% CI)
① P53	4	(2)	12	(6)	0.07	NA	9	(14)	0.001	7.88 (2.34–26.53)	19	(22)	<0.001	13.49 (4.44–41.05)
② P53 + IMP1	6	(3)	18	(8)	0.06	NA	16	(25)	<0.001	10.56 (3.95–28.39)	30	(34)	<0.001	16.72 (6.64–42.15)
③ P53 + IMP1 + P16	8	(4)	22	(10)	0.02	2.75 (1.20–6.33)	19	(29)	<0.001	9.91 (4.09–24.06)	37	(42)	<0.001	17.41 (7.64–39.70)
④ P53 + IMP1 + P16 + cyclin B1	8	(4)	26	(12)	0.004	3.32 (1.47–7.52)	23	(35)	<0.001	13.14 (5.50–31.40)	43	(49)	<0.001	22.93 (10.09–52.15)
⑤ P53 + IMP1 + P16 + cyclin B1 + P62	8	(4)	32	(15)	<0.001	4.22 (1.89–9.40)	24	(37)	<0.001	14.05 (5.90–33.48)	50	(57)	<0.001	31.58 (13.86–71.95)
⑥ P53 + IMP1 + P16 + cyclin B1 + P62 + C-myc	12	(6)	38	(18)	<0.001	3.38 (1.71–6.68)	25	(38)	<0.001	9.79 (4.53–21.11)	56	(64)	<0.001	27.42 (13.25–56.75)
⑦ P53 + IMP1 + P16 + cyclin B1 + P62 + C-myc + Survivn	13	(7)	38	(18)	0.001	3.12 (1.60–6.02)	25	(38)	<0.001	8.99 (4.24–19.07)	56	(64)	<0.001	25.17 (12.37–51.22)
⑧ P53 + IMP1 + P16 + cyclin B1 + P62 + C-myc + Survivn + Koc	15	(8)	40	(19)	0.001	2.84 (1.51–5.32)	25	(38)	<0.001	7.71 (3.73–15.93)	56	(64)	<0.001	21.58 (10.91–42.70)

*P values relative to NOR.
†OR (95% CI) was only shown if the P value ≤0.05.
BCH, basal cell hyperplasia; CI, confidence interval; DYS, dysplasia; ESCC, esophageal squamous cell carcinoma; NA, not applicable; NOR, normal epithelium; OR, odds ratio.

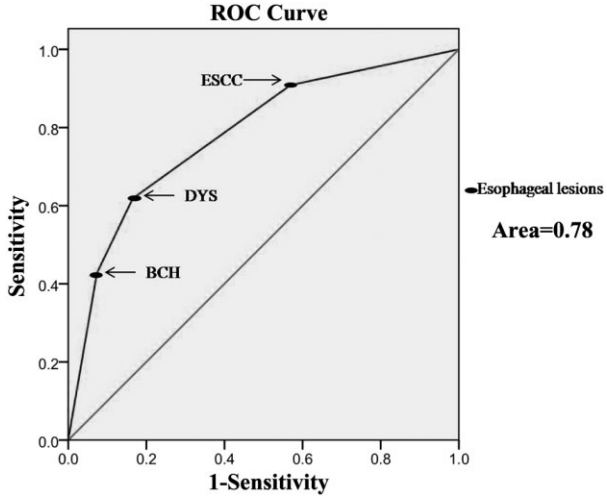

Fig. 1 Receiver operating characteristic (ROC) curve analysis of autoantibodies to successive addition of six tumor-associated antigens in serum from subjects with different degree of esophageal lesions. BCH, basal cell hyperplasia; DYS, dysplasia; ESCC, esophageal squamous cell carcinoma.

95% CI 1.71–6.68; DYS vs. NOR: OR = 9.79, 95% CI 4.53–21.11; ESCC vs. NOR: OR = 27.42, 95% CI 13.25–56.75). The area under the ROC curve for cumulative autoantibodies was 0.78 (95% CI 0.74–0.83) with a sensitivity of 64% and a specificity of 94% (Fig. 1). With the addition of more antigens (Survivn and Koc) to the panel, no further increase in sensitivity was observed, but the specificity decreased to 92%.

Expression of eight TAAs in esophageal epithelial tissues with different lesions

The positive immunostaining was located in cell nucleus (P53 and P16) or in cytoplasm (IMP1, P62, and Koc), whereas the positive immunostaining for cyclin B1, C-myc, and Survivn was located in both cell nucleus and cytoplasm. The positive expression of the eight TAAs was much higher in ESCC tissue than that in normal tissue (Table 4). The overexpression of C-myc in tissues significantly increased the risk of DYS and ESCC (DYS vs. NOR: OR = 3.49, 95% CI 1.25–9.77; ESCC vs. NOR: OR = 8.80, 95% CI 3.06–25.49). Comparing with NOR tissue, the expression of Survivn, P53, IMP1, and Koc was positively correlated with ESCC (for Survivn: OR = 28.50, 95% CI 9.74–83.40; for P53: OR = 7.97, 95% CI 3.95–16.05; for IMP1: OR = 6.00, 95% CI 1.69–21.26; for Koc: OR = 3.50, 95% CI 1.04–1.73). No significant difference was found between the expression of cyclin B1, P16, and P62, and the progression of ESCC.

To correlate the TAA expression in the tissues by immunohistochemistry with corresponding autoantibody expression in sera by ELISA, we found that the positive immunostaining rate of the eight TAAs

Table 4 The expression of eight tumor-associated antigens in esophageal tissues*

Antigen	NOR		BCH			DYS			ESCC		
	%	(*n*/*N*)	%	(*n*/*N*)	*P*	OR (95% CI)†	%	(*n*/*N*)	*P*	OR (95% CI)	%	(*n*/*N*)	*P*	OR (95% CI)
P53	40	(58/126)	47	(34/73)	0.33	NA	47	(22/47)	0.39	NA	84	(63/75)	<0.001	7.97 (3.95–16.05)
IMP1	33	(10/30)	57	(26/46)	0.05	2.60 (1.00–6.77)	30	(8/27)	8/27	NA	75	(15/20)	0.006	6.00 (1.69–21.26)
P16	7	(2/28)	3	(1/31)	0.51	NA	17	(4/23)	0.27	NA	13	(3/24)	0.52	NA
Cyclin B1	41	(13/32)	52	(24/46)	0.32	NA	36	(10/28)	0.70	NA	65	(13/20)	0.09	NA
P62	45	(15/33)	64	(30/47)	0.11	NA	34	(10/29)	0.38	NA	28	(5/18)	0.22	NA
C-myc	16	(8/51)	22	(18/81)	0.36	NA	39	(13/33)	0.02	3.49 (1.25–9.77)	62	(18/29)	<0.001	8.80 (3.06–25.49)
Survivn	18	(6/33)	24	(11/46)	0.54	NA	38	(11/29)	0.08	NA	87	(76/88)	<0.001	28.50 (9.74–83.41)
Koc	36	(12/33)	51	(24/47)	0.20	NA	31	(9/29)	0.66	NA	67	(12/18)	0.04	3.50 (1.04–11.73)

*P values relative to NOR.
†OR (95% CI) was only shown if the P value ≤0.05.
BCH, basal cell hyperplasia; CI, confidence interval; DYS, dysplasia; ESCC, esophageal squamous cell carcinoma; NA, not applicable; NOR, normal epithelium; OR, odds ratio.

expect P62 in esophageal tissues was much higher than the detection frequency of the corresponding anti-TAA autoantibodies in sera ($P < 0.05$). Moreover, there was a good correlation between the expression of TAAs in esophageal tissue and the detection frequency of corresponding anti-TAA autoantibodies in sera for P53, C-myc, and Survivn ($r = 0.95$, $P = 0.05$). In addition, although the expression of both Survivn and Koc in ESCC tissues was significantly higher than that in normal tissue ($P < 0.05$) (Fig. 2A–D), the detection frequency of autoantibodies to Survivn and Koc showed no difference between sera from ESCC patients and normal individuals. In contrast, despite the detection frequency of autoantibodies to P16 and P62 was much higher in sera from ESCC patients than that from normal individuals ($P < 0.05$), the positive immunostaining rate of P16 and P62 showed no difference in ESCC tissues comparing with that in normal tissues (Fig. 2E–H).

DISCUSSION

In this study, we investigated the possibility for high-risk subjects screening and early ESCC detection by determining autoantibodies against eight tumor-related antigens of P53, IMP1, P16, cyclin B1, P62, C-myc, Survivn, and Koc in sera. The advantage of the current study is that we examined the autoantibodies assays in sera by ELISA and protein expression of their relevant TAAs in the corresponding tissues by immunohistochemistry on 479 symptom-free subjects from mass survey in high-incidence area for ESCC. All these subjects have been performed endoscopic and mucosal biopsy examinations and histologically diagnosed as NOR ($n = 200$) and esophageal precancerous lesions (BCH and DYS, $n = 279$).

Of particular interest, the present study demonstrates the possibility of using autoantibodies to a panel of TAAs as serum biomarker for high-risk subjects screening and ESCC detection. The positive detection rate of autoantibodies to the panel of eight TAAs, except Survivn and Koc, was positively correlated with the progression of esophageal carcinogenesis. When the autoantibodies successively accumulated to six TAAs (P53, IMP1, P16, cyclin B1, P62, and C-myc), the positive detection rate increased stepwise (6% for normal, 18% for BCH, 38% for DYS, and 64% for ESCC) and significantly increased the risk of BCH, DYS, and ESCC for 3-, 9-, and 27-folds, respectively. The optimal sensitivity and specificity were 64% and 94%, respectively, for ESCC.

All the TAAs in this study were selected based on literature reports by our laboratory and others, which were related with ESCC[13–18] or cancers.[12,19] Although the mechanisms related with autoantibody

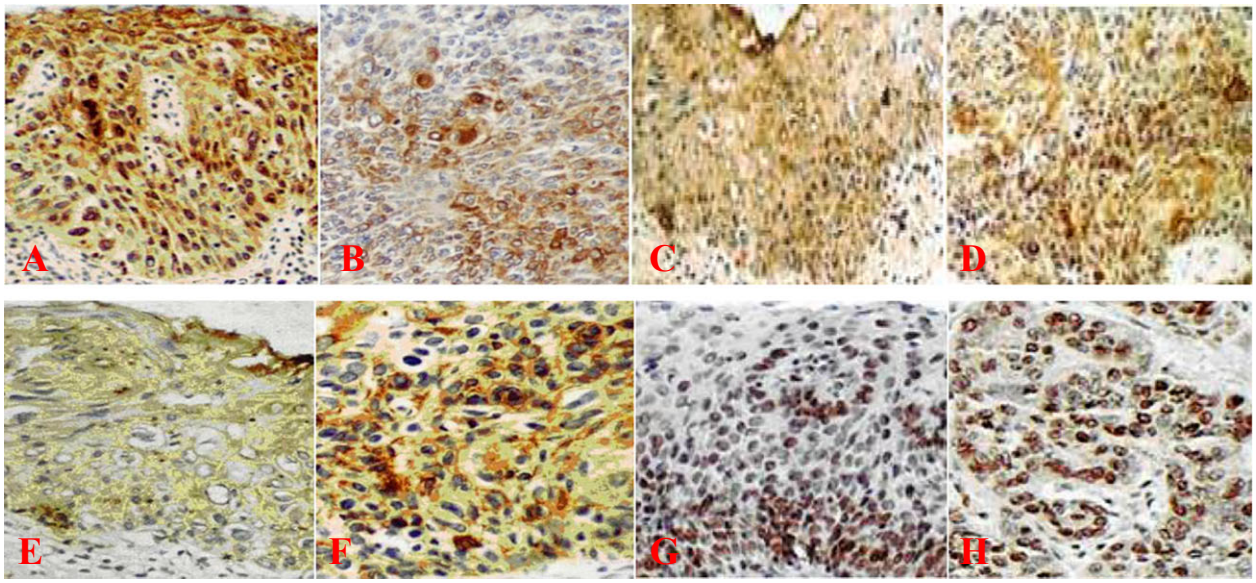

Fig. 2 The protein expression of Survivun, Koc, P62, and P16 in esophageal tissues by immunohistochemistry.

presentation in sera is not clear, accumulated evidences have indicated the positive correlation of autoantibody in sera with relevant TAA overexpression in the corresponding tissue in many human cancers, including head and neck,[20] lung[21] cancers, even with survival.[15] In ESCC, several studies have been reported for sera autoantibody assay for P53[13–16], P16[17] and P62,[18] and suggested that part of these serum biomarkers could be used for diagnosis and mass screening purpose. Comparing with our study, the previous reports have some disadvantages either because of relatively small sample size or limited serum biomarkers analyzed.[13–16] Moreover, most reports are focused on ESCC and normal subjects, lack of precancerous lesion analysis. Our results clearly showed a good correlation for autoantibodies to P53, IMP1, P16, cyclin B1, P62, and C-myc in sera and esophageal carcinogenesis from normal to BCH to DYS and to ESCC, which suggest that these serum biomarkers could be used as one of the supplemental strategies for high-risk subjects screening and early ESCC detection, and then may increase the targeted endoscopic screening.

It could not be overemphasized for the usefulness and crucial role of endoscopic and mucosal biopsy examinations, especially together with iodine staining for targeted biopsy, in the high-risk subject screening and early ESCC detection on the population in high-incidence areas for ESCC.[22] However, the limitations for this powerful technique clinical application are also obvious because of uncomfortable experience, which result unacceptable by symptom-free high-risk population, and complicate procedures, e.g. each subject costs at least 1 hour for examination and sterilization, only less than 10 subjects could be examined 1 day for each endoscopist, which is far from the requirement by mass screening for high-risk population.[7] Moreover, based on our experience, with endoscopic and mucosal biopsy screening for high-risk subjects (with severe precancerous lesions) and early ESCC detection on the symptom-free subjects in high-incidence areas, only 2–3% of ESCC patients and 10–14% of patients with esophageal DYS could be identified.[6] In other words, more than 80% of the subjects could be considered overexamined. It is much desirable to establish a simple, economic, and patient-acceptable screening method as serum biomarker to screening first the high-risk subjects for further endoscopic examination.

It is noteworthy that our earlier study has demonstrated the high-frequency alteration of P53 mutation and protein accumulation in esophageal precancerous and cancerous lesion, even in histological normal epithelia cells around the papilla region, indicating that P53 may be a promising early indicator for esophageal carcinogenesis in Chinese population.[9] The present results confirmed the high frequency of P53 protein accumulation in esophageal carcinogenesis and further indicated that serum autoantibody to P53, together with other five serum biomarkers, may be a desirable panel of indicators for high-risk subject screening. Furthermore, the consistent presentation for high P53 autoantibody level in sera and P53 protein accumulation in the corresponding tissues suggests the possibility that unstable or mutated P53 protein may be one of the mechanisms for induced autoantibody to P53 in sera.

The present results demonstrated that a combination of panel serum biomarkers could apparently increase sensitivity and specificity for high-risk subject screening and ESCC detection. However, when the combination was over six serum

biomarkers, no further increase in sensitivity was observed in the present study, indicating the importance for the serum biomarker assemble in clinical application.

Interestingly, the immunohistochemical results showed that the positive immunostaining rates for the eight TAAs except P16 and P62 in this study increased significantly in ESCC tissues comparing with NOR tissues. Furthermore, the overall positive immunostaining rates for TAAs in tissues were higher than the positive detection rates of corresponding autoantibodies in sera (e.g. 84% for P53 positive immunostaining rate in ESCC tissue vs. 22% for P53 autoantibody in ESCC sera).

In summary, the present study demonstrates the possibility of using autoantibodies to a panel of six TAAs of P53, IMP1, P16, cyclin B1, P62, and C-myc as serum biomarkers for high-risk subjects screening and ESCC detection. A stepwise increasing of autoantibody detection rate was observed in esophageal carcinogenesis when the autoantibody assay successively increased to a total of six TAAs indicates the importance for the serum biomarker assemble in clinical application. The consistent presentation for high P53 autoantibody level in sera and P53 protein accumulation in the corresponding tissues suggest the possibility that unstable or mutated P53 protein may be one of the mechanisms for induced autoantibody to P53 in sera.

Acknowledgments

We thank Dr. Jian Yang Zhang of University of Texas, El Paso, TX, USA for his kindness to donate antibodies and experimental guidance. We thank Dr. Jun Yan Hong at UMDNJ (University of Medicine and Dentistry of New Jersey) in New Jersey to help us improve the scientific writing. This project was funded by 863 HighTech Key Projects (2012AA02A503, 2012AA02A209, and 2012AA02A201), National Natural Science Foundations (30971133, 81071783), and Innovation Scientists and Technicians Troop Construction Projects of Henan Province.

References

1 Jemal A, Bray F, Center M M *et al*. Global cancer statistics. CA Cancer J Clin 2011; 61: 69–90.
2 Ke L. Mortality and incidence trends from esophagus cancer in selected geographic areas of China circa 1970–90. Int J Cancer 2002; 102: 271–4.
3 Enzinger P C, Mayer R J. Esophageal cancer. N Engl J Med 2003; 349: 2241–52.
4 Headrick J R, Nichols F C III, Miller D L *et al*. High-grade esophageal dysplasia: longterm survival and quality of life after esophagectomy. Ann Thorac Surg 2002; 73: 1697–702.
5 Fan Y J, Song X, Li J L *et al*. Esophageal and gastric cardia cancers on 4238 Chinese patients residing in municipal and rural regions: a histopathological comparison during 24-year period. World J Surg 2008; 32: 1980–8.
6 Wang L D, Zhou Q, Feng C W *et al*. Intervention and follow-up on human esophageal precancerous lesions in Henan, northern China, a high-incidence area for esophageal cancer. Gan To Kagaku Ryoho 2002; 29 (Suppl 1): 159–72.
7 Wang L D. Limitation and strategies on endoscopic examination in high-risk subject screening for esophageal cancer in high-incidence area. J Zhengzhou Univ (Med Sci) 2009; 44: 11–2. (In Chinese.).
8 Wang L D, Zhou Q, Gou R Y *et al*. Reproducibility of an esophageal biopsy sampling procedure in a high-incidence area for esophageal cancer in northern China. Cancer Epidemiol Biomarkers Prev 1996; 5: 405–6.
9 Wang L D, Hong J Y, Qiu S L *et al*. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. Cancer Res 1993; 53: 1783–7.
10 Zhang J Y. Tumor-associated antigen array to enhance antibody detection for cancer diagnosis. Cancer Detect Prev 2004; 28: 114–8.
11 Houghton A N. Cancer antigens: immune recognition of self and altered self. J Exp Med 1994; 180: 1–4.
12 Shi F D, Zhang J Y, Liu D *et al*. Preferential humoral immune response in prostate cancer to cellular proteins p90 and p62 in a panel of tumor-associated antigens. Prostate 2005; 63: 252–8.
13 Yu G Q, Zhou Q, Ivan D *et al*. Changes of p53 protein blood level in esophageal cancer patients and normal subjects from a high incidence area in Henan, China. World J Gastroenterol 1998; 4: 365–6.
14 Shimada H, Takeda A, Arima M *et al*. Serum p53 antibody is a useful tumor marker in superficial esophageal squamous cell carcinoma. Cancer 2000; 89: 1677–83.
15 Bergqvist A S, Bergqvist M, Brattstrom D *et al*. Serum p53 autoantibodies as prognostic marker in patients with oesophageal carcinoma. Anticancer Res 2001; 21: 4141–5.
16 Chen W, Abnet C C, Wei W Q *et al*. Serum markers as predictors of esophageal squamous dysplasia and early cancer. Anticancer Res 2004; 24: 3245–9.
17 Looi K, Megliorino R, Shi F D *et al*. Humoral immune response to p16, a cyclin-dependent kinase inhibitor in human malignancies. Oncol Rep 2006; 16: 1105–10.
18 Su Y, Qian H, Zhang J *et al*. The diversity expression of p62 in digestive system cancers. Clin Immunol 2005; 116: 118–23.
19 Zhang J Y, Megliorino R, Peng X X *et al*. Antibody detection using tumor-associated antigen mini-array in immunodiagnosing human hepatocellular carcinoma. J Hepatol 2007; 46: 107–14.
20 Brennan J A, Boyle J O, Koch W M *et al*. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. N Engl J Med 1995; 332: 712–7.
21 Iizasa T, Fujisawa T, Saitoh Y *et al*. Serum anti-p53 autoantibodies in primary resected non-small-cell lung carcinoma. Cancer Immunol Immunother 1998; 46: 345–9.
22 Wang L D, Qiu S L, Yang G R *et al*. A randomized double-blind intervention study on the effect of calcium supplementation on esophageal precancerous lesions in a high risk population in China. Cancer Epidemiol Biomarkers Prev 1993; 2: 71–8.