

## Open

## Autoantibodies as Potential Biomarkers for the Early Detection of Esophageal Squamous Cell Carcinoma

Yi-Wei Xu, MSM<sup>1,2</sup>, Yu-Hui Peng, BSM<sup>2</sup>, Bo Chen, MSM<sup>1</sup>, Zhi-Yong Wu, MD<sup>3,4</sup>, Jian-Yi Wu, BSc<sup>3</sup>, Jin-Hui Shen, MD<sup>5</sup>, Chun-Peng Zheng, MSM<sup>4</sup>, Shao-Hong Wang, MD<sup>5</sup>, Hai-Peng Guo, MD<sup>6</sup>, En-Min Li, PhD<sup>3</sup> and Li-Yan Xu, PhD<sup>1</sup>

**OBJECTIVES:** Esophageal squamous cell carcinoma (ESCC) is one of the most frequent causes of cancer death worldwide and effective diagnosis is needed. We assessed the diagnostic potential of an autoantibody panel that may benefit early diagnosis.

**METHODS:** We analyzed data for patients with ESCC and normal controls in a test cohort and a validation cohort. Autoantibody levels were measured against a panel of six tumor-associated antigens (p53, NY-ESO-1, matrix metalloproteinase-7 (MMP-7), heat shock protein 70 (Hsp70), peroxiredoxin VI (Prx VI), and BMI1 polycomb ring finger oncogene (Bmi-1)) by enzyme-linked immunosorbent assay.

**RESULTS:** We assessed serum autoantibodies in 513 participants: 388 with ESCC and 125 normal controls. The validation cohort comprised 371 participants: 237 with ESCC, and 134 normal controls. Autoantibodies to at least 1 of 6 antigens demonstrated a sensitivity/specificity of 57% (95% confidence interval (CI): 52–62%)/95% (95% CI: 89–98%) and 51% (95% CI: 45–57%)/96% (95% CI: 91–99%) in the test and validation cohorts, respectively. Measurement of the autoantibody panel could differentiate early-stage ESCC patients from normal controls (sensitivity 45% (95% CI: 32–59%) and specificity 95% (95% CI: 89–98%) in the test cohort; 46% (95% CI: 35–58%) and 96% (95% CI: 91–99%) in the validation cohort). In either cohort, no significant differences were seen when patients were subdivided by age, gender, smoking status, size of tumor, site of tumor, depth of tumor invasion, histological grade, lymph node status, TNM stage, or early-stage and late-stage groups.

**CONCLUSIONS:** Measurement of an autoantibody response to multiple tumor-associated antigens in an optimized panel assay, to help discriminate early-stage ESCC patients from normal controls, may aid in early detection of ESCC.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at <http://www.nature.com/ajg>

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## INTRODUCTION

Esophageal cancer is the eighth most common malignant disease and the sixth leading cause of cancer-related death worldwide (1). China is one of the regions with the highest incidence rates, and 90% of cases are esophageal squamous cell carcinomas (ESCCs) (2). ESCC mortality and incidence rates are similar, and the 5-year overall survival rate is below 15% (1,3). This outcome is partly because of the lack of a screening strategy for timely diag-

nosis. Patients with localized disease have better survival rates than those with metastatic disease (4). However, most cases of ESCC often present at an advanced stage at the time of diagnosis (5). Failure of local control remains a significant clinical problem. Therefore, a noninvasive method for the early detection of ESCC is urgently needed and would represent an important clinical advancement in the management of patients. Current blood tests mainly identify circulating tumor antigens that are elevated

<sup>1</sup>Institute of Oncologic Pathology, The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou, Guangdong, China; <sup>2</sup>Department of Clinical Laboratory, the Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China; <sup>3</sup>Department of Biochemistry and Molecular Biology, The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou, Guangdong, China; <sup>4</sup>Department of Oncology Surgery, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou, Guangdong, China; <sup>5</sup>Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou, Guangdong, China; <sup>6</sup>Department of Head and Neck Surgery, the Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China. **Correspondence:** Li-Yan Xu, PhD, Institute of Oncologic Pathology, Shantou University Medical College, No. 22, Xinling Road, Shantou 515041, Guangdong, China or En-Min Li, PhD, Department of Biochemistry and Molecular Biology, Shantou University Medical College, No. 22, Xinling Road, Shantou 515041, Guangdong, China. E-mail: lyxu@stu.edu.cn or nmli@stu.edu.cn

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most commonly in patients with metastatic disease and appear to reflect tumor bulk. Serum markers for ESCC in clinical use, such as carcinoembryonic antigen, squamous cell carcinoma antigen, and CYFRA21-1, are not sufficiently sensitive for early diagnostic purposes (6–8).

Evidence of the humoral immune response, in the form of autoantibodies, to tumor-associated antigens (TAAs) has created opportunities for exploiting the immune system as a source of cancer biomarkers. Autoantibodies have been found to precede manifestations of symptomatic cancer by several months to years (9–12), making their identification of particular relevance for early detection. Nonetheless, measurement of a single autoantibody may lack sufficient sensitivity required for cancer screening and diagnosis (13). In order to overcome this problem, subsequent studies have provided better sensitivity in the diagnosis of cancer by screening for multiple autoantibodies toward a panel of TAAs (10,14–21). However, most of these reports had limitations, such as small study size and single-cohort study design. This underscores the difficulty in gaining access to human samples.

In this study, we assessed the diagnostic accuracy of autoantibodies to a panel of TAAs for ESCC, and validated the results in an independent population. A panel of six antigens was selected for investigation and comprised a number of well-recognized TAAs (p53, NY-ESO-1, matrix metalloproteinase-7 (MMP-7), heat shock protein 70 (Hsp70), peroxiredoxin VI (Prx VI), and BMI1 polycomb ring finger oncogene (Bmi-1)) that have been shown to induce the production of autoantibodies in ESCC (22–27). In brief, p53 is a tumor-suppressor gene that was described as the first antigen to elicit autoantibodies in cancer (28). Importantly, autoantibodies to this protein have also been detected in some cases before cancer diagnosis (11,12). Furthermore, p53 autoantibodies have been detected in different cancer types, including ESCC (29). Cancer/testis antigen 1B (NY-ESO-1), whose expression is present in some solid tumors, has previously been shown to induce autoantibodies in esophageal cancer (27,30). MMP-7, the smallest of the matrix-degrading metalloproteinases that play an important part in degradation of extracellular matrix, is highly expressed in early stages of cancer and has been described as capable of inducing an autoantibody response in ESCC (26,31). Autoantibodies to Prx VI, a member of the thiol-specific antioxidant protein family, have been considered to be a specific serologic marker for ESCC (22). The Bmi-1, a transcriptional repressor belonging to the polycomb group family, has been described as eliciting an autoantibody response in the sera of patients with ESCC as well (25). The final antigen in the panel, Hsp70, has been previously identified in sera from patients with ESCC by using a proteomics-based approach and has been shown to be a highly immunogenic antigen in ESCC (23).

## METHODS

### Study population

We recruited patients with ESCC to a test cohort, from the Department of Thoracic Surgery, the Cancer Hospital, Shantou University, Guangdong, China, from July 2011 to July 2012. A validation cohort comprising patients with ESCC was recruited

from the Department of Oncological Surgery, Shantou Central Hospital, Sun Yat-sen University, Guangdong, China, from October 2007 to July 2012. The normal controls in the test cohort were from the staff of the Cancer Hospital, and the normal controls in the validation cohort were eligible blood donors with no previous malignant disease (**Table 1**).

ESCC was defined on the basis of gastroscopy and spiral computed tomography, and was confirmed by histopathology. Tumor stage, determined from resected tumors, was defined according to the seventh edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (32). Tumor stage of patients without tumor resection was defined according to the proposal of the Chinese panel of experts for nonsurgical treatment of esophageal cancer on clinical stages (33). For the purpose of this study, we classified tumors with AJCC stage 0+I+IIA as early-stage ESCC.

Patients from the two centers were all newly diagnosed. Sera of these patients were obtained at the time of diagnosis before any treatment. Sera of patients in the validation cohort were routinely obtained and kept in a bank for research. Peripheral blood samples were centrifuged at 1250 g for 5 min and stored at  $-80^{\circ}\text{C}$  until use. Patients with esophageal adenocarcinoma were excluded from the study. Two independent researchers (Yu-Hui Peng and Chun-Peng Zheng) were responsible for data collection.

Before the use of these clinical materials for investigation, approval for the study from the institutional ethics review committee at each study center and informed consent of patients were obtained.

### Expression and purification of recombinant TAAs

See **Supplementary Materials and Methods** online for detail.

### Autoantibody detection

Enzyme-linked immunosorbent assay for serum autoantibodies was performed by two researchers (Yi-Wei Xu and Bo Chen) who had no access to patient clinical information. Briefly, purified recombinant antigens, p53, NY-ESO-1, MMP-7, Hsp70, Prx VI, and Bmi-1, were diluted in 50 mM bicarbonate buffer (pH 9.6) to a final protein concentration of 0.1, 0.1, 0.6, 0.8, 1.5, and 0.6  $\mu\text{g}/\text{ml}$ , respectively. Serum samples and quality control samples (a pooled plasma sample collected randomly from 100 patients with ESCC) were diluted 1:110 in blocking buffer, and then incubated at  $37^{\circ}\text{C}$  for 1 h, as well as were appropriate control rabbit polyclonal antibodies (Immunosoft, Zhoushan, China) specific for capture proteins. After washing, horseradish peroxidase-conjugated goat anti-human IgG or anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies at the dilution recommended by the manufacturer. After a 60-min incubation, the plates were washed, and ready prepared 3,3',5,5'-tetramethylbenzidine (InTec PRODUCTS, Xiamen, China) and hydrogen peroxide (InTec PRODUCTS) were added. Color formation was allowed to proceed for 15 min and then stopped with 0.5 M  $\text{H}_2\text{SO}_4$ . The absorbance of each well was read at 450 nm within 5 min by a plate microplate reader (Thermo Fisher Scientific, Boston, MA).

**Table 1. Patient details and clinicopathological characteristics**

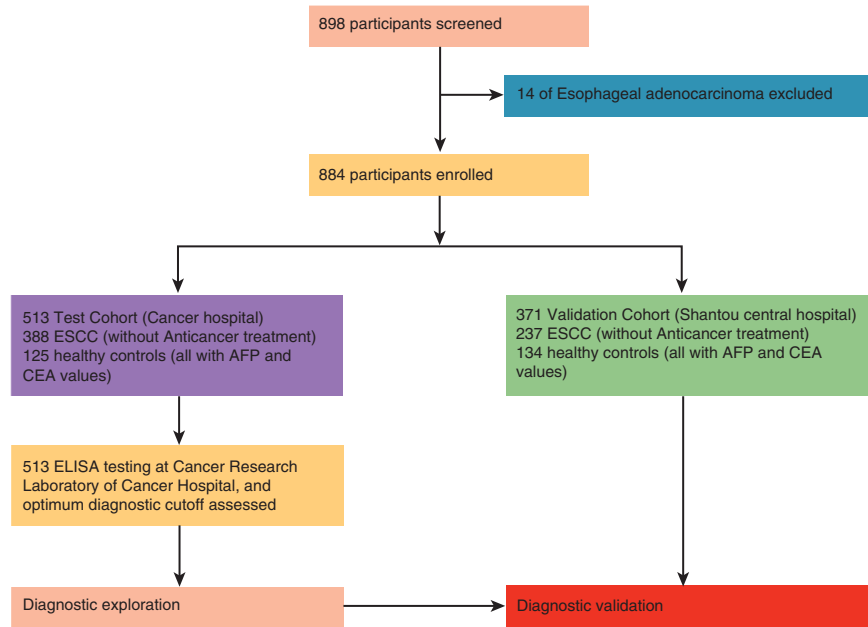
Group	Test cohort		Validation cohort	
	ESCC	Normal	ESCC	Normal
Number	388	125	237	134
Female, <i>n</i> (%)	97 (25%)	63 (50%)	61 (26%)	44 (33%)
Mean age $\pm$ s.d. (years)	59 $\pm$ 8	52 $\pm$ 9	59 $\pm$ 9	51 $\pm$ 10
Age range (years)	39–82	40–80	42–79	41–73
Smokers, <i>n</i> (%)	280 (72%)	45 (36%)	156 (66%)	Unknown
<i>TNM stage</i>				
0	2 (2) <sup>a</sup>		2	
I	29 (23) <sup>a</sup>		31	
II (IIA+IIB) <sup>b</sup>	96 (28+38) <sup>a</sup>		114 (43+71)	
III	229 (135) <sup>a</sup>		90	
IV	27		—	
Unknown	5		—	
<i>Histological grade</i>				
High (grade 1)	80 (77) <sup>a</sup>		56	
Middle (grade 2)	114 (111) <sup>a</sup>		160	
Low (grade 3)	23 (22) <sup>a</sup>		21	
Unknown	171 (16) <sup>a</sup>		—	
<i>Depth of tumor invasion</i>				
Tis	2 (2) <sup>a</sup>		2	
T1+T2	68 (50) <sup>a</sup>		58	
T3+T4	310 (174) <sup>a</sup>		177	
Unknown	8			
<i>Lymph node metastasis</i>				
Positive	234 (125) <sup>a</sup>		103	
Negative	146 (101) <sup>a</sup>		134	
Unknown	8		—	
<i>Size of tumor</i>				
<5 cm	138 (104) <sup>a</sup>		185	
$\geq$ 5 cm	197 (112) <sup>a</sup>		52	
Unknown	53			
<i>Site of tumor</i>				
Cervical esophagus	8		—	
Upper thorax	59 (28) <sup>a</sup>		18	
Middle thorax	270 (164) <sup>a</sup>		155	
Lower thorax	47 (34) <sup>a</sup>		62	
Unknown	4		2	

ESCC, esophageal squamous cell carcinoma.

<sup>a</sup>Data shown in parentheses from the test cohort represent patients with tumor resection.

<sup>b</sup>Denotes only patients with tumor resection can be classified with American Joint Committee on Cancer (AJCC) stages IIA and IIB.

ESCC data in the validation cohort are all from patients with tumor resection.



**Figure 1.** Study profile. AFP,  $\alpha$ -fetoprotein; CEA, carcinoembryonic antigen; ESCC, esophageal squamous cell carcinoma; ELISA, enzyme-linked immunosorbent assay.

All cancer and normal samples were interspersed on the plates and run in duplicate. Quality control samples were run to ensure quality control monitoring of the assay runs by using Levey–Jennings plots. With the purpose of minimizing an intraassay deviation, the ratio of the difference between duplicated sample optical density values to their sum was used to assess precision of the assay. If the ratio was  $>10\%$ , the test of this sample was treated as being invalid and the sample was repeated.

#### Assay cutoff values

The cutoff value designating positive reactivity was defined as an optical density value greater than the mean plus 3 s.d. of the normal controls from the test cohort (14,19). Specificity of the assay was calculated as the percentage of normal controls who gave a negative result.

#### Statistical analysis

All analyses were done using SPSS (version 17.0, Chicago, IL), Microsoft Excel (Redmond, WA), or GraphPad Prism software (La Jolla, CA). The number and proportion of positive samples were presented with 95% exact confidence interval (95% CI) for binomial proportions (34). The false positive rate, false negative rate, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio, all with 95% CIs, were presented to improve clinical interpretation. The  $\chi^2$  tests or Fisher's exact tests were carried out to flag where the proportion of positive results was significantly different between cancer groups and the normal controls, and to identify correlations of individual and combined antibody assay positivity with clinical parameters. In all tests, we considered  $P$  values of  $<0.05$  (two sided) to be significant.

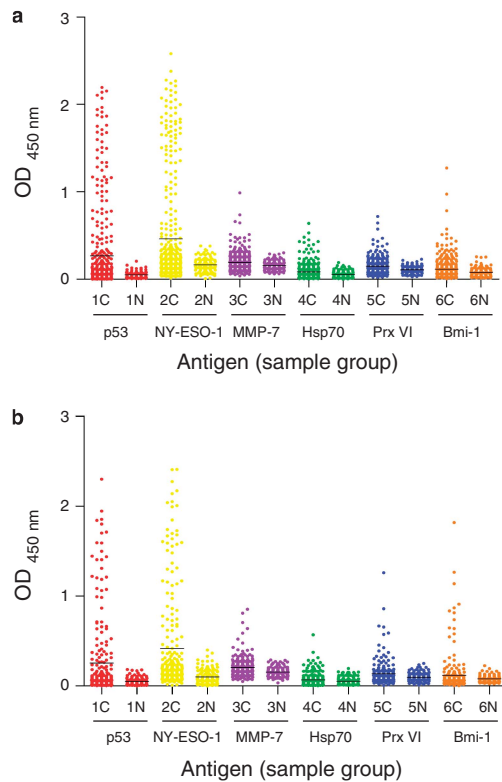
## RESULTS

### Autoantibodies in ESCC

In total, 884 participants were recruited, 513 in the test cohort and 371 in the validation cohort (**Figure 1**). Patient and normal control demographics and tumor clinicopathological characteristics are shown in **Table 1**.

The presence of autoantibodies to all TAAs in both cohorts is shown for one concentration of antigen in the scatter plots in **Figure 2**. When compared with the sera of normal controls, elevated levels of autoantibodies for all six TAAs were clearly present and similar in ESCC individuals in both cohorts.

Levels of autoantibodies to individual antigens in the ESCC groups and normal controls are shown in **Table 2**. In the test cohort, patients with ESCC were significantly different from the normal controls in individual autoantibodies. Individual autoantibodies were elevated between 9% (95% CI: 6–12%) and 30% (95% CI: 25–35%) in ESCC sera. Use of these 6 autoantibody assays provided an enhanced panel sensitivity of 57% (95% CI: 52–62%) and panel specificity of 95% (95% CI: 89–98%). The importance of autoantibody responses to individual antigens in the panel assay varied. There was a higher percentage of positive reactions to p53 antigen and NY-ESO-1 antigen, whereas positive reactions to MMP-7, Hsp70, Prx VI, and Bmi-1 were lower. Some TAA signals overlapped in cancer cases. In 23% of the seropositive individuals in panel 1 of 6, autoantibodies were raised to a second antigen in ESCC samples in the test cohort (**Supplementary Figure S1** online). This was reflected in the observation that a restricted panel consisting of the four antigens p53, NY-ESO-1, Hsp70, and Prx VI exhibited only a slightly reduced sensitivity of 55% (95% CI: 50–60%), but with an increased specificity of 98% (95% CI: 93–99%) in the test cohort (**Tables 2** and **3**).



**Figure 2.** Enzyme-linked immunosorbent assay (ELISA) antibody titers of individual patients and normal controls for tumor-associated antigens (TAAs). (a) Scatter plots of optical density (OD) values of autoantibodies from ESCC sera (388) and normal sera (125) in the test cohort. (b) Scatter plots of OD values of autoantibodies from ESCC sera (237) and normal sera (134) in the validation cohort. Black horizontal lines are means. Bmi-1, BMI1 polycomb ring finger oncogene; C, cancer; ESCC, esophageal squamous cell carcinoma; Hsp70, heat shock protein 70; MMP-7, matrix metalloproteinase-7; N, normal; Prx VI, peroxiredoxin VI.

With use of the cutoff values for individual autoantibodies from the test cohort, we observed similar results in the validation cohort to those in the test cohort. In the validation cohort, ESCC patients were also significantly different from the normal controls for each individual autoantibody in the panel assay. Individual autoantibodies were elevated between 8% (95% CI: 5–11%) and 29% (95% CI: 23–35%) in the ESCC group compared with between 0% (95% CI: 0–2%) and 3% (95% CI: 1–8%) in the normal controls. In addition, the validation cohort confirmed the ability of the autoantibody panel to diagnose ESCC with 51% (95% CI: 45–57%) sensitivity and 96% (95% CI: 91–99%) specificity. A restricted panel assay exhibited a sensitivity of 48% (95% CI: 42–54%) with a specificity of 96% (95% CI: 91–99%) in the validation cohort (Tables 2 and 3).

#### Autoantibody panel for early detection

In the test cohort, 53 patients with ESCC had early-stage disease (AJCC stage 0+I+IIA in patients with tumor resection). Positive frequencies of the autoantibody panel were significantly higher in these patients than those in normal controls ( $P < 0.0001$ , Table 4). The ability of the autoantibody panel to distinguish early-stage patients from normal controls was confirmed in the validation cohort (Table 4). In the test cohort, panel sensitivity for the detection of early-stage ESCC was 45% (95% CI: 32–59%), with specificity at 95% (95% CI: 89–98%), and similar results were obtained with the validation cohort (Table 5). Restriction of the panel to the presence of p53, NY-ESO-1, Hsp70, and Prx VI autoantibodies exhibited almost the same diagnostic performance for early-stage ESCC (Table 5).

In both the test cohort and validation cohort, within the early-stage groups, 4 patients with tumor resection were confirmed to have stage 0 disease, and the autoantibody panel gave a positive

**Table 2.** Frequency of autoantibodies to tumor-associated antigens (TAAs)

Group	p53	NY-ESO-1	MMP-7	Hsp70	Prx VI	Bmi-1	Panel	Panel of 4
<i>Test</i>								
ESCC	30 (25–35)*	26 (22–30)*	9 (6–12)**	11 (8–14)**	11 (8–14)**	11 (8–14)***	57 (52–62)*	55 (50–60)*
Normal controls	2 (1–7)	0 (0–2)	0 (0–2)	1 (0–5)	0 (0–2)	2 (1–7)	5 (2–10)	2 (1–7)
Specificity	98 (93–99)	100 (98–100)	100 (98–100)	99 (95–100)	100 (98–100)	98 (93–99)	95 (90–98)	98 (93–99)
<i>Validation</i>								
ESCC	29 (23–35)*	24 (19–29)*	10 (6–14)**	8 (5–11)***	10 (6–14)**	8 (5–11)**	51 (45–57)*	48 (42–54)*
Normal controls	3 (1–8)	1 (0–5)	0 (0–2)	1 (0–5)	0 (0–2)	0 (0–2)	4 (1–9)	4 (1–9)
Specificity	97 (92–99)	99 (95–100)	100 (98–100)	99 (95–100)	100 (98–100)	100 (98–100)	96 (91–99)	96 (91–99)

Bmi-1, BMI1 polycomb ring finger oncogene; ESCC, esophageal squamous cell carcinoma; Hsp70, heat shock protein 70; MMP-7, matrix metalloproteinase-7; Prx VI, peroxiredoxin VI.

All values are given in percentage positivity with 95% confidence interval (CI) in each group.

Panel: autoantibody positivity to any one of the six antigens.

Panel of 4: autoantibody positivity to any one of the four antigens (p53, NY-ESO-1, Hsp70, and Prx VI).

$P$  value is relative to normal controls ( $\chi^2$  tests). \* $P < 0.0001$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.01$ .

**Table 3.** Results for measurement of the autoantibody panel and panel of 4 in the diagnosis of ESCC

ESCC vs. NC	Sensitivity	Specificity	FPR	FNR	PPV	NPV	PLR	NLR
<i>Test cohort</i>								
Panel	57% (52–62%)	95% (89–98%)	5% (2–11%)	43% (38–48%)	97% (94–99%)	42% (36–48%)	11.92 (5.43–26.15)	0.45 (0.40–0.50)
Panel of 4	55% (50–60%)	98% (93–99%)	2% (1–7%)	45% (40–50%)	99% (96–100%)	41% (35–47%)	22.87 (7.54–70.21)	0.46 (0.41–0.52)
<i>Validation cohort</i>								
Panel	51% (45–57%)	96% (91–99%)	4% (1–9%)	49% (43–55%)	96% (90–99%)	53% (46–59%)	13.68 (5.74–32.63)	0.51 (0.45–0.58)
Panel of 4	48% (42–54%)	96% (91–99%)	4% (1–9%)	52% (46–58%)	96% (90–98%)	51% (45–57%)	12.89 (5.40–30.77)	0.54 (0.48–0.61)

CI, exact confidence interval; ESCC, esophageal squamous cell carcinoma; FNR, false negative rate; FPR, false positive rate; NC, normal controls; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value.

All values are given with 95% CI in each group.

Panel: autoantibody positivity to any one of the six antigens.

Panel of 4: autoantibody positivity to any one of the four antigens (p53, NY-ESO-1, heat shock protein 70 (Hsp70), and peroxiredoxin VI (Prx VI)).

**Table 4.** Positive rates of the autoantibody panel and panel of 4 in early-stage ESCC

Group	N	Panel		Panel of 4	
		Positive (%; 95% CI)	P value	Positive (%; 95% CI)	P value
<i>Test cohort</i>					
Early-stage ESCC (0+I+IIA)	53	24 (45, 32–59)	$P < 0.0001$	24 (45, 32–59)	$P < 0.0001$
Normal controls	125	6 (5, 2–10)		3 (2, 1–7)	
<i>Validation cohort</i>					
Early-stage ESCC (0+I+IIA)	76	35 (46, 35–57)	$P < 0.0001$	34 (45, 33–57)	$P < 0.0001$
Normal controls	134	5 (4, 1–9)		5 (4, 1–9)	

CI, exact confidence interval; ESCC, esophageal squamous cell carcinoma.

Statistical significance was determined using the  $\chi^2$  test. Data were from patients with tumor resection.

Panel: autoantibody positivity to any one of the six antigens.

Panel of 4: autoantibody positivity to any one of the four antigens (p53, NY-ESO-1, heat shock protein 70 (Hsp70), and peroxiredoxin VI (Prx VI)).

result in 75% (95% CI: 19–99%) of stage 0 cancers, although the numbers were small.

#### Effect of clinicopathological features on autoantibody assay sensitivity and specificity

The effect of patient demographics or tumor characteristics on variation in the specificity/sensitivity is demonstrated in **Figure 3** and **Supplementary Table S1** online. In the test cohort, there were no significant differences in sensitivity of the autoantibody panel when the ESCC samples were subdivided by patient age, gender, smoking status, size of tumor, site of tumor, depth of tumor invasion, histological grade, lymph node status, TNM stage or early-stage and late-stage groups ( $P > 0.05$ , **Figure 3** and **Supplementary Table S1**). Sensitivity of the autoantibody panel according to patient demographics or tumor characteristics was further confirmed with no significant difference in the validation cohort ( $P > 0.05$ , **Figure 3** and **Supplementary**

**Table S1**). Although statistical analysis showed that correlations of positivity for the panel were not significant between early-stage patients and late-stage patients, there seemed to be a higher incidence of autoantibodies in patients with later-stage tumors than in the early-disease group, especially in the test cohort. Because of the small number of stage IV cases in patients not undergoing tumor resection, and no cases of stage IV cases in the validation cohort, statistical analysis was performed on stage I, stage II, and stage III (patients with tumor resection) only. The sensitivity of the autoantibody panel in stage IV cancers was 63% (17 of the 27, 95% CI: 42–81%), which was similar to stage III cancers (patients with tumor resection). In two cohorts, there were almost no significant differences among tumor or patient characteristics between the different autoantibody positivities (**Supplementary Tables S2 and S3**). The correlation of the autoantibody panel with the available demographic data in early-stage patients was further evaluated. However, there was

**Table 5.** Results for measurement of the autoantibody panel and panel of 4 in the diagnosis of early-stage ESCC

Early-stage ESCC vs. NC	Sensitivity	Specificity	FPR	FNR	PPV	NPV	PLR	NLR
<i>Test cohort</i>								
Panel	45% (32–59%)	95% (89–98%)	5% (2–11%)	55% (41–68%)	80% (61–92%)	80% (73–86%)	9.43 (4.09–21.74)	0.57 (0.45–0.73)
Panel of 4	45% (32–59%)	98% (93–99%)	2% (1–7%)	55% (41–68%)	89% (70–97%)	81% (73–87%)	18.87 (5.94–59.97)	0.56 (0.44–0.72)
<i>Validation cohort</i>								
Panel	46% (35–58%)	96% (91–99%)	4% (1–9%)	54% (42–65%)	88% (72–95%)	76% (69–82%)	12.34 (5.05–30.17)	0.56 (0.46–0.69)
Panel of 4	45% (33–57%)	96% (91–99%)	4% (1–9%)	55% (43–67%)	87% (72–95%)	75% (68–82%)	11.99 (4.90–29.36)	0.57 (0.47–0.70)

CI, exact confidence interval; ESCC, esophageal squamous cell carcinoma; FNR, false negative rate; FPR, false positive rate; NC, normal controls; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value.

All values are given with 95% CI in each group.

Panel: autoantibody positivity to any one of the six antigens.

Panel of 4: autoantibody positivity to any one of the four antigens (p53, NY-ESO-1, heat shock protein 70 (Hsp70), and peroxiredoxin VI (Prx VI)).

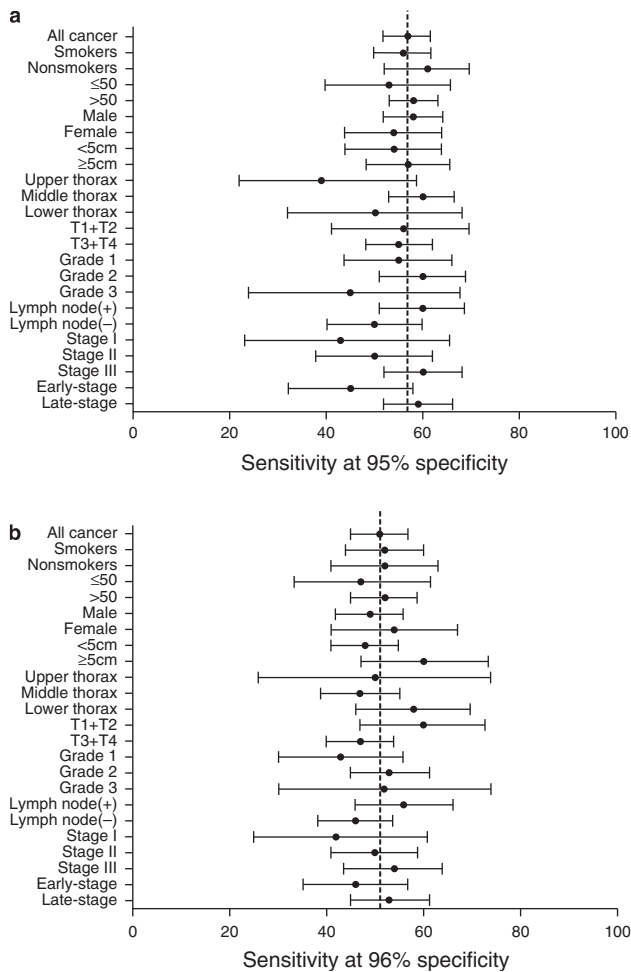
no correlation with any of the variables (**Supplementary Table S4**).

## DISCUSSION

Because of the lack of specific symptoms in patients with early-stage ESCC and the lack of reliable noninvasive screening tests, early detection is hampered. Currently, the diagnosis of ESCC is based on endoscopic examination followed by histological analysis of biopsy, an invasive technique not suitable for the screening of asymptomatic populations. In this study, we have demonstrated that the detection of autoantibodies to selected tumor-associated antigens in the peripheral blood has potential diagnostic value for ESCC. More than half of the ESCC patients examined had autoantibodies to at least 1 of the 6 TAAs, with a specificity of at least 95%. The sensitivity and specificity of the autoantibody panel in this study are in broad agreement with published data for individual autoantibody assays in other cancers such as lung, breast, colorectal, gastric, and prostate cancers, and further confirm that application of several autoantibodies would detect cancer with higher efficiency than a single biomarker (14,16–20,22,23,26,35). Consistent with prior reports (16,17,19), no significant difference was seen in autoantibody detection of cancer when patients were subdivided by tumor characteristics. This is in contrast to detection of the tumor-associated antigens that are markers of tumor burden and not useful for the early detection of ESCC (6–8).

Identification of novel serum biomarkers for the diagnosis of cancer, especially for detection and screening in early-stage cancer, is an important means to improve clinical outcome (36). Previous publications about other tumors have given prominence to the potential value of a panel of autoantibodies for the early detection of cancer (10,14–20). Using a panel of antigens, autoantibodies can be detectable as early as 5 years before radiographic detection

on incidence screening in lung cancer and can be detected in the asymptomatic stage of breast cancer up to 5 years before the onset of disease (10,37). Our study further supports previous publications indicating that the induction of autoantibodies occurs early in the process of carcinogenesis. The positive frequencies of our autoantibody panel in the early-stage ESCC group were 45% (95% CI: 32–59%) in the test cohort and 46% (95% CI: 35–58%) in the validation cohort. Thus, our autoantibody panel improves the diagnostic performance in early-stage ESCC samples, compared with other biomarkers (e.g., CYFRA21-1 and squamous cell carcinoma antigen) currently used in clinical practice (6–8). For example, positive frequencies of CYFRA21-1 are reported to be only 4.7% and 25% in stage I and stage II cancers, respectively (7). For squamous cell carcinoma antigen, positive frequencies in patients with stage 0 + I and stage II have been reported to be 10.8% and 24.0%, respectively (8). However, the measurement of the autoantibody panel does not seem sensitive enough, with false negative rates of 43% (95% CI: 38–48%) and 55% (95% CI: 41–68%) in patients with ESCC and early-stage ESCC in the test cohort, respectively. Similar results were observed in the validation cohort (**Table 3**). Such a high false negative frequency will prevent the timely diagnosis of ESCC for some patients, particularly the symptomless, early-stage patients. Thus, a further search for autoantibodies with high sensitivity will be the focus of future studies. This autoantibody panel assay has excellent specificity for ESCC, especially for the early stage. The robust specificity of the test, as a prerequisite to be useful to aid in early detection, indicates that our autoantibody panel should make a significant contribution to the diagnosis and screening of ESCC patients. However, in screening a large population where the prevalence of ESCC is low, as a result of low sensitivity and large population, even with a very low false positive rate, many false positive results will be obtained. Therefore, if measurement of the autoantibody panel is used as a screen for ESCC,



**Figure 3.** Forest plot showing the sensitivity at a fixed specificity by patient demographics and tumor characteristics. **(a)** Forest plot for the test cohort showing the sensitivity at a fixed specificity of 95%. Data concerning size of tumor, site of tumor, depth of tumor invasion, histological grade, lymph node status, TNM stage, and early stage and late stage were from patients undergoing tumor resection. Data about age, gender, and smoking status were from all esophageal squamous cell carcinoma (ESCC) patients. Line shows sensitivity of 57%. **(b)** Forest plot for the validation cohort showing the sensitivity at a fixed specificity of 96%. All data were from patients with tumor resection. Line shows sensitivity of 51%.

especially for early-stage patients in a general population, further diagnosis should involve additional imaging tests, such as gastroscopy. As a screen, our autoantibody test may be more appropriate for regions with high incidence rates of ESCC. Whether measurement of autoantibodies may have a role in populations with different ESCC prevalences needs further validation. In the future, we will use our autoantibody test to screen for ESCC in regions with high incidence rates in a prospective study. Patients with a positive autoantibody test would undergo gastroscopy or another imaging technology to detect early tumor lesions. If esophageal (or other) cancer is verified by imaging, then definitive treatment could be carried out earlier.

Each antigen in the panel has been shown to be capable of inducing autoantibodies in patients with ESCC. However, measurement

of autoantibodies to MMP-7, Prx VI, Hsp70, and Bmi-1 did not significantly improve the assay. The low levels of autoantibodies to MMP-7, Prx VI, Hsp70, and Bmi-1 emphasize that the combination of an optimized panel to measure these autoantibodies has to be selective for high sensitivity and specificity. In contrast, measurement of the autoantibody responses to p53 and NY-ESO-1 antigens is integral to the panel assays. Previous studies, investigating the presence of autoantibodies to NY-ESO-1 antigen in lung cancer and breast cancer, demonstrate similar levels of sensitivity and specificity as reported here (16,17). The clinical use of autoantibodies for screening and early diagnosis appears to depend on finding an optimized panel of autoantibodies. The restricted panel consisting of the four antigens p53, NY-ESO-1, Hsp70, and Prx VI exhibited almost the same diagnostic performance as when screening for all six autoantibodies. Taking into account the cost-benefit, autoantibody assays against the restricted panel is acceptable for aiding the early detection of ESCC. The advantage of this method is that the full range of heterogeneous esophageal cancers can be detected by increasing the number of TAAs or altering the antigens used in the panel. Many proteomics-based technologies, such as serological analysis of tumor antigens by recombinant complementary DNA expression cloning (38), phage display (39), and serological proteome analysis (40), have been used for the detection of antigen-specific antibodies. Therefore, an increasing number of other cancer-specific immunoreactive antigens is continually being described. Recent publications have reported additional antigens, such as the ATP-binding cassette, sub-family C, member 3 (ABCC3), and cell division cycle 25B (CDC25B), that induce the production of autoantibodies in ESCC (41,42). Whether these antigens can enhance the diagnostic efficiency of the autoantibody panel remains to be investigated.

The sample size and the proportion of patients with different tumor characteristics in our test cohort were different from those in the validation cohort (**Figure 1** and **Table 1**). Therefore, to a certain extent, the groups differed in diagnostic performance (**Tables 2–5** and **Figures 2** and **3**). For example, because the number of patients with later-stage tumors, in which the autoantibody responses to the panel seemed more prevalent, was larger in the test cohort than that in the validation cohort, the sensitivity of the autoantibody panel differed between two cohorts (**Tables 2** and **3**). Despite these differences, the diagnostic performance of the autoantibody panel was generally similar in the test and validation cohorts. We believe that the autoantibody panel has diagnostic potential for ESCC because its value in the test cohort was confirmed in an independent validation cohort.

In summary, this is a large study to report the clinically diagnostic relevance of an autoantibody assay using a panel of specific autoantibodies as potential serum markers for ESCC. Our results reveal that an autoantibody blood test, which is noninvasive, cost effective, and has no side effects, may act as an aid to diagnose ESCC, especially in the early-stage ESCC.

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### CONFLICT OF INTEREST

**Guarantor of the article:** Li-Yan Xu, PhD.

**Specific author contributions:** Yi-Wei Xu designed the study, searched the literature, performed the experiments, analyzed and interpreted the data, and wrote the manuscript; Yu-Hui Peng collected patient samples and clinical data, analyzed, and interpreted the data; Bo Chen did the experiments; Zhi-Yong Wu and Jian-Yi Wu provided patient samples and clinical data; Chun-Peng Zheng collected patient samples and clinical data; Hai-Peng Guo, Jin-Hui Shen, and Shao-Hong Wang analyzed and interpreted clinical data; Li-Yan Xu and En-Min Li conceptualized and designed the study, supervised the project, and revised the paper. All authors vouch for the respective data and analysis, and have approved the final version and agreed to publish the manuscript.

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## Study Highlights

### WHAT IS CURRENT KNOWLEDGE

- ✓ Esophageal squamous cell carcinoma (ESCC) has a poor prognosis partly because of the lack of early diagnosis.
- ✓ Whether measurement of an autoantibody panel could aid in early diagnosis of ESCC remains to be revealed.

### WHAT IS NEW HERE

- ✓ This autoantibody assay enabled discrimination between early-stage ESCC and normal controls.
- ✓ The diagnostic value of the autoantibody panel for ESCC was confirmed in an independent validation cohort.

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