EBioMedicine 73 (2021) 103674

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.elsevier.com/locate/ebiom

Tumor-associated autoantibodies in ESCC screening: Detecting prevalent early-stage malignancy or predicting future cancer risk?

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ARTICLE INFO

Article History:
Received 24 August 2021
Revised 19 October 2021
Accepted 19 October 2021
Available online xxx

Keywords:
Early warning biomarker
ESCC
Tumor-associated autoantibodies
Early-detection
Risk prediction

ABSTRACT

Background: To assess potential roles for tumor-associated autoantibodies (TAAs) in esophageal squamous cell carcinoma (ESCC) screening: detecting early-stage malignancy, and predicting future cancer risk.

Methods: Thirteen candidate autoantibodies identified in previous literatures were measured using multiplex serological assays in sera from cases and matched controls nested in two population-level screening cohorts in China. To evaluate the role of TAAs in detecting prevalent esophageal malignant lesions, an identification set (150 cases vs. 560 controls) and an external validation set (34 cases vs. 121 controls) were established with pre-screening sera collected $\leq$ 12 months prior to screening-related diagnosis. To explore the role of TAAs in predicting future ESCC risk, an exploration set (105 cases vs. 416 controls) with pre-diagnostic sera collected $>$ 12 months before clinical diagnosis was established. Two models, the questionnaire-based model and full model additionally incorporating TAA markers, were constructed. Area under the receiver operating characteristic curve (AUC) and net reclassification improvement (NRI) were calculated to compare the performance of the two models.

Findings: In the identification set, NY-ESO-1 (OR=2·12, 95% CI=1·02-4·40) and STIP1 (OR=1·83, 95% CI=1·10-3·05) were positively associated with higher risk of esophageal malignancy. Elevated MMP-7 was associated with higher risk of malignancy in females (OR_{female}=5·07, 95% CI=1·30-19·71). The estimates in validation set were consistent with these results, but were close to null in exploration set. Integration of selected TAAs improved the performance of questionnaire-based models in detecting prevalent esophageal malignancy (female: $AUC_{full\ model}$=0·745, 95% CI=0·675-0·814, $AUC_{questionnaire\text{-}based\ model}$=0·658, 95% CI=0·585-0·732, NRI=0·604, $P<0·0001$; male: $AUC_{full\ model}$=0·662, 95% CI=0·596-0·728, $AUC_{questionnaire\text{-}based\ model}$=0·619, 95% CI=0·548-0·690, NRI=0·357, P=0·0028). This improvement was also seen in validation set, but was not similarly effective in distinguishing long-term incident cases from healthy controls.

Interpretation: Serological autoantibodies against NY-ESO-1, STIP1, and MMP-7 perform well in detecting early-stage esophageal malignancy, but are less effective in predicting future ESCC risks.

Funding: This work was supported by the National Science & Technology Fundamental Resources Investigation Program of China (2019FY101102), the National Natural Science Foundation of China (82073626), the National Key R&D Program of China (2016YFC0901404), the Beijing-Tianjin-Hebei Basic Research Cooperation Project (J200016), the Digestive Medical Coordinated Development Center of Beijing Hospitals Authority (XXZ0204), and the Natural Science Foundation of Beijing Municipality (7182033).

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https://doi.org/10.1016/j.ebiom.2021.103674

Research in context

Evidence before this study

Tumor-associated autoantibodies (TAAs) are promising as blood-based markers for early warning of cancer given the properties of biological specific productivity, stability, and blood accessibility. We searched PubMed with the terms (1) "esophageal", or "oesophageal", or "esophagus"; and (2) "cancer", or "neoplasm", or "carcinoma", or "malignancy", or "tumor"; and (3) "autoantibody" or "antibody", or "immunoglobulin"; and (3) "detection", or "diagnosis", or "screen", or "screening", or "biomarker", or "marker" for studies up to Dec 31, 2020. Over 30 kinds of blood TAAs have been reported as candidate early-warning biomarkers for esophageal cancer.

Added value of this study

This is the first study embedded in real-world population-level screening cohorts to systematically evaluate the potential early-warning roles of TAAs in ESCC screening: 1) detection of prevalent early-stage malignancy, and 2) prediction of future risk of developing cancer. We showed that serological autoantibodies against NY-ESO-1, STIP1, and MMP-7 performed well in detecting early-stage malignancy in the esophagus, but did not effectively predict future ESCC cases.

Implications of all the available evidence

Serological autoantibodies against NY-ESO-1, STIP1, and MMP-7 coupled with data for traditional risk factors for ESCC allow non-invasive detection of early-stage malignant lesions in the esophagus, which may result in down-staging and improve survival for patients with ESCC. For research on cancer early-warning, it is essential to conduct studies in population-based screening settings rather than clinical settings. In TAA-related research, more importance should be attached to early-stage malignancy warning than long-term risk predicting .

1. Introduction

Esophageal cancer (EC) is one of the most common and lethal cancer worldwide, with 604,100 incident cases and 544,076 deaths in 2020 [1]. In China, EC ranks as the sixth most frequent cancer and fourth leading cause of cancer death, [2] and more than 90% of the EC cases are of esophageal squamous cell histologic type [3, 4]. Early detection has been shown to improve survival and reduce mortality from this disease, and Lugol's chromoendoscopy is the current standard technique for population-level esophageal squamous cell carcinoma (ESCC) screening [5]. However, endoscopic screening has limitations which include high cost, use of an invasive procedure and potential for adverse events (e.g. perforation, hemorrhage, and severe allergic reaction to iodine) [6]. Noninvasive methods, such as blood-based markers are therefore needed to identify individuals at high-risk for ESCC. This will help achieve precision screening for risk-prediction.

According to current evidence, tumor-associated autoantibodies (TAAs) show promise as blood-based markers for early warning of ESCC. It is speculated that TAA production is triggered by increased immunogenicity of corresponding antigens, such as proteins which are mutated, aberrantly expressed, misfolded or overexpressed in early stages of carcinogenesis [7–9]. In addition, several properties of TAAs allow their application as early-warning biomarkers, including stability, accessibility in blood specimens, and detectability which sometimes precedes clinical diagnosis by several months to years [8]. TAAs have already been tested in a pilot study as early-warning markers for lung cancer. A blood-based screening test called "EarlyCDT-Lung" which incorporates seven autoantibodies was developed to detect prevalent lung cancer [10].

Over 30 kinds of blood TAAs have been reported as candidate early-warning biomarkers for esophageal cancer [11–14]. However, there are several problems with these studies. First, almost all previous studies enrolled patients with obvious symptoms and convenient controls directly from hospitals, which typically recruited a high proportion of advanced stage cases. This kind of study design may result in false-positive findings, which cannot be validated in a real-world screening setting. Second, the specific role TAAs play in early-warning of ESCC, including detection of early-stage malignancy, or prediction of future risk of developing cancer, was not clarified in these previous studies. Third, when evaluating the independent association of TAAs and ESCC, previous studies seldom made adequate adjustment for potential confounding influences such as body mass index (BMI), family history of EC, cigarette smoking and alcohol consumption.

In this prospective nested case-control study which is based on two large-scale population-level ESCC screening cohorts in a high-risk area in rural China, we measured the serum reactivity of candidate TAAs using multiplex serological assays. Our aim was to assess the role of early-warning by TAAs in detection of prevalent esophageal malignancy and prediction of future ESCC risk.

2. Methods

2.1. Parent study

This study was nested in two population-level screening cohorts (Endoscopic Screening for Esophageal Cancer in China, *ESECC* [ClinicalTrials.gov identifier: NCT01688908]; [15] Anyang Esophageal Cancer Cohort Study, *AECCS* [16]) in the "Taihang Mountain Region", which is a high-risk area for ESCC in rural China. The design of these two parent studies has been described elsewhere.

***ESECC* cohort**. In 2012-2016, 668 randomly selected villages in Hua County in rural Anyang were allocated to either the screening arm or the control arm of the study at a ratio of 1:1 through blocked randomization. A total of 33,948 village residents aged 45-69 years were enrolled. In the baseline investigation, a blood sample was collected from each participant and a questionnaire interview was conducted. Participants in the screening arm underwent endoscopic examination with iodine staining. Incident cancer cases were identified through annual door-to-door interviews or passive linkage with local medical insurance claims data up to September 15th 2019 [17, 18].

***AECCS* cohort**. In 2006-2013, 8112 residents aged 25-65 years from 6 representative villages in rural Anyang were invited for three rounds of endoscopic examination, blood sample collection, and questionnaire interview [16]. Incident cancer cases were identified in the same way as in the *ESECC* trial.

2.2. Study design

Study population. To evaluate the performance of TAAs in detecting prevalent malignant esophageal lesions, an identification set and an independent external validation set were established, enrolling participants from the screening arm of the *ESECC* trial and the *AECCS* cohort. Pre-screening serum samples were collected $\leq$ 12 months prior to screening-related diagnosis of early-stage esophageal cancer after endoscopy (Figure 1) [19].

To explore the performance of TAAs in prediction of long-term (more than 12 months) ESCC risk, an exploration set was established, enrolling participants from the two arms of the *ESECC* trial using pre-

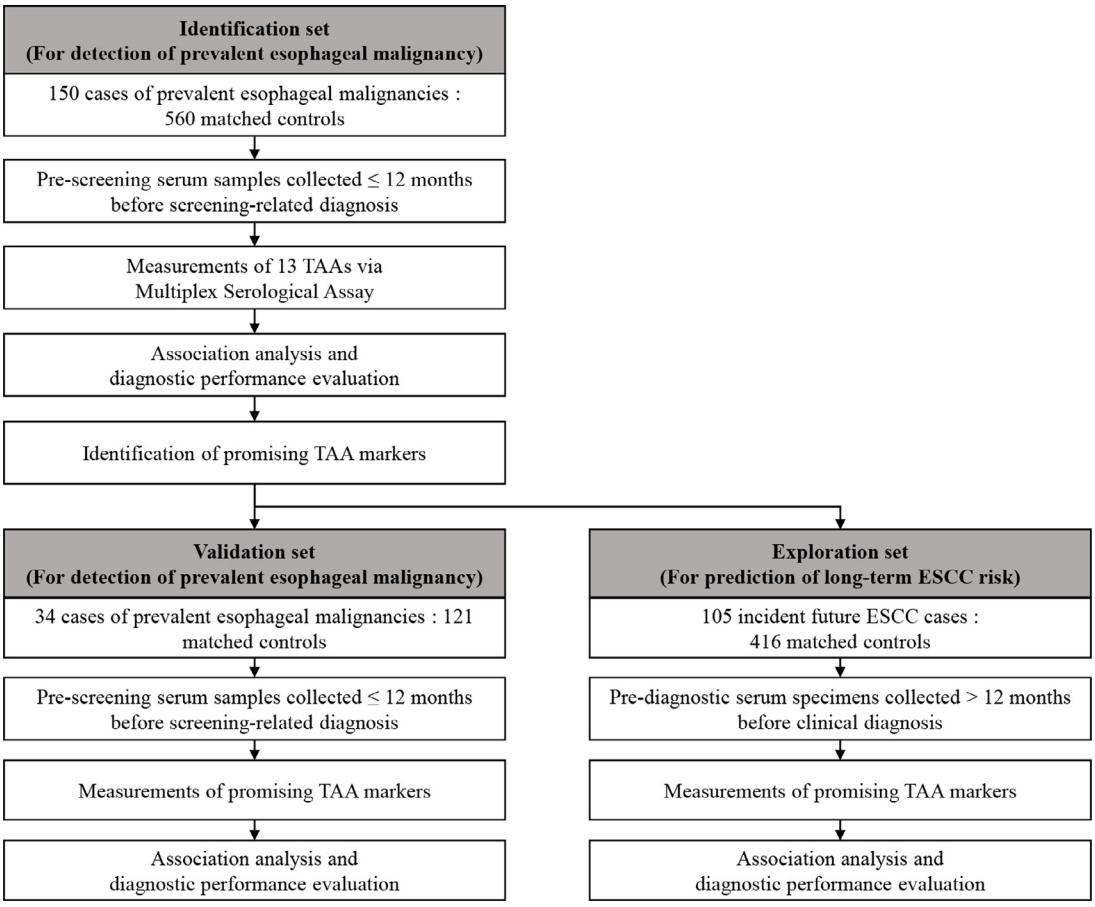

Fig. 1. Flow chart for TAA evaluation in the identification set, validation set and exploration set. Prevalent esophageal malignancy (in the identification set and validation set) were defined as severe dysplasia and above (SDA) lesions in the esophagus, including severe dysplasia, carcinoma in situ, or ESCC identified at endoscopic examination or clinically diagnosed at hospitals in 12 months or less of blood sample collection. Incident future ESCC cases (in the exploration set) were defined as clinically diagnosed ESCC patients who were diagnosed more than 12 months after blood collection. For each case, up to 4 controls were randomly selected using incidence density sampling, matching on the source cohort, allocated arm (for *ESECC* trial), sex, age at enrollment (5-year age group), community, calendar year of enrollment and calendar year of the blood sample collection. The identification set included 150 cases of prevalent esophageal malignancy and 560 matched controls; the validation set included 34 prevalent esophageal malignant lesions and 121 matched controls; and the exploration set included 105 long-term incident cases and 416 matched controls. Abbreviations: ESCC: esophageal squamous cell carcinoma; TAA: tumor-associated autoantibody.

diagnostic serum specimens collected > 12 months before clinical diagnosis.

The inclusion criteria for these three sets were: 1) completion of the questionnaire, and 2) provision of qualified blood samples at baseline investigation or before endoscopic screening.

Case identification. Two endpoints were defined for evaluation of the two putative roles of TAAs in early-warning . Prevalent malignant lesions (in the identification set and the validation set) included severe dysplasia and above (SDA) lesions in the esophagus, including severe dysplasia, carcinoma in situ, and ESCC identified at endoscopic examinations, or clinically diagnosed at hospitals at a time point less than or equal to 12 months after blood sample collection [19]. Future incident cases (in the exploration set) were defined as ESCC patients who were clinically diagnosed more than 12 months after blood sampling. All available cases were used in this study.

Control selection. For each case, up to 4 controls were randomly selected using incidence density sampling. These controls were matched for source cohort, allocated arm (for ESECC trial), sex, age at enrollment (5-year age group), community, calendar year of enrollment and calendar year of blood sample collection.

3. Autoantibody detection methods

Serum specimen collection. A fasting blood sample of ~ 5 mL was collected from each participant prior to endoscopic examination (screening arm of *ESECC* cohort, *AECCS* cohort) or at enrollment (control arm of *ESECC* cohort). After clotting, the blood samples were centrifuged at 2000 g for 5 minutes and stored at -80°C until testing was carried out.

Bead-based multiplex serological assays. We selected 13 candidate TAAs based on previous literature (Cancer/testis antigen 1B, NY-ESO-1; Baculoviral IAP repeat-containing protein 5, Survivin; Stress-induced-phosphoprotein 1, STIP1; Peroxiredoxin-6, Prx VI; Fragment of DNA topoisomerase I, TOPO48; Cellular tumor antigen p53, p53; Polycomb complex protein BMI-1, Bmi-1; Heat shock 70 kDa protein 1A, HSP70; Matrix metallopeptidase 7, MMP-7; G2/mitotic-specific cyclin-B1, cyclinB1; LETM1 domain-containing protein 1, HCCR; Insulin-like growth factor 2 mRNA-binding protein 1, IMP1; and Insulin-like growth factor 2 mRNA-binding protein 2, p62) [12, 20–23]. Detailed information regarding these 13 candidate TAAs, fusion protein preparation, and development of multiplex serological assays is presented in the Supplementary Materials. In short, targeted tumor-associated antigens were cloned and expressed with N-terminal glutathione S-transferase (GST) and C-terminal FLAG tag. Autoantibodies were measured with multiplex serological assays in which glutathione casein-coated microspheres [24] were used to capture GST-X-FLAG fusion proteins (see Supplementary Figure 1) [24, 25]. Serum samples were diluted at 1:150 and added into a mixture of coupled beads for overnight incubation at 4°C. After incubation with biotinylated donkey anti-human immunoglobulin G (H+L) secondary

Table 1
Baseline characteristics of cases and controls in the identification set, validation set and exploration set

Variables	Identification set (For detection of prevalent esophageal malignancy)			Validation set (For detection of prevalent esophageal malignancy)			Exploration set (For prediction of long-term ESCC risk)		
	Control n (%)	Case n (%)	*P* value[a]	Control n (%)	Case n (%)	*P* value[a]	Control n (%)	Case n (%)	*P* value[a]
n[b]	560	150	-	121	34	-	416	105	-
Age at blood draw									
Mean (SD)	62·45 (4·72)	62·99 (4·77)	0·22	54·79 (9·15)	55·29 (9·13)	0·78	63·02 (3·89)	62·24 (3·96)	0·61
Sex									
Female	269 (48·04)	74 (49·33)	0·78	43 (35·54)	13 (38·24)	0·77	177 (42·55)	45 (42·86)	0·95
Male	291 (51·96)	76 (50·67)		79 (64·46)	21 (61·76)		239 (57·45)	60 (57·14)	
Family history of esophageal cancer									
No	495 (88·39)	120 (80·00)	0·0073	109 (90·08)	27 (79·41)	0·094	377 (90·63)	87 (82·86)	0·023
Yes	65 (11·61)	30 (20·00)		12 (9·92)	7 (20·59)		39 (9·38)	18 (17·14)	
Body mass index									
≤22 kg/m^2	105 (18·75)	45 (30·00)	0·0027	22 (18·18)	10 (29·41)	0·15	85 (20·43)	26 (24·76)	0·33
kgm^2	455 (81·25)	105 (70·00)		99 (81·82)	24 (70·59)		331 (79·57)	79 (75·24)	
Cigarette smoking									
No	426 (76·07)	111 (74·00)	0·60	65 (53·72)	17 (50·00)	0·70	289 (69·47)	78 (74·29)	0·33
Yes	134 (23·93)	39 (26·00)		56 (46·28)	17 (50·00)		127 (30·53)	27 (25·71)	
Alcohol consumption									
No	425 (75·89)	111 (74·00)	0·63	96 (79·34)	27 (79·41)	0·99	296 (71·15)	77 (73·33)	0·66
Yes	135 (24·11)	39 (26·00)		25 (20·66)	7 (20·59)		120 (28·85)	28 (26·67)	

[a] *P* values were derived using the Chi-square test (categorical variables) or the Wilcoxon rank-sum test (continuous variables).
[b] Individuals with complete questionnaire data and qualified blood specimens were included in the present study.
Abbreviations: ESCC, esophageal squamous cell carcinoma; SD, standard deviation.

antibody and streptavidin-conjugated R-phycoerythrin, antibody reactivity was quantified by the fluorescence of these beads and expressed as median fluorescence intensity (MFI) based on reading at least 100 beads per region per well on a Bio-plex 200 analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Final antigen-specific MFI values were calculated by subtracting individual bead background values (a GST-Flag fusion protein without intervening tumor-associated antigen) [24, 25].

Quality control. In assay development phase, standard curves were prepared by serial dilution of commercially available antibodies, and critical immunoassay parameters were evaluated according to standards (Supplementary Table 1-4, Supplementary Figure 2). Multiplex assays showed good precision with intra-batch coefficients of variation (CV) of 3·12%-6·04% and inter-batch CV of 4·90%-15·40% across the 13 analytes. These assays showed good accuracy with recovery ranging from 73·90% to 106·88% for the 13 analytes. In the detection phase, all serum samples were tested blindly in duplicate, and samples from any given case-control pentad were analyzed in the same batch to minimize batch effect.

4. Statistical analysis

Participant characteristics of cases and controls were compared using the Chi-square test (categorical variables) or the Wilcoxon rank-sum test (continuous variables). Cutoff points for each autoantibody were calculated based on the MFI values of the controls from the identification set, where signals higher than the mean plus standard deviation (SD) were considered seropositive as defined in other studies. [12,14] Univariable and multivariable conditional logistic regression models were applied to evaluate the association between each TAA marker and risk of malignant esophageal lesions. The multivariable model adjusted for identified risk factors of ESCC including age (continuous form), family history of EC, BMI, smoking, and alcohol consumption, which were detailed in Supplementary Table 5. Potential effect modification was evaluated by adding an interaction term for the marker with each of the above-mentioned risk factors one at a time in the multivariable model, with application of stratified analysis when significant interactions were detected. TAAs which showed promise were further evaluated in the validation set and the exploration set using the same coding rules for seropositive and seronegative as the identification set.

We developed two models to assess the diagnostic performance of TAA markers. The questionnaire-based model contained well-recognized risk factors for ESCC only (age, family history of EC, BMI, smoking and alcohol consumption), and the full model additionally integrated the panel of TAAs identified in the identification set. Area under the receiver operating characteristic curve (AUC) for these two models was calculated to evaluate the models' discriminatory performance. We also calculated the net reclassification improvement (NRI) to determine whether selected TAA markers promoted the performance of questionnaire-based models. Comparison of the two models was also carried out in the validation set and the exploration set using the same model coefficients derived from the identification set.

Statistical analysis was performed using STATA (Version 13·1; Stata Corp LLC, TX, USA). All tests were 2-sided and had a significance level of 0·05 unless otherwise specified.

5. Ethics statement

This study was approved by the Institutional Review Board of the Peking University School of Oncology, China (Approval number: 2011101110, 2006020). All participants in this study provided written informed consent.

6. Role of the funding source

The funders were not involved in study design, data collection, analysis, interpretation or writing.

7. Results

The current study included three datasets (Fig. 1): 1) an identification set including 150 cases of prevalent esophageal malignancy (137 screening-detected cases and 13 cases which were clinically diagnosed within 12 months of blood sample collection) and 560 matched controls from the screening arm of the *ESECC* trial; 2) a validation set including 34 prevalent esophageal malignant lesions (32

Table 2
The association of three autoantibody markers (NY-ESO-1, STIP1, MMP-7) and risk of esophageal malignancy in the identification set, validation set and exploration set

TAA target[a]	Classification	Identification set (For detection of prevalent esophageal malignancy)			Validation set (For detection of prevalent esophageal malignancy)			Exploration set (For prediction of long-term ESCC risk)		
		Cases / Controls	Crude OR (95% CI)	Adjusted OR (95% CI)[b]	Cases / Controls	Crude OR (95% CI)	Adjusted OR (95% CI)[b]	Cases / Controls	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
NY-ESO-1[c]	**Negative**	129/508	Ref.	Ref.	29/115	Ref.	Ref.	92/368	Ref.	Ref.
	Positive	21/52	2·20 (1·08-4·48)	2·12 (1·02-4·40)	5/6	3·65 (0·96-13·97)	3·47 (0·82-14·59)	13/48	1·09 (0·56-2·11)	1·05 (0·54-2·03)
STIP1[c]	**Negative**	116/476	Ref.	Ref.	16/88	Ref.	Ref.	90/369	Ref.	Ref.
	Positive	34/84	1·79 (1·10-2·91)	1·83 (1·10-3·05)	18/33	2·70 (1·27-5·76)	3·68 (1·50-9·03)	15/47	1·30 (0·69-2·44)	1·27 (0·67-2·42)
MMP-7[d]										
Female	**Negative**	68/262	Ref.	Ref.	11/39	Ref.	Ref.	43/173	Ref.	Ref.
	Positive	6/7	4·18 (1·15-15·18)	5·07 (1·30-19·71)	2/4	1·77 (0·32-9·78)	1·71 (0·30-9·88)	2/4	1·90 (0·35-10·41)	0·98 (0·14-6·69)
Male	**Negative**	75/274	Ref.	Ref.	18/74	Ref.	Ref.	57/224	Ref.	Ref.
	Positive	1/17	0·22 (0·03-1·68)	0·20 (0·03-1·56)	3/4	2·88 (0·64-12·88)	4·02 (0·58-27·70)	3/15	0·76 (0·20-2·85)	0·67 (0·18-2·55)
***P* value for interaction**[e]			0·016	0·016		0·67	0·54		0·40	0·69

[a] A total of 13 candidate TAAs (Cancer/testis antigen 1B, NY-ESO-1; Baculoviral IAP repeat-containing protein 5, Survivin; Stress-induced-phosphoprotein 1, STIP1; Peroxiredoxin-6, Prx VI; Fragment of DNA topoisomerase I, TOPO48; Cellular tumor antigen p53, p53; Polycomb complex protein BMI-1, Bmi-1; Heat shock 70 kDa protein 1A, HSP70; Matrix metallopeptidase 7, MMP-7; G2/mitotic-specific cyclin-B1, cyclinB1; LETM1 domain-containing protein 1, HCCR; Insulin-like growth factor 2 mRNA-binding protein 1, IMP1; Insulin like growth factor 2 mRNA binding protein 2, p62) were evaluated and only TAAs with identified independent associations are listed in the table.

[b] In the multivariable conditional logistic regression model, adjustments were made for ESCC risk factors including age (continuous form), family history of esophageal cancer, body mass index (BMI), smoking and alcohol consumption.

[c] The association of NY-ESO-1, STIP1 and risk of esophageal malignancy was evaluated among all participants in each dataset.

[d] The association of MMP-7 and risk of esophageal malignancy was evaluated separately in female and male participants.

[e] The *P* value was calculated to test the effect modification in MMP-7 and risk of esophageal malignancy by sex.

Abbreviations: CI, confidential interval; MMP-7, Matrix metallopeptidase 7; NY-ESO-1, Cancer/testis antigen 1B; OR, odds ratio; STIP1, Stress-induced-phosphoprotein 1; TAA, tumor-associated autoantibody.

cases screening-detected cases and 2 cases which were clinically diagnosed within 12 months of blood sample collection) and 121 matched controls from the *AECCS* cohort; 3) an exploration set including 105 long-term incident cases (clinically diagnosed cases identified more than 12 months after blood sample collection; median lead-time = 43 months) and 416 matched controls from the two arms of the *ESECC* trial.

Selected participant characteristics are shown in Table 1. In the identification set, cases and controls were of similar age and sex distribution (matching variables). Cases were more likely to have a family history of EC and lower BMI as compared with controls. A similar pattern was observed in the validation and exploration sets.

The association of these 13 candidate autoantibody markers and risk of esophageal malignancy was first estimated in the identification set (Supplementary Table 6). Of these 13 markers, two autoantibodies (NY-ESO-1 and STIP1) were positively associated with having prevalent malignant esophageal lesions after controlling for potential confounding factors (Table 2, NY-ESO-1: odds ratio [OR]=2·12, 95% confidential interval [CI]=1·02-4·40; STIP1: OR=1·83, 95% CI=1·10-3·05). In the pairwise interaction test, sex was found to modify the association of MMP-7 and risk of prevalent esophageal malignancy. Stratified analysis showed that MMP-7 was significantly increased in female cases as compared with female controls (OR_{female}=5·07, 95% CI=1·30-19·71, P value for interaction=0·016). In the external independent validation set, similar positive association patterns were observed for these three markers, which showed comparable ORs but wider confidential intervals (NY-ESO-1: OR=3·47, 95% CI=0·82-14·59; STIP1: OR=3·68, 95% CI=1·50-9·03; MMP-7: OR_{female}=1·71, 95% CI=0·30-9·88). However, OR estimates were closer to null values using serum samples collected more than 12 months prior to clinical diagnosis in the exploration set (NY-ESO-1: OR=1·05, 95% CI=0·54-2·03; STIP1: OR=1·27, 95% CI=0·67-2·42; MMP-7: OR_{female}=0·98, 95% CI=0·41-6·69).

The performance of the questionnaire-based model was compared with that of the full model which integrated the selected TAA markers. Given the interaction of sex and MMP-7, three markers (NY-ESO-1, STIP1, MMP-7) were added to the full models for females, and only two (NY-ESO-1, STIP1) were added to the full models for males. In the identification set, adding TAA markers improved the discrimination performance of questionnaire-based models (Fig. 2 & Table 3, female: $AUC_{full\ model}$=0·745, 95% CI=0·675-0·814, $AUC_{questionnaire\text{-}based\ model}$=0·658, 95% CI=0·585-0·732, NRI=0·604, $P<0·0001$; male: $AUC_{full\ model}$=0·662, 95% CI=0·596-0·728, $AUC_{questionnaire\text{-}based\ model}$=0·619, 95% CI=0·548-0·690, NRI=0·357, P=0·0028). Similar improvement was observed with addition of TAAs in the external independent validation set (female: $AUC_{full\ model}$=0·644, 95% CI=0·484-0·804, $AUC_{questionnaire\text{-}based\ model}$=0·563, 95% CI=0·363-0·762, NRI=0·472, P=0·068; male: $AUC_{full\ model}$=0·702, 95% CI=0·563-0·842, $AUC_{questionnaire\text{-}based\ model}$=0·671, 95% CI=0·534-0·808, NRI=0·513, P=0·019). However, the power of the questionnaire-based models to discriminate future ESCC cases from healthy controls was not increased by addition of TAA markers as seen in the exploration set (female: $AUC_{full\ model}$=0·625, 95% CI=0·530-0·719, $AUC_{questionnaire\text{-}based\ model}$=0·626, 95% CI=0·531-0·722, NRI=0·179, P=0·14; male: $AUC_{full\ model}$=0·527, 95% CI=0·447-0·607, $AUC_{questionnaire\text{-}based\ model}$=0·539, 95% CI=0·455-0·623, NRI=0·096, P=0·25).

8. Discussion

In this nested case-control study, we systematically evaluated the potential early-warning roles of TAAs in ESCC screening based on real-world population-level screening cohorts for the first time. Integration of a panel of serological autoantibodies against NY-ESO-1, STIP1 and MMP-7 was found to improve the performance of questionnaire-based models in detecting prevalent esophageal malignancy (diagnosed $\leq$ 12 months after blood sample collection).

Table 3
Improvement in AUC and NRI[a] resulting from addition of TAA panel to questionnaire-based models in the identification set, validation set and exploration set

Dataset	AUC (95% CI) (full model[b])	AUC (95% CI) (questionnaire-based model[c])	NRI (*P* value)
Total			
Identification set (for detection of prevalent esophageal malignancy)	0·670 (0·619-0·719)	0·639 (0·588-0·690)	0·229 (0·013)
Validation set (for detection of prevalent esophageal malignancy)	0·703 (0·594-0·812)	0·627 (0·514-0·739)	0·817 (<0·0001)
Exploration set (for prediction of long-term ESCC risk)	0·575 (0·512-0·638)	0·577 (0·514-0·640)	0·141 (0·20)
Female			
Identification set (for detection of prevalent esophageal malignancy)	0·745 (0·675-0·814)	0·658 (0·585-0·732)	0·604 (<0·0001)
Validation set (for detection of prevalent esophageal malignancy)	0·644 (0·484-0·804)	0·563 (0·363-0·762)	0·472 (0·068)
Exploration set (for prediction of long-term ESCC risk)	0·625 (0·530-0·719)	0·626 (0·531-0·722)	0·179 (0·14)
Male			
Identification set (for detection of prevalent esophageal malignancy)	0·662 (0·596-0·728)	0·619 (0·548-0·690)	0·357 (0·0028)
Validation set (for detection of prevalent esophageal malignancy)	0·702 (0·563-0·842)	0·671 (0·534-0·808)	0·513 (0·019)
Exploration set (for prediction of long-term ESCC risk)	0·527 (0·447-0·607)	0·539 (0·455-0·623)	0·096 (0·25)

[a] AUC was calculated to evaluate the discriminatory performance of these models. NRI was calculated to determine whether selected TAA markers improve the diagnostic performance of traditional questionnaire-based models.
[b] The full model consisted of TAA markers (NY-ESO-1, STIP1, MMP-7 in females and NY-ESO-1, STIP1 in males) as well as the aforementioned risk factors.
[c] The questionnaire-based model contained only well-recognized risk factors for ESCC (age, family history of esophageal cancer, body mass index, smoking and alcohol consumption).
Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidential interval; ESCC, esophageal squamous cell carcinoma; MMP-7, Matrix metallopeptidase 7; NRI, net reclassification improvement; NY-ESO-1, Cancer/testis antigen 1B; STIP1, Stress-induced-phosphoprotein 1; TAA, tumor-associated autoantibodies.

However, these biomarkers did not perform well in predicting long-term ESCC risk (diagnosed > 12 months after blood sample collection).

To provide advance "warning" of cancer development, early-warning biomarkers must have the capacity to distinguish early-stage patients from healthy controls. Most previous studies identified "promising" biomarkers by comparing patients with obvious symptoms (typically at an advanced stage) and convenient controls recruited directly from hospitals. As a result, the biomarkers found in these studies may reflect only changes that occur a long time after the initiation of cancer, and whether they have early-warning value is incompletely determined. Therefore, the evaluation of early-warning biomarker performance must make use of early-stage cases and comparable controls selected from real-world screening settings.

The clinical use of early-warning biomarkers falls into two categories: 1) prediction of long-term risk of developing clinically recognizable cancer before the biologic onset of the disease, and this approach may be applied as a risk mitigation tool for monitoring high-risk individuals; and 2) detection of early-stage malignancy in asymptomatic individuals or patients with early symptoms, and this would be expected to promote utilization of additional screening tests or further diagnostic evaluation in high-risk populations, and to shift stage distribution to earlier stages where better survival may be expected. [26, 27]

In this study, we evaluated both of these potential roles of TAAs in esophageal cancer screening. In detection of prevalent malignancies, NY-ESO-1 and STIP1 were positively associated with risk of having present esophageal malignancy. Sex modified the association of MMP-7 and risk of ESCC; MMP-7 was significantly elevated only in female cases. Adding a panel of selected TAAs improved the performance of questionnaire-based models to detect the presence of esophageal malignancy at an early stage (identification set: NRI=0·604, $P<0·0001$ in females, NRI=0·357, $P=0·0028$ in males; validation set: NRI=0·472, $P=0·068$ in females, NRI=0·513, $P=0·019$ in males).

In the three TAAs identified in this study, NY-ESO-1 is a cancer-testis family antigen which is typically restricted to germ cells, but is also frequently expressed in cancer cells. [28] Given this restricted expression, corresponding autoantibodies were considered to have promise as early-detection biomarkers, and have been widely evaluated in esophageal cancer, [29, 30] gastric cancer, [31] colorectal cancer [32] and lung cancer. [10] STIP1 is co-chaperone molecule which regulates the heat shock protein 90 and heat shock protein 70 chaperone machinery, and this molecule participates in diverse biologic functions including RNA splicing, transcription, protein folding and cell cycle regulation. [33] Overexpression of STIP1 has been identified in tumorigenesis in several kinds of cancers, [34–36] and autoantibody titers against STIP1 may be significantly elevated in cancer patients as compared with healthy controls. [37, 38] MMP-7 belongs to the matrix metalloproteinase family and is capable of degrading the extracellular matrix. The biologic mechanism of effect modification by sex is not clear, but previous studies have reported that MMP-7 is more abundant in serum from females, [39] and MMP-7 is expressed at higher levels in females with idiopathic pulmonary fibrosis. [40]

Although NY-ESO-1, STIP1 and MMP-7 performed well in detection of early-stage malignant lesions in the esophagus, these molecules showed limited ability for predicting risk of ESCC occurring more than a year after blood collection. With serum samples collected long before the diagnosis (median lead-time ≈ 4 years, exploration set), the estimated association of reactivity of TAAs and risk of developing ESCC were close to null, and little improvement in the accuracy of distinguishing future cancer cases from healthy controls was gained by incorporating TAA panels into questionnaire-based models ($AUC_{full\ model}$=0·625, $AUC_{questionnaire\text{-}based\ model}$=0·626, NRI=0·179, P=0·14; male: $AUC_{full\ model}$=0·527, $AUC_{questionnaire\text{-}based\ model}$=0·539, NRI=0·096, P=0·25). TAA-only model revealed similar results that TAAs performed better in detecting prevalent malignancy than predicting future risk of ESCC (Supplemental Figure 3). This was consistent with findings in ovarian cancer. [41, 42] These observations suggest that high levels of autoantibodies may represent elevated tumor burden. Previous researches support several different aspects of this supposition: 1) production of TAAs is considered to be triggered by increased immunogenicity of the corresponding tumor-associated antigen, [7–9] 2) autoantibody titers are positively correlated with the tumor mass or metastases in both human researches [43] and animal experiments, [44, 45] and 3) antibody responses decrease after tumor excision or surgery. [46] Consequently, for individuals with malignancies that have accumulated enough malignant transformations, the level of TAAs may be adequately high to allow

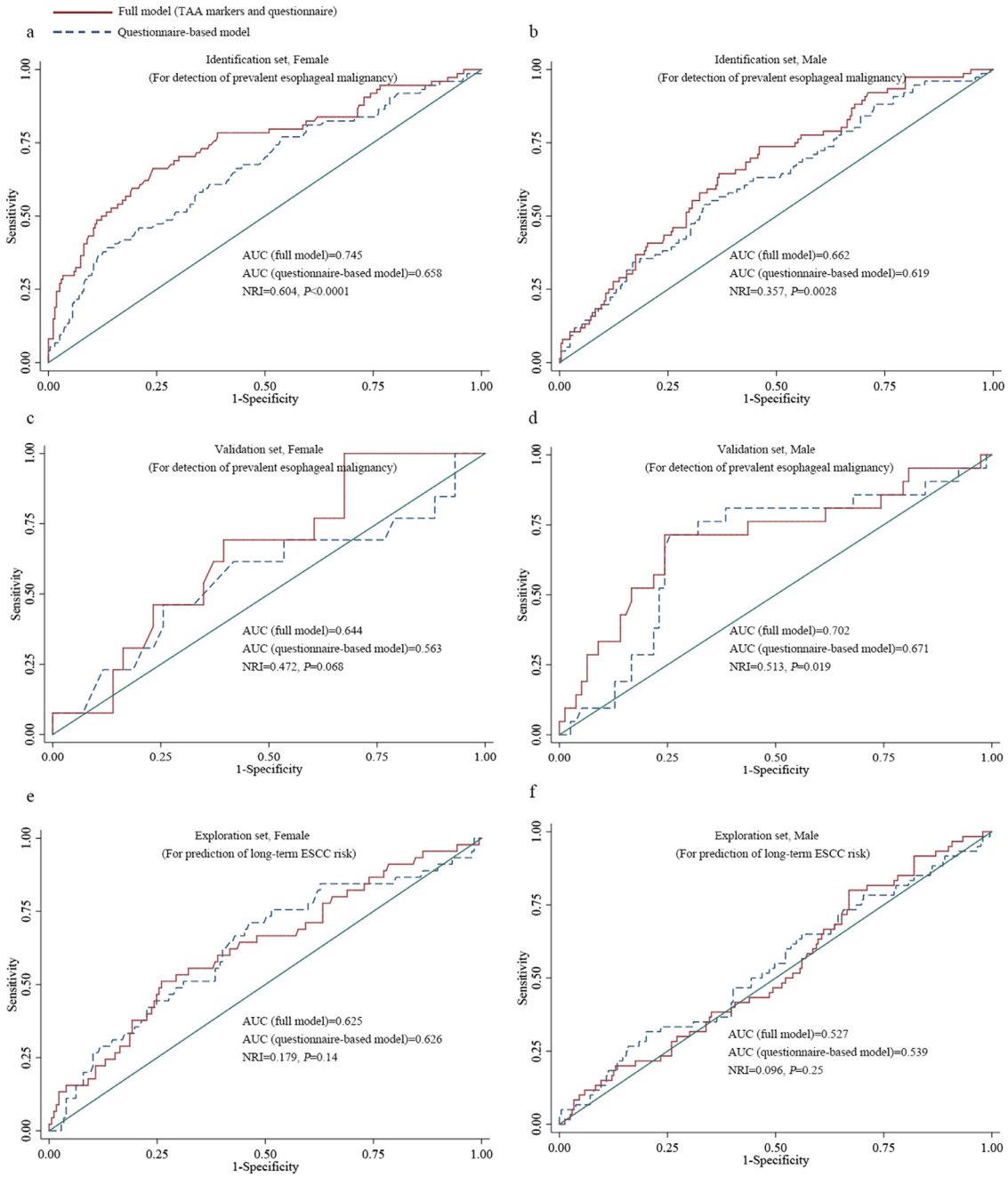

Fig. 2. Improvement in AUC and NRI resulting from integrating TAA panels to questionnaire-based models in the identification set, validation set and exploration set. AUC and NRI of questionnaire-based models and full models were calculated and compared to evaluate the improvement in performance by integrating the TAA panel in the identification set (females [a, 74 cases and 269 matched controls], males [b, 76 cases and 291 matched controls]), validation set (females [c, 13 cases and 43 matched controls], males [d, 21 cases and 78 matched controls]), and exploration set (females [e, 45 cases and 177 matched controls], males [f, 60 cases and 239 matched controls]). The questionnaire-based model contained only well-recognized risk factors for ESCC (age, family history of EC, body mass index [BMI], smoking and alcohol consumption), and the full model additionally incorporated the panel of TAAs (NY-ESO-1, STIP1, MMP-7 in females, and NY-ESO-1, STIP1 in males). In the identification set, adding TAA markers improved the discrimination performance of questionnaire-based models (female: NRI=0·604, $P<0·0001$; male: NRI=0·357, $P=0·0028$). Similar improvement was observed with addition of TAAs in the external independent validation set (female: NRI=0·472, $P=0·068$; male: NRI=0·513, $P=0·019$). The discrimination power was not increased by addition of TAA markers as seen in the exploration set (female: NRI=0·179, $P=0·14$; male: NRI=0·096, $P=0·25$). Abbreviations: AUC, area under the receiver operating characteristic curve; ESCC, esophageal squamous cell carcinoma; NRI, net reclassification improvement; TAA, tumor associated autoantibody.

distinction from healthy controls. In patients who may develop cancer in the future, however, autoantibodies would fail in prediction of long-term risk due to insufficient tumor load.

This study has several points which warrant emphasis. First, owing to the unique research platform based on two large-scale prospective real-world screening cohorts, we were for the first time able to simultaneously evaluate the role of TAAs in early detection of prevalent malignancy and in prediction of future risk. Second, the availability of comprehensive questionnaire interviews enabled us to fully adjust for traditional risk factors in evaluation of the independent association of TAAs and esophageal malignancy. Third, TAA markers were measured through multiplex serological assays with rigorous quality control measures.

At the same time, some limitations of this study must be acknowledged. First, this is a single-center study representing a single high-risk area in rural China. Second, the number of esophageal cancer

cases was limited despite the fact that over 38,000 participants were recruited and over 30,000 endoscopies were performed in these two cohorts. Further multi-center studies with greater statistical power in real-world screening cohorts are needed to confirm the improvement to detect esophageal malignancies by incorporating TAAs. A screening tool including demographic and lifestyle factors, TAAs, and other biomarkers may be constructed and evaluated in future population-level studies.

In summary, TAAs coupled with data for traditional risk factors for ESCC allow non-invasive detection of early-stage malignant lesions in the esophagus, resulting in down-staging and improved survival for patients with ESCC. TAAs have limited performance capacity for predicting future ESCC cases, possibly due to low tumor burden. For research on cancer early-warning, it is essential to conduct studies in population-based screening settings rather than clinical settings. In TAA related research, more importance should be attached to early-stage malignancy warning than predicting long-term risk of occurrence of future cancers.

Contributors

Conception and design: Yang Ke, Zhonghu He. Acquisition of data: Minmin Wang, Fangfang Liu, Yaqi Pan, Ruiping Xu, Fenglei Li, Anxiang Liu, Haijun Yang, Liping Duan, Lin Shen, Qi Wu, Ying Liu, Mengfei Liu, Zhen Liu, Zhe Hu, Huanyu Chen, Hong Cai. Analysis and interpretation of data: Minmin Wang, Fangfang Liu, Zhonghu He, Yang Ke. Verification of the underlying data: Minmin Wang, Fangfang Liu, Zhonghu He, Yang Ke. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Acknowledgments

This work was supported by the National Science & Technology Fundamental Resources Investigation Program of China (2019FY101102), the National Natural Science Foundation of China (82073626), the National Key R&D Program of China (2016YFC0901404), the Beijing-Tianjin-Hebei Basic Research Cooperation Project (J200016), the Digestive Medical Coordinated Development Center of Beijing Hospitals Authority (XXZ0204), and the Natural Science Foundation of Beijing Municipality (7182033). We would like to thank Dr. Borgia A. Jeffery from Rush University Medical Center for assistance in the development of multiplex serological assays. We also would like to thank Dr. Michael A. McNutt for editing and correction of this manuscript.

Data sharing statement

The datasets and/or analytic methods used during the current study will be made available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103674.

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