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ORIGINAL ARTICLE

Retrospective Study

Plasma DNA methylation detection for early screening, diagnosis, and monitoring of esophageal adenocarcinoma and squamous cell carcinoma

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Abstract

BACKGROUND

The early diagnosis rate of esophageal cancer (EC), one of the most prevalent digestive tract cancers worldwide, remains low.

AIM

To investigate the utility of plasma *SHOX2*, *SEPTIN9*, *EPO*, and *RNF180* methylation in the clinical diagnosis and monitoring of EC.

METHODS

Plasma samples were collected from 210 patients at Hubei Cancer Hospital, and TaqMan polymerase chain reaction was employed to detect plasma *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* methylation. The area under the curve was used to estimate their diagnostic value for EC. Cox and logistic regression analyses were used to estimate the independent screening risk factors for patients with EC.

RESULTS

The sensitivity and specificity of combined assessment of plasma *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* methylation for adenocarcinoma, squamous cell carcinoma (SCC), and EC detection were 66.67% and 86.27%, 77.40% and 85.29%, and 76.19% and 86.27%, respectively; the area under the curve values for diagnosing adenocarcinoma, SCC, and EC were 0.737 [95% confidence interval (CI): 0.584–0.89], 0.824 (95%CI: 0.775–0.891), and 0.864 (95%CI: 0.809–0.92), respectively.

CONCLUSION

According to our findings, plasma *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* methylation exhibits appreciated sensitivity for diagnosing EC. The precise measurement of plasma *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* methylation can improve EC diagnosis and therapy efficacy monitoring.

Key Words: Methylation; Tumor markers; Esophageal squamous cell carcinoma; Adenocarcinoma; Diagnosis

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Core Tip: This study for the first time analyzed the clinical value of detecting plasma *SHOX2*, *SEPTIN9*, *EPO*, and *RNF180* methylation for diagnosis and monitoring of esophageal cancer (EC). We revealed that the sensitivity and specificity of combined assessment of methylation of the four genes in plasma for detecting EC were 76.19% and 86.27%, respectively; the area under the curve value of this combination was 0.864 (95% confidence interval: 0.809–0.92). The accurate measurement of methylation of these four genes in plasma might aid monitoring of therapy efficacy in both esophageal adenocarcinoma and squamous cell carcinoma.

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INTRODUCTION

Esophageal cancer (EC), a prevalent digestive tract tumor, claims the lives of approximately 604000 individuals worldwide annually. It exhibits varying morbidity and mortality rates across different countries. China reportedly has the highest incidence of EC globally, with an average annual death toll of approximately 576000 people[1]. In recent years, EC has surged rapidly in China, particularly affecting the younger population[1]. Progressive dysphagia is the hallmark symptom of EC; however, its diagnosis is frequently delayed due to nonspecific symptoms in the early stages of tumor development. Dysphagia onset often correlates with an advanced disease, carrying a 5-year survival rate of < 15%[1,2].

Clinical diagnosis of EC primarily relies on double-contrast barium X-ray and endoscopy[3]. However, endoscopic population screening lacks widespread acceptance in China[4]. The early diagnosis rate of EC remains low, contributing to poor prognoses for patients with EC diagnosed at advanced clinical stages[5,6]. Currently, there are no established tumor markers for EC. Traditional markers for esophageal squamous cell carcinoma (SCC) and adenocarcinoma, such as SCC antigen (SCCA), carbohydrate antigen 199 (CA199), and carcinoembryonic antigen (CEA), exhibit limited sensitivity and specificity. In recent years, the clinical significance of circulating tumor DNA (ctDNA) as tumor markers in cancer diagnosis has gained recognition. ctDNA is released into the bloodstream by tumor cells, encompassing cancer-related genetic mutations and epigenetic variations[7-9]. DNA methylation variations emerge early in cancer, representing dynamic epigenetic changes that evolve with tumor progression. ctDNA methylation in liquid biopsy offers advantages such as non-invasiveness, stable target molecules, cost-effectiveness, high diagnostic performance, and broad applicability[10,11]. Monitoring ctDNA methylation levels aids in early tumor diagnosis and prognosis assessment, with proven application and potential in the clinical diagnosis of various cancers. Noteworthy examples include the use of *SHOX2* promoter methylation for lung cancer screening[12,13]. Further, an analysis of *EPO* promoter methylation revealed frequent hypermethylation of regulatory sequences in tumors, leading to epigenetic silencing of *EPO* in cancer cells[14]. *SHOX2* exhibits high methylation levels in patients with lung cancer, with its dynamic increase markedly associated with elevated cancer risk in high-risk populations[15,16]. Moreover, *SEPT9* methylation, employed for colorectal cancer (CRC) screening, stands out as the first ctDNA liquid biopsy indicator approved by the United States

Food and Drug Administration. It demonstrates superior diagnostic performance than protein tumor markers and fecal immunochemical tests[17,18], with its sensitivity progressively increasing with cancer stage. According to previous studies, the sensitivity and specificity of circulating methylated Septin 9 (mSEPT9) are estimated to be 50%-70% and ≥ 90%, respectively, for detecting CRC. *RNF180* is a novel preferentially methylated gene in gastric adenocarcinoma[19,20], and the area under the curve (AUC) values of methylated *RNF180* is 0.723 [95% confidence interval (CI): 0.694-0.752] [sensitivity: 46.2% (95%CI: 42.3%-50.1%); specificity: 87.3% (95%CI: 84.1%-89.9%)]. The sensitivity of mSEPT9 for detecting EC was 43.1% (AUC = 0.69), with a 95.6% specificity[21,22]. Despite these promising findings, the application of methylation detection in early screening for EC remains limited in reported studies. Consequently, it is imperative to develop non-invasive detection methods that facilitate effective early screening of EC. The present study for the first time explored the clinical utility of combined detection of methylation of plasma *SHOX2*, *SEPTIN9*, *EPO*, and *RNF180* in diagnosis and monitoring of esophageal adenocarcinoma and SCC.

MATERIALS AND METHODS

Genome data

Cancer genome atlas data were retrieved from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and The Cancer Genome Atlas (http://www.ualcan.path.uab.edu) databases.

Patients and healthy controls

We retrospectively enrolled patients (n = 210) from Hubei Cancer Hospital, China between June 2022 and July 2023 to evaluate the clinical performance of plasma *SHOX2*, *SEPTIN9*, *EPO*, and R*NF180* gene methylation in diagnosing EC. The screening population had one of the following characteristics: Barrett's esophagus history; family history of EC; dysphagia; chronic uncured gastroesophageal reflux disease; and corrosive esophageal burn. All patients underwent an upper endoscopy, and some received pathological examination if necessary. Thirty-five patients were initially diagnosed as having EC, and the other 175 EC patients were diagnosed based on pathological evidence according to World Health Organization criteria and TNM classification. Patients who had not undergone prior anticancer therapy were included. Healthy controls (n = 72) were obtained from the health examination center, and cases of benign esophageal diseases (n = 30) were collected from Wuhan Hospital of Integrated Traditional Chinese and Western Medicine, China. Ethical approval was obtained from the Hubei Cancer Hospital ethics committee.

Blood samples

To ensure sample integrity, whole blood samples (10 mL/case) were collected from each participant with Streck Cell-Free DNA BCT (Streck, United States) and centrifuged at 1600 g for 20 min at room temperature to obtain the plasma. All plasma samples were stored at -80 °C. To mitigate the impact of sample storage duration on DNA quality, all samples were analyzed within 1 mo after collection. The BioChain Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, United States) was used to extract cfDNA from plasma. CpG island cytosine methylation in *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* genes in human plasma was detected by TaqMan polymerase chain reaction (PCR) after treating DNA with sulfite. Unmethylated cytosine was converted to uracil using sodium bisulfite to ensure that only methylated cytosine was replicated. Bisulfite-converted DNA was then captured on the magnetic particles, purified by washing steps and eluted in a 55-μL elution buffer for real-time PCR. DCt, calculated as Ct (gene of interest) − Ct (internal control), was used to assess the co-methylation levels of these genes. Samples were included in our analyses when $18 \leq Ct_{ACTB} \leq 30$. They were classified as methylation-positive when DNA methylation levels of at least one of the four genes met the following quantitative criteria: $Ct_{SHOX2} < 32$ and $DCt_{SHOX2} \leq 9$; $Ct_{EPO} < 35$ and $DCt_{EPO} \leq 12$; $Ct_{SEPTIN9} < 40$ and $DCt_{SEPTIN9} \leq 9$; and $Ct_{RNF180} < 40$ and $DCt_{RNF180} \leq 9$. All other samples were classified as methylation-negative. Methylation reagents for *SHOX2* and *EPO* genes were sourced from LungMe Assay (Tellgen Corporation, Shanghai, China), and those for *SEPTIN9* and *RNF180* were from a methylation test kit (Biochain Science Technology Inc, Beijing, China).

Serum SCCA, CA199, and CEA level determination

Serum levels of SCCA, CEA, and CA199 were detected using a chemiluminescence immunoassay and associated reagents (Snibe Diagnostic Company Ltd., Shenzhen, China). Abnormal levels were determined based on the following predefined thresholds: SCCA > 2.7 mg/L, CEA > 5 μg/mL, and CA199 > 41 U/mL.

Statistical analysis

SPSS 25 (IBM, Armonk, NY, United States) was used for statistical analyses. The normality of the distribution was determined using the Shapiro–Wilk test. Differences between groups were analyzed using the Mann–Whitney U-test, Kruskal–Wallis H-test, one-way analysis of variance, independent t-test, or χ^2 test, as appropriate. The association between individual patient variables was analyzed with Spearman's rank correlation significance test. $P < 0.05$ indicated statistical significance. Specificity and sensitivity were calculated using receiver operating characteristic (ROC) curves. Differences between groups with different results were analyzed using the t-test or χ^2 test. Univariate analysis and multivariate Cox regression were employed to identify the association of different variables with EC.

Table 1 Clinical characteristics of 210 patients with esophageal cancer

Clinicopathological data	*n*	*SHOX2* methylation	*Septin9* methylation	*EPO* methylation	*RNF180* methylation	Combined methylation detection
Tumor pathology						
Adenocarcinoma	33					
Squamous cell carcinoma	177					
P value		0.354	0.19	0.524	0.098	0.527
Gender						
Male	122					
Female	88					
Male *vs* female		0.416	0.379	0.439	0.279	0.464
Age at diagnosis						
≤ 60 years	73					
> 60 years	137					
Median age (years)	65.6					
Mean age (years)	63.5					
≤ 60 *vs* > 60 years		0.665	0.011	0.021	0.211	0.001
Tumor stage						
I-II	19					
III-IV	191					
I-II *vs* III-IV		0.019	0.022	0.001	0.149	0.001
Follow-up						
Follow-up available	23					

RESULTS

Study cohort

This study comprised 210 patients with EC and 102 controls from the screening population at Hubei Cancer Hospital between June 2022 and July 2023. The study cohort consisted of 122 men [average age: 65.6 years (51–87 years)] and 88 women [average age: 69.9 years (54–89 years)]. Disease characteristics of the cohort are presented in Table 1.

Methylation percentage and association with EC diagnosis

According to TCGA data analysis, *EPO* and *SHOX2* expression in EC tissue samples was significantly higher than that in normal tissue samples, while *RNF180* expression in EC tissue samples was lower (P < 0.05). Promoter methylation levels of *EPO*, *SHOX2*, and *RNF180* in normal (n = 16) and EC (n = 185) tissue samples were indicated by beta values ranging from 0 (unmethylated) to 1 (fully methylated). Beta values between 0.7 and 0.5 indicated hypermethylation and those between 0.3 and 0.25 indicated hypomethylation (Figure 1).

Figure 2 and Figure 3 depict the frequency distribution of methylation for the four aforementioned genes for detecting esophageal adenocarcinoma and SCC. In EC patients with advanced stage disease, *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* DNA methylation was more prevalent than in EC patients with early stage disease (Figure 2). However, there were no significant differences in the positive percentages of *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation in EC patients with SCC and adenocarcinoma (Figure 3). The sensitivity and specificity of *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation for diagnosing EC were 18%–56% and 80%–90%, respectively (Figure 4). Figure 4 depicts the ROC curves for methylation of these genes. The sensitivity and specificity of *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* methylation for diagnosing esophageal adenocarcinoma were 18.18% and 94.12%, 42.42% and 88.24%, 42.42% and 86.27%, and 45.45% and 88.24%, respectively, while the sensitivity for diagnosing esophageal SCC was 33.90%, 55.93%, 54.80%, and 23.73%, respectively. When combining the methylation of the four genes, the sensitivity and specificity for diagnosing adenocarcinoma, SCC, and EC were 66.67% and 86.27%, 77.40% and 85.29%, and 76.19% and 86.27%, respectively. The AUC values of *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* methylation for diagnosing esophageal SCC were 0.639 (95%CI: 0.575–0.703), 0.765 (95%CI: 0.710–0.820), 0.731 (95%CI: 0.673–0.789), and 0.559 (95%CI: 0.491–0.626), and the corresponding values for diagnosing esophageal adenocarcinoma were 0.601 (95%CI: 0.484-0.719), 0.691 (95%CI: 0.577-0.805), 0.626 (95%CI: 0.504-0.749), 0.697 (95%CI: 0.582-0.813), respectively (Figure 4). The AUC values for the combined evaluation to diagnose adenocarcinoma, SCC, and EC were 0.737 (95%CI: 0.584–0.89), 0.824 (95%CI: 0.775–0.891), and 0.864 (95%CI: 0.809–0.92), respectively

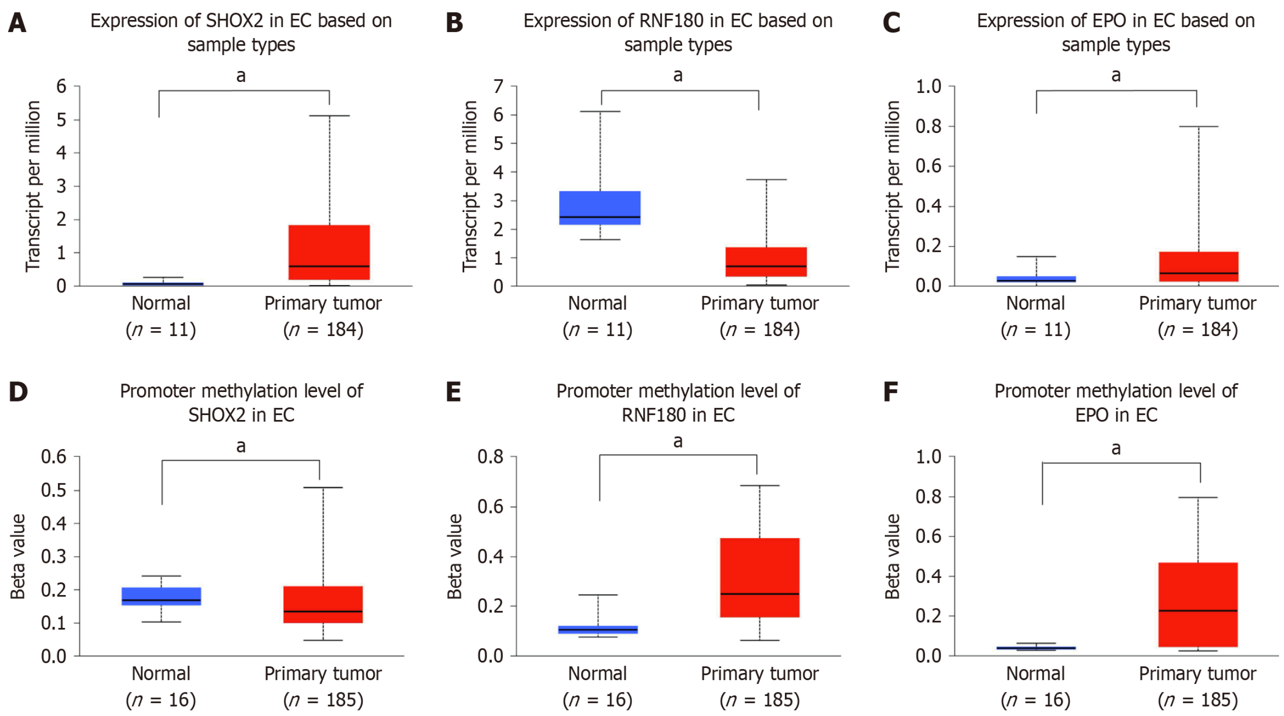

Figure 1 *SHOX2*, *RNF180*, and *EPO* expression and methylation in normal esophageal tissue (*n* = 11) and esophageal cancer tissue (*n* = 181) samples from previously reported genome data. A, C, and E: Expression of *SHOX2*, *RNF180*, and *EPO* was up- or downregulated in esophageal cancer (EC) tissues (*n* = 181); B, D, and F: Promoter methylation levels of *SHOX2*, *RNF180*, and *EPO* in normal esophageal tissues (*n* = 11) and EC tissues (*n* = 181), as indicated by beta values ranging from 0 (unmethylated) to 1 (fully methylated). $^aP < 0.05$. EC: Esophageal cancer.

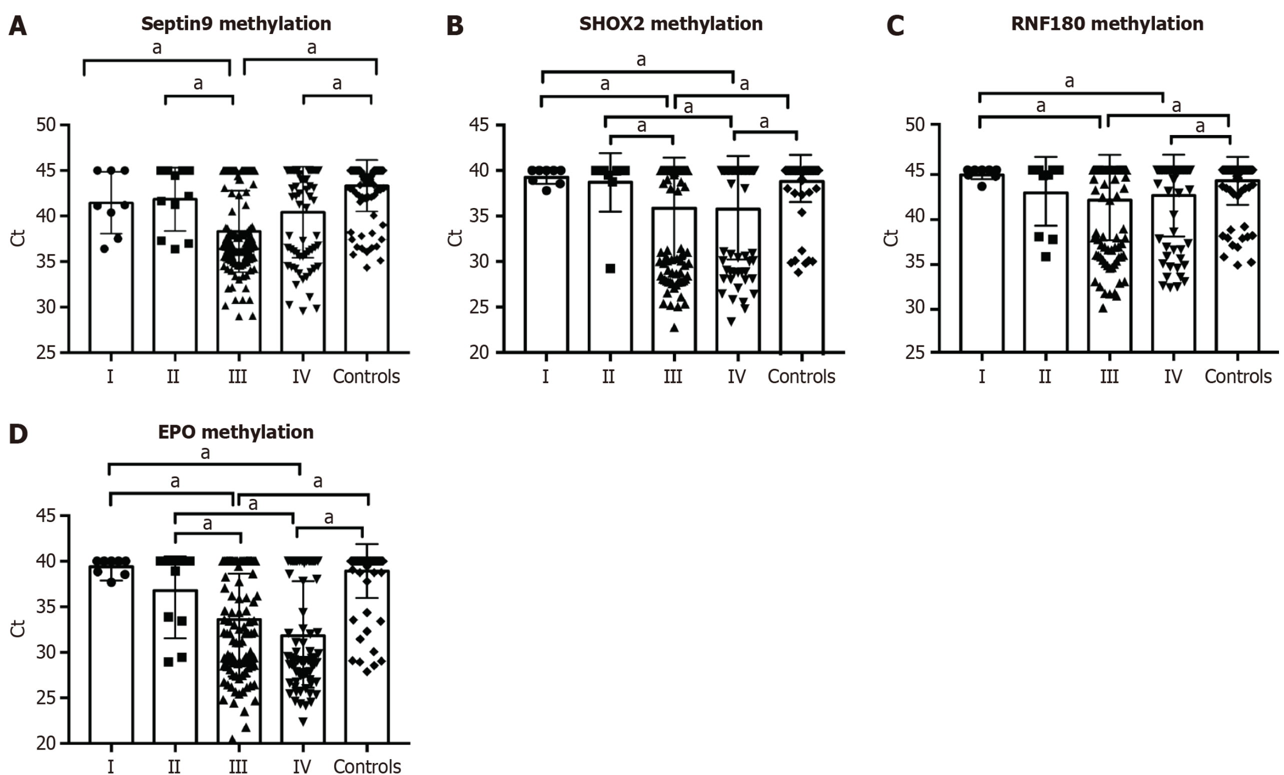

Figure 2 Quantitative analysis of *SEPTIN9*, *SHOX2*, *RNF180* and *EPO* methylation in plasma specimens of controls (*n* = 102) and patients with different stages of esophageal cancer (*n* = 210). A-D: Quantitative analysis of *SEPTIN9* (A), *SHOX2* (B), *RNF180* (C), and *EPO* (D) methylation in esophageal cancer patients with different stages and controls. The positive rates of *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* DNA methylation in EC patients with advanced stages were higher than those of EC patients with early stages and controls. $^aP < 0.05$.

Table 2 Results of logistic regression analysis (Cox proportional hazard models)

Univariate analysis		Number of patients	OR (95%CI)	*P* value
Gender	(Male *vs* female)	210	0.65 (0.32-1.34)	0.568
Age at diagnosis	(≤ 60 *vs* > 60 years)	210	2.32 (1.45-6.61)	0.021
SCCA	(≤ 3.5 *vs* > 3.5 U/mL)	210	3.01 (1.54-6.45)	0.023
EPO methylation	(*EPO*− *vs* *EPO*+)	210	7.89 (3.28-18.21)	0.001
SEPT9 methylation	(*SEPT9*− *vs* *SEPT9*+)	210	7.15 (2.86-17.63)	0.001

SCCA: Squamous cell carcinoma antigen; OR: Odds ratio.

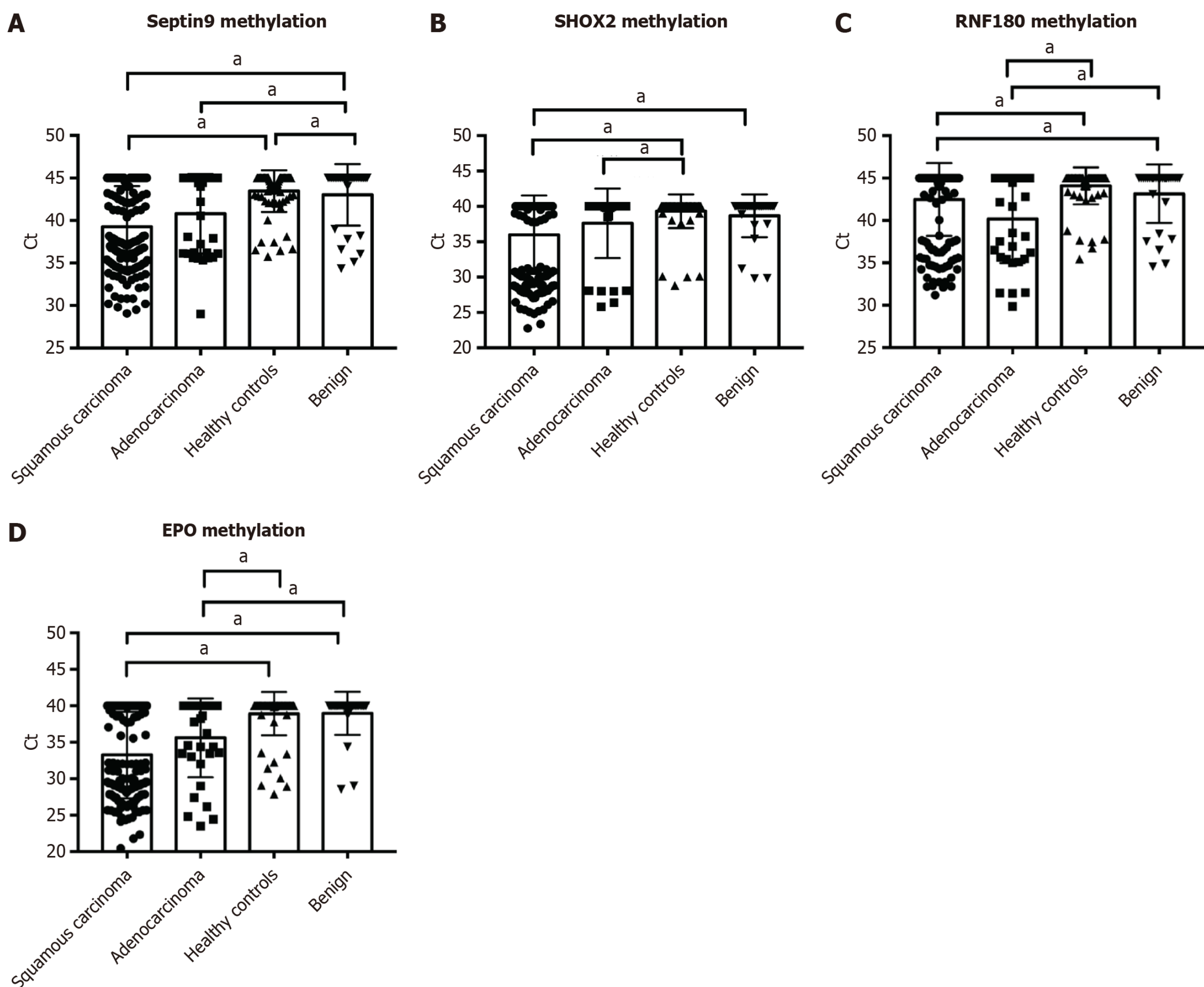

Figure 3 Quantitative analysis of *SEPTIN9*, *SHOX2*, *RNF180*, and *EPO* methylation in plasma specimens of controls (*n* = 102) and patients with esophageal adenocarcinoma (*n* = 33) and squamous cell carcinoma (*n* = 177). A-D: Quantitative analysis of *SEPTIN9* (A), *SHOX2* (B), *RNF180* (C), and *EPO* (D) methylation in esophageal adenocarcinoma, squamous cell carcinoma (SCC), and controls. No significant differences were observed in the positive rates of *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation in esophageal cancer patients with SCC and adenocarcinoma, while they exceeded those of healthy controls and benign patients. [a]$P < 0.05$.

(Figure 5).

Correlations between DNA methylation and serum SCCA, CA199, and CEA for early detection of EC

Of the 210 patients with EC, 19 had stage I-II and 191 had stage III-IV disease (Table 1). The sensitivity and specificity of *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* methylation for diagnosing stage I-II EC were 52.63% and 86.54%, respectively.

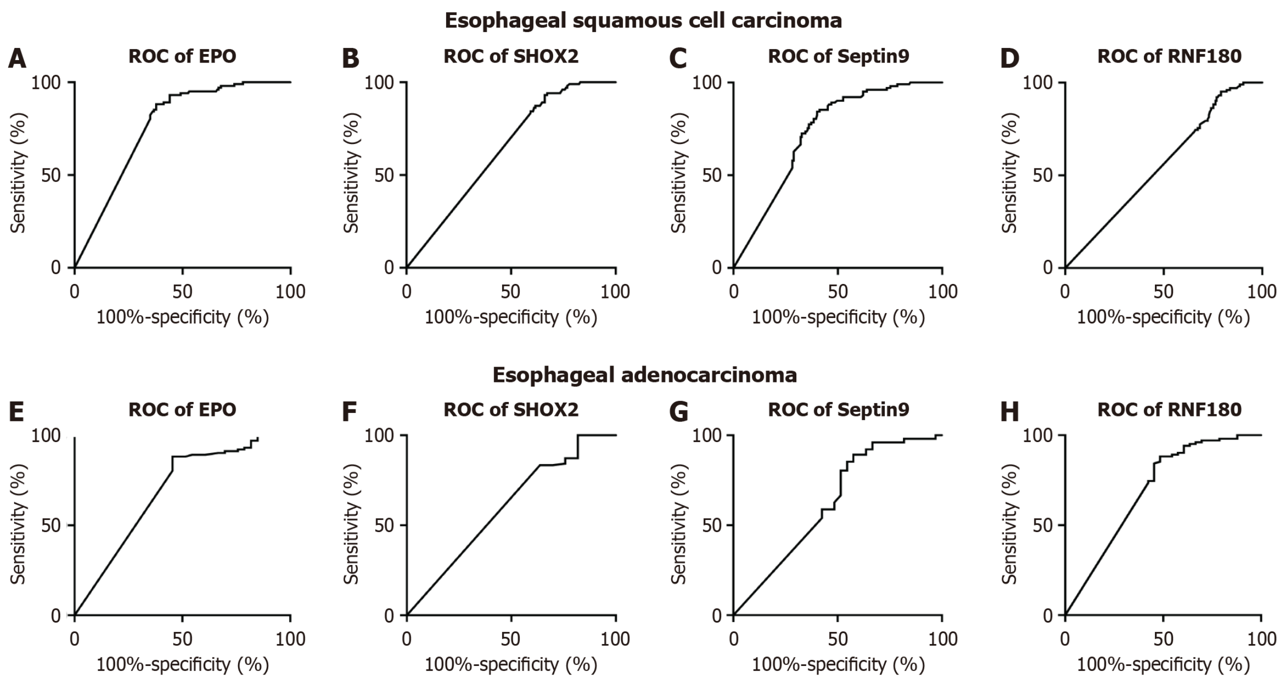

Figure 4 Receiver operating characteristic curve analysis of *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation biomarkers. A-D: Receiver operating characteristic (ROC) curves of methylation for squamous cell carcinoma. Area under the curve (AUC)-*SHOX2* = 0.639 (95%CI: 0.575–0.703), AUC-*SEPTIN9* = 0.731 (95%CI: 0.673–0.789), AUC-*EPO* = 0.765 (95%CI: 0.710–0.820), and AUC-*RNF180* = 0.559 (95%CI: 0.491–0.626); E-H: ROC curve analysis of methylation for adenocarcinoma. AUC-*SHOX2* = 0.601 (95%CI: 0.484–0.719), AUC-*SEPTIN9* = 0.626 (95%CI: 0.504–0.749), AUC-*EPO* = 0.691 (95%CI: 0.577–0.805), and AUC-*RNF180* = 0.697 (95%CI: 0.582–0.813). ROC: Receiver operating characteristic.

Serum SCCA demonstrated a 28.81% sensitivity and 90.20% specificity for detecting esophageal SCC, while CEA demonstrated a 19.2% sensitivity. CA199 and CEA demonstrated a 18.18% and 21.21% sensitivity and 87.25% and 86.27% specificity, respectively, for diagnosing adenocarcinoma. The AUC values of SCCA, CEA, and complex makers to diagnose esophageal SCC were 0.516 (95%CI: 0.448–0.583), 0.626 (95%CI: 0.557–0.695), and 0.648 (95%CI: 0.584-0.711), respectively (Figure 5). For diagnosing adenocarcinoma, the AUC value of the CEA, CA199, and complex markers were 0.683 (95%CI: 0.572–0.793), 0.408 (95%CI: 0.297-0.519), and 0.693 (95%CI: 0.588-0.798), respectively. The AUC value of the combined tumor markers (SCCA and CEA) and *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* methylation was 0.864 (0.820–0.908) for diagnosing esophageal SCC, while it was 0.798 (95%CI: 0.714-0.883) for diagnosing adenocarcinoma (Figure 5).

Evaluation of methylation for EC monitoring

Figure 6 depicts the frequency distribution of plasma DNA methylation before and after therapy in 23 patients. DNA methylation in the plasma of 10 patients exhibited a considerable negative change post-therapy.

Predictive models

The multivariate logistic regression model revealed that methylation of the four genes [*SHOX2* methylation: odds ratio (OR) = 3.22, 95%CI: 1.76–6.02; *EPO* methylation: OR = 9.52, 95%CI: 4.93–18.02; *RNF180* methylation: OR = 2.33, 95%CI: 1.19–4.51; and *SEPTIN9* methylation: OR = 7.62, 95%CI: 3.99–14.04), age (> 60 years: OR = 2.54, 95%CI: 1.52–4.28), smoking status (current smoker: OR = 1.61, 95%CI: 0.95–2.77), alcohol intake status (OR = 2.04, 95%CI: 1.23–3.32), high SCCA levels (> 2.7 mg/L: OR = 3.72, 95%CI: 1.84–7.52), and high CEA levels (> 5.0 μg/mL: OR = 2.19, 95%CI: 1.07–4.44)] were positively associated with an esophageal SCC risk (Figure 7). While the independent risk factors for adenocarcinoma were *SHOX2* methylation (OR = 3.56, 95%CI: 0.98–12.7) *EPO* methylation (OR = 5.53, 95%CI: 2.28–13.84), *RNF180* methylation (OR = 6.25, 95%CI: 2.39–15.63), *SEPTIN9* methylation (OR = 4.63, 95%CI: 1.80–10.81), age (> 60 years: OR = 2.60, 95%CI: 1.14–5.76), and alcohol intake status (OR = 2.50, 95%CI: 1.10–5.87) (Figure 7). In addition, age-adjusted logistic regression analysis indicated a positive association between EC risk and *EPO* and *SEPTIN9* methylation as well as SCCA levels (Table 2).

In this study, we employed two sets of models: The epidemiological model, which included four established predictors of EC (age, alcohol intake status, tumor markers, and ctDNA methylation), and the full model, which, through stepwise logistic regression, further incorporated two metabolic markers—SCCA and ctDNA methylation.

DISCUSSION

Esophagoscopy plays a crucial role in the early screening of EC[21]. However, EC diagnosis is often delayed due to occult symptoms in the early stages of tumor development. As esophagoscopy is not a routine option in normal physical

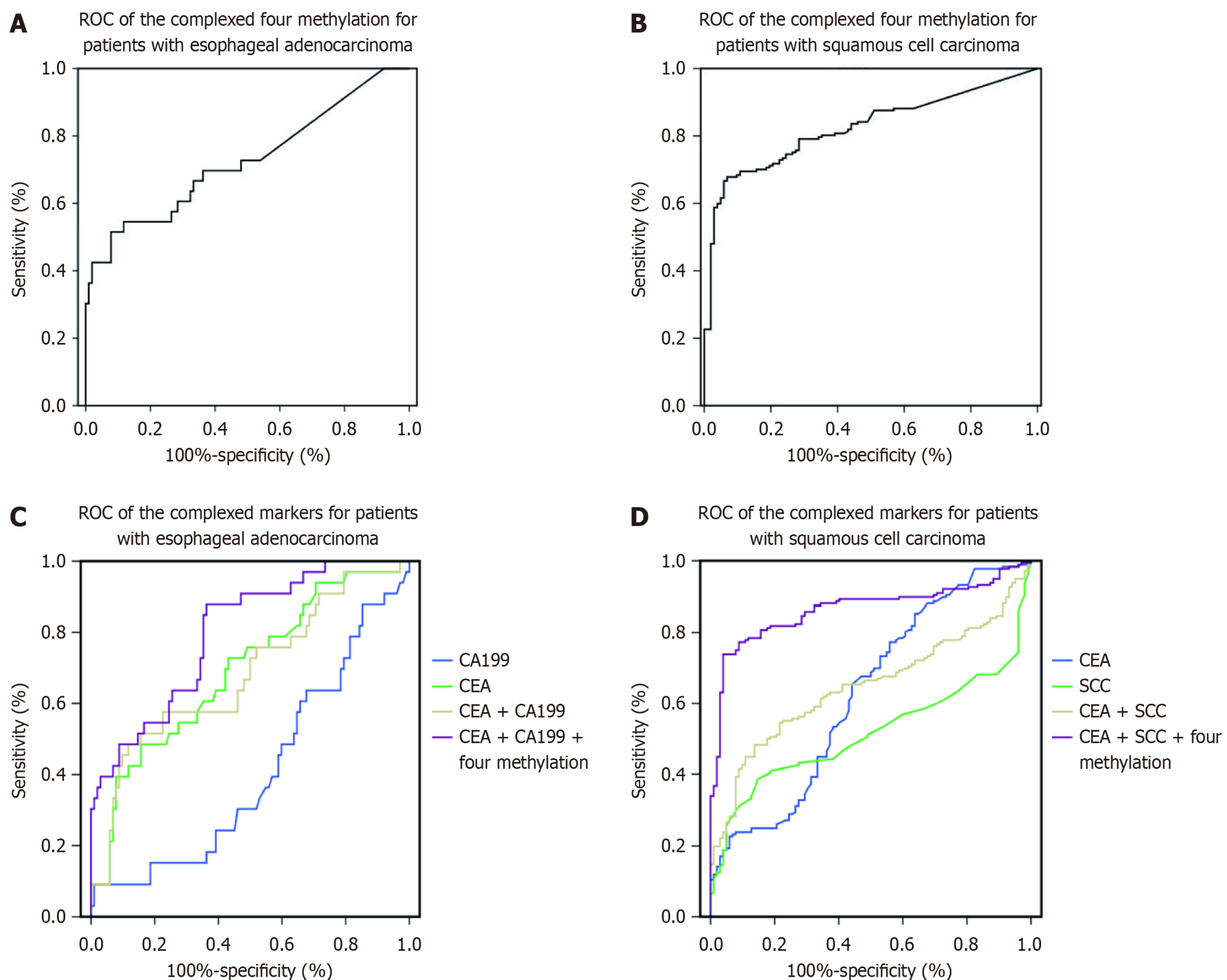

Figure 5 Receiver operating characteristic curve analysis of the tumor markers squamous cell carcinoma antigen, carbohydrate antigen 199, and carcinoembryonic antigen as well as methylation of the four genes as evaluated using plasma samples of patients with esophageal cancer. A and C: Receiver operating characteristic (ROC) curves of methylation of the four genes (A) and tumor markers (C) in 33 patients with esophageal adenocarcinoma. Area under the curve (AUC)-complex methylation = 0.737 [95% confidence interval (CI): 0.584–0.89], AUC-carbohydrate antigen 199 (CA199) = 0.408 (95%CI: 0.297–0.519), AUC-carcinoembryonic antigen (CEA) = 0.683 (95%CI: 0.572–0.793), AUC-CEA + CA199 = 0.693 (95%CI: 0.588–0.798), and AUC-tumor markers and *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation = 0.798 (95%CI: 0.714–0.883); B and D: ROC curves of methylation of the four genes (B) and tumor markers (D) in 177 patients with squamous cell carcinoma (SCC). AUC-complex methylation = 0.824 (95%CI: 0.775–0.891), AUC-SCC antigen (SCCA) = 0.516 (95%CI: 0.448–0.583), AUC-CEA = 0.626 (95%CI: 0.557–0.695), AUC-SCCA + CEA = 0.648 (95%CI: 0.584–0.711), and AUC-tumor markers and *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation = 0.864 (95%CI: 0.820–0.908).

examinations, and a significant number of patients with EC are diagnosed at advanced stages in China, leading to a poor prognosis[23], there is a critical need to identify an ideal screening method for EC that is cost-effective, well-tolerated, and applicable in primary care. Plasma samples offer an easily obtainable, less invasive, and highly acceptable alternative.

ctDNA methylation is a potentially valuable tumor marker for EC. Herein we explored the clinical significance of *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* methylation for the early screening, clinical diagnosis, and monitoring of esophageal adenocarcinoma and SCC. Our results indicated that the combined analysis of *SHOX2*, *SEPTIN9*, *RNF180*, and *EPO* promoter methylation effectively distinguished esophageal adenocarcinoma and SCC, and EC and controls without any tumors. For EC, the area under the operating characteristic curve value of combined detection of methylation of the four genes was 0.864, which was significantly higher than those for SCCA, CA199, and CEA. For esophageal adenocarcinoma and SCC, the area under the operating characteristic curve values of the combined methylation detection were 0.737 and 0.824, respectively. Moreover, the sensitivity and specificity of the combined methylation detection for early EC diagnosis were 40% and 80%, respectively. Our study was the first study on ctDNA methylation in detection of early-stage disease. Moreover, the incorporation of SCCA, CA199, CEA, and ctDNA methylation as metabolic markers satisfactorily improved the discriminatory performance of ctDNA methylation alone (C-statistic for the full model, 0.735). As all the indicators in this model can be easily acquired from general clinical or screening assays, the potential for translation into practical use is considerable. Internal validation indicated that the model may perform well with regard to model discrimination when applied to other populations.

Clearly, the role of ctDNA methylation in screening early EC is superior (> 50%). A blood-based test could be an ideal screening platform for implementation in primary care settings. ctDNA methylation holds greater value in diagnosing

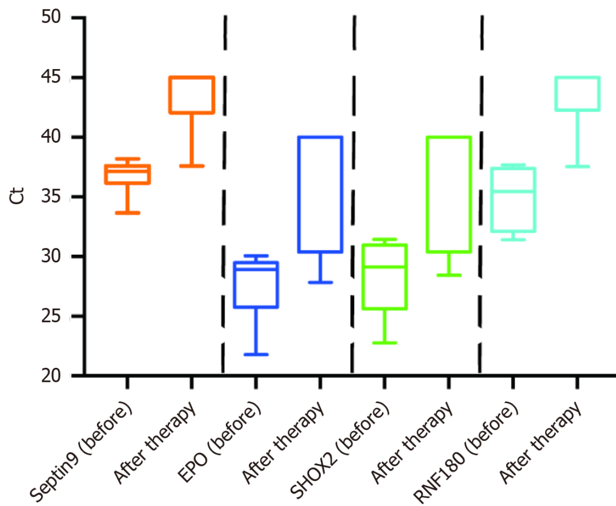

Figure 6 Frequency distributions of plasma *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation before and after therapy in patients with esophageal cancer (*n* = 23). Frequency distributions of plasma *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation were less after therapy than before therapy.

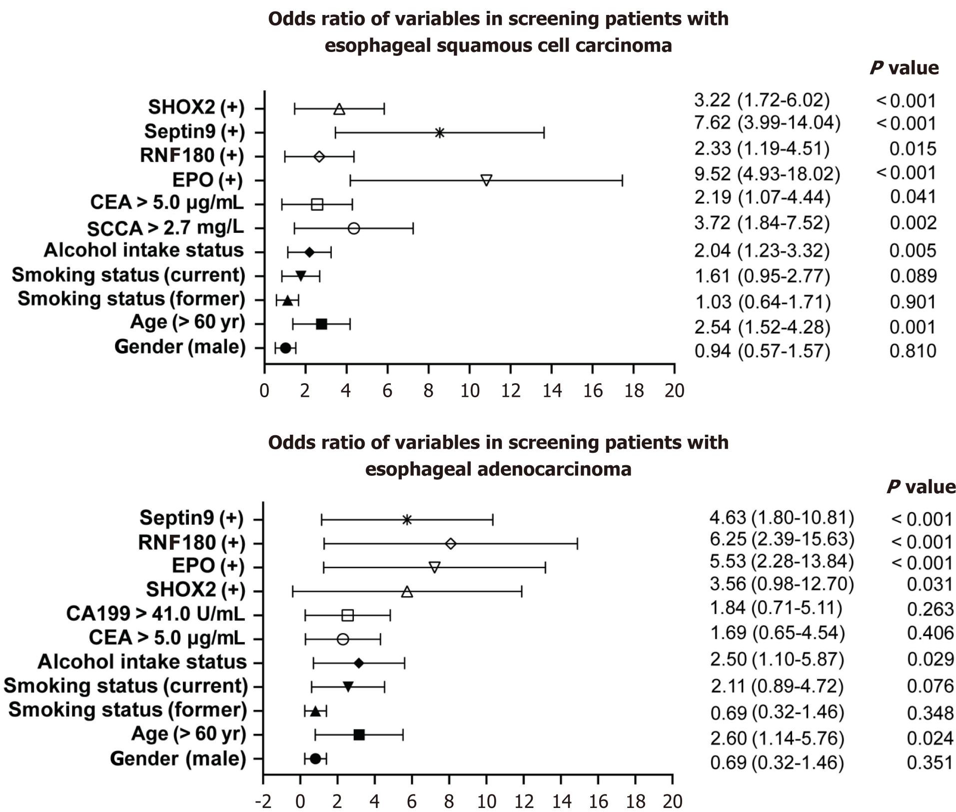

Figure 7 Forest plots of age, gender, smoking status, alcohol intake status, biomarker levels, and *SHOX2*, *RNF180*, *SEPTIN9*, and *EPO* methylation in screening patients with esophageal cancer. Variables are on the left axis, while *P* values are on the right. OR: Odds ratio; SCCA: Squamous cell carcinoma antigen; CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen 199.

advanced EC and shows promise for evaluating efficacy and monitoring disease progression. Plasma DNA methylation, utilized to predict the recurrence of EC, reflects transient changes in treatment response and tumor load, enabling accurate tumor staging and early diagnosis of recurrence. The combined detection of multiple methylation targets can enhance diagnostic performance in EC. The combined methylation of *EPO*, *SHOX2*, *SEPTIN9*, and *RNF180*, as jointly detected in our study, effectively distinguished EC and benign esophageal diseases from healthy subjects with high sensitivity and specificity. These promising results suggest the potential use of these DNA methylation markers in plasma to complement current EC detection methods and monitor EC in patients with primary sclerosing cholangitis. However, we found no significant differences in the methylation of *SHOX2*, *EPO*, *SEPTIN9*, and *RNF180* between adenocarcinoma and SCC. It is presumable that the common four methylation markers may be applicable for all EC types, not limited to a

specific subtype at a particular anatomic location.

Over the years, investigators have explored esophageal cell collection devices that can be deployed either *via* a catheter or swallowed by patients[24]. Unfortunately, the accuracy for detecting Barrett's esophagus was deemed too low for clinical utility, primarily due to a low cell yield and reliance on standard cytology for identifying cell atypia[24-26]. Cytology samples can undergo additional testing, such as DNA methylation assays, so as to increase diagnostic accuracy and facilitate risk stratification[26,27]. In this study, the methylation status of EC cytology samples was positive, while that of tissue samples from benign esophageal diseases was negative. Combining cytology with DNA methylation assays appears to be superior to plasma methylation for early EC screening.

CONCLUSION

EC remains relatively concealed, necessitating patient selection for screening based on symptoms, family history, and other risk factors to enhance cost-effectiveness. It is imperative to conduct studies in relevant populations to avoid misleading estimates of sensitivity and specificity in detection. Monitoring ctDNA methylation in addition to traditional tumor markers seems effective for EC diagnosis and treatment. Herein the combined detection of methylation of *EPO*, *SHOX2*, *SEPTIN9*, and *RNF180* could effectively distinguish EC and benign esophageal diseases. We believe that cytology combined with DNA methylation assays may prove superior for early EC screening.

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FOOTNOTES

Author contributions: Yu J, Liu XJ, and Pi GL had access to all the clinical data generated by the study and take responsibility for data integrity and accuracy of the data analysis; Yu J and Zeng H contributed to study concept and design; Wang S, Kai JD, Yu HF, and Shi HW contributed to acquisition, analysis, or interpretation of the data; Yu J and Liu XJ contributed to manuscript preparation.

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