A multicenter prospective clinical trial reveals cell free DNA methylation markers for early esophageal cancer

Ruixiang Zhang[1,#], Yongzhan Nie[2,#], Xiaobing Chen[3,#], Tao Jiang[4,#], Jinhai Wang[5,#], Yuhui Peng[6,#], Guangpeng Zhou[7,#], Yong Li[1], Lina Zhao[8], Beibei Chen[3], Yunfeng Ni[4], Yan Cheng[5], Yiwei Xu[6], Zhenyu Zhu[7], Xianchun Gao[2], Zhen Wu[7], Tianbao Li[7], Jie Zhao[7], Cantong Liu[6], Gang Zhao[5], Jiakuan Chen[4], Jing Zhao[3], Gang Ji[2,9,*], Xiaoliang Han[7,*], Jie He[1,*], Yin Li[1,*]

[1]Department of Thoracic Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

[2]State Key Laboratory of Holistic Integrative Management of Gastrointestinal Cancers, National Clinical Research Center for Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

[3]Department of Oncology, the Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan 450008, China

[4]Department of Thoracic Surgery, Tangdu Hospital, The Air Force Military Medical University, Xi'an, Shaanxi 710038, China

[5]Department of Gastroenterology, the second affiliated hospital, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China

[6]Department of Clinical Laboratory Medicine, the Cancer Hospital of Shantou University Medical College, Shantou, Guangdong 515041, China

[7]BioChain (Beijing) Science & Technology Inc., Beijing 102600, China.

[8]Department of Radiation Oncology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

[9]Department of Digestive Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

# These authors contributed equally to this work.

* Correspondence authors:

Yin Li, National Cancer Center, 17 Panjiayuan Nanli, Chaoyang District, Beijing, 100021, China. Phone: 86.010.87788053; Email: liyin@cicams.ac.cn;

Jie He, National Cancer Center, 17 Panjiayuan Nanli, Chaoyang District, Beijing, 100021, China. Phone: 86.010.87788713; Email: hejie@cicams.ac.cn;

Xiaoliang Han, BioChain (Beijing) Science & Technology Inc., No.18 Hongda South Road, Daxing District, Beijing, 100176, China. Phone: 86.18612824530; Email: sean.han@biochainbj.com;

Gang Ji, Xijing Hospital, Changle West Road, Xi'an, Shaanxi, 710032, China. Phone: 86.029.84771518; Email: Jigang@fmmu.edu.cn

Abstract

Background: Current methods for detecting esophageal cancer (EC) are generally invasive or exhibit limited sensitivity and specificity, especially for the identification of early-stage tumors.

Methods: We identified potential methylated DNA markers (MDM) from multiple genomic regions in a discovery cohort and a diagnostic model was developed and verified in a model-verification cohort of 297 participants. The accuracy of the MDM panel was validated in a multicenter, prospective cohort (n = 1429). The clinical performance of identified MDMs were compared with current tumor-associated protein markers.

Results: From 31 significant differentially methylated EC-associated regions identified in the marker discovery, we trained and validated a 3-MDM diagnostic model that could discriminate among EC patients and Non-EC volunteers in a multicenter clinical prospective cohort with a sensitivity of 85.5% and a specificity of 95.3%. This panel showed higher sensitivity in diagnosing early-stage tumors, with sensitivities of 56% for Stage 0 and 77% for Stage I, comparing with the performance of current biochemical markers. In population with high risk for EC, the sensitivity and specificity are 85.68% and 93.61% respectively.

Conclusion: The assessment of tumor-associated methylation status in blood samples can facilitate non-invasive, and reliable diagnosis of early-stage EC, which warrants further development to expand screening and reduce mortality rates.

Trial registration number: ChiCTR2400083525

Keywords: Esophageal cancer; Early Diagnosis; DNA methylation; Plasma-based

Introduction

Esophageal squamous cell carcinoma (ESCC) remains the predominant histological subtype of esophageal cancer (EC) worldwide, representing nearly 90% of the 604,000 new cases reported in 2020(1). As a result of the absence of common clinical manifestations and physical indicators during the initial phases of esophageal cancer, a large proportion of patients in China are diagnosed at intermediate to advanced stages, contributing to the unfavorable overall prognosis for individuals with this condition(2). However, regions in China where screening is routine demonstrate notably higher survival rates compared to those without screening (40.6% vs 32.8%)(3). In addition, substantial improvements in survival are observed when the disease is confined to superficial mucosal layers, with rates exceeding 80% after endoscopic or surgical intervention(4), suggesting the critical value of early detection in clinical practice . However, endoscopy, the gold-standard technique for EC diagnosis, is not suitable for population-based screening due to its relatively high cost and invasiveness. In addition, although the preinvasive stage of esophageal squamous dysplasia is well-described and could serve as a reliable basis for development of less invasive, blood-based, early detection strategies, currently available biomarkers have shown generally insufficient accuracy and efficacy(5).

To overcome the deficiency, cancer-specific DNA methylation modifications have been proposed as potentially promising biomarkers for EC detection due to their prominent role in dysregulation of tumor

suppressor genes or oncogenes, and consequently, tumorigenesis(6). Aberrant methylated DNA markers (MDMs) have demonstrated to be an early event in oncogenesis and presented less heterogeneous than gene mutations, and could thus serve as ideal tools for early detection of different malignancy types, including EC. For example, MDMs identified from esophageal cytology specimens obtained via sponge sampling devices showed nearly perfect performance in detecting Barrett's esophagus, achieving 92% sensitivity and 94% specificity(7). This high accuracy suggests that MDMs could provide high diagnostic power for early detection of EC. Another study has shown that a panel of five MDMs (FER1L4, ZNF671, ST8SIA1, TBX15, ARHGEF4) identified from tissue samples could also to detect EC in plasma-based assays from limited clinical samples (8). It is necessary to prove that EC at early stage can be detected in plasma, such as at stage 0, and to validate the early diagnosis of EC in a clinical trial with sufficient clinical samples including EC at stage 0.

To improve early detection of EC, in this current study, we developed a method for detecting methylated DNA markers in multiple genomic regions in blood samples from EC at early stage. We applied this method in a training cohort, which resulted in a diagnostic model based on three markers for EC at early stage. Then we validated the model in a multi-center clinical cohort including diagnosed group and diagnosing group with high risk of EC to simulate applications of methylation testing in real clinical situation. Furthermore, we systematically validated the diagnostic performance of these EC-specific methylation markers for detecting early-stage EC by comparison with current protein-based markers.

Results

The study was conducted in three main phases: 1) Marker Discovery, a phase in which methylation markers were screened from tissue and plasma samples; 2) Model Verification, a phase in which the probes were optimized, and model was constructed; and 3) Clinical Validation, a phase wherein diagnostic performance for early EC was evaluated in a cohort of 1429 participants and control subjects **(Figure 1)**.

Marker Discovery

In the biomarker discovery phase, we analyzed whole-genome bisulfite sequencing (WGBS) data from plasma samples of 56 esophageal cancer (EC) patients and 107 healthy controls. This dataset was augmented with the methylation data of 108 ESCC samples and 356 healthy individuals sourced from public databases to delineate differential methylation patterns. Our analysis revealed that overall methylation predominated in relatively rare genomic regions **(Supplementary Figure 1)**. We identified 31 differentially methylated regions associated with esophageal cancer and some of the selected genes were shown in Integrative Genomics Viewer **(Figure 2A and Supplementary Figure 2)**. Using logistic regression modeling, we preliminarily identified 6 differentially methylated regions, which were annotated to the 6 genes, Epo, MT-1A, PDGFRA, HOXB13, TRIM15, and Septin9, as potential diagnostic markers for esophageal cancer. These six potential diagnostic markers were performed further validation through quantitative methylation-specific PCR (qMSP) in Hela cell DNA, WBC (white blood cell) DNA and plasma samples collected from a subset of participants, including 20 EC patients, 12 healthy individuals, and 10 patients with benign esophageal diseases. The original data of qMSP assays

based on these plasma samples were presented in **Supplementary Table 1**.The methylation level of the differentially methylated region on TRIM15 gene showed relative high background signal in WBC DNA and the amplification signal of the differentially methylated region on PDGFRA gene in Hela cell DNA was below the limit of detection, which led us to focus on the remaining four potential markers (Epo, MT-1A, HOXB13, and Septin9) for further verification **(Supplementary Figure 3)**. Notably, the Ct values in qMSP assays showed that the differentially methylated regions on Septin9, MT-1A, and Epo genes were significantly lower in esophageal cancer samples compared with controls (Wilcoxon test, $P<0.05$), indicating these potential markers had a higher methylation status in cancer samples (**Figure 2B**). Through this validation process, three highly unique differentially methylated regions emerged as candidate methylated DNA markers (MDMs).

Model Verification

In the Model Verification phase, the three candidate MDMs were assessed by qMSP in plasma samples of the Model Verification cohort, including 87 patients with esophageal cancer, 5 patients with High-grade intraepithelial neoplasia, 16 patients with other types of cancers, and 189 healthy individuals.

The combination of candidate MDMs was employed by logistic regression and parallel techniques and evaluated by the diagnostic performance from a Model Verification cohort. The area under curve (AUC) values for the individual ROC curves of Septin9, Epo, and MT-1A were 0.857, 0.853, and 0.837, respectively. When combined, they reached AUC values of 0.947(logistic regression) and 0.948(parallel techniques), indicating improved diagnostic accuracy **(Figure 3A and Supplementary Table 2)**. In the integration of three MDMs, the AUC values of logistic regression and parallel techniques were similar,

with slightly higher AUC for the parallel technique. Therefore, we opted for the parallel technique in our combined approach. Comparison of predicted (by qMSP and parallel techniques) versus observed classifications by confusion matrix showed that this panel of candidate MDMs provided 95.29% accuracy in discriminating between EC and healthy controls, suggesting relatively high consistency between the model and actual clinical diagnoses **(Figure 3B**, kappa=0.89).

Clinical Validation

1. Demographics in the Clinical Cohort

The Clinical Validation cohort consisted of 641 participants with EC, while the control group comprised 788 participants without EC, including healthy controls and participants with benign esophageal diseases or other types of cancer. Participants with EC were categorized according to the AJCC staging system, with 32 participants at stage 0, 106 participants at stage I, 111 participants at stage II, 204 participants at stage III, 117 participants at stage IV, and 71 participants with unknown staging information (**Table 1**).

For assessing the performance of diagnostic marker in confirmed participants with EC and illustrating its applicability for diagnosing EC in high-risk individuals, participants were assigned to the diagnosed group (including participants diagnosed with esophageal cancer and healthy controls before methylation testing, N=534) and the diagnosing group (including participants in diagnosing esophageal cancer, benign esophageal diseases, and healthy controls after methylation testing, N=697) as detailed in Table 1. There are 12 participants at stage 0, 48 participants at stage I, 40 participants at stage II, 88 participants

at stage III, 32 participants at stage IV, and 37 participants with unknown staging information in the diagnosed group and 20 participants at stage 0, 58 participants at stage I, 71 participants at stage II, 116 participants at stage III, 85 participants at stage IV, and 34 participants with unknown staging information in the diagnosing group. Besides, the Other Cancers Group comprises 198 cases (Age 60.39±10.2, 130 Male and 68 Female) and the distributions of cancer types are as follows: lung cancer accounted for 14.14% with 28 cases, liver cancer represented 9.6% with 19 cases, colorectal cancer accounted for 25.76% with 51 cases, breast cancer accounted for 15.15% with 30 cases, and gastric cancer was the most prevalent at 35.35% with 70 cases.

2. ROC analysis

In the clinical validation phase, the MDMs were again validated by qMSP in plasma samples from 609 participants with EC, 32 participants with high-grade intraepithelial neoplasia (EC at Stage 0), 298 participants with benign esophageal diseases, 198 participants with other types of cancer, and 292 healthy participants. The original data of qMSP assays were presented in **Supplementary Table 3**. We assessed the accuracy of the three MDMs by ROC analysis in the clinical cohort (n=641 EC samples; n=788 non-EC samples). Among the 1429 clinical samples, ROC curve analysis of qMSP Ct values in individual or multiplex detection assays of Septin9, Epo, and MT-1A yielded AUC values of 0.793, 0.758, and 0.795, respectively, and 0.904 for all three markers together (**Figure 4A**). These results suggested that multiplex detection using these candidates could provide higher diagnostic accuracy compared to detection of any individual MDM. Further comparison by confusion matrix of classifications predicted by qMSP assays

of the three-MDM panel with the observed clinical diagnoses showed an accuracy value of 90.17% **(Figure 4B**, kappa=0.80).

3. Sensitivity

To further assess whether the diagnostic efficacy of the three-MDM panel differed among stages of EC, we compared its sensitivity among participants in the clinical cohort stratified by disease stage. Multiplex qMSP analysis of Septin9, Epo, and MT-1A showed detection sensitivities of 85.49% (95%CI: 82.55%~88.01%) for overall (n=641) patients and the detection performance showed positive correlation with the tumor progression. The sensitivity of stage 0 (n=32), stage I (n=106), stage II (n=111), stage III (n=204), stage IV (n=117) were 56.25% (95%CI: 39.06%~73.44%), 77.36% (95%CI: 69.39%~85.32%), 86.69% (95%CI: 80.13%~92.85%), 89.70% (95%CI: 85.54% ~93.88%), 94.02% (95%CI: 88.06%~97.56%) respectively **(Supplementary Figure 4)**. The sensitivity in different age groups showed no significant difference in the range of 40 to over 80 **(Supplementary Table 4).** The sensitivity performance of MDMs in differentiation groups of different tumor were analysis, the medium-high differentiation and Medium differentiated groups showed highest performance of 92.31% (95%CI: 66.69%~98.63%) **(Supplementary Table 5).**

4. Specificity

In assays testing whether our multiplex qMSP method could distinguish control subjects, including both healthy individuals and benign esophageal disease patients, the three-MDM panel achieved a specificity

95.25% (93.21%~96.82%). More specifically, healthy individuals could be identified with 97.26% specificity (95% CI, 94.67%-98.81%), while benign esophageal diseases were diagnosed with 93.29% specificity (95% CI, 89.82%-95.85%). We assembled a cohort of heterogeneous cancer types, including liver, colorectal, breast and lung cancers to examine the specificity of these three candidate markers for discriminating EC from other cancer types. Specificity decreased to 56.86% (95% CI, 43.27%-70.46%) among colorectal cancer patients. By contrast, the specificity in detecting lung cancer reached 100.00% (95% CI, 87.94%-100.00%), 100.00% for breast cancer (95% CI, 88.43%-100.00%), 78.95% for liver cancer (95% CI, 60.62%-97.28%), and a specificity of 70.00% for discriminating gastric cancer (95% CI, 59.26%-80.74%) **(Figure 4C and Supplementary Figure 5)**.

5. PPV and NPV

The positive predictive value (PPV) in overall clinical cohort is 95.14% (548/576), while the negative predictive value (NPV) was 85.80% (562/655), while the Septin9 alone showed the highest PPV of 96.72% and the best single marker NPV was detected by Epo with 65.15% **(Table 2)**.

6. Comparison with conventional tumor markers

Further comparison of the Septin9, Epo, and MT-1A MDM panel with conventional tumor markers in diagnosing different tumor stages in the clinical cohort showed positive detection rates of 56.25%, 77.36%, 86.49%, 89.71%, and 94.02% for cancer stages 0 to IV, respectively, notably higher than those of the conventional tumor markers, CEA, SCC, CA199 and NSE **(Figure 5A and Supplementary Figure 6)**. In addition, we also calculated the Youden Index of each tumor markers, and the results

showed that the three-MDM panel detection method was optimal. The respective Youden Indexes of CEA, SCC, CA199, NSE and three-MDM panel were 0.11, 0.22, 0.03, -0.02, 0.76. Furthermore, the sensitivity of three-MDM panel for squamous cell carcinoma, adenocarcinoma and other rare cancers showed no significant difference, and can be applied to all types of esophageal cancers.

7. Performance for high-risk populations in EC

To further evaluate whether the esophageal cancer methylation detection method in this study has the potential to be used for screening or as an adjunct diagnostic tool in populations with high-risk for esophageal cancer, we analyses performance of the three-marker panel in the diagnosed group (esophageal cancer diagnosed before methylation testing and healthy controls, N=534) and the diagnosing group (esophageal cancer diagnosed after methylation testing, benign esophageal diseases and healthy controls, N=697) as detailed in Table 1, the sensitivity, specificity, PPV and NPV in the diagnosed group were 85.21% (80.27%~89.31%) , 97.11%(94.39%~98.74%), 96.48% (93.17% ~ 98.47%) and 87.62% (83.41% ~ 91.09%) respectively, while the 85.68% (81.77%~89.02%), 93.61% (90.30%~96.05%), 94.27% (91.29% ~ 96.46%) and 84.20% (79.93% ~ 87.87%) were in the diagnosing group **(Table 3)**. The diagnosing group includes populations with high-risk for esophageal cancer, while the diagnosed group includes participants with confirmed esophageal cancer as control for the diagnosing group. There is no statistically difference between the diagnosed group and the diagnosing group in terms of sensitivity, specificity, PPV and NPV, which indicates that the three-marker panel can be used for screening or diagnosing populations with high-risk for esophageal cancer.

8. Treatment Monitoring

Additionally, examination of postoperative methylation levels in a subset of participants who underwent complete surgical resection revealed that 29 of 32 (90.6%) participants tested negative for methylation in the Septin9, Epo, and MT-1A promoter regions on the third day post-surgery. The methylation risk scores (45-ΔCT) of most participants included in the treatment monitoring decreased post-surgery compared to pre-surgery, showing statistical significance (Wilcoxon test, $P<0.001$) **(Figure 5B)**.

Discussion

This study introduces a non-invasive approach for the detection of esophageal cancer (EC) using gene methylation profiles in plasma samples, offering marked advantages over the conventional invasive endoscopic and pathological examinations which are often painful and less accessible, thus impeding early diagnosis and treatment. By screening esophageal cancer methylation chip data in public databases, along with internal plasma WGBS data including EC at early stage, 31 differentially methylated regions were identified. Subsequent logistic regression analysis of 31 differentially methylated regions in esophageal and non-esophageal cancer samples pinpointed six differentially methylated regions with a strong association with EC. Subsequently, they were verified using qMSP technology in cancer cell lines and clinical plasma samples, leading to the selection of three genes—MT-1A, Epo, and Septin9—for the development of a methylation-based detection method for EC.

In our study, preclinical plasma sample verification was conducted, followed by a case-control and multicenter clinical study with sufficient participants to validate the effectiveness of this method. The results indicated high consistency with clinical gold standard, with superior sensitivity and specificity

compared to existing studies and commonly used tumor markers, particularly for early-stage esophageal cancer at stage 0 and I. And the approach by combining the detection of MT-1A, Epo, and Septin9 gene methylation for esophageal cancer diagnosis, which introduced by this study, is a strategy not previously documented. The combining method was more effective in the diagnosing esophageal cancer compared to single gene methylation detection.

From the perspective of clinical study design, this research utilized a multi-center trial approach, which enables the inclusion of a larger number of participants within the same timeframe compared to a single-center trial, thereby reducing the duration of the clinical trial. Multi-center trials involve collaboration among various regions, different trial institutions and numerous clinical researchers, leading to conclusions that are often broadly representative.

In this clinical validation stage, we employed a strategically designed two-group cohort to thoroughly evaluate our biomarkers' diagnostic performance. The diagnosed group included patients who had already been diagnosed with esophageal cancer at the time of methylation testing. By comparing the methylation detection results with confirmed diagnoses in this group, we could robustly assess the accuracy and reliability of the DNA methylation markers. The diagnosing group comprised high-risk individuals who had not yet been definitively diagnosed with esophageal cancer at the time of methylation testing. After performing the methylation testing on this group, the diagnosis was definitively made to simulate applications of methylation testing in real clinical situation. We later correlated the results of methylation testing with the definitive diagnostic outcomes and conducted integrated analysis. This setup allowed us to examine the practical application of the DNA methylation

testing in the assessment of EC high-risk individuals. Remarkably, the performance characteristics observed in the diagnosing group were consistent with those in the diagnosed group, demonstrating a promising tool for early detection in high-risk populations. Therefore, this two-group design provides compelling evidence that our biomarkers can be effectively utilized for both clinical diagnosis and early screening of esophageal cancer in high-risk individuals, highlighting the broad diagnostic potential.

Regarding the gene characters and functions, the metallothionein (MT) family, is a low molecular-weight protein family known for its strong affinity towards metal ions(9). This protein family consists for isomers and plays a crucial role in regulating the homeostasis and oxidation of transition metal ions with cells. Among its various functions are the maintenance of cellular balance, as well as involvement in processes such as cell proliferation, differentiation, and apoptosis. MT-1A is one of the four isoforms in MT family and aberrant MT expression has been observed in several human tumors, including esophageal cancer, gallbladder cancer, B-cell lymphoma, breast cancer, liver cancer, skin cancer, papillary thyroid cancer and prostate cancer(10). Studies have demonstrated that overexpression of MT can shield cancer cells from free DNA damage and lipid peroxidation induced by free radicals(11). Recent investigations have highlighted that elevated expression of MT in squamous cell carcinoma, suggesting its potential utility as a diagnostic marker for esophageal squamous cell carcinoma(12).Erythropoietin (Epo) is a glycoprotein hormone(13). Chan K et al. discovered that Epo can rapidly induce the expression of the proto-oncogene c-myc, exert anti-apoptotic effects, and promote cell survival(14). Septin is a conserved family of skeleton protein genes with GTPase activity found in all eukaryotes except plants, playing a role in cell division. In humans, the family comprises 14 members

designated as SEPT 1 to 14. Research has indicated a direct association between Septin9 and tumor development, with varying expression and function across different tumor types(15). Particularly, Septin9 is highly expressed in gastrointestinal tumors(16, 17), serving as a reliable marker for their detection.

Numerous studies have explored gene methylation markers for esophageal cancer. For instance, Qin Y et al. used quantitative allele-specific real-time target and signal amplification technology to develop a diagnostic model based on five methylation genes, achieving a specificity of 91%, detecting 74% of 84 esophageal cancers, with a sensitivity of 43% for 14 stage I cancer and no cancer at stage 0. Although they used a similar framework, our study employed Whole Genome Bisulfite Sequencing and included both tissue and plasma samples from a larger cohort in marker discovery stage. These methodological differences in data coverage, sample types, and sample sizes led to distinct gene signatures, emphasizing the robustness and specificity of our approach in identifying reliable biomarkers (8). Li D et al. established a diagnostic methylation classifier based on 12 CpG sites, effectively distinguish BE, EAC and ESCC from normal tissues (AUC = 0.992) (18). However, this study was solely based on bioinformatics analysis without validation using clinical plasma samples. Salta S et al. utilized quantitative methylation-specific PCR to assess the efficacy of detecting esophageal cancer tissue using two methylated gene combinations(19). Their study achieved the identification accuracy of 82.29% for adenocarcinoma and 81.73% for squamous cell carcinoma tissue, which was lower than that showed in our study. Qiao G et al. employed targeted methylation sequencing technology and a support vector machine algorithm to develop an early detection classifier for esophageal cancer based on 921

differentially methylated regions by sophisticated deep targeted sequencing, with a sensitivity of 74.7% and a specificity of 95.9% in 181 clinical samples(20). The sensitivity for detecting stage 0 to II esophageal cancer was lower than that observed in the current research.

Conventional tumor markers commonly used for adjunctive diagnostic, prognosis, and therapeutic monitoring purposes in esophageal cancer include cytokeratin-21-1-fragment (CYFRA21-1), carcinoembryonic antigen (CEA), squamous epithelial cell carcinoma Antigen (Squamous cell carcinoma antigen, SCC) and tissue polypeptide specific antigen (TPS), etc. While combined application of these tumor markers may enhance efficiency in the intermediate and advanced stages of esophageal cancer, the individual sensitivity of them for esophageal cancer at early stage is generally below 20%. Our study not only confirmed the high diagnostic accuracy of the methylation-based approach but also demonstrates its superiority over conventional tumor markers like CYFRA21-1, CEA, SCC, and TPS. Furthermore, there was a notable enhancement in specificity among individuals exhibiting symptoms of esophageal cancer but not gastrointestinal (GI) cancer.

In the study, there were 534 individuals in the diagnosed group and 697 individuals in the diagnosing group. The sensitivity results observed were consistent, suggesting that the screening method is suitable for identifying esophageal cancer in suspected and high-risk populations. The accuracy of esophageal cancer methylation detection method in Model Verification cohort (95.29%) was higher than that in Clinical Validation cohort (90.17%). It possibly due to differences in sample size, sample heterogeneity, and experimental errors. The findings from methylation testing for pre- and post-surgery patients indicated that 90.6% of patients exhibited a negative methylation status following the surgical procedure,

leading to a notable reduction in overall methylation levels. However, when we compared the methylation risk scores of EC patients before and after surgery, we observed that three patients did not experience a decline after treatment. For one patient, the pre-operative methylation levels of the biomarkers were already very low, resulting in a false-negative diagnosis before surgery. This might be attributed to individual differences, as some esophageal cancer (EC) patients do not exhibit abnormal methylation in peripheral blood cfDNA. Consequently, this patient's post-operative methylation risk score did not decrease. For the other two patients, the exact mechanisms underlying the lack of a decline in methylation risk scores remain unclear. In future studies, we intend to conduct a dedicated investigation to evaluate the performance of this esophageal cancer biomarker in treatment monitoring and better understand the clinical implications of changes in methylation risk scores during the treatment process.

Conclusions

In conclusion, there exists a robust association between the integration of MT-1A, Epo, and Septin9 methylation testing and the development of esophageal cancer. And we offer a promising, highly accurate method not only for the early detection of EC and individuals with high-risk for EC, but also for the therapeutic monitoring.

Methods

Sex as a biological variable

Our study examined male and female human beings, but the sex was not considered as a biological

variable.

Study design and patient cohorts

Two cohorts of participants are enrolled in this study (the model-verification cohort containing 297 participants and the clinical validation cohort containing 1429 participants). The participants are prospectively recruited, including participants with esophageal cancer (EC), participants with benign lesions, healthy controls, and participants with other cancers, from multiple centers in China.

The inclusion criteria are as follows:

(1) Esophageal cancer. participants at high risk of esophageal cancer (EC) who were advised by clinicians to undergo endoscopy. The included participants were those over 40 years old who satisfied any of the following criteria: participants with long-term residence in areas with high EC incidence or with family disease history. participants with symptoms of upper gastrointestinal discomfort. participants with presence of precancerous lesions of EC. And participants with a strong clinical suspicion of EC or high-grade intraepithelial neoplasia (HGIN) based on endoscopic, imaging, or pathological biopsy findings. And participants with benign digestive system diseases intending to perform endoscopy test or with prior endoscopic findings. All the included participants in this classification are with no history of esophageal cancer surgery and no prior treatment.

To validate the performance of the diagnostic marker in confirming esophageal cancer (EC) patients in this study, while also demonstrating its potential for diagnosing EC in high-risk populations. The included patients of the clinical validation cohort were divided into a diagnosed group and a diagnosing group. In the diagnosed group (esophageal cancer definitively diagnosed before methylation testing), the

participants were diagnosed definitively first, then provided blood for subsequent methylation testing. In the diagnosing group (esophageal cancer definitively diagnosed after methylation testing), the participants provide blood for methylation testing first, then start definitive diagnosing procedures to simulate applications of methylation testing in real clinical situation. EC was diagnosed based on characteristics observed during upper gastrointestinal endoscopy, computed tomography (CT), or magnetic resonance imaging (MRI) and confirmed through histopathology. Tumor staging was determined according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) 8th edition system. During all the double-blinded experimental processes, the participants information was kept confidential from experimental operators and researchers to ensure the credibility and reliability of clinical trial outcomes.

(2) Benign esophageal conditions. Participants are clinically diagnosed with other esophageal diseases (such as reflux esophagitis, achalasia, esophageal hiatal hernia, diffuse esophageal spasm, and irregular esophageal spasm) based on laboratory tests (tumor markers or bronchoscopy or imaging, etc.), with no evidence of esophageal cancer, requiring further evaluation and management.

(3) Other types of cancer. Participants are clinically diagnosed with other cancers, such as gastric cancer, colorectal cancer, etc., who have not undergone treatment or surgery.

(4) Healthy controls. Participants with no history of malignant tumors, clinically confirmed to be free of esophageal cancer (EC), other digestive diseases and those with substantial medical conditions such as hepatitis, cirrhosis, and chronic obstructive pulmonary disease. All healthy volunteers will undergo a series of routine health assessments, including complete blood counts, urinalysis, blood biochemistry

tests, electrocardiograms, low-dose chest computed tomography (CT), and abdominal ultrasound examinations.

Sample collection and preparation

The blood samples from esophageal cancer patients and other groups were collected from multiple hospitals in China. Blood samples collected from all participating hospitals were processed following the same protocol by trained technicians. A 5ml K_2EDTA anticoagulant tube (BD Vacutainer®) was used to collect a 5ml peripheral blood sample to ensure the accuracy of the tests. Samples were processed and transported following the guidelines for nucleic acid extraction reagent (BioChain (Beijing) Science & Technology, Inc.) Plasma was separated from whole blood by centrifugation within 4 hours of blood sample collection and stored immediately at −80°C. Plasma was tested within 2 weeks of collection.

DNA extraction and bisulfite conversion

DNA extraction and bisulfite conversion were carried out following the instructions provided in the manufacturer's manual for the nucleic acid extraction reagent.

Quantitative methylation-specific PCR (qMSP)

When designing primers and probes for qMSP, primers are designed to include at least one CpG site in both forward and reverse primers, as well as in the probe binding sequence, ensuring that only methylated DNA templates are amplified. We extract cfDNA from cell-free plasma to prepare DNA templates. We simultaneously detect a reference gene, ACTB, when testing target genes. Through extensive clinical sample validation, we have established that cfDNA content in plasma should not fall below 0.9 ng/mL. Consequently, we set a reference ACTB Ct value threshold of $\leqslant$34.8. If the reference gene meets this

criterion, the sample is deemed suitable for analysis, allowing us to determine the presence or absence of methylation in the marker gene. The sulfite-modified DNA served as the template for quantitative methylation-specific PCR (qMSP), following the detailed procedures outlined in the MT-1A, Epo, and Septin9 methylated gene detection kit, which employs the PCR fluorescent probe method. The amplification reactions were conducted in a total volume of 50 μL, consisting of 25 μL of reaction buffer and 25 μL of sulfite-modified DNA template. The amplification process was conducted using either the Applied Biosystems 7500 Fast Real-Time PCR System or the SLAN-96S Fully Automatic Medical PCR Analysis System. Each experimental batch included patient DNA samples, positive controls and negative controls to maintain stringent quality control throughout the analysis.

Marker Discovery

In this study, a total of 108 EC cancer tissue samples, 107 adjacent normal tissue samples, and 249 healthy human WBC (white blood cell) samples from public datasets were analyzed. To integrate data from two methylation detection chips, a mapping and matching process was conducted for the detection probes based on specific criteria. These criteria included ensuring that the probe design intervals overlapped in the genome coordinates or that the maximum distance between probes did not exceed 150 base pairs. Additionally, probes from the Human Methylation 450 chip were aligned with those from the GoldenGate chip. Cancer-specific hypermethylation markers were identified based on the following criteria: adjusted p-value (p. adj)<1e-2, delta_T2N (difference in methylation levels between tumor and adjacent normal tissues) > 0.1 and mean_wbc (mean methylation level in WBCs) < 0.1.

The public data sets utilized in this research, including GSE51287, GSE26784, GSE40279, GSE52826

and GSE74693, were obtained from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/gds). These datasets exclusively consisted of methylated Chip data based on two kinds of platforms, the Human Methylation 450 chip and the GoldenGate chip. Notably, GSE40279 specifically included samples from individuals aged between 30 and 60 years.

Furthermore, a subset of the inhouse plasma samples were utilized as a discovery-step validation set to confirm the markers previously selected based on the public datasets mentioned before. The inhouse samples were from 107 healthy individuals and 56 patients with esophageal cancer at early stage. These samples underwent WGBS assay. Given the presence of strong background signals in plasma detection outcomes, a one-hot approach was utilized to delineate the identification of cancer-specific hypermethylation signal patterns in plasma. It is a method for categorizing methylation signals based on a predefined threshold. For each methylation DNA region, if the methylation level in a sample exceeds the detection threshold, it is classified as "detected," with the signal value set to the methylation level; otherwise, it is classified as "not detected," with a signal value of zero. The threshold was set at the 95th percentile of plasma methylation levels in individuals without cancer to achieve 95% specificity in these control participants, minimizing false positives and allowing for the identification of suitable candidate markers. During this screening process, a total of 31 marker intervals were examined.

We utilized the methylation levels of esophageal cancer differential methylation regions and the sample type (esophageal cancer vs. non-esophageal cancer) to establish a logistic regression diagnostic model for esophageal cancer. The variables included in the model will be used as highly relevant candidate biomarkers for esophageal cancer for subsequent validation through quantitative methylation-specific

PCR (qMSP) analysis.

For the qMSP analysis, we used DNA from HeLa cell (75ng), which was verified by Sanger sequencing to be highly methylated for Epo, MT-1A, PDGFRA, HOXB13, TRIM15, and Septin9, as the positive control, and WBC DNA (35ng) as the negative control. Clinical blood samples from patients with esophageal cancer (n = 20), benign esophageal diseases (n = 10), and healthy individuals (n = 12) were also tested. This screening process effectively validated the methylation biomarkers related to esophageal cancer.

Model verification

To evaluate the diagnostic efficacy of the methylation-based markers for esophageal cancer (EC), a model-verification cohort comprising various participant groups was assembled, including patients diagnosed with EC, individuals with benign esophageal conditions, patients with other types of cancer, and healthy individuals. The model-verification cohort was consisted of 87 patients with EC, 5 patients with high-grade intraepithelial neoplasia, 16 patients with other cancers, 189 healthy individuals. The blood samples were collected from individuals of this cohort to perform qMSP. The experimental details were described above.

Clinical Validation

A multi-center, parallel comparison, blinded clinical trial design was utilized, with inclusion criteria consistent described above. The samples were used to assess the diagnostic efficacy of esophageal cancer methylation gene detection technology.

The clinical trial was approved prior to the commencement of the study. This Clinical Validation cohort

consisted of 609 patients with EC, 32 patients with high-grade intraepithelial neoplasia, 298 patients with benign esophageal disease, 198 patients with other cancers, 292 healthy individuals. The blood samples were collected from individuals of this cohort to perform qMSP. The experimental details were described above.

The pre-specified primary outcomes of this clinical trial were sensitivity and specificity, which are the key measures of diagnostic performance for the biomarkers being evaluated. The analyses presented in the manuscript focus on the pre-specified primary outcomes (sensitivity and specificity).

Statistical analysis

A descriptive analysis of the demographic characteristics and initial data of the participants was conducted. Categorical variables were summarized using frequency and percentage composition, while quantitative variables were summarized using measures such as mean, standard deviation, median. The diagnostic efficacy was assessed through diagnostic test evaluation, including comparison with the gold standard, calculation of Kappa values and their corresponding 95% CI (21). Sensitivity was defined as the proportion of correctly identified positive esophageal cancer cases among all esophageal cancer cases, while specificity was defined as the proportion of correctly identified negative cases among all normal/ esophageal benign disease and other cancer cases. Positive predictive value (PPV) and negative predictive value (NPV) were calculated to determine the probability of a positive or negative disease test result, respectively. Receiver operating characteristics (ROC) curves were generated using R software, and the area under the ROC curve (AUC) were analyzed. For sample sizes less than 5, the association between test positivity and demographic characteristics was assessed using either the chi-square test or

Fisher's exact test. Statistical significance was defined as $P < 0.05$.

Study approval

Prior to sample collection, all participants in this research provided written consent and were duly informed about the utilization of sample and test outcomes. Approval for this study was obtained from the Medical Ethics Committee of all the hospitals participated in the study. The ethical numbers are as follows:

Cancer Hospital of the Chinese Academy of Medical Sciences, 21/223-2894

The First Affiliated Hospital of the Air Force Medical University, QX20211043-x-1

The Second Affiliated Hospital of the Air Force Medical University, 202110-09

Henan Provincial Cancer Hospital, 2021-240B-001

The Second Affiliated Hospital of Xi'an Jiaotong University, （2021）Ethical（056）

The Affiliated Cancer Hospital of Shantou University Medical College, 2021019

The ethical approvals were obtained from six ethic committees of hospitals in 2021. The recruitment of participants and sample collection were conducted after obtaining ethical approvals. And the trial was registered on the Chinese Clinical Trial Registry (ChiCTR) website in April 2024.

Data availability

The data values associated with the data points shown in graphs and values behind any reported means

were presented in the “Supporting data values” file. The qMSP data and the detailed Ct values in the three stages of present study are displayed in the “Supporting data values” file. The WGBS data of the inhouse cohort utilized in marker discovery step can be viewed in the Genome Sequence Archive (GSA) database under accession codes PRJCA035851 (https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA035851).

Author Contributions

Ruixiang Zhang, Yongzhan Nie, Xiaobing Chen, Tao Jiang, Jinhai Wang, Yuhui Peng and Guangpeng Zhou are co-first anthors of the article. These co-first authors conducted the experiments and had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Gang Ji, Xiaoliang Han, Jie He and Yin Li were responsible for the study conception and design. Yong Li, Lina Zhao, Beibei Chen, Yunfeng Ni, Yan Cheng and Yiwei Xu were responsible for managing patients and data acquisition. Analysis and interpretation of the data were conducted by Zhenyu Zhu, Xianchun Gao, Zhen Wu, Tianbao Li, Jie Zhao, Cantong Liu, Gang Zhao, Jiakuan Chen, Jing Zhao. Drafting the manuscript was performed by Ruixiang Zhang, Yongzhan Nie, Xiaobing Chen, Tao Jiang, Jinhai Wang, Yuhui Peng, Guangpeng Zhou, Gang Ji, Xiaoliang Han, Jie He and Yin Li. Ruixiang Zhang, Zhenyu Zhu, Zhen Wu, Jie Zhao, Xiaoliang Han and Yin Li checked and confirmed the conclusion and the final manuscript.

Footnotes

Author Details

[1]Department of Thoracic Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China. [2]State Key Laboratory of Holistic Integrative Management of Gastrointestinal Cancers, National Clinical Research Center for Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China. [3]Department of Oncology, the Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan 450008, China. [4]Department of Thoracic Surgery, Tangdu Hospital, The Air Force Military Medical University, Xi'an, Shaanxi 710038, China. [5]Department of Gastroenterology, the second affiliated hospital, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China. [6]Department of Clinical Laboratory Medicine, the Cancer Hospital of Shantou University Medical College, Shantou, Guangdong 515041, China. [7]BioChain (Beijing) Science & Technology Inc., Beijing 102600, China. [8]Department of Radiation Oncology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China. [9]Department of Digestive Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

Clinical Trial Information

Clinical trial number, ChiCTR2400083525

Medical Device Registration Information

NMPA Registration number 20243401368

Conflict of Interest

The authors declare the following potential conflict of interest with respect to the research, authorship, and/or publication of this article: Dr. Guangpeng Zhou, Zhenyu Zhu, Zhen Wu, Dr. Tianbao Li, Dr. Jie Zhao, Dr. Xiaoliang Han are current employees of BioChain (Beijing) Science & Technology, Inc. The other authors have no potential conflict of interest to report.

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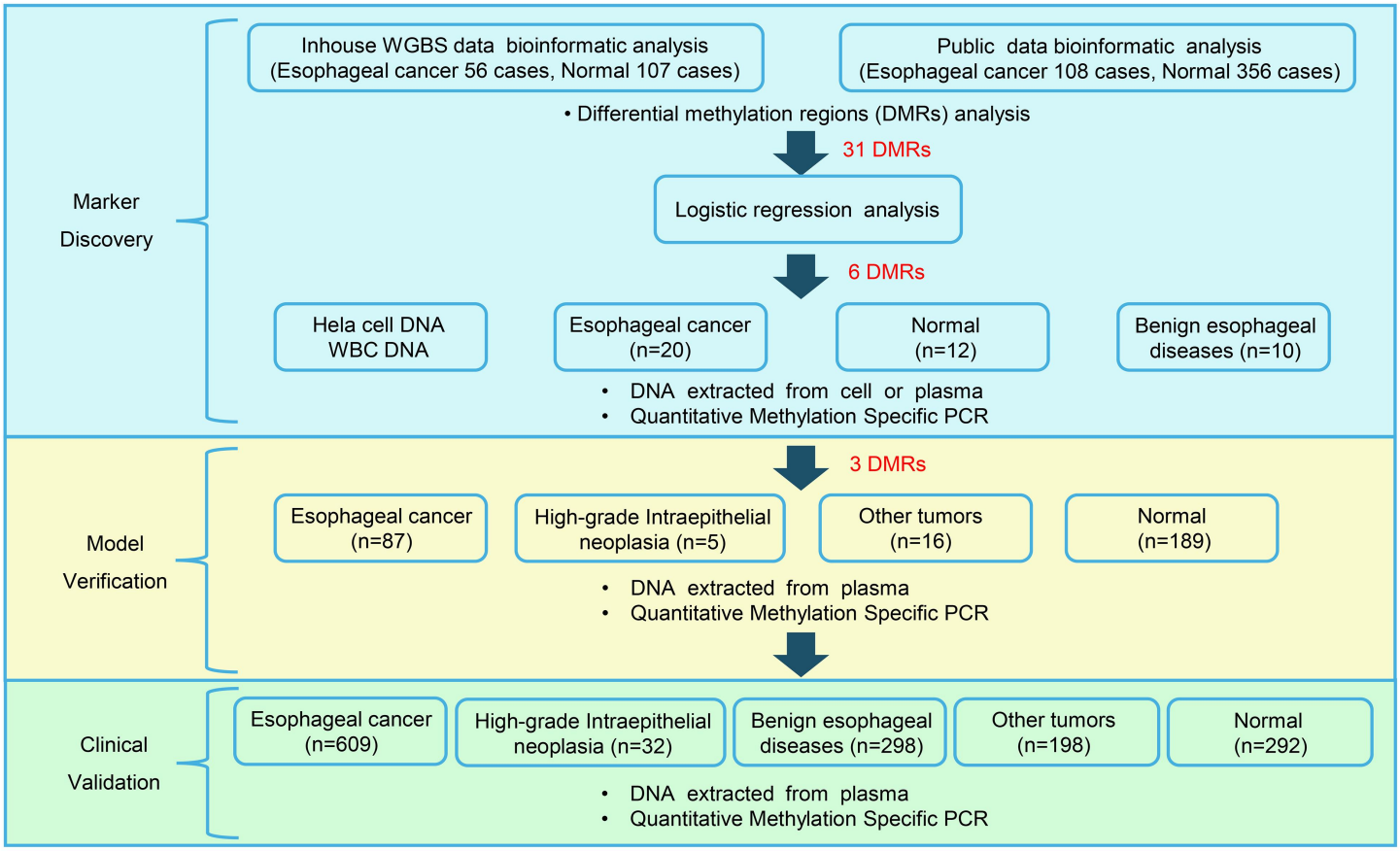

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614 **Figure 1**. Workflow of the three stages study design, including Marker Discovery, Model Verification,

615 and Clinical Validation.

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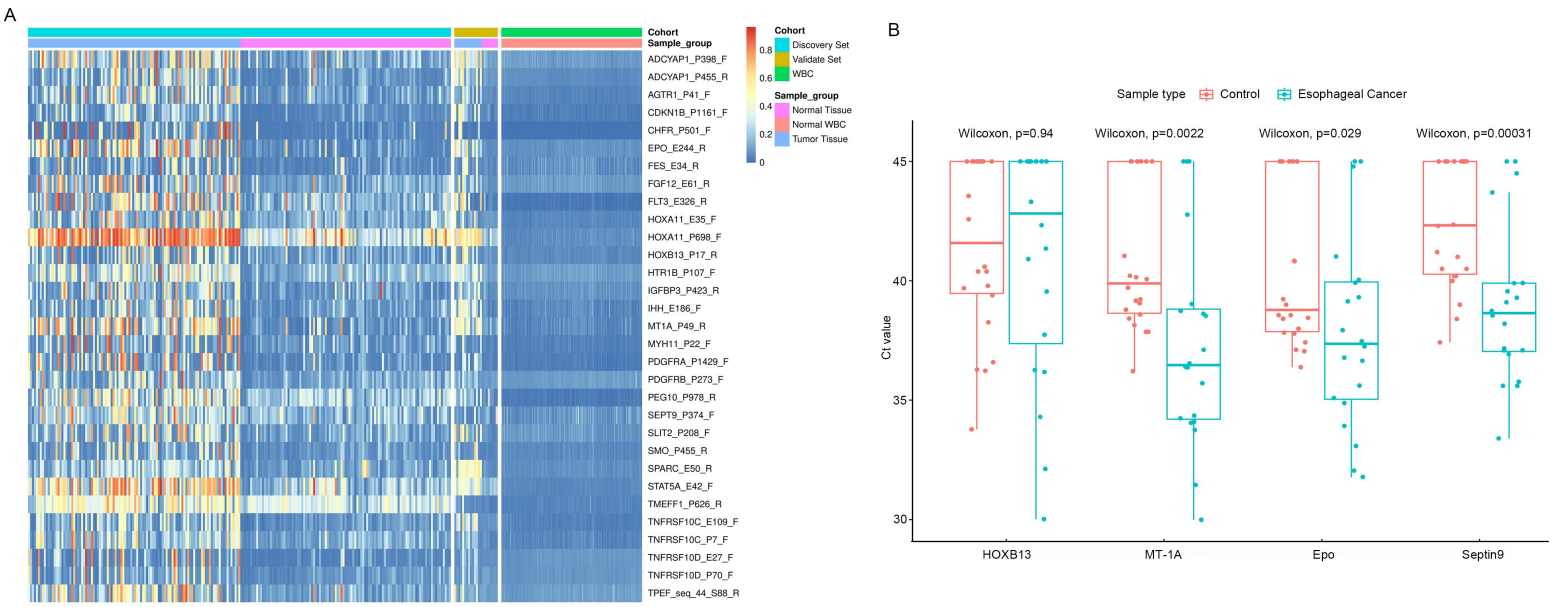
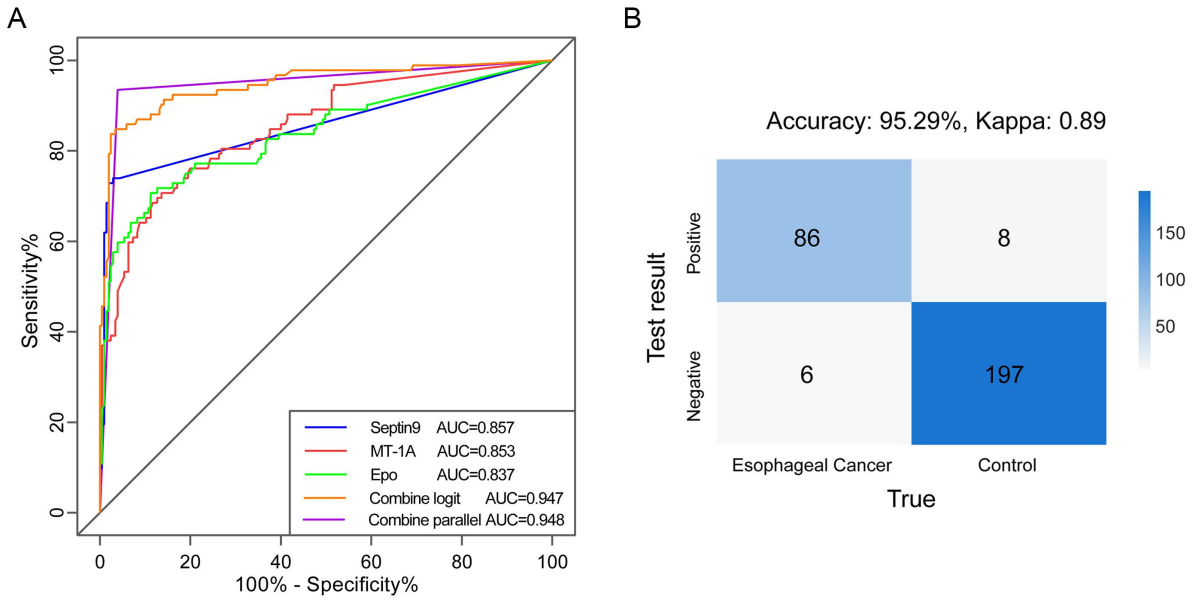


Figure 2. Differential Methylation of Candidate DNA Markers between esophageal cancer patients and normal individuals. (A) Methylation levels of 31 differentially methylated regions between ESCC tumor tissue (n = 108) and non-esophageal cancer cases (normal tissue and WBC) (n = 356) derived from public datasets, illustrating distinct methylation profiles between cancerous and non-cancerous samples. (B) Box plot presenting the qMSP Ct values for the selected 4 potential markers in blood samples of esophageal cancer patients (n =20) and control cases (n = 22) in the Marker Discovery cohort. Control cases including benign esophageal diseases and normal individuals. The box-and-whisker plot illustrates the interquartile range (IQR), with the line within the box denoting the median of the data and the whiskers extending from the box to the minimum and maximum values within 1.5 times the IQR. Each point represents one sample. The Wilcoxon test was used for pairwise comparison.



629 **Figure 3.** MDM model detection of methylation status by qMSP in the model verification cohort. (A)
630 Diagnostic efficacy of the three candidate MDMs and combined panel in samples from the model
631 verification cohort. The ROC curves indicated the performance for distinguishing esophageal cancer (n
632 = 92, including 87 patients with esophageal cancer and 5 patients with high-grade intraepithelial
633 neoplasia) from non-esophageal cancer (n = 205, including 16 patients with other cancers and 189 healthy
634 individuals. (B) Confusion matrix comparing true-observed classifications (reference detection methods)
635 with three-MDM panel-predicted diagnoses in the model verification cohort. Esophageal cancer (n = 92)
636 according to true-observed classifications including 87 patients with esophageal cancer and 5 patients
637 with high-grade intraepithelial neoplasia. Control group (n = 205) according to true-observed
638 classifications including 16 patients with other cancers and 189 healthy individuals.
639

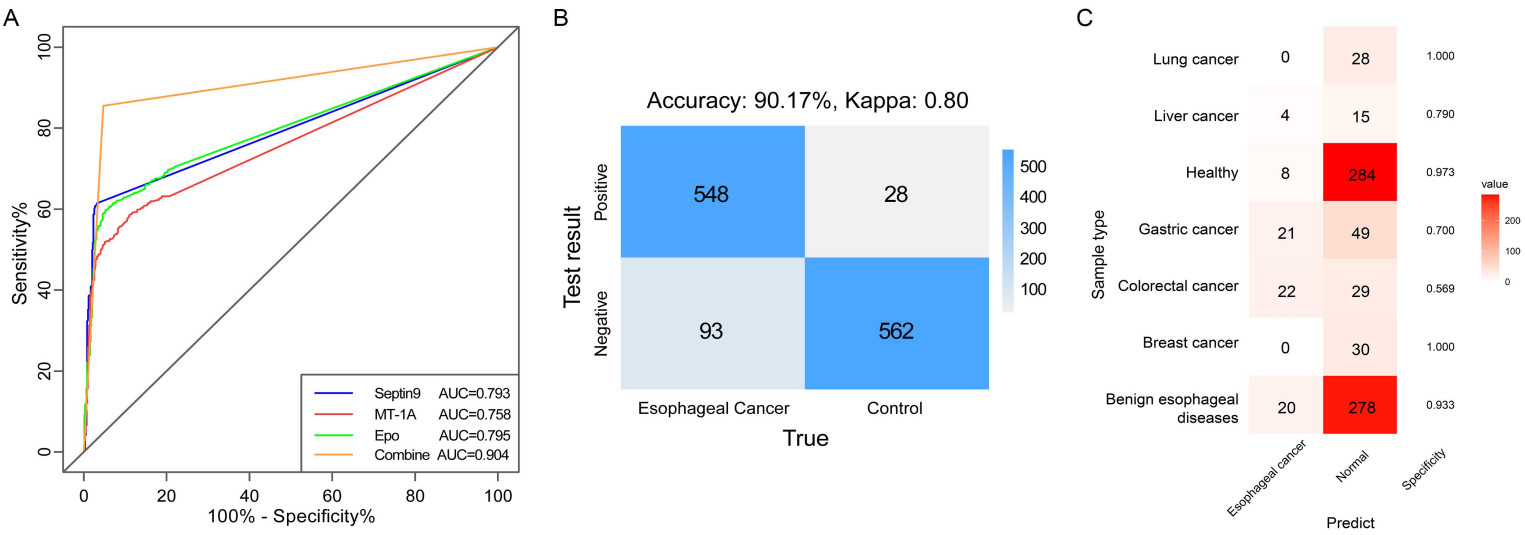
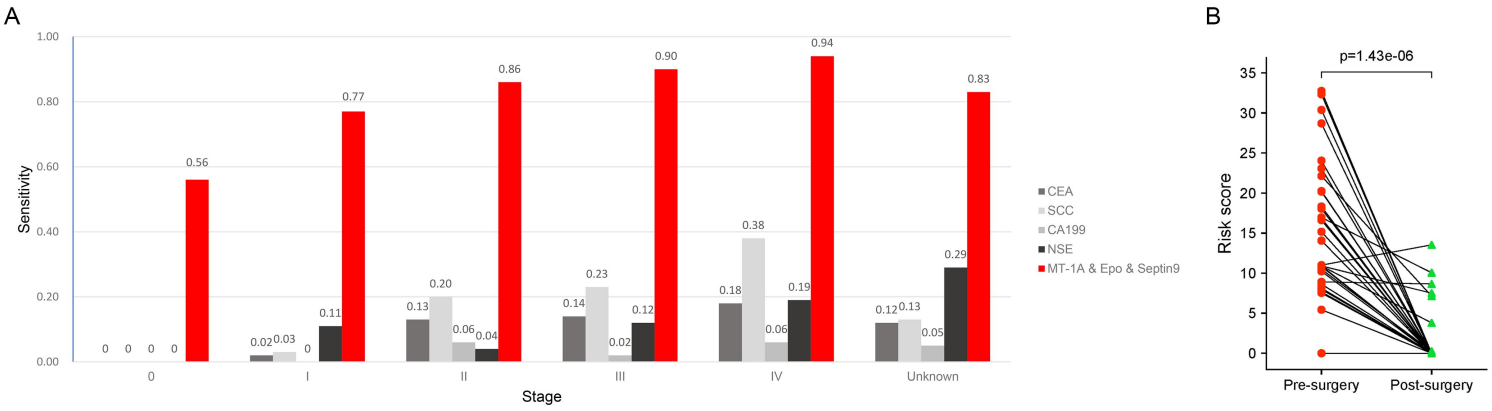


Figure 4. Multiplex detection of Septin9, Epo, and MT-1A methylation status by qMSP in the clinical validation cohorts. (A) Diagnostic efficacy of the MDMs and combined panel in the clinical validation cohort. The ROC curves indicated the performance for distinguishing esophageal cancer (n = 641, including 609 patients with esophageal cancer and 32 patients with high-grade intraepithelial neoplasia) from non-esophageal cancer (n = 788, including 198 other cancer participants, 292 healthy participants, and 298 participants with benign esophageal diseases). (B) Confusion matrix comparing true-observed classifications (reference detection methods) with three-MDM panel-predicted diagnoses in the clinical validation cohort. Esophageal cancer (n = 641) according to true-observed classifications including 609 patients with esophageal cancer and 32 patients with high-grade intraepithelial neoplasia. Control cases (n = 590) according to true-observed classifications including 298 patients with benign esophageal diseases and 292 healthy individuals. (C) Specificities of the three-MDM panel in each cancer type of 198 other cancer participants, in 292 healthy participants, and in 298 participants with benign esophageal diseases. Different sample types are listed along the vertical axis and predictive results are shown in the heatmap, while the corresponding specificity for each sample type is on the right vertical axis.



655 **Figure 5.** The three-MDM panel comparing with conventional markers and the application in treatment
656 monitoring. (A) Comparison of sensitivity between the three-MDM panel detection method and
657 conventional tumor protein markers in different cancer stages of the clinical validation cohort. The total
658 number of esophageal cancer samples is 609, with the sample sizes for each stage listed in Table 1. (B)
659 Pre-operative and Post-operative methylation levels in a subset of patients (n =32) who underwent
660 complete surgical resection. The Wilcoxon test was used for pairwise comparison.

661

662

Table 1. Patient demographics in the clinical cohort

	Overall			Diagnosed Group			Diagnosing Group			
	Cancer	Control		Cancer	Control		Cancer	Benign	Control	Total
Case Number	(n=641)	(n=788)	Total (n=1429)	(n=257)	(n=277)	Total (n=534)	(n=384)	(n=298)	(n=15)	(n=697)
Age (years)	64.5 ± 8.0	58.0 ± 11.0	61.0 ± 10.3	63.9±7.9	60.11±8.5	62.17±8.4	64.99±8.1	56.06±12.4	58.1±8.9	61.03±11.1
Sex (Male, n%)	516(80.50)	477(60.53)	993(69.49)	198(77.04)	133(48.01)	331(61.98)	318(82.81)	203(68.12)	11(73.33)	532(76.33)
AJCC stage, n (%)										
0	32(4.99)			12(4.67)			20(5.21)			
I	106(16.54)			48(18.68)			58(15.10)			
II	111(17.32)			40(15.56)			71(18.49)			
III	204(31.82)			88(34.24)			116(30.21)			
IV	117(18.25)			32(12.45)			85(22.14)			
Unknown	71(11.08)			37(14.40)			34(8.85)			

Table 2. The performance in clinical validation cohort

Group	Sensitivity(%)	Specificity(%)	PPV(%)	NPV(%)	Accuracy(%)
MT-1A	46.02(295/641)	97.12(573/590)	94.55(295/312)	62.35(573/919)	70.51(868/1231)
Epo	52.11(334/641)	97.29(574/590)	95.43(334/350)	65.15(574/881)	73.76(908/1231)
Septin9	40.49(257/641)	98.31(580/590)	96.72(295/305)	62.63(580/926)	71.08(875/1231)
MT-1A+Epo+Septin9	85.49(548/641)	95.25(562/590)	95.14(548/576)	85.80(562/655)	90.17(1110/1231)

Table 3. The detection performance of the three-MDM panel in diagnosed group and diagnosing group, respectively.

Indicators	Diagnosed Group	Diagnosing Group
Sensitivity (%) (95% CI)	85.21 (80.27~89.31)	85.68 (81.77~89.02)
Specificity (%) (95% CI)	97.11 (94.39~98.74)	93.61 (90.30~96.05)
Accuracy (%) (95% CI)	91.39 (88.68~93.62)	89.24 (86.70~91.44)
Positive Predictive Value (%) (95% CI)	96.48 (93.17~98.47)	94.27 (91.29~96.46)
Negative Predictive Value (%) (95% CI)	87.62 (83.41~91.09)	84.20 (79.93~87.87)