Received: 30 June 2022 | Accepted: 29 September 2022

DOI: 10.1111/1759-7714.14690

ORIGINAL ARTICLE

WILEY

A RASSF8-AS1 based exosomal lncRNAs panel used for diagnostic and prognostic biomarkers for esophageal squamous cell carcinoma

Kai Xie[1,2] | Chao Zheng[3,4] | Wenfeng Gu[5] | Zhisheng Jiang[5] | Chao Luo[6] | Jing Luo[5] | Yifei Diao[5] | Gaoming Wang[7] | Zhuangzhuang Cong[5] | Xinyue Yao[6] | Liwen Hu[1,5] | Yi Shen[1,3,5,6]

[1]Department of Cardiothoracic Surgery, Jinling Hospital, School of Nanjing Medical University, Nanjing, China

[2]Department of Thoracic Surgery, Suzhou Dushu Lake Hospital of Soochow University, Suzhou, China

[3]Department of Cardiothoracic Surgery, Jinling Hospital, Medical School of Southeast University, Nanjing, China

[4]Department of Thoracic Surgery, National Cancer Center, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

[5]Department of Cardiothoracic Surgery, Jinling Hospital, Medical School of Nanjing University, Nanjing, China

[6]Department of Laboratory Medicine, Jinling Hospital, Clinical School of Medical College, Nanjing University, Nanjing, China

[7]Department of Thoracic Surgery, Xuzhou Central Hospital, Xuzhou, China

Correspondence
Yi Shen, Department of Cardiothoracic Surgery, Jinling Hospital, School of Nanjing Medical University, 305 East Zhongshan Road, Nanjing, China.
Email: dryishen@nju.edu.cn

Liwen Hu, Department of Cardiothoracic Surgery, Jinling Hospital, Medical School of Nanjing, University, 305 East Zhongshan Road, Nanjing, China.
Email: lw_hoo@163.com

Xinyue Yao, Department of Laboratory Medicine, Jinling Hospital, Clinical School of Medical College, Nanjing University, Nanjing, China.
Email: xinghai0903@163.com

Funding information
China Postdoctoral Science Foundation, Grant/Award Numbers: 2018M643884, 2020T130128ZX; National Natural Science Foundation of China, Grant/Award Number: 81702444

Abstract

Background: Exosomal long non-coding RNA (lncRNA) has been shown to be potential biomarker for cancer diagnosis and follow up. However, little is known about its application in esophageal squamous cell carcinoma (ESCC) detection. Here, we sought to develop a novel diagnostic model based on serum exosomal lncRNAs to improve ESCC screening efficiency.

Methods: A multiphase, case-control study was conducted among 140 ESCC patients and 140 healthy controls. Microarray screening was performed to acquire differentially expressed exosomal lncRNAs in the discovery phase. The diagnostic model Index I was constructed based on a panel of three lncRNAs using logistic regression in the training phase, and were confirmed in a subsequent validation phase. A receiver operating characteristic (ROC) curve was generated to calculate the diagnostic value. The effects of the selected lncRNAs level on ESCC mortality were evaluated using a Cox hazard regression model and Kaplan-Meier curve analysis, and the expression level with clinicopathological features was also calculated. Finally, we explored the oncogenic potential of candidate lncRNA RASSF8-AS1 in vitro and by target mRNA sequencing.

Results: Index I was able to discriminate ESCC patients from healthy controls, and showed superiority to classic tumor biomarkers. Moreover, serum levels of the exosomal lncRNAs correlated with clinicopathological features and prognosis. The in vitro assays showed that RASSF8-AS1 played an oncogenic role in ESCC. Target mRNA scanning results suggested involvement of RASSF8-AS1 in tumor immunity and metabolism.

Kai Xie, Chao Zheng, and Wenfeng Gu contributed equally to this work.

Conclusion: The newly identified serum exosomal lncRNAs could be used as new biomarkers for ESCC, and showed oncogenic potential in ESCC.

KEYWORDS
circulating lncRNAs, esophageal squamous cell carcinoma, exosome; liquid biopsy, prognosis

INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth leading cause of malignancy deaths worldwide.[1] Squamous cell carcinoma is the main pathology type in China. The current standard for esophageal squamous cell carcinoma (ESCC) diagnosis depends on endoscopy and pathological biopsy.[2] However, the operation is relatively invasive and uncomfortable, resulting in delay or missing the best chance for most ESCC diagnosis and therapy. Liquid biopsy provides an alternative way of ESCC screening, but the traditional biomarkers used in the diagnosis of ESCC, such as serum squamous cell carcinoma antigen (SCC), cancer antigen 125 (CA125), carcinoembryonic antigen (CEA), and cytokeratin-19-fragment (CYFRA21-1),[3,4] lack enough sensitivity and specificity, which limits their clinical application. For example, SCC may be also applied in other types of squamous cell carcinoma, such as lung, head and neck, and cervical squamous cell carcinoma. CEA is mainly used for the detection of colorectal cancer, while CYFRA21-1 is usually used as a biomarker for diverse subtypes of lung and bladder cancer.

Exosomes are small (50–150 nm) membrane vesicles inside the cell, which may fuse with the membrane and are then released into the circulation.[5,6] They are detected in various body fluids such as serum, plasma, urine and saliva.[7,8] Exosomes execute communication functions intercellularly by transporting various biomolecules (such as DNAs, RNAs and proteins),[9,10] and the contents are protected by a phospholipid bilayer, making them suitable for liquid biopsy. Long noncoding RNAs (LncRNAs) is one of the exosome cargos,[11] and previous studies considered that lncRNAs in exosomes may be more stable for exosome protection against RNase.[12,13] LncRNAs detected in body fluids have been shown to be ideal noninvasive biomarkers for cancer diagnosis and prognosis. For example, lncRNA HOTAIR and SBF2-AS1 in serum exosomes have been implicated for the diagnosis and prognosis assessment of glioblastoma.[14,15] Similarly, Xu et al.[16] found in an independent cohort study that ENSG00000258332.1 and LINC00635 in serum had higher sensitivity and specificity for the diagnosis of hepatocellular carcinoma in combination with AFP. Recently, Tao et al.[17] found that the expression levels of TBILA and AGAP2-AS1 in the serum of NSCLC patients were significantly increased, and the combination with serum tumor biomarkers could further improve the diagnostic accuracy of NSCLC patients. Compared with the increasing evidence for exosomal lncRNAs in other types of cancer, little is known about their roles in ESCC. Furthermore, it has previously been reported that a joint model based on the union of multiple biomarkers may improve the diagnostic power, which prompted our study design.[18,19]

Here, we used a multistep study to investigate the possibility that novel serum exosomal lncRNAs act as potential biomarkers for ESCC detection. We selected candidate lncRNAs by integrating microarray results derived from serum and tissue samples separately, as a previous study reported that serum exosomal lncRNAs may be derived from tumor tissues. We deduced that the lncRNAs simultaneously overexpressed in serum and tissue samples may be more likely to meet the criteria. Finally, a diagnostic model based on three lncRNAs panel was established and the association of the novel panel with clinicopathological features or prognosis was also evaluated. Furthermore, we found involvement of candidate lncRNA RASSF8-AS1 in ESCC malignant phenotypes, and the combined results provide new insights into ESCC diagnosis and target therapy.

METHODS

Patients and samples

From 2017 to 2021, 140 ESCC patients and 140 healthy individuals were randomly recruited from Jinling Hospital of Nanjing Medical University, each of whom provided 5 ml of whole blood at indicated timepoints. Neither patient received preoperative chemo−/radiotherapy, and the overall survival (OS) was measured from the date of diagnosis to the date of death due to any cause or the date of the most recent follow-up. All patients were followed every 3 months for the first year, every 6 months for the next 2 years, and then annually. The end of the follow-up was November 31, 2021. The study was approved by the ethics committee of Jinling hospital, and all enrolled participants signed informed consent. All serum samples were stored at −80°C until use for total RNA extraction. The pathological stage was determined according to the eighth edition of the Union for International Cancer Control TNM staging system.

Exosome extraction and identification

A total of 1.5 ml of serum was centrifuged at 2000 × *g*, 4°C for 30 min, and the supernatant was then recentrifuged at 10 000 × *g*, 4°C for 45 min. The supernatant was then collected and filtered through a membrane of 0.22 μm pore size. The filtrate was collected and further subjected to ultracentrifugation at 10 000 × g, 4°C for 70 min. The precipitate was resuspended in 10 ml of precooling 1 × PBS, and

ultracentrifugation repeated. Finally, the precipitate was resuspended in 1.5 ml of precooling 1 × PBS. The isolated exosomes were characterized using transmission electron microscopy (TEM). Exosome concentration-particle size testing (nanoparticle tracking analysis [NTA]) was performed by Zeta View (Particle Metrix Ltd.).

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted from exosomes or tissues using an exosome RNA isolation kit according to protocols (Invitrogen). The extracted RNA was synthesized into cDNA by the PrimeScript RT Master Mix (Takara). RT-qPCR was performed to detect the relative RNA levels of target genes. β-actin was used as an internal reference gene for analysis. The relative expression levels of the lncRNAs were calculated using $2^{-\Delta\Delta Ct}$.[20,21] The sequences of primers used for qRT-PCR of the lncRNAs are listed in Table S1.

Microarray screening and analysis

Microarray screening was performed with Arraystar LncRNA_8 × 60 k (Arraystar), which contains both lncRNA and mRNA probes. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX version 12.1 software package (Agilent Technologies). After quantile normalization of the raw data, LncRNAs and mRNAs that at least four out of eight samples have flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed LncRNAs and mRNAs with statistical significance between the two groups were identified through *p*-value/FDR filtering.

Cell culture

Esophageal squamous cell carcinoma cell lines (TE1, TE13, KYSE150, KYSE140) and normal human esophageal epithelial cells (HET-1A) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). These cells were cultured in 1640 medium (KeyGene) containing 10% fetal bovine serum (FBS) (KeyGene), at 37°C in a 5% CO_2 atmosphere.

Stability analysis for exosomal lncRNAs panel

Exosome samples ($n = 15$) were allocated to aliquots for different processing, including storage under different temperature, treated with RNase A, and subjected to freeze–thaw cycles and acidic/alkaline environments, to test to the stability of exosomal lncRNAs, which is essential for clinical use.

Transfection and RNA interfering

Cells were seeded on six-well plates (2×10^5/well) with 1640 medium supplemented with 10% FBS overnight. Then, siRNA was transfected at a final concentration of 100 nM using lipofectamine 2000 (Invitrogen), and 24 h post transfection cells were harvested and subsequently RNA was extracted by Trizol reagent and the interfering efficiency was determined by RT-qPCR. Sequences of siRNAs and negative control are provided in Table S2.

Cell proliferation, migration, and invasion assays

Transfected cells were collected and grown in 96-well plates (2×10^3 cells per well) overnight and cell proliferation was detected using cell counting kit-8 (CCK-8). The reaction products were measured at 450 nm according to the manufacturer's instructions. For migration/invasion assays, 5×10^4 cells were incubated using Transwell kits (8 mm PET, 24-well Millicell) or matrix-coated inserts (BD Biosciences). After 24 or 48 h of incubation, cells were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotim). Imaging and counting of migrated cells were conducted by microscopy.

RNA sequencing and enrichment analysis

Cell transfection and RNA interfering were performed as described above. Then, 24 h post transfection, cells were harvested and RNA was extracted using Trizol reagent (Invitrogen). RNA-seq libraries were prepared using the Illumina RNA-seq preparation kit and sequenced on illumina nova seq 6000 sequencer. The differentially expressed RNAs (fold change >1.5, $p < 0.05$) were retrieved and subjected to gene ontology and KEGG pathway analysis.

Statistical analysis

All data were statistically analyzed with SPSS 26.0 (Chicago) and visualized with GraphPad Prism version 8.0 (GraphPad Software). Differences in lncRNAs expression between groups were determined using the Mann–Whitney unpaired *t*-test or paired *t*-test. The χ^2 or Fisher's exact test was used to compare the clinical characteristics of the groups. Receiver operating characteristic (ROC) curves were used to assess the diagnostic performance of selected biomarkers. The optimal cutoff point was determined using Youden's

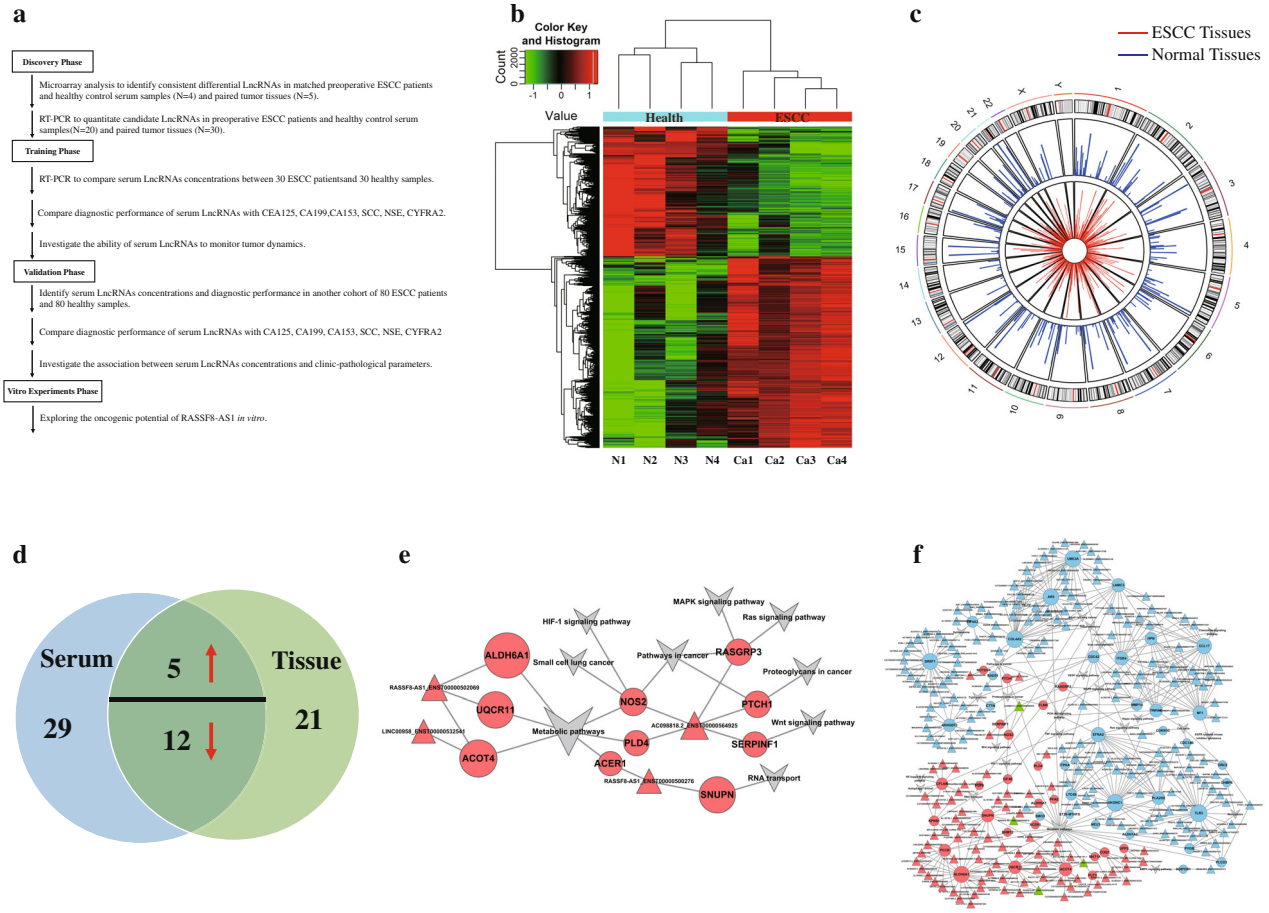

FIGURE 1 Microarray screening results of differential lncRNAs and function prediction. (a) Experimental design overview. (b) Heatmap results of microarray analysis for four preoperative esophageal squamous cell carcinoma (ESCC) patients and matched normal healthy serum samples. (c) Circos plots of lncRNAs in the human genome (hg19). The outer tracks represent the cytoband ideogram of chromosome. For the two tracks, the outer one (blue) represents the levels of lncRNAs in normal tissues and the inner one (red) represents the levels in ESCC tissues. (d) Venn diagram showing consistent upregulation of five lncRNAs in both microarray analyses. (e, f) Signal pathway networks of mRNAs involved in lncRNAs-mRNAs relationships.

index. DeLong's test was used to evaluate the statistical significance when comparing differences of AUCs. Using the binary status of enrolled participants (patients or controls) as the dependent variable, the regression coefficient for each lncRNA was estimated by a univariate logistic regression model, and was used as the weighting to construct the diagnostic index. The diagnostic index developed in the training phase was also directly applied to the validation phase to evaluate the clinical utility of the identified lncRNAs individually or in combination. A forward conditional logistic regression model was used to analyze the association between selected biomarkers and ESCC mortality. The long-term prognostic values of selected biomarkers were evaluated using Cox's hazard regression model and Kaplan–Meier curve analysis. Univariate analysis was used to explore clinicopathological factors (i.e., tumor depth, stage, size) associated with OS. Factors with p-value <0.05 were selected for the multivariate analysis with Cox's proportional hazard regression model, hazard ratio and 95% confidence interval (CI) were calculated. All p-values <0.05 were considered statistically significant.

RESULTS

Identification of exosomes in serum

A total of 280 participants, including 140 ESCC patients and 140 healthy controls were enrolled in the study, as shown in Table S3. To verify whether the exosomes were successfully isolated from serum, we characterized the isolated exosomes using TEM and NTA. The results showed that the isolated exosomes had a bilayer membrane structure (Figure S1 A), and the particle size distribution of exosomes was about 30–150 nm in diameter (Figure S1 B), meeting the criteria of exosome patterns.

Discovery phase: Genome-wide profiling to identify differentially expressed lncRNAs between ESCC and healthy individual controls

The overall workflow of this study is illustrated in Figure 1a. In the discovery phase, we performed LncRNA microarray

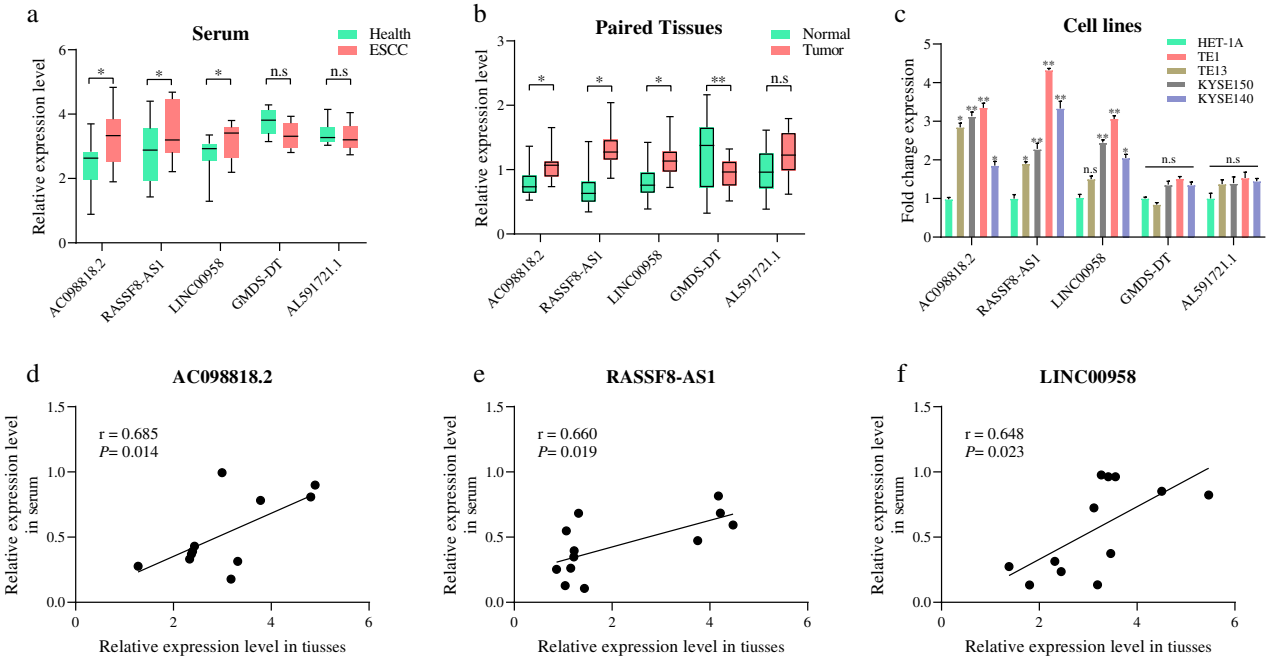

FIGURE 2 Identification of candidate five lncRNAs expression. (a) The expression levels of the five candidate lncRNAs in esophageal squamous cell carcinoma (ESCC) serum ($n = 20$) and normal serum samples ($n = 20$). (b) The expression levels of the five candidate lncRNAs in paired tumors and adjacent normal tissues ($n = 30$). (c) The expression levels of the five candidate lncRNAs in ESCC cell lines (TE1, TE13, KYSE140 and KYSE150) and the human esophageal epithelial cells line (HET-1A). (d–f) Spearman's correlation analysis between expression levels of lncRNAs in tumor tissues and paired serum samples of ESCC. $^{*}p < 0.05$, $^{**}p < 0.01$.

analysis on serum exosome samples from four preoperative ESCC patients and matched healthy controls. The top 100 differential lncRNAs identified in serum exosome samples from four preoperative ESCC patients and healthy control are shown in Figure 1b (FDR <0.005, Fold change >2, GEO accession number: GSE192662). Circos plots globally and genome-widely displayed kinds of detected lncRNAs in ESCC and control serum samples (Figure 1c). A previous microarray comparing five pairs of tumor and paracancerous tissues (GSE89102) was also included for further analysis. To achieve more reliable aberrantly expressed lncRNAs, we adopted a more restrictive filtering criteria (FDR <0.005, fold change >5) for both datasets, and integrated the two datasets to finally obtain five (AC098818.2, RASSF8-AS1, LINC00958, GMDS-DT and AL591721.1) candidate genes with significant overexpression in ESCC serum samples and cancerous tissues (Figure 1d).

To investigate the potential role of the candidate lncRNAs in ESCC, we analyzed mRNA results from the same microarray chip derived from serum samples and performed pathway analysis (Figure S2 A–D). By lncRNAs-mRNA coexpression network analysis, most of the candidate lncRNAs-related mRNAs could be enriched in pathways such as MAPK, Ras, Wnt, metabolic pathways and HIF-1α, which have been identified to be involved in ESCC carcinogenesis (Figure 1e,f).

To validate the microarray data, we examined the expression of five candidate lncRNAs in a pilot cohort containing serum exosome samples ($n = 20$ for ESCC and healthy control separately), and 30 paired tissues, respectively. The qRT-PCR results showed that the expression of AC098818.2, RASSF8-AS1 and LINC00958 being significantly increased in ESCC patients' serum compared with healthy, while no significant difference in the expression of AL591721.1 and GMDS-DT (Figure 2a). Compared with normal esophageal tissues, AC098818.2, RASSF8-AS1 and LINC00958 expression was significantly upregulated in tumor tissues, whereas the expression level of GMDS-DT was significantly downregulated, and no significant difference was seen in the expression of AL591721.1 (Figure 2b). Moreover, we examined the expression levels of these five lncRNAs in ESCC cell lines (TE1, TE13, KYSE140 and KYSE150) and normal epithelial cell HET-1A. Compared with HET-1A, the expression of AC098818.2, RASSF8-AS1 and LINC00958 was upregulated in the ESCC cell lines, while there was no significant difference in the expression of AL591721.1 and GMDS-DT (Figure 2c). Meanwhile, by big data mining through ExoRbase, we found that AC098818.2, RASSF8-AS1 and LINC00958 were significantly overexpressed in ESCC blood exosomes compared with healthy donors and other types of cancers (Figure S3 A-C), indicating excellent specificity in ESCC screening. By searching in the GEPIA database which obtained expression data from TCGA, a gradually increased trend of RASSF8-AS1 expression toward TNM stages was found (Figure S3 D). Furthermore, we detected expression level of the three candidates in 12 pairs of serum samples and corresponding cancerous tissues, and found moderate correlation for each lncRNA (Figure 2d-f).

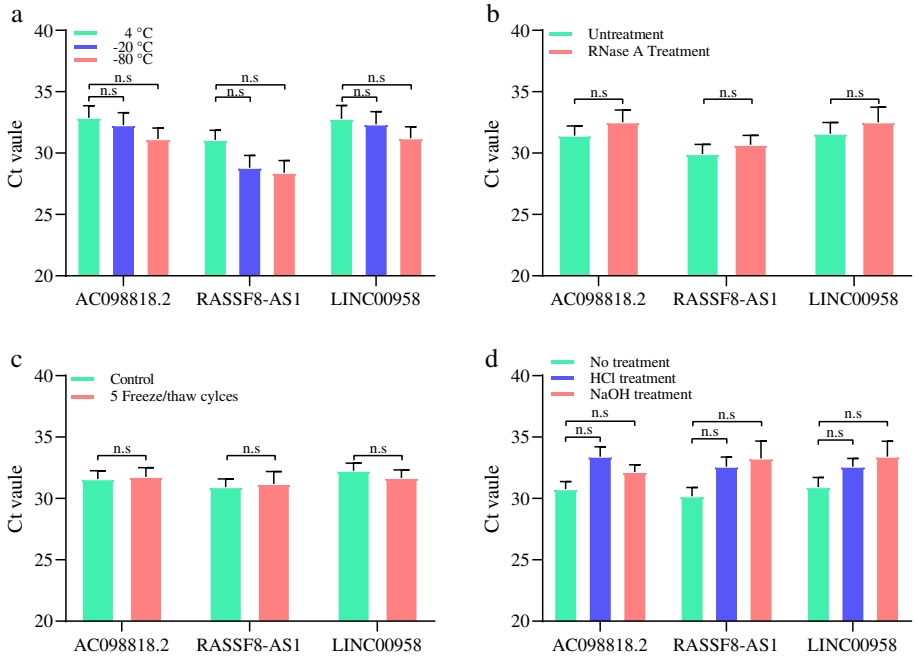

FIGURE 3 Stability of plasma lncRNAs under extreme conditions. (a) Incubation at different temperature for 3 h. (b) Repeated freeze–thaw cycles. (c) RNase A digestion for 3 and 6 h. (d) Exposure to acidic or alkaline environments.

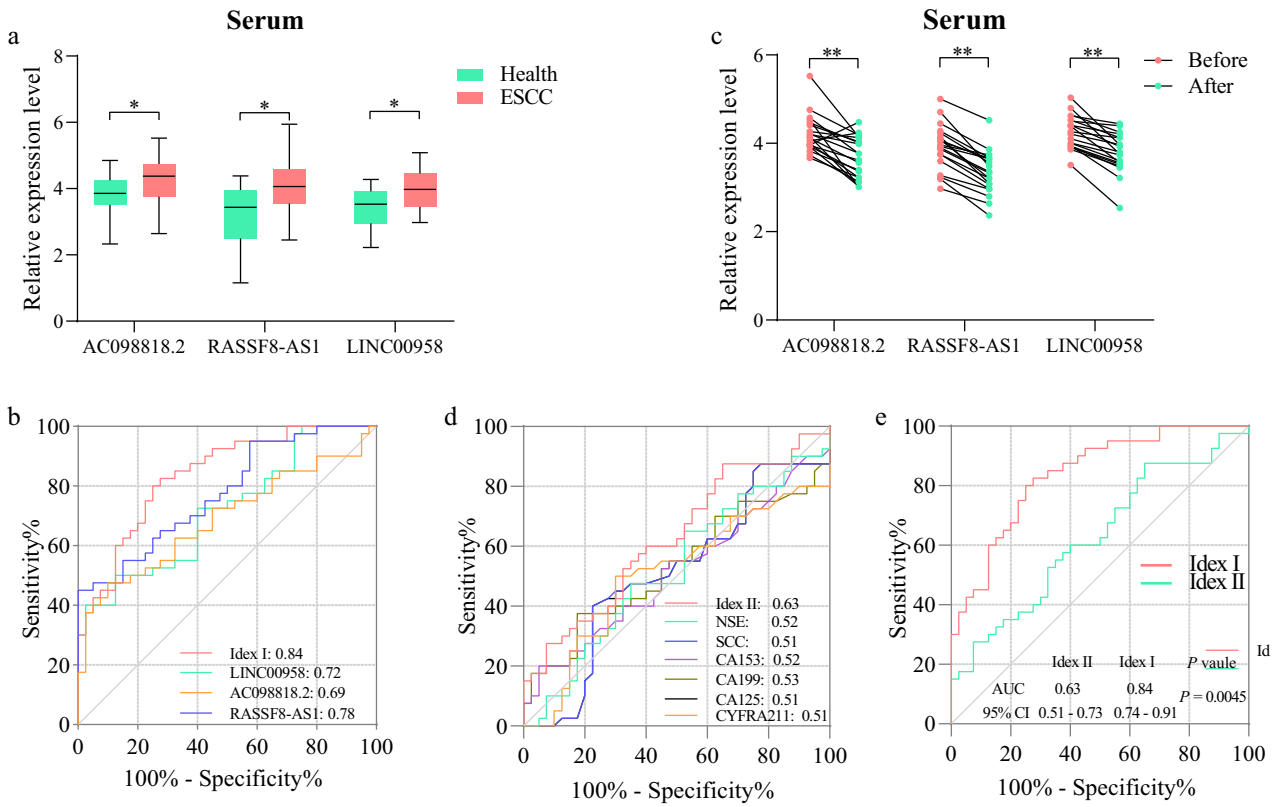

FIGURE 4 Training phase: A panel of serum lncRNAs-based diagnostic indicators Index I. (a) The expression levels of the three candidate lncRNAs in esophageal squamous cell carcinoma (ESCC) serum ($n = 40$) and normal serum samples ($n = 40$). (b) Receiver operating characteristic (ROC) curves to evaluate the sensitivity and specificity of three candidate lncRNAs to discriminate ESCC from healthy controls. (c) Paired comparison of the three candidate lncRNAs level before and after surgery ($n = 20$). (d) ROC curves to evaluate the sensitivity and specificity of traditional tumor markers to discriminate ESCC from healthy control. (e) Comparison of the diagnostic performance of Index I and Index II. *$p < 0.05$, **$p < 0.01$.

Evaluation of stability of exosomal lncRNA in serum samples under different conditions

Given that stability is a key prerequisite for body fluid tumor biomarkers, we evaluated the stability of exosomal lncRNAs in serum samples under different conditions. Serum samples were subjected to extreme conditions, such as incubation at different temperatures for 3 h, repeated freeze–thaw cycles, RNase A digestion for 3 and 6 h, and exposure to acidic or alkaline environments. Interestingly, these treatments had little or no effect on the level of these serum lncRNAs (Figure 3), indicating the lncRNAs panel was valuable for clinical application.

Training phase: A panel of serum lncRNAs-based diagnostic indicators Index I

To further investigate the diagnostic ability of the three candidate lncRNAs (AC098818.2, RASSF8-AS1 and LINC00958) for ESCC, we determined the relative expression levels of these three lncRNAs in serum samples of 40 ESCC patients and 40 healthy individuals and performed ROC curve analysis to evaluate the diagnostic capability for each lncRNA alone. The results showed that the expression levels of these lncRNAs were significantly elevated in ESCC patients (Figure 4a), with AUC values ranging from 0.69 to 0.78 (Figure 4b). Table S4 summarizes in detail the AUC values and 95% CI of each individual lncRNA. The optimal threshold for each lncRNA was determined by the Youden's index.[22] Serum lncRNA RASSF8-AS1 was the best diagnostic marker, with AUC values of 0.78 and 95% CI: 0.67–0.88, respectively.

To evaluate whether the expression levels of the lncRNAs panel could be used to monitor tumor dynamics, we collected paired serum samples from 20 pre- and postoperative patients. By paired comparison, we found that all three lncRNAs levels descended significantly after surgery (Figure 4c), reflecting potential utility in tumor burden monitoring during the therapy period.

Meanwhile, we explored the diagnostic value of several commonly used tumor markers (CEA125, CA199, CA153, SCC, NSE, CYFRA21-1) in ESCC detection. The ROC curves showed that the AUC values of these markers ranged from 0.51 to 0.53 (Figure 4d), and Table S4 showed that CA199 had the best diagnostic ability with AUC values of 0.53 and 95% CI: 0.40–0.66, which is inferior to lncRNAs.

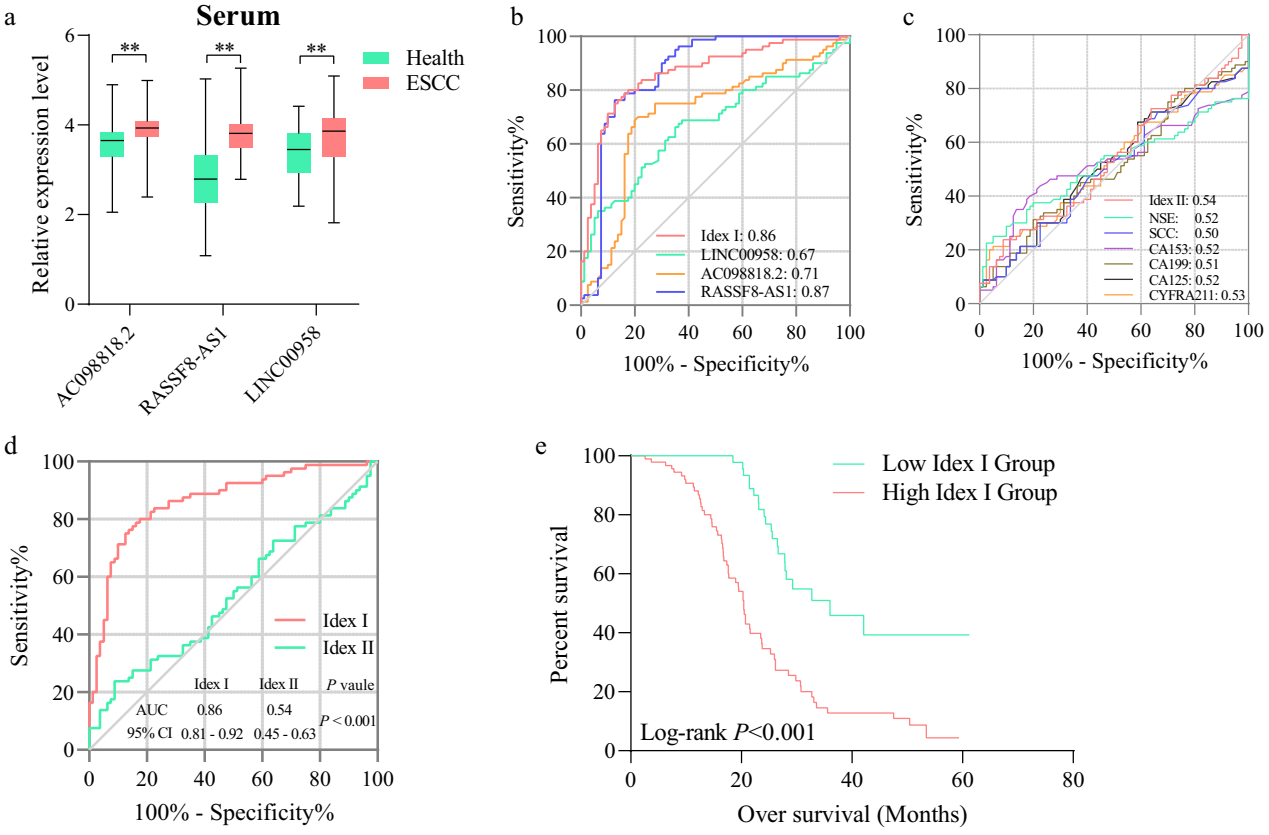

FIGURE 5 Validation phase: A panel of serum lncRNAs-based diagnostic indicators index I. (a) The expression levels of the three candidate lncRNAs in esophageal squamous cell carcinoma (ESCC) ($n = 80$) and control's serum samples ($n = 80$). (b) Receiver operating characteristic (ROC) curves of candidate lncRNAs to discriminate ESCC from healthy control. (c) ROC curves of traditional tumor markers. (d) Comparison of the diagnostic performance of Index I and Index II. (e) Kaplan–Meier survival curves of ESCC patients with low and high Index I.**$p < 0.01$.

Furthermore, we developed logistic regression models for each lncRNA and commonly used tumor markers, and calculated the regression coefficients separately. We subsequently used the regression coefficients as weights to establish the combined index. Interestingly, both combined indices outperformed single lncRNA alone or traditional biomarkers alone in terms of ESCC detection (Table S4). Next, we plotted the ROC curves and compared AUC values by the DeLong's test.[23] Index I had a significantly higher AUC value than Index II ($p = 0.0045$) with an AUC value of 0.84 (95% CI: 0.74–0.91), showing remarkably better diagnostic performance (Figure 4e and Table S4; $p = 0.0045$). The above results suggested that the combination of these three lncRNAs exhibited superior discriminatory power than traditional biomarkers.

TABLE 1 The correlation between the serum Index I expression and clinicopathological factors of ESCC

Characteristics	*n*	High Index I expression	Low Index I expression	*p*-value
Total (%)	140	70	70	—
Sex				
Male (%)	109	56	53	0.5414
Female (%)	31	14	17	
Age				
<65 (%)	44	21	23	0.8668
≥65 (%)	96	49	57	
Tumor depth				
T1–2 (%)	61	24	37	**0.0267**
T3–4 (%)	79	46	33	
Stage				
I–II (%)	65	29	46	**0.0040**
III–IV (%)	75	41	24	
Lymph node metastasis				
Negative (%)	67	29	48	**0.0012**
Positive (%)	73	41	22	
Tumor size				
<10 cm^3	78	38	40	0.1761
≥10 cm^3	62	32	30	

Abbreviations: ESCC, esophageal squamous cell carcinoma; —, no data available.
Bold values means *p*-value < 0.05.

Validation phase: The lncRNAs based model can distinguish ESCC from healthy control and the levels of lncRNA panel correlated with clinicopathological features

We further analyzed the serum lncRNAs levels in 80 ESCC patients and matched healthy samples. Being consistent with the results of the training phase, the expression levels of lncRNAs were significantly higher in ESCC patients than in healthy individuals (Figure 5a). Then, we plotted ROC curves to compare the diagnostic performance between lncRNAs and classic biomarkers, with maximum AUC values of 0.86 and 0.54 for lncRNAs and classic biomarkers, respectively (Figure 5b,c). Table S4 showed in detail the AUC values and 95% CI of lncRNAs and tumor markers during the training phase.

Being consistent with the results of the training phase, the lncRNAs-based Index I obtained the greatest diagnostic performance with an AUC value of 0.86 (95% CI: 0.81–0.92) (Table S4). In addition, the AUC values of lncRNAs-based Index I were significantly outperformed to that from Index II (Figure 5d and Table S4; $p < 0.001$).

To investigate Index I related clinicopathological factors, we collected ESCC patients from the discovery, training, and

TABLE 2 Associations between three serum-derived lncRNAs, classic tumor markers and ESCC mortality

		Univariate analysis		Multivariate analysis	
Parameters	Categories	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Tumor depth	T1–2 vs. T3–4	1.763 (0.892–3.485)	0.103	—	—
Stage	I–II vs. III–IV	1.621 (0.832–3.192)	0.162	—	—
Lymph node metastasis	Negative vs. Positive	1.158 (0.591–2.270)	0.67	—	—
Tumor size	<10 cm^3 vs. >10 cm^3	3.354 (1.629–6.907)	0.001	2.560 (1.085–6.043)	0.032
RASSF8-AS1 expression	<Mean vs. >mean	5.062 (2.421–10.555)	<0.0001	4.534 (1.894–10.852)	0.001
AC098818.2 expression	<Mean vs. >mean	0.702 (0.357–1.379)	0.304	—	—
LINC00958 expression	<Mean vs. >mean	0.037 (0.176–0.678)	0.002	—	—
CEA125 expression	<Mean vs. >mean	0.702 (0.357–1.379)	0.304	—	—
CA199 expression	<Mean vs. >mean	0.732 (0.574–2.205)	0.732	—	—
CA153 expression	<Mean vs. >mean	0.790 (0.403–1.550)	0.493	—	—
SCC expression	<Mean vs. >mean	0.702 (0.357–1.379)	0.304	—	—
NSE expression	<Mean vs. >mean	0.623 (0.316–1.227)	0.171	—	—
CYFRA2 expression	<Mean vs. >mean	0.552 (0.280–1.091)	0.087	0.407 (0.177–0.937)	0.035

Abbreviations: CI, confidence interval; ESCC, esophageal squamous cell carcinoma; OR, odds ratio; —, no data available.

TABLE 3 Univariate and multivariate Cox analysis of overall survival in ESCC patients

Parameters	Categories	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Tumor depth	T1–2 vs. T3–4	1.627 (1.039–2.549)	0.033	—	—
Stage	I–II vs. III–IV	1.465 (0.943–2.276)	0.089	—	—
Lymph node metastasis	Negative vs. Positive	2.269 (1.457–3.533)	<0.0001	—	—
Tumor size	<10 cm^3 vs. >10 cm^3	1.091 (0.707–1.684)	0.694	—	—
RASSF8-AS1 expression	<Mean vs. >mean	2.976 (1.879–4.714)	<0.0001	2.976 (1.879–4.714)	<0.0001
AC098818.2 expression	<Mean vs. >mean	1.020 (0.658–1.582)	0.928	—	—
LINC00958 expression	<Mean vs. >mean	0.764 (0.490–1.193)	0.236	—	—
CEA125 expression	<Mean vs. >mean	0.733 (0.474–1.132)	0.161	—	—
CA199 expression	<Mean vs. >mean	0.903 (0.584–1.395)	0.644	—	—
CA153 expression	<Mean vs. >mean	0.883 (0.571–1.365)	0.575	—	—
SCC expression	<Mean vs. >mean	0.733 (0.474–1.132)	0.161	—	—
NSE expression	<Mean vs. >mean	0.787 (0.509–1.217)	0.282	—	—
CYFRA2 expression	<Mean vs. >mean	0.887 (0.572–1.377)	0.594	—	—

Abbreviations: CI, confidence interval; ESCC, esophageal squamous cell carcinoma; HR, hazard ration; —, no data available.

TABLE 4 The correlation between the serum RASSF8-AS1 expression and clinicopathological factors of ESCC

Characteristics	*n*	High RASSF8-AS1 expression	Low RASSF8-AS1 expression	*p*-value
Total (%)	140	70	70	—
Sex				
Male (%)	109	50	49	0.1721
Female (%)	31	20	11	
Age				
<65 (%)	44	26	18	0.1453
≥65 (%)	96	44	52	
Tumor depth				
T1–2 (%)	61	22	49	**<0.0001**
T3–4 (%)	79	58	21	
Stage				
I–II (%)	65	25	40	**0.0110**
III–IV (%)	75	45	30	
Lymph node metastasis				
Negative (%)	67	27	40	**0.0278**
Positive (%)	73	43	30	
Tumor size				
<10 cm^3	78	26	52	**<0.0001**
≥10 cm^3	62	44	18	

Abbreviation: ESCC, esophageal squamous cell carcinoma; —, no data available.
Bold values means *p*-value < 0.05.

validation phases and classified them into high and low levels using the median of the Index I index as the threshold value. As shown in Table 1, higher Index I levels were associated with greater depth of infiltration ($p = 0.0267$), lymph node metastasis ($p = 0.0012$), advanced tumor stage ($p = 0.0040$), and poor prognosis (Figure 5e; $p < 0.001$).

Prognostic significance of three serum lncRNAs panel

We then analyzed the associations between 140 ESCC mortality and both three serum-derived lncRNAs (AC098818.2, RASSF8-AS1 and LINC00958) and classic tumor markers (CEA125, CA199, CA153, SCC, NSE, CYFRA21-1) using a forward conditional logistic regression model. As shown in Table 2, in the univariable analysis, tumor size, RASSF8-AS1 expression and LINC00958 expression were associated with ESCC mortality. However, in the multivariable analysis, only RASSF8-AS1 expression and CYFRA21-1 expression were independently associated with ESCC mortality. The long-term prognostic values of three serum-derived lncRNAs (AC098818.2, RASSF8-AS1 and LINC00958) and classic tumor markers (CEA125, CA199, CA153, SCC, NSE, CYFRA21-1) were analyzed using a Cox regression model and the results are listed in Table 3. In the univariable analysis, tumor depth, lymph node metastasis and RASSF8-AS1 expression were significantly associated with long-term mortality. However, in the multivariable analysis, only RASSF8-AS1 expression was independently associated with long-term mortality. In conclusion, RASSF8-AS1 expression was an independent prognostic factor for both short- and long-term outcomes of ESCC.

In vitro analysis of the oncogenic potential of RASSF8-AS1 and target scanning

As lncRNA RASSF8-AS1 showed good performance for ESCC diagnosis and RASSF8-AS1 expression was an independent prognostic factor for both short- and long-term outcomes of ESCC. We speculated that RASSF8-AS1 plays an oncogenic role in ESCC. We then determined that higher RASSF8-AS1 was associated with clinicopathological factors

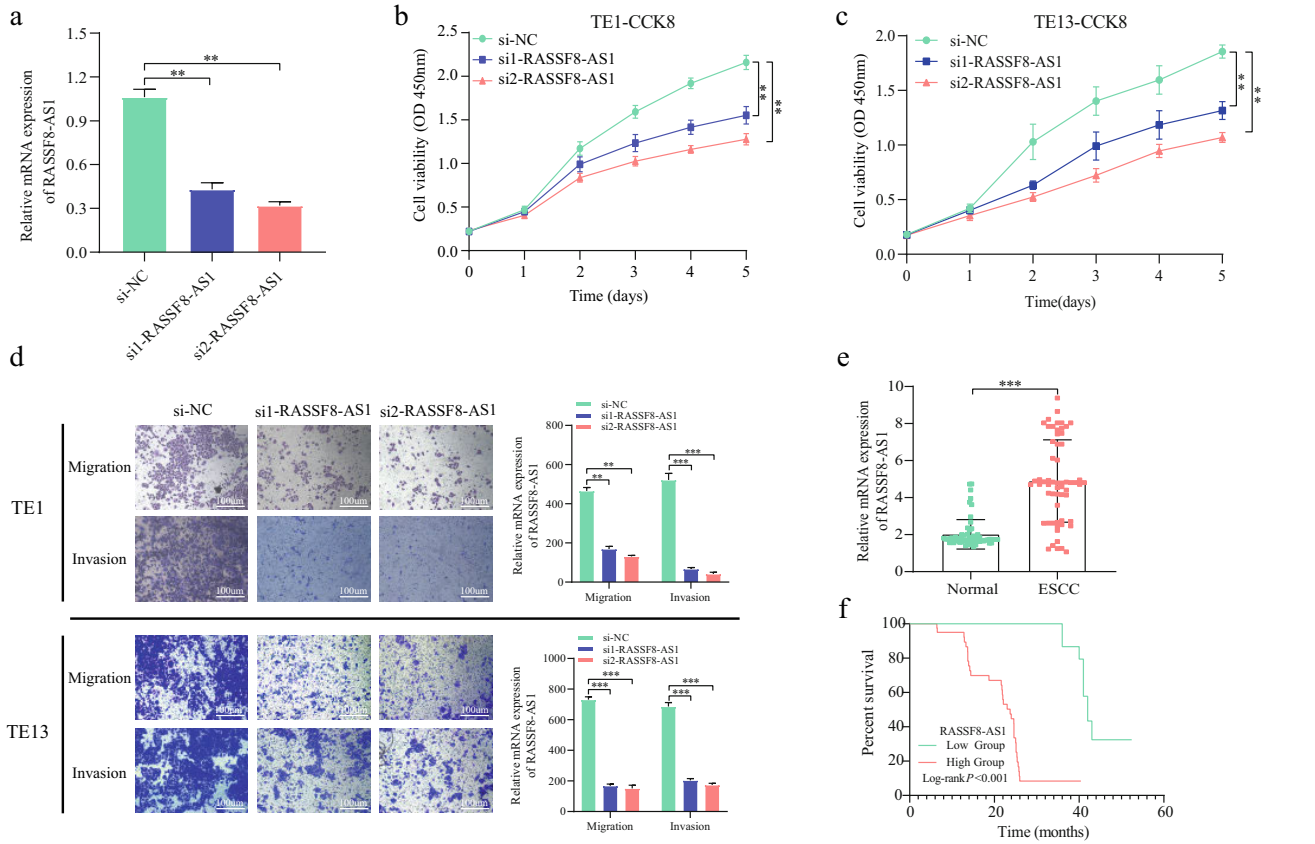

FIGURE 6 RASSF8-AS1 promotes esophageal squamous cell carcinoma (ESCC) cell proliferation, migration and invasion in vitro. (a) The mRNA expression of RASSF8-AS1 after transfection by two siRNAs. (b, c) Assessment of knockdown of RASSF8-AS1 on cell growth of TE1 and TE13 cells. (d) Assessment of knockdown of RASSF8-AS1 on cell migration and invasion. (e) The mRNA expression of RASSF8-AS1 in normal tissues and ESCC tissues. (f) Kaplan–Meier survival curves of ESCC patients with low and high RASSF8-AS1 expression. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Table 4), such as larger tumor size ($p < 0.0001$), greater depth of infiltration ($p < 0.0001$), lymph node metastasis ($p = 0.0278$), and advanced tumor stage ($p = 0.0110$). The expression of RASSF8-AS1 tended to decrease significantly after tumor resection surgery in ESCC patients (Figure 4c), and RASSF8-AS1 was found to be significantly higher in ESCC in the mid-late stage than in stage I through the GEPIA database (Figure S3 D). The above data suggested that RASSF8-AS1 plays a protumor role for ESCC.

Subsequently, we explored the oncogenic function of RASSF8-AS1 by knocking down RASSF8-AS1 in TE1 and TE13 cells (Figure 6a). In vitro assays showed that the knockdown of RASSF8-AS1 inhibited cell proliferation (Figure 6b,c), motility and invasiveness (Figure 6d), respectively.

In addition, we performed qRT-PCR on 60 pairs of tumor and adjacent normal tissues, and found that RASSF8-AS1 was likewise markedly elevated in ESCC tissues (Figure 6e). Next, we divided the 60 ESCC samples into RASSF8-AS1 low expression group ($n = 30$) and high expression group ($n = 30$) according to the median level of RASSF8-AS1 to explore the prognostic significance of RASSF8-AS1 in ESCC. Kaplan–Meier analysis showed that patients with high RASSF8-AS1 expression had a better dismal OS (Figure 6f).

Finally, we screened RASSF8-AS1 targets by RNA-sequencing and performed gene ontology (GO) and pathway enrichment analysis. The result found 303 positively regulated (Figure S4 C, D) and 430 negatively regulated targets (Figure 4e,f). GO results suggested RASSF8-AS1 was positively involved, mainly in immune response and cell migration, and KEGG pathway analysis indicated the positively regulated targets mostly enriched in cell metabolism (Figure S4 E, F), all of which have been proven to be essential for tumor progression.

DISCUSSION

Delayed diagnosis is the main cause for poor prognosis and rapid progression of ESCC. The five-year survival rate for patients with early diagnosis of ESCC is >90%, while survival rate with late detection is <20%.[24] Endoscopic surveillance and biopsy examination are current applied approaches for ESCC diagnosis and monitoring, but are not popular because of the high cost and level of discomfort, while imaging technology is limited for the low sensitivity, thereby highlighting the urgent need to develop new, relatively noninvasive and reliable tools to improve the early diagnosis of ESCC.

There are currently no widely accepted serum biomarkers for ESCC screening, surveillance for treatment response and recurrence. Traditional tumor biomarkers are inadequate in ESCC detection as they have poor sensitivity and specificity.

In recent years, the emergence of novel liquid tumor markers has provided a new solution for the early screening of tumors. The discovery of circulating tumor cells (CTC),[25] circulating tumor cell DNA (ctDNA),[26] circulating cell-free DNA (cfDNA),[27] and exosomes can further improve the efficacy of noninvasive screening. These humoral markers reflect the molecular and genetic information, as well as temporal and spatial heterogeneity of tumors, making the dynamic monitoring of tumor status possible. Among the currently acceptable noninvasive markers, the total amount of CTC is relatively low, while the specificity of ctDNA and cfDNA is insufficient, making the clinical application full of challenges. With the development of exosome isolation and enrichment technologies, exosome diagnosis has become a rising star in the field of liquid biopsy. Benefiting from the protection of the phospholipid bilayer on the surface of exosomes, the exosome contents have good stability, especially the RNAs can be protected by the membrane to avoid being degraded by RNase. The discovery of noncoding RNAs has enriched the understanding of tumorigenesis mechanisms. The role of exosome-transported noncoding RNAs in tumor metastasis, drug resistance, and radio-resistance across cells has received increasing attention. Studies have shown that the number of LncRNAs is huge, dozens of times that of currently known mRNAs, with high tissue specificity, which provides ideal conditions for the development of noninvasive screening. It has been reported that serum exosomal biomarkers may be derived from tumor tissues,[28] therefore in this study we also incorporated the upregulated lncRNAs from tissue microarray. The linear association between serum and tissue samples for the candidate lncRNAs confirmed the assumption. Finally, we identified a novel panel of exosomal lncRNAs for ESCC liquid biopsy through a multiphase study.

The lncRNAs-based composite Index I showed significantly higher diagnostic power than traditional tumor biomarker based composite Index II in two independent cohorts. Furthermore, the novel lncRNAs signature was stably detectable in serum, supporting the clinically applicable value. We also noted serum lncRNAs declined post-surgery, indicating that Index I may reflect tumor burden of ESCC patients, which should be used as a follow-up parameter for ESCC.

Among the newly identified lncRNA panel, RASSF8-AS1 showed the best diagnostic power for all ESCC patients, suggesting its oncogenic role in ESCC. Currently, the function of RASSF8-AS1 has only been reported in laryngeal squamous cell carcinoma.[29] Our results showed its oncogenic role in ESCC for the first time and revealed the underlying mechanisms by target mRNA scanning. Similarly, the role of LIN00958 in ESCC has been recently reported, which also verified the oncogenic role of LIN00958, suggesting our results are reasonable.[30]

We also note the limitations of this study. First, we included only a relatively small number of patients and healthy controls, and the ESCC patients were diagnosed with cancer as they had already been diagnosed pathologically earlier, which should be verified in a much larger sample size. Second, we conducted a retrospective, single-center, cross-sectional study, which may have introduced unavoidable selection bias. A prospective multicenter study is needed to further confirm the diagnostic efficiency of Index I, and to check the superiority to traditional biomarkers. Next, how lncRNA enters the exosome, how lncRNA encapsulated exosomes play a role in tumor progress and distant migration call for more attention. In recent years, the role of exosomes in mediating distant tumor metastasis has gained emphasis,[31,32] and our study revealed high expression of serum lncRNA RASSF8-AS1 was also correlated with metastasis, and our sequencing results suggested its involvement in immune response and cell metabolism. How our newly discovered exosomal lncRNAs play a role in distant metastasis of ESCC deserves further investigation.

ACKNOWLEDGMENTS

The authors thank the Jiangsu Institute of Cancer Research and Jiangsu Key Laboratory of Molecular and Translational Cancer Research for providing technical platform support. And gratefully acknowledge GEPIA and ExoRbase for open access to their database.

FUNDING INFORMATION

This research was supported by the National Natural Science Foundation for Youth of China (no. 81702444), China Postdoctoral Science Foundation 2018M643884 and 2020T130128ZX.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

ORCID

Liwen Hu https://orcid.org/0000-0002-5219-8464
Yi Shen https://orcid.org/0000-0002-2144-882X

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–49.
2. Pennathur A, Farkas A, Krasinskas AM, Ferson PF, Gooding WE, Gibson MK, et al. Esophagectomy for T1 esophageal cancer: outcomes in 100 patients and implications for endoscopic therapy. Ann Thorac Surg. 2009;87(4):1048–54. (discussion 1054-1045).
3. Kosugi S, Nishimaki T, Kanda T, Nakagawa S, Ohashi M, Hatakeyama K. Clinical significance of serum carcinoembryonic antigen, carbohydrate antigen 19-9, and squamous cell carcinoma antigen levels in esophageal cancer patients. World J Surg. 2004;28(7):680–5.
4. Mroczko B, Kozlowski M, Groblewska M, Lukaszewicz M, Niklinski J, Jelski W, et al. The diagnostic value of the measurement of matrix metalloproteinase 9 (MMP-9), squamous cell cancer antigen (SCC)

and carcinoembryonic antigen (CEA) in the sera of esophageal cancer patients. Clin Chim Acta. 2008;389(1–2):61–6.
5. Urbanelli L, Magini A, Buratta S, Brozzi A, Sagini K, Polchi A, et al. Signaling pathways in exosomes biogenesis, secretion and fate. Genes (Basel). 2013;4(2):152–70.
6. Li X, Wang Y, Wang Q, Liu Y, Bao W, Wu S. Exosomes in cancer: small transporters with big functions. Cancer Lett. 2018;435:55–65.
7. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci. 2011;68(16):2667–88.
8. Niu L, Song X, Wang N, Xue L, Song X, Xie L. Tumor-derived exosomal proteins as diagnostic biomarkers in non-small cell lung cancer. Cancer Sci. 2019;110(1):433–42.
9. Dai J, Su Y, Zhong S, Cong L, Liu B, Yang J, et al. Exosomes: key players in cancer and potential therapeutic strategy. Signal Transduct Target Ther. 2020;5(1):145.
10. Rashed MH, Bayraktar E, Helal GK, Abd-Ellah MF, Amero P, Chavez-Reyes A, et al. Exosomes: from garbage bins to promising therapeutic targets. Int J Mol Sci. 2017;18(3):538.
11. Kowal J, Tkach M, Thery C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol. 2014;29:116–25.
12. Boukouris S, Mathivanan S. Exosomes in bodily fluids are a highly stable resource of disease biomarkers. Proteomics Clin Appl. 2015;9(3–4):358–67.
13. Gezer U, Ozgur E, Cetinkaya M, Isin M, Dalay N. Long non-coding RNAs with low expression levels in cells are enriched in secreted exosomes. Cell Biol Int. 2014;38(9):1076–9.
14. Tan SK, Pastori C, Penas C, Komotar RJ, Ivan ME, Wahlestedt C, et al. Serum long noncoding RNA HOTAIR as a novel diagnostic and prognostic biomarker in glioblastoma multiforme. Mol Cancer. 2018;17(1):74.
15. Zhang Z, Yin J, Lu C, Wei Y, Zeng A, You Y. Exosomal transfer of long non-coding RNA SBF2-AS1 enhances chemoresistance to temozolomide in glioblastoma. J Exp Clin Cancer Res. 2019;38(1):166.
16. Xu H, Chen Y, Dong X, Wang X. Serum exosomal long noncoding RNAs ENSG00000258332.1 and LINC00635 for the diagnosis and prognosis of hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev. 2018;27(6):710–6.
17. Tao Y, Tang Y, Yang Z, Wu F, Wang L, Yang L, et al. Exploration of serum exosomal LncRNA TBILA and AGAP2-AS1 as promising biomarkers for diagnosis of non-small cell lung cancer. Int J Biol Sci. 2020;16(3):471–82.
18. Han L, Shi WJ, Xie YB, Zhang ZG. Diagnostic value of four serum exosome microRNAs panel for the detection of colorectal cancer. World J Gastrointest Oncol. 2021;13(8):970–9.
19. Xia D, Gu X. Plasmatic exosome-derived circRNAs panel act as fingerprint for glioblastoma. Aging (Albany NY). 2021;13(15):19575–86.
20. Zhang K, Shi H, Xi H, Wu X, Cui J, Gao Y, et al. Genome-wide lncRNA microarray profiling identifies novel circulating lncRNAs for detection of gastric cancer. Theranostics. 2017;7(1):213–27.
21. Dong L, Qi P, Xu MD, Ni SJ, Huang D, Xu QH, et al. Circulating CUDR, LSINCT-5 and PTENP1 long noncoding RNAs in sera distinguish patients with gastric cancer from healthy controls. Int J Cancer. 2015;137(5):1128–35.
22. Hajian-Tilaki K. The choice of methods in determining the optimal cut-off value for quantitative diagnostic test evaluation. Stat Methods Med Res. 2018;27(8):2374–83.
23. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics. 1988;44(3):837–45.
24. Saeki H, Nakashima Y, Zaitsu Y, Tsuda Y, Kasagi Y, Ando K, et al. Current status of and perspectives regarding neoadjuvant chemoradiotherapy for locally advanced esophageal squamous cell carcinoma. Surg Today. 2016;46(3):261–7.
25. Saini M, Szczerba BM, Aceto N. Circulating tumor cell-neutrophil tango along the metastatic process. Cancer Res. 2019;79(24):6067–73.
26. Abbosh C, Birkbak NJ, Swanton C. Early stage NSCLC—challenges to implementing ctDNA-based screening and MRD detection. Nat Rev Clin Oncol. 2018;15(9):577–86.
27. Kerachian MA, Poudineh A, Thiery JP. Cell free circulating tumor nucleic acids, a revolution in personalized cancer medicine. Crit Rev Oncol Hematol. 2019;144:102827.
28. Xiao K, Dong Z, Wang D, Liu M, Ding J, Chen W, et al. Clinical value of lncRNA CCAT1 in serum extracellular vesicles as a potential biomarker for gastric cancer. Oncol Lett. 2021;21(6):447.
29. Liu T, Meng W, Cao H, Chi W, Zhao L, Cui W, et al. lncRNA RASSF8AS1 suppresses the progression of laryngeal squamous cell carcinoma via targeting the miR664b3p/TLE1 axis. Oncol Rep. 2020;44(5):2031–44.
30. Wang B, Tang D, Liu Z, Wang Q, Xue S, Zhao Z, et al. LINC00958 promotes proliferation, migration, invasion, and epithelial-mesenchymal transition of oesophageal squamous cell carcinoma cells. PLoS One. 2021;16(5):e0251797.
31. Sun H, Wang C, Hu B, Gao X, Zou T, Luo Q, et al. Exosomal S100A4 derived from highly metastatic hepatocellular carcinoma cells promotes metastasis by activating STAT3. Signal Transduct Target Ther. 2021;6(1):187.
32. Zhao S, Mi Y, Guan B, Zheng B, Wei P, Gu Y, et al. Tumor-derived exosomal miR-934 induces macrophage M2 polarization to promote liver metastasis of colorectal cancer. J Hematol Oncol. 2020;13(1):156.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Xie K, Zheng C, Gu W, Jiang Z, Luo C, Luo J, et al. A RASSF8-AS1 based exosomal lncRNAs panel used for diagnostic and prognostic biomarkers for esophageal squamous cell carcinoma. Thorac Cancer. 2022;13(23):3341–52. https://doi.org/10.1111/1759-7714.14690