Original Paper

Three Circulating LncRNA Predict Early Progress of Esophageal Squamous Cell Carcinoma

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Key Words

ESCC • Plasma circulating biomarker • ROC analysis

Abstract

Background/Aims: Previous studies revealed that circulating (either from plasma or serum) long non-coding RNA may predict the occurrence or prognosis of multiple human malignant tumors. In this study, we mainly explored whether circulating lncRNAs can be utilized as biomarkers predicting the development of human esophageal squamous cell carcinoma (ESCC). **Methods:** LncRNA microarray was applied to screen the potential biomarkers for ESCC. Each group contained three individual plasma samples. A multi-stage validation and risk score formula detection were used for validation. **Results:** Eleven dysregulated lncRNAs were obtained after Venny analysis. Further validation in a larger cohort including 205 ESCC patients, 82 patients suffering from esophagus dysplasia and 210 healthy controls confirmed that increased Linc00152, CFLAR-AS1 and POU3F3 might be potential biomarkers for predicting the early progress with an area under curve (AUC) of 0.698, 0.651 and 0.584, respectively. The merged AUC of the three factors and merged with CEA was 0.765 and 0.955, respectively. We also revealed that circulating levels of three lncRNAs were associated with poor post-surgery prognosis of ESCC patients. **Conclusions:** The three circulating lncRNAs might serve as potential biomarkers for predicting the early occurrence of ESCC.

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Introduction

Esophageal carcinoma is one of the most deadly malignant tumors in the world. It has become the sixth leading cause of death and the eighth most frequently diagnosed cancer [1-3]. Esophageal carcinoma includes two main types: esophageal adenocarcinoma (EADC) and esophageal squamous cell carcinoma [4, 5]. ESCC is the most common subtype with a poor

treatment and survive rate with a 5-year overall survival rate about 14% [6, 7]. Traditional biomarker in diagnosis ESCC mainly including CEA, CA19-9 and SCCA; However, due to the limitation of lacking sufficiently high diagnostic sensitivity or specificity, the early diagnosis of ESCC still remains poor which leading to the early metastasis before diagnosis and poor prognosis [8, 9]. Therefore, developing new diagnosis methods or new biomarkers for ESCC early diagnosis are very necessary.

Long non-coding RNA (lncRNAs) were characterized with the relatively stable style with their secondary structure in body fluids, thus, the detection of lncRNAs in human plasma or urine was possible for researchers [10-12]. Based on this, lncRNAs have been previously investigated for their potential role as cancer biomarkers in body fluids (either in plasma, serum or urine) [13-18]. For example, the lnc-RNA UCA1 (urothelial carcinoma associated 1) is a lncRNA revealed as a potential biomarker for bladder cancer [19]. Also in prostate cancer patients, fragments from different regions of MALAT1 transcript were detected in plasma at higher copy number than in non-prostate cancer patients [20]. This lncRNA was proposed as a biomarker for prostate cancer diagnosis with a sensitivity and specificity of 58.6% and 84.8%, respectively, corresponding to a promising AUC of 0.836.

During the new approach exploring in ESCC diagnosis, researchers have demonstrated that four plasma miRNAs that could potentially serve as novel diagnostic biomarkers for ESCC. Moreover, specific miRNAs, such as miR-16 and miR-21, can predict poor survival in ESCC [21]. In addition, the circulating plasma POU3F3 could serve as a potential biomarker for diagnosis of ESCC in particular for early tumor screening based on a detailed mechanism study regarding POU3F3 [22, 23]. However, other potential lncRNAs based on high throughput detection still remain unclear. In this study, we aimed to characterize the genome-wide lncRNAs expression profile in plasma from ESCC patients and dysplasia patients comparing with the cancer-free controls to identify a panel of plasma lncRNAs that might serve as novel biomarkers for diagnosis of ESCC.

Materials and Methods

Samples

The study totally enrolled 205 patients who had been pathologically diagnosed with ESCC as well as 82 patients suffering from dysplasia in Huai'an First People's Hospital between 2011 and 2013. Patients with other malignant disorders, previous history of cancers, radiotherapy and chemotherapy were excluded. The cancer-free control subjects were from the routine physical examination individuals who have been confirmed without any malignant disorders or congenital diseases. The study was approved by the Institutional Ethics Committee of Huai'an First People's Hospital. All research was performed in compliance with government policies and the Helsinki Declaration. Experiments were undertaken with the understanding and written consent of each subject.

The clinicopathological relevance analyses of total 497 patients were summarized in Table 1. All 205 ESCC patients enrolled in this study were clinically and pathologically diagnosed. There were no significant differences in the distribution of age and sex between the cancer/dysplasia patients and the cancer-free controls.

Real Time Polymerase Chain Reaction

Blood samples were collected from each donor were placed in the EDTA-anticoagulant tube. The plasma was separated by centrifugation at 800g for 10 min at room temperature, followed by a 15-min high-speed centrifugation at 10000g at room temperature to completely remove cell debris. For mRNA detection, total RNAs (500 ng) were reverse transcribed using the reverse transcription kit through an Oligo-DT approach (with poly A) or random hexanucleotide primer method (without poly A) from TAKARA (Tokyo, Japan). β -actin was used as an internal control. Quantitative RT-PCR was performed using ABI Prism 7900HT (Applied Biosystems, CA, USA) according to the direction of the reagents

Microarray analysis of lncRNAs

For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. In brief, total RNA containing small RNA was extracted from plasma by using the Trizol reagent (Invitrogen, CA, USA) and purified with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to manufacturer's protocol. The purity and concentration of RNA were determined from OD260/280 readings using

Table1. Clinicopathological analysis of ESCC patients, Dysplasia patients and cancer-free control samples. ^aStudent t-test, ^bChi-square test

	ESCC	Dysplasia	Control	P value
N	205	82	210	
Age Mean (SE) year	54.29(9.22)	53.82(7.28)	55.18(4.28)	0.64 ^a
Sex (male/female)	158/47	55/27	162/48	0.16 ^b
Location				
Middle	95			
Lower	100			
Differentiation grade				
Well	67			
Moderate	104			
Poorly	34			
Tumor Size(cm)				
≤5cm	118			
>5cm	87			
TNM stage				
I-II	133			
III-IV	72			
Metastasis				
Yes	124			
No	81			

spectrophotometer (NanoDrop ND-1000). RNA integrity was determined by 1% formaldehyde denaturing gel electrophoresis. Double-stranded cDNAs (containing the T7 RNA polymerase promoter sequence) were synthesized from total RNA using the CbcScript reverse transcriptase with cDNA synthesis system according to the manufacturer's protocol (Capitalbio, Beijing China) with the T7 Oligo (dT) and T7 Oligo (dN). After completion of double-stranded cDNA (dsDNA) synthesis using DNA polymerase and RNase H, the dsDNA products were purified using a PCR NucleoSpin Extract II Kit (MN) and eluted with elution buffer. The amplified cRNA was purified using the RNA Clean-up Kit (MN). For the hybridization, Agilent Gene Expression Hybridization Kit (Agilent p/n 5188-5242, CA, USA) was used. CDNA labeled with a fluorescent dye (Cy3-dCTP) was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reaction. The procedure has been improved by using CapitalBio cRNA Amplification and Labeling Kit (CapitalBio, Beijing, China) for producing higher yields of labeled cDNA. After having washed the slides, the arrays were scanned by the Agilent Scanner G2505C (Agilent, CA, USA). 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 v12.0 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs that at least 3 out of 9 samples have flags in present or marginal ("All Targets Value") were chosen for further RT-PCR and risk score analysis. The raw data was uploaded in the ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress>) with ArrayExpress accession E-MTAB-5084.

Risk score analysis

Risk score analysis was performed to evaluate the associations between the concentrations of the plasma lncRNAs expression levels. The upper 95% reference interval of each lncRNA value in controls group was set as the threshold to code the expression level of the corresponding lncRNA for each sample as 0 and 1 in the training set. A risk score function (RSF) to predict ESCC group was defined according to a linear combination of the expression level for each lncRNA. For example, the RSF for sample i using information from three lncRNAs was: $rsfi = \sum 3j - 1Wj.sij$. In the above equation, sij is the risk score for lncRNA j on sample i, and Wj was the weight of the risk score of lncRNA j.

Statistical analysis

Venny online tool (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) was employed in screening the increased lncRNA in ESCC group and dysplasia group. All data was expressed as Mean ± SEM. Statistical analysis was performed with the Student's t-test for comparison of two groups and analysis of variance (ANOVA) for multiple comparisons. The statistical significance of microarray results was analyzed in terms

of fold change using the Student's t-test. False discovery rate (FDR) was calculated to correct the P-value. Fold changes ≥ 4 , or ≤ 0.25 ($P < 0.01$) were used as threshold values used to screen differentially expressed lncRNAs. Risk score analysis was applied in calculating the potential ability for predicting the ESCC Receiver operating characteristic curve was created by STATA 10. Kaplan–Meier survival curves were plotted and the log rank test was done. The significance of various variables for survival was analyzed by the Cox proportional hazards model in a multivariate analysis. Statistical analysis was performed using STATA 10, and presented with GraphPad Prism 5.0 software. Results were considered statistically significant at $P < 0.05$.

Results

Aberrant lncRNA expression profile in ESCC and dysplasia patients

Human lncRNA Array v3.0 was used to detect the lncRNAs derived from plasma of 3 patients with ESCC, 3 patients with dysplasia and 3 cancer-free controls in this study. Clustering analysis revealed an aberrant different expression lncRNAs profile in these three groups (Fig. 1A). A total of 380 lncRNAs transcripts were specifically dysregulated in ESCC patients and 217 lncRNAs in dysplasia patients comparing with the control group. Among these, only 11 lncRNAs were selected based on an aberrant increased level in both ESCC patients and dysplasia patients with the cutoff 4/0.25 (Fig. 1B).

Fig. 1. High throughput screening of circulating lncRNA in ESCC and dysplasia patients. (A) Hierarchical cluster analysis of lncRNA microarray detected in plasma from three ESCC patients, three dysplasia patients and three controls. (B) Venn analysis of dysregulated lncRNA in ESCC group and Dysplasia group comparing with controls. (C–E) Expression of lnc00152 (panel C), CFLAR-AS1 (panel D) and POU3F3 (panel E) in 205 ESCC patients, 82 dysplasia patients and 210 controls. Data was presented as mean $\pm$ SEM. Data was analyzed with Student t test. * indicated $p < 0.05$; ** indicated $p < 0.01$.

Fig. 2. Validation of the candidate lncRNAs. Data was presented as mean $\pm$ SEM and was analysis with Student t test. * indicated $p < 0.05$; ** indicated $p < 0.01$; n.s. indicated no significant.

Fig. 3. ROC analysis. Receiver operating characteristics (ROC) curves were drawn with the data of plasma lncRNAs separately, merged or combined with CEA from 205 patients with ESCC, 82 dysplasia patients and 210 healthy controls. Merged indicated the combination of three lncRNAs while CEA indicated the combination of both the three lncRNAs and CEA.

Multiphase validation

The 11 selected lncRNAs were further amplified in a larger sample including 205 ESCC patients, 82 dysplasia patients and 210 healthy controls. Only three lncRNAs (Linc00152, CFLAR-AS1 and POU3F3) was validated as significant up-regulated in both ESCC patients and dysplasia patients comparing with the control group while the rest 8 lncRNAs presented a disqualification as presented in Fig. 1A and Fig. 2.

To assess the diagnostic value of the three lncRNAs

Table 2. Risk score analysis. ^aPPV, positive predictive value, ^bNPV, negative predictive value

Score	0-6.228	6.228-9.763	PPV ^a	NPV ^b
Training set			0.95	0.90
ESCC	1	19		
Control	18	2		
Validation set			0.88	0.94
ESCC	23	162		
Control	178	12

Fig. 4. Stability detection of lncRNAs in human plasma. Human plasmas obtained from healthy controls were incubated at room temperature for 12h, 24h, 48h or subjecting it to up to 3 cycles of freezing and thawing. Data was presented as mean $\pm$ SEM.

Fig. 5. Prognosis predicting ability of circulating lncRNA. Five-year survival analysis reflecting the diagnosis value of these three indices on the prognosis was carried out by using Kaplan–Meier curve according to the follow up data. The upper 95% CI in control group was used as cutoff. (A) Linc00152, (B) POU3F3, (C) CFLAR.

profiling system, we used a risk score formula to calculate the risk score function for cases and control samples. Taken the three lncRNAs as candidates, we further chose 60 individuals in the three groups mentioned above (each group contained 20 individuals) randomly as training set. The expression of the three lncRNAs was calculated through a risk score analysis. Based on the risk score analysis we obtained the optimal cutoff value (Value=5.828) with the value of sensitivity + specificity considered to be maximal, plasma samples were then divided into a high-risk group, representing the possible ESCC group, and a low-risk group, representing the predicted controls. The positive predictive value and negative predictive value was 95% and 90% in the training set, respectively. Similarly, when the same cutoff value was applied to calculate the risk score of samples in the larger validation sets (the rest samples in each group), the positive predictive value and negative predictive value was 88% and 94% (Table 2).

We further analyzed the ROC curves to test the sensitivity and specificity of the three-lncRNAs signature for ESCC. As presented in Fig. 3, the areas under the curve (AUC) of Linc00152, CFLAR-AS1 and POU3F3

and the merged factor were 0.698, 0.651, 0.584, and 0.765, respectively. After merging the three lncRNAs with CEA, the AUC was remarkably increased and up to 0.955 (Fig. 3).

The expression stability of three lncRNA in plasma

We next amplified the three lncRNAs in ten healthy controls at room temperature for 12h, 24h, 48h, or subjecting it to up to 3 cycles of freezing and thawing. As presented in Fig. 4, all of the process had minimal effects on the concentrations of the three lncRNAs, demonstrating that these lncRNAs might be stably expressed in human plasma and be detectable which is an important requirement for a biomarker.

Circulating level of lncRNAs were associate with poor post-surgery prognosis of ESCC patients

Lastly, a 5-years survival analysis reflecting the diagnosis value of these three indices on the prognosis was carried out by using Kaplan–Meier curve according to the follow up data. All patients were sub-divided into high and low expression groups according to up 95% CI in normal control group. All three lncRNAs indices were bad prognosis indicators for post-surgery ESCC patients. Among them, survival difference was most significant in Linc00152 ($P=0.0028$, $HR=1.888$, 95% CI: 1.220 to 2.581) (Fig. 5A). However, other two indices also relatively ideal in predicting the prognosis, with $P=0.0062$ for POU3F3 ($HR=1.819$ with 95% CI: 1.166 to 2.508) (Fig. 5B) and $P=0.0141$ for CFLAR ($HR=1.675$, with 95% CI: 1.076 to 2.320) (Fig. 5C).

Discussion

Patterns of lncRNAs deregulation in primary tumor tissues were mirrored in various bodily fluids, including plasma, serum and urine, even gastric juice [24, 25]. With recent improvements in technology associated with RNA analysis, higher sensitivity may in the future be achieved and in time lncRNAs may be recognized as cancer biomarkers with utility in the clinical setting [26]. So far, plasma/serum and urine were the bodily fluids which have been most commonly used for lncRNA profiling in cancer patients, however other fluids have also been tested. For instance, expression of the lncRNA AA174084 was evaluated in gastric juice for the diagnosis of gastric cancer patients. In this fluid, the levels of the lncRNAs were significantly higher than in healthy individuals and patients with other gastric mucosa lesions, corresponding to an AUC of 0.848 for gastric cancer diagnosis. AA174084 in digestive fluids presented in this way as a robust biomarker, allowing the diagnosis of the disease with a sensitivity of only 0.46 but a specificity of 0.93. Most interestingly, the levels of the same lncRNA in the plasma could not distinguish gastric cancer patients from healthy individuals [24].

Researchers have found a potential ability of circulating POU3F3 as a biomarker in ESCC, in this study, through a microarray screening; we also found an increased level of POU3F3 in the circulating microenvironment of ESCC patients as well as the dysplasia patients, which was consistent with previous report. They also demonstrated that the combination of POU3F3 with SCCA, the AUC could up to 0.926; however, based on a larger sample size, we obtained a lower sensitivity and specificity; we thought this might be reasonable due to the individual difference. POU3F3 has been identified highly with the development of ESCC based on an epigenetically modification with Notch signaling pathway [22]. Besides, Linc00152 was also involved in the pathogenesis of HCC by promoting proliferation targeting EpCAM via the mTOR signaling pathway. It was also confirmed that Linc00152 might act as novel biomarkers in predicting diagnosis of hepatocellular carcinoma [27, 28]; however, no evidence was been mentioned in the occurrence of ESCC.

In conclusion, we have identified three lncRNAs aberrant expressed in plasma of the ESCC patients compared with those of healthy controls. These findings indicated for the first time that the lncRNAs in plasma might be used as a novel and a rapid early diagnostic biomarker for ESCC.

Abbreviations

ESCC (esophageal squamous cell carcinoma); lncRNA (long non-coding RNA); PCR (Polymerase Chain Reaction); RSF (A risk score function); ROC (Receiver Operating Characteristic); AUC (areas under the curve).

Availability of Data and Materials

The detailed information of the microarray was submitted to ArrayExpress (accession code E-MTAB-3553).

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Disclosure Statement

The authors declared that they have no financial conflict of interest.

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