Identification of palsma exosomal lncRNA as a biomarker for early diagnosis of gastric cancer

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Abstract

Background, There were about 1,090,000 gastric cancer cases in 2020 in China. The incidence and mortality rates ranked the fifth and third among all kinds of cancers in China. Early diagnosis plays an important role in the treatment and prognosis of gastric cancer.plasma exosome lncRNAs, has become a promissing biomarkers with high specificity and sensitivity for early diagnosis of cancers. In this study, the exosomes in the plasma of patients with early gastric cancer were isolated by a commercial kit. After identified by electron microscopy observation, particle size analysis and Western-blot verification, the lncRNAs in the exosomes were extracted. The lncRNAs differentially expressed in the plasma exosomes of patients with gastric cancer were analysized by high-throughput RNA sequencing. The differentially expressed lncRNAs were verified by RT-qPCR in 93 patients with early gastric cancer and 49 normal controls. Electron microscopy, particle size analysis and Western blot showed that exosomes were successfully isolated from plasma. RNA-Seq results show that 76 lncRNAs were up-regulated and 260 lncRNAs were down regulated in plasma exosomes of early gastric cancer patients compared with normal controls. RT-qPCR analysis indicated that a total of 6 lncRNAs were significantly and differentially expressed in gastric cancer patients compared to normal controls, with 2 highly expressed and 4 lowly expressed ($p < 0.05$). The survival curve analysis indicated that lncmstrg.2441832.8 and lncmstrg.2312697 had higher sensitivity and specificity for the diagnosis of gastric cancer, respectively and AUC curve areas were 0.6211 and 0.631, $p < 0.05$, respectively, which were greater than the traditional clinical detection indexes CEA (0.61) and AFP (0.57). When combined lncmstrg.2441832.8 and lncmstrg.2312697 in gastric cancer diagnosis, AUC curve area reached 0.73, which was greater than CA199 (0.71). Conclusion, lncmstrg.2441832.8 and lncmstrg.2312697 may be a potential and promissing biomarkers for early diagnosis of gastric cance

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Abstract

Background, There were about 1,090,000 gastric cancer cases in 2020 in China. The incidence and mortality rates ranked the fifth and third among all kinds of cancers in China. Early diagnosis plays an important role in the treatment and prognosis of gastric cancer. In recent years, noninvasive diagnosis, especially plasma exosome lncRNAs, has become a promissing biomarkers with high specificity and sensitivity for early diagnosis of cancers. ***Methods*****,** In this study, the exosomes in the plasma of patients with early gastric cancer were isolated by a commercial kit. After identified by electron microscopy observation, particle size analysis and Western-blot verification, the lncRNAs in the exosomes were extracted. The lncRNAs differentially expressed in the plasma exosomes of patients with gastric cancer were analysized by high-throughput RNA sequencing(RNA-Seq). The differentially expressed lncRNAs were verified by RT-qPCR in 93 patients with early gastric cancer and 49 normal controls. ***Results,*** Electron microscopy, particle size analysis and Western blot showed that exosomes were successfully isolated from plasma. RNA-Seq results show that 76 lncRNAs were up-regulated and 260 lncRNAs were down regulated in plasma exosomes of early gastric cancer patients compared with normal controls. RT-qPCR analysis indicated that a total of 6 lncRNAs were significantly and differentially expressed in gastric cancer patients compared to normal controls, with 2 (lncmstrg. 1319590,Lncmstrg. 2312697) highly expressed and 4 lowly expressed (lncmstr-g.1004024.1, lncmstrg. 2441832.8, lncmstrg. 315376.1, lncmstrg. 907985.2,)($p < 0.05$). The survival curve analysis indicated that lncmstrg.2441832.8 and lncmstrg.2312697 had higher sensitivity and specificity for the diagnosis of gastric cancer, respectively and AUC curve areas were 0.6211 and 0.631, $p < 0.05$, respectively, which were greater than the traditional clinical detection indexes CEA (0.61) and AFP (0.57). When combined lncmstrg.2441832.8 and lncmstrg.2312697 in gastric cancer diagnosis, AUC

curve area reached 0.73, which was greater than CA199 (0.71). ***Conclusion,*** lncmstrg.2441832.8 and lncmstrg.2312697 may be a potential and promissing biomarkers for early diagnosis of gastric cancer.

Key words: gastric cancer, exosome, lncRNA, diagnosis biomarker

Introduction

Cancer is the second leading cause of human death in the world, and the number of deaths and morbidity are increasing year by year. Gastric cancer is one of the most important cancers that harm human health. According to 2020 cancer burden data released by IARC, the world's newest gastric cancer cases are 1 million and 90 thousand cases and 770 thousand deaths in 2020. Incidence rate and mortality rate are thefifth and fourth of all kinds of tumors. China had 480 thousand new cases and 370 thousand deaths of gastric cancers and the incidence rate and mortality rate are the third of all kinds of tumors in the world[1]. At present, the specificity and sensitivity of commonly used tumor diagnosis markers such as CA199 and CEA are quietly low. Although gastroscopy is a gold standard for the diagnosis of gastric cancer, many people unwell to do it because gastroscopy is an invasive examination. Additionally, the early symptoms of gastric cancer are hidden and difficult to detect. Therefore, these result in a low rate of early diagnosis of gastric cancer.

Exosome is an important subgroup of extracellular vesicles [2], which are usually considered as membranous vesicular bodies with a diameter of 30 ~ 150 nm released to outside the cell through the fusion of multivesicles and cell membrane [3]. It was found that exosomes exist in a variety of human fluids, such as serum (plasma), saliva, urine, amniotic fluid, milk, etc. [4].

Exosomes contain abundant bioactive molecules, such as protein, mRNA, lncRNA and lipids, which participate in a variety of biological processes *in vivo*, such as intercellular material transport and signal transmission, angiogenesis, histone modification, immune activation / inhibition, cell growth and apoptosis. A large number of studies have shown that exosomes are closely related to the occurrence, growth, invasion, metastasis and metabolism of a variety of tumors.

lncRNAs are RNA molecules that do not encode or rarely encode proteins between 200 nt-100 kb in length [5-7]. lncRNAs play a key role in the regulation of chromatin dynamics, gene expression, cell growth and differentiation [17]. lncRNAs participate in a variety of cellular or biological processes by interacting with various biological macromolecules such as DNA, proteins and RNAs (including mRNAs,

microRNAs and other lncRNAs) [8-12]. lncRNAs have been identified to be involved in many complex cellular processes, such as cell death, growth, differentiation, apoptosis, epigenetic regulation, genomic imprinting, splicing, post transcriptional gene expression regulation, chromatin modification, inflammation, etc. [13-21]. Recent studies have shown that some lncRNAs are highly expressed in various human cancers and play an important role in tumorigenesis, apoptosis, invasion and metastasis [22-24]. In view of the high stability of exosome lncRNAs in body fluids, exosomal lncRNAs have a wide application potentiality in early cancer diagnosis.

Patients and Methods

Patients

Ninety three plasma samples were collected from the patients with early gastric cancer in the Beijing 301 Hospital from 2018 to 2019 and 49 normal control plasma samples were obtained from the Jiangsu Oilfield General Hospital. All gastric cancer patients should be: (1) cancers by pathological diagnosis belong to early stage; (2) none received any cancer treatment. The following patients were excluded: (1) patients who were suffering from other cancers at the same time; (2) patients who were suffering from functional damage to their heart, liver and kidney. Clinical information was available for all gastric cancer patients and normal controls involved in the study. The study was approved by the hospital ethics committee, and all subjects signed the informed consent form. The plasma was isolated by centrifuged at 2000g for 10 minutes within two hours after obtaining the blood samples. The separated plasma was stored in a refrigerator at - 80℃.

Isolation of exosomes

1.5 ml plasma samples were first passed through a 0.8 μm diameter filter membrane (Millipore). Then, exosomes and exosomal RNA were isolateted according to the procedure of exorneasy MIDI Kit (Qiagen). The obtained exosomes were resuspended with 40μl PBS and stored in -80 ℃ refrigerator for further analysis.

Identification of exosomes

NTA analysis

The concentration and particle size of exosomes were analyzed by Nanoparticle Tracking Analysis(NTA） with ZetaView(Particle Metrix,German).

Transmission Electron Microscopy(TEM) analysis

First the copper mesh containing 10μl freshly isolated exosome samples were placed on the filter paper. After sucking the excess samples with the filter paper, the samples were dried in the air for 5 minutes. Then 1% uranyl acetate and negative dye were added on samples and standed for 2 minutes. After sucking the excess dye and drying in the air for 40 minutes, the samples were observed under transmission electron microscope(G2 F30 S-TWIN，FEI).

Western Blot

Exosomes were lysed in standard RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). The amount of proteins was measured with a BCA protein assay kit (Beyotime, China). The protein was solubilized with loading buffer (5×) and heated at 100°C for 10 min. Proteins were separated by SDS-PAGE and then transferred to a 0.2-μm PVDF membrane (BioRad, USA). After blocking with Odyssey Blocking Buffer (Li-COR Biosciences, USA), the membrane was incubated with primary antibody (1:1000) at 4°C overnight, then incubated with secondary antibodies (1:5000, LI-COR Biosciences, USA).

High throughput sequencing of exosomal RNAs

Plasma exosomal RNAs from 5 patients with early gastric cancer and 5 normal controls were used for high-throughput RNA sequencing. Exosomal RNAs were extracted using exorneasy MIDI Kit (Qiagen) and quantified with Nanodrop. The quality of RNA was assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). High throughput RNA sequencing was completed by Baimike company (Beijing) and RNA sequencing was done using Illumina hiseq platform.

Real-time quantitative PCR

Purified RNA was reversely transcribed into cDNA using the Hiscript Q RT SuperMix. Then, qRT-PCR was performed using SYBR Green assays (Vazyme) on a

BIO-RAD CFX Connect system. The reactions were incubated at 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. All experiments were conducted in triplicate, and the products were confirmed by melting curve analysis following each reaction. The level of each candidate lncRNA was normalized to that of GAPDH. The primers used for PCR are as followings:

lncMSTRG.1319590: (forward) 5′-AGAGTCTCGTTCGTTATCG-3′ and (reverse) 5′-CGGACAGGATTGACAGATT-3′;

lncMSTRG.2312697: (forward) 5′-TCCATCCATCCATCATCTATC-3′ and (reverse) 5′-ATGCTGGATGAATGGAGAAT-3′;

lncENSG00000095932 : (forward) 5′-TCCATCCATCCATCATCCA-3′ and (reverse) 5′-AGTGGGTGAATGGGTGAG-3′;

lncENSG00000182162：（forward) 5′-ATGAATGAGTGAGTGAATGG-3′ and (reverse) 5′-CATCCATCCATCCATCCAT-3′.;

lncENSG0000014881: (forward) 5′-CACTTACACCCACCCTTAC-3′ and (reverse) 5′-GGCGTGGAGGTAGATGTA-3′;

lncENSG00000214226: (forward) 5′-CACCATCAGCACCATCAC-3′ and (reverse) 5′-TTGTGGTGGTGGTAATAGTG-3′;

GAPDH: (forward) 5′-CCGGGAAACTGTGGCGTGATGG-3′ and (reverse) 5′-AGGTGGAGGAGTGGGTGTCGCTGTT-3′.

The relative expression levels of the lncRNAs were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analyses were performed with SPSS 22.0 and GraphPad Prism 8.0 Software. The difference of the expression levels of plasma exosomal lncRNAs between GC patients and normal controls were evaluated by a Student's *t*-test or one-way ANOVA. Receiver operating characteristic curve (ROC) and area under curve (AUC) were used to estimate the diagnostic value of each index for GC. A combined ROC was calculated on the basis of the logistic regression model. $P < 0.05$ was regarded as statistically significant.

Results

Screening of differentially expressed exosomal lncRNAs in early gastric cancer by high throughput RNA sequencing assay

We extracted plasma exosomal RNA from 5 patients with early gastric cancer and 5 normal controls for high-throughput RNA sequencing assay. The sequencing results showed that 76 lncRNAs were up-regulated and 260 lncRNAs were down-regulated compared with the normal controls (fold change≥ 1.5, P<0.05). The difference of lncRNA expression levels between gastric cancer group and control group and t can be seen volcano map (Figure 1). Nine lncRNAs with the largest expression difference were selected for the further verification.

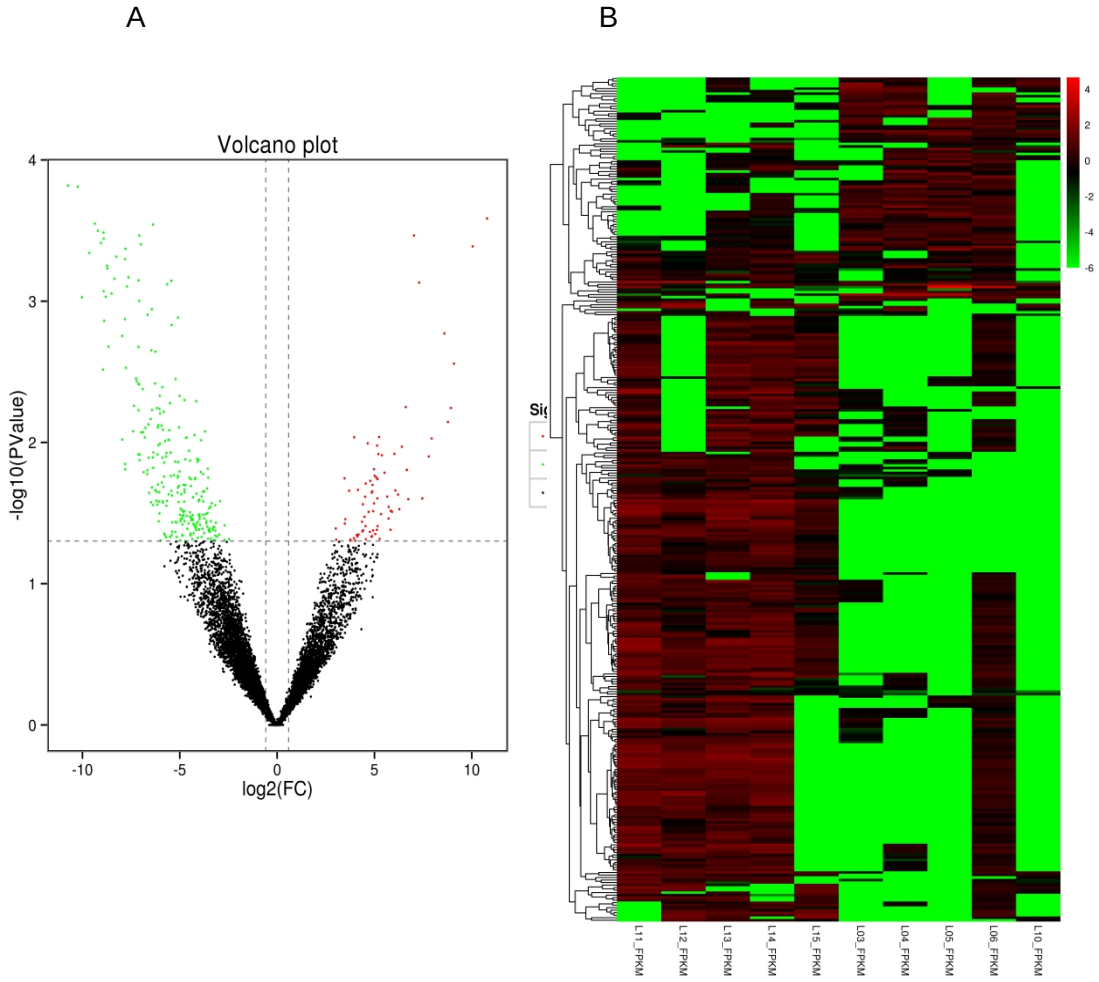
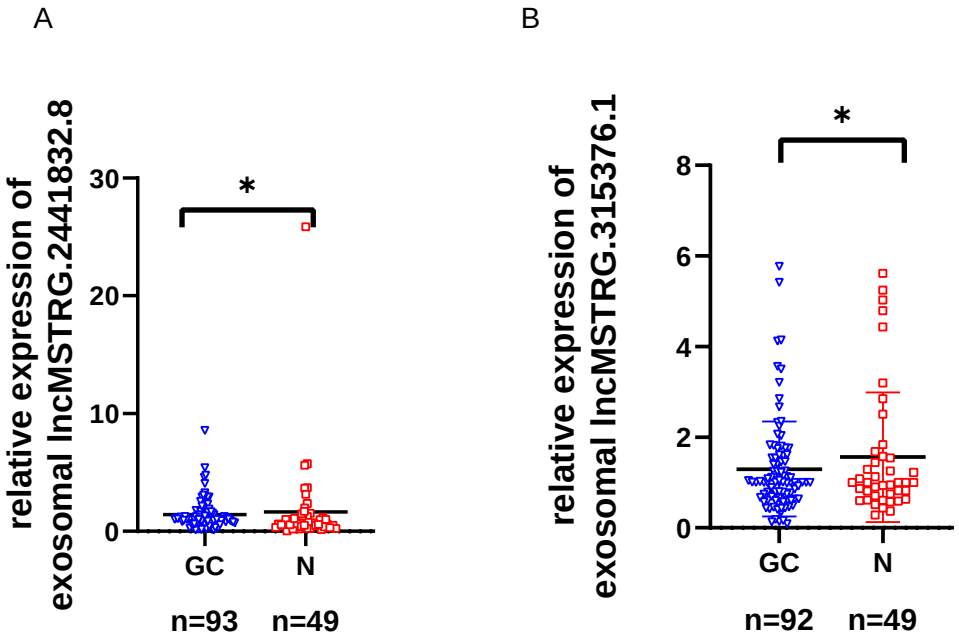


Figure 1. Screening of differentially expressed lncRNAs by RNA-seq. (A) A total of 336 differential expression lncRNAs were obtained between GC groups and normal control (B) Heatmap result of differential expression lncRNAs based on RNA-seq.

Verification of selected differentially expressed exosomal lncRNAs in plasma samples of gastric cancer patients

To verify the differential expression of exosomal lncRNAs in early gastric cancer patients，plasma exosomes and RNA were isolated from 93 patients with early gastric cancer and 49 normal controls. Seven lncRNAs with the largest differential expression were selected from 336 up-regulated lncRNAs. These lncRNAs were further confirmed by RT-qPCR (Figure 2A-F).The results indicated that 6 lncRNAs showed significant differences in the expression levels between the gastric cancer group and the control group (P<0.05), of which 2 lncRNAs (lncmstrg1319590 and lncmstrg2312697) were highly expressed and 4 were lowly expressed in the plasma exosomes of gastric cancer patients (lncmstrg 1004024.1, lncmstrg2441832.8, lncmstrg315376.1, lncmstrg907985.2).



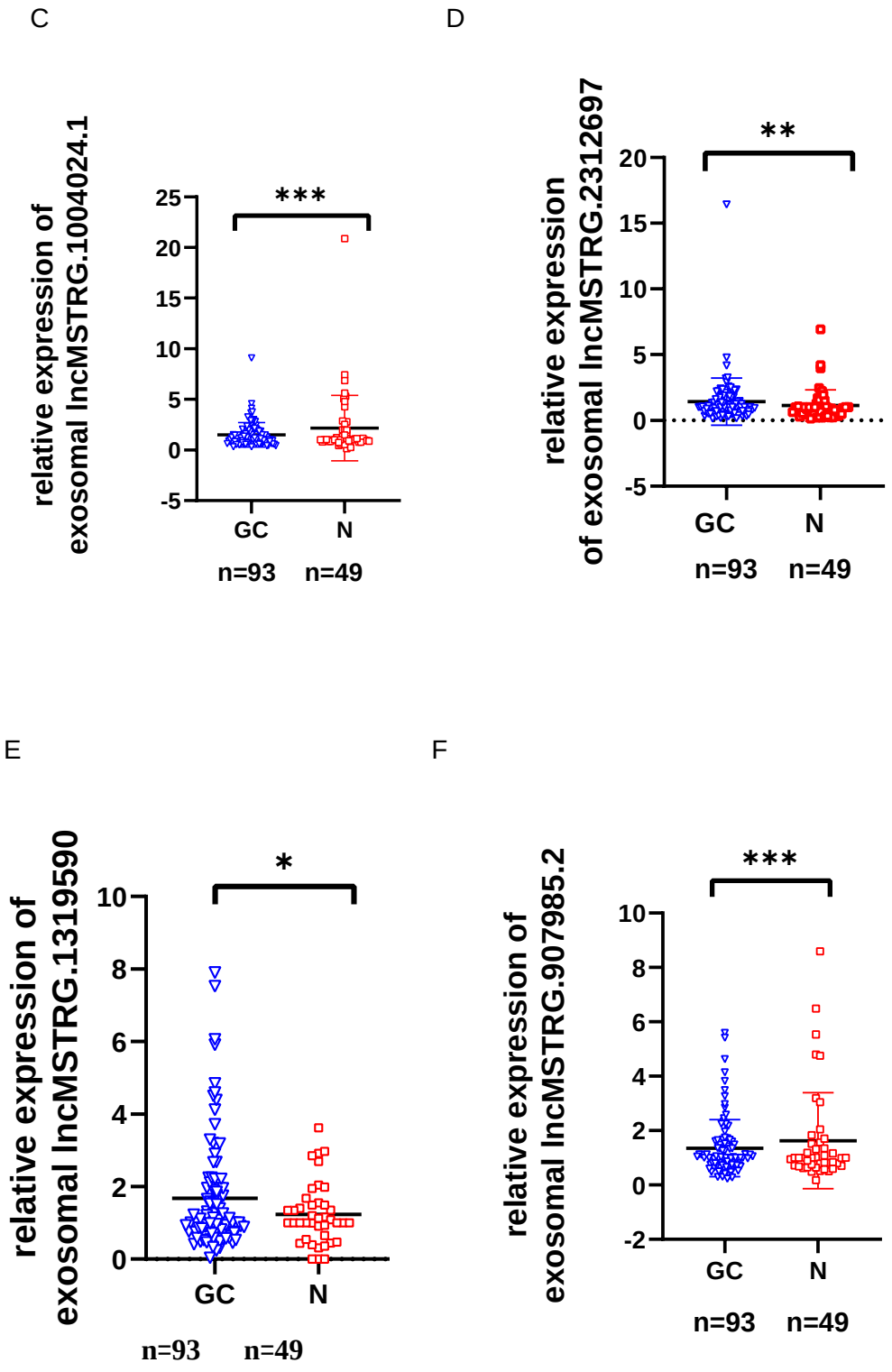


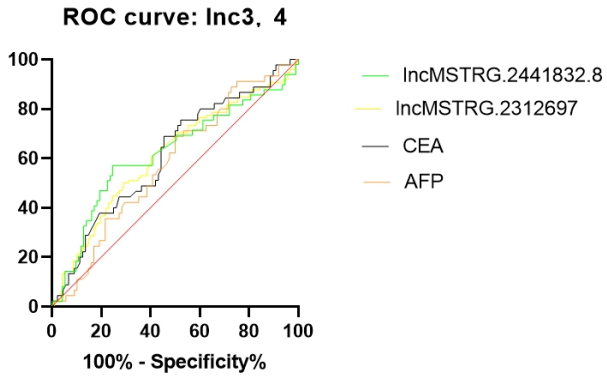
Figure 2 Relative expression levels of 6 exosomal lncRNA in GC patients and normal control differential expressed lncRNAs were validated in clinical samples. Relative expression levels of 79 exosomal lncRNAs were examined by RT-qPCR

in GC patients and normal control. (A) Expression levels of lncMSTRG.2441832.8 in GC patients and normal control. (B) Expression levels of lncMSTRG.315376.1 in GC patients and normal control. (C)Expression levels of lncMSTRG.1004024.1 in GC patients and normal control. (D)Expression levels of lncMSTRG.2312697 in GC patients and normal control. (E)Expression levels of lncMSTRG.1319590 in GC patients and normal control. (F)Expression levels of lncMSTRG.907985.2 in GC patients and normal control.

The verification of differentially expressed exosomal lncRNAs in clinical samples

Based on the six differentially expressed lncRNAs in the plasma exosomes of early gastric cancer patients with the largest difference, we used the receiver operating characteristic curve (ROC) to calculate the specificity and sensitivity of the differentially expressed lncRNAs for the early diagnosis of gastric cancer and decided whether they would have diagnostic advantage compared with the traditional tumor markers like CEA, CA199 and AFP. ROC results indicated that lncmstrg.2441832.8 and lncmstrg.2312697 had higher sensitivity and specificity for the diagnosis of gastric cancer, respectively. The Area Under Curves (AUCs) were 0.6211 and 0.631 ($P < 0.05$), while AUCs of CEA and AFP were 0.61 and 0.57, respectively. When we combined lncmstrg.2441832.8 with lncmstrg.2312697 to diagnose early gastric cancer, the AUCreached 0.73, was greater than 0.71 of CA199 (Fig. 3A-B). These results indicate that lncmstrg.2441832.8 and lncmstrg.2312697 may be used as potential biomarkers for early diagnosis of gastric cancer.

A



B

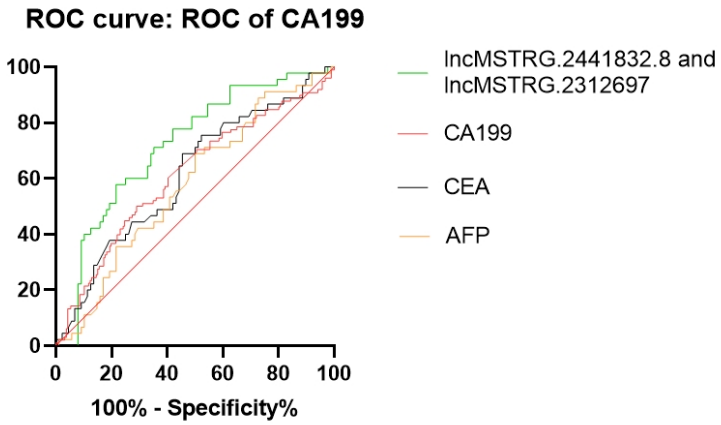


Figure 3 ROCs of plasma exosomal lncMSTRG.2441832.8， lncMSTRG.2312697 compared with traditional biomarkers CEA, AFP and CA19-9. (A) ROCs of plasma exosomal lncMSTRG.2441832.8, lncMSTRG.2312697 and plasma CEA, AFP in GC patients versus healthy controls. (B) ROCs of the combination of plasma exosomal lncMSTRG.2441832.8 with lncMSTRG.2312697, compared with plasma CEA, CA19-9, and AFP in GC patients versus healthy controls.

DISCUSSION

Gastric cancer is one of the common malignant tumors endangering human health. Because the current commonly used tumor markers can not accurately diagnose gastric cancer, especially early gastric cancer, gastric cancer is usually found in the middle and late stage, which seriously affects the treatment and prognosis of patients. The five-year survival rate is usually only 10-30%[25-26]

The rise and development of liquid biopsy technology provides a technical means

for early detection of tumor specific markers in patients' blood. Its noninvasive, sensitivity and accuracy bring new hope for early tumor diagnosis. After early detection and standardized treatment, the five-year survival rate of gastric cancer can reach more than 90%.Exosome detection is an important part of liquid biopsy technology. Exosomes are vesicle like bodies actively secreted by a variety of living cells. There are a variety of bioactive substances in exosomes, including DNA, protein, mRNA, miRNA, lnRNA, etc. Exosomes are representative of source cells and can carry specific biological macromolecules of source cells, including tumor cells, such as protein, lncRNA *etc*. It can reflect the pathophysiological state of tumor patients and the nature of primary tumors, and is expected to be used in the clinical diagnosis of malignant tumors [27].

Exosome encapsulated lncRNA has good stability in blood and is usually considered as a biomarker for early diagnosis of gastric cancer. Studies have shown that the high expression of hottip in serum exosomes is positively correlated with tumor size, pathological stage, metastasis and prognosis of patients with gastric cancer. Hottip can mediate cell proliferation by inhibiting p21 or leading to miRNA silencing. The expression of hottip in plasma exosomes of patients with gastric cancer was increased significantly, and the area under AUC curve was 0.827, which was significantly higher than clinical biomarkers such as CEA and CA199 [28].Through sequencing analysis of plasma exosomal RNA, it was found that plasma exosomal lnceegc1 was significantly up-regulated in patients with early gastric cancer, and the product under the subject curve was 0.84, which was much higher than conventional markers such as CEA [29]. In addition, linc00152, zfas1, ufc1 and hotair in plasma (serum) exosomes have also been proved to be potential biomarkers for early diagnosis of gastric cancer.

In this study, 336 differentially expressed lncRNAs were obtained by high-throughput sequencing analysis of RNA in exosomes of early gastric cancer and normal control, of which 76 were up-regulated and 269 were down regulated in gastric cancer samples.We selectedNine 9 lncRNAs with the largest differential expression changes were selected for further verification, and further obtained 6 differentially

expressed lncRNAs, including 2 with higher expression and 5 with lower expression were obtained..Through ROC analysis, we infered that lncmstrg.2441832.8 was finally obtained, and lncmstrg.2312697 maycan be used as lncRNA biomarkers for early diagnosis biomarkers of gastric cancer. To our knowledge, .At present, as far as we know, the above two potential lncRNA biomarkerss have not been reported so farfound by other studies, and their biological functions and target genes need to be further studied. Additionally, The number of gastric cancer and controls included in this study is not enough. Wwe need to increase the sample size to deeply analyze the diagnostic specificity and sensitivity of lncmstrg.2441832.8 and lncmstrg.2312697. In conclusion, we obtained the differentially expressed lncRNAs in plasma exosomes of early gastric cancer patients through high-throughput sequencing., and verified them in large-scale samples. Finally, We further obtained identify two exosomal lncRNA biomarkers that may are expected to be used as potential biomarkers for early diagnosis of gastric cancer.

Funding

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