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ORIGINAL RESEARCH

Serum Exosomal Long Noncoding RNA pcsk2-2:1 As A Potential Novel Diagnostic Biomarker For Gastric Cancer

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Chenchen Cai[1,*]
Haoliang Zhang[2,*]
Yingxing Zhu[2,*]
Peiming Zheng[3]
Yinhai Xu[2]
Jingfang Sun[2]
Miaomiao Zhang[1]
Ting Lan[1]
Bing Gu[1,2]
Shibao Li[1,2]
Ping Ma[1,2]

[1]Department of Medical Technology, Xuzhou Medical University, Xuzhou 221004, People's Republic of China; [2]Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, People's Republic of China; [3]Department of Laboratory Medicine, Henan Provincial People's Hospital, Zhengzhou 450003, People's Republic of China

*These authors contributed equally to this work

Purpose: Exosome-shuttled bioactive long non-coding RNA, as novel non-invasive biomarkers for cancer diagnosis, has received increasing attention. Here, we aimed to investigate the expression of serum exosomal long non-coding RNA pcsk2-2:1 (Exo-Lnc RNApcsk2-2:1) in patients of gastric cancer and evaluate its diagnostic value as a marker.

Patients and methods: Exosomes were isolated from serum sample of gastric cancer using HiPure Exosomekits and identified via transmission electron microscopy, Western blotting, and nanoparticle tracking analysis. The total exosomal RNA was extracted and reverse transcribed to cDNA. The expression of Exo-Lnc RNA PCSK2-2:1 was detected in serum exosomes of 29 healthy people and 63 gastric cancer patients by real-time quantitative reverse transcription PCR (qRT-PCR), and the relationship between the expression level of Exo-Lnc RNA PCSK2-2:1 and clinicopathological parameters of patients was analyzed. Finally, a receiver operating characteristic curve was used to evaluate the clinical value of Exo-Lnc RNA PCSK2-2:1 as an auxiliary diagnostic marker for gastric cancer.

Results: Transmission electron microscopy, nanoparticle size analysis, and Western blotting showed successful separation of serum exosomes. qRT-PCR results revealed that compared with the healthy control, Lnc RNA PCSK2-2:1 expression level in serum exosomes of gastric cancer patients was significantly downregulated (p=0.006). Moreover, the expression level of Exo-Lnc RNA PCSK2-2:1 was correlated with tumor size (p=0.0441), tumor stage (p=0.0061), and venous invasion (p=0.0367). The area under the curve of Exo-Lnc RNA PCSK2-2:1 was 0.896. At the optimal cut-off value, the diagnostic sensitivity and specificity were 84% and 86.5%, respectively.

Conclusion: Our data indicate that Exo-Lnc RNA PCSK2-2:1 may perform a vital role in the progression of gastric cancer and can be used as a potential marker for the diagnosis of gastric cancer.

Keywords: exosome, lncRNA, diagnose, biomarker

Introduction

Gastric cancer, a non-negligible malignant cancer, is prevalent worldwide, making it the fifth most commonly diagnosed cancer and the third leading cause of cancer death. There were more than 1 million new cases in 2018, causing approximately 783,000 deaths.[1] Clinical data show that the 5-year survival rate of patients with advanced gastric cancer is only 30% to 40%, while that of early gastric cancer is 70% to 90%.[2] Therefore, a clear early diagnosis of gastric cancer can have a positive impact on the follow-up treatment and prognosis of patients. Up to now, pathological examination is still the gold standard for the diagnosis of gastric cancer; however, disadvantage is an

Correspondence: Shibao Li; Ping Ma
Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, 99 West Huaihai Road, Xuzhou 221002, People's Republic of China
Tel +8615895212960; +8613685127799
Email sdjnshlb@126.com; pingm62@aliyun.com

invasive examination and limited tissue sample.[3] In addition, CEA, CA199 and CA724, the traditional clinical indicators, have many disadvantages in terms of lack of diagnostic sensitivity and specificity. Therefore, it is of great socioeconomic significance to find a highly sensitive and specific diagnostic marker for early gastric cancer.

Exosome, a small type of extracellular vesicle with a diameter between 30 and 120 nm and containing a variety of components, is mainly divided into three categories – protein, lipid, and nucleic acid. Due to different cell origins, the secreted exosomes are highly heterogeneous in size and composition.[4] LncRNA, a non-coding RNA of more than 200 bp in length, is aberrancy abundant in many exosomes and related to the development and progress of various tumors including bladder cancer,[5] cervical cancer,[6] lung cancer,[7] colorectal cancer,[8] and hepatocellular carcinoma,[9] especially gastric cancer.[10] For example, Li et al revealed that Long intergenic non-protein-coding RNA 152 (LINC00152) is highly expressed in serum of patients with gastric cancer.[11] Zhao et al also observed that serum exosomal Lnc RNA HOTTIP was significantly upregulated compared with the healthy control group.[12] Moreover, a recent study on the exosomal RNA profiling identified that tumor-originated exosomal LncUEGC1 can serve as a circulating biomarker for early stage gastric cancer.[13] Those data indicate that exosomal LncRNAs may serve as an auxiliary biomarker for gastric cancer.

Lnc RNApcsk2-2:1, also known as ribosomal protein S27a pseudogene 2, is located on chromosome 20 and has a length of 465 bp. Yet even now, there have been no reports on Lnc RNApcsk2-2:1 research. In this study, we aim to assess the expression of serum exosomal LncRNApcsk2-2:1 in healthy and gastric cancer patients and evaluated its prognostic value.

Materials And Methods

Specimens

A total of 63 blood specimens of patients with newly diagnosed gastric cancer were collected from the affiliated hospital of Xuzhou Medical University from April 2018 to October 2018, including 45 males with a median age of 62 years and 18 females with a median age of 65.5 years. Meanwhile, controlled blood samples also were collected from 29 healthy volunteers. Procoagulant blood collection tubes with separation gel (Jiangsu Kangjian, Jiangsu, China) were employed to collect peripheral blood samples and centrifuged (3000×g for 10 mins) within 20 mins after collection. The serum was separated into a 2 mL RNase-free centrifuge tube (Axygen, Union, CA) then stored at −80°C until RNA isolation. No radiotherapy or chemotherapy treatment was conducted in these patients. All patient specimens were confirmed by pathological examination. Tumors were staged according to the AJCC TNM staging system (8thed). This study was conducted in accordance with the Declaration of Helsinki and approved by the hospital ethics committee (XYFY2017-QL007-1).

Exosome Extraction

Serum exosomes were isolated from frozen serum specimens using HiPure Exosome kits based on the manufacturer's protocol. Briefly, the collected frozen serum samples were firstly thawed in a 25°C water bath, and then placed on ice for use. The cells and cell debris were removed by centrifugation at 2000 g for 30 mins at 4°C. The supernatant was immediately transferred to a new ribozyme-free tube with 0.5 volume of Exosome Precipitation Solution, vortexed, and mixed. After the precipitation at 2–8°C for 30 mins, the supernatant was centrifuged at 10,000 g for 10 mins at room temperature. Finally, the exosomes were obtained via discarding the supernatant.

Exosome Identification

Transmission electron microscopy was used to capture the morphological features of exosome. The isolated exosomes were resuspended in PBS, and 10 μL of the diluted mixture was adsorbed on a copper mesh for 10 mins. Subsequently, the copper mesh was transferred to a 3% glutaraldehyde droplet and suspended for 5 mins. The copper mesh was then transferred to a drop of deionized water for cleaning, 10 cycles for 2 mins each time. Finally, staining with uranyl acetate for 1 min. After drying naturally, a transmission electron microscope (Thermo scientific, New York, USA) was used to take a picture. Fresh exosome specimens were tested for nanoparticle size using nanoparticle tracking analysis (PSS, USA). Subsequently, Western blotting was used to detect exosome surface marker proteins involved TSG101 (Proteintech, Chicago, USA) and CD54 (CST, USA).

Exosomal RNA Preparation And qRT-PCR Detection

Total exosomal RNA was extracted from serum exosomes using HiPure Exosome RNA kits (Magen, Guangzhou,

China) as described in the manufacturer's protocol. RNA concentration and purity were measured by NanoDrop2000 photometer. Then, the total RNA was reverse-transcribed to cDNA according to the instructions of the reverse transcription kit. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect the level of Lnc PCSK2-2:1. The sequences of the PCR primers for β-actin were 5′-TCCTCTCCCAAGTCCACACA-3′ (forward) and 5′-GCACGAAGGCTCATCATTCA-3′ (reverse) and 5′-TGGAGGATGGATGTGCTTTGT-3′ (forward) and 5′-TTAGCACCACCACGAAGTCTC-3′ (reverse) for Lnc PCSK2-2:1. The conditions were as follows: 95°C, 10 mins for pre-denaturation, then 45 cycles at 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s. The ΔCt method was used to calculate the level of Exo-Lnc RNA PCSK2-2:1. All results were expressed as the mean ± SD of three independent experiments.

Detection Of CA724, CEA, And CA199 In Serum

In this project, CEA, CA199, and CA724 were detected by Roche COBAS E602 analysis requirements. The reference intervals of CEA, CA199, CA724 are 0–5 ng/mL, 0–35 U/mL, and 0–6.9 U/mL, respectively.

Statistical Analysis

Data are expressed as mean ± standard deviation. The unpaired *t*-test was used to investigate the significance of Exo-lncRNA PCSK2-2:1 expression whether correlated with clinicopathologic characteristics in gastric cancer. All statistical analyses were performed by Statistical Program for Social Sciences (SPSS) 22.0 software (SPSS, Chicago, IL) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). $p<0.05$ is considered as statistically significant.

Results

Identification Of Exosomes

Transmission electron microscopy showed that the vesicles isolated from the serum had a distinct bilayer membrane and a typical cup-shaped structure (Figure 1A). Subsequently, the nanoparticle tracking analysis was used to re-identify the vesicles in size. The results of particle size analysis revealed that the average diameter of the extracted serum vesicles was 59.8 nm (Figure 1B), which was consistent with the conventional size of exosomes. Finally, the exosome marker proteins TSG101 and CD54 were verified by Western blotting (Figure 1C). The above evidence indicated that serum exosomes were successfully isolated.

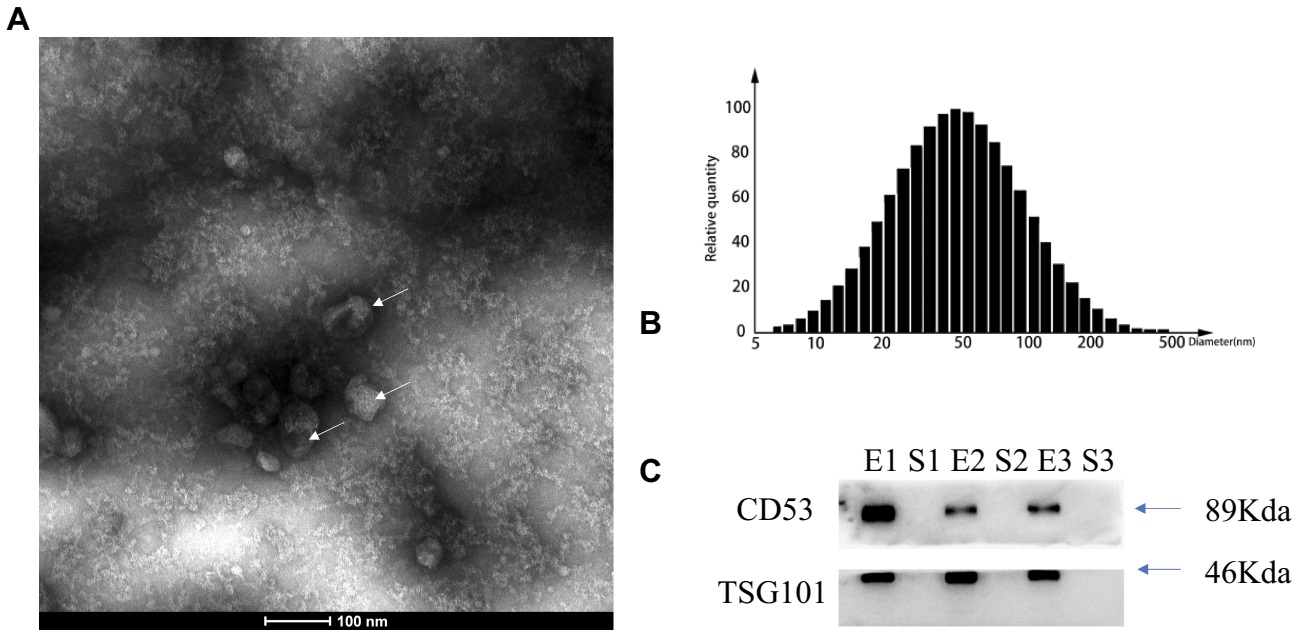

Figure 1 Identification of serum-extracted exosomes. (**A**) Transmission electron microscopy showed the morphological characteristics of exosomes.The three white arrows represent tipical exosomes in a single field of view under TEM, Scale bar = 100 nm. (**B**) Use a nanoparticle size analyzer to detect exosome size. (**C**) Verification of exosomal surface marker protein by Western blotting, E represents the exosomal sample and S is used as the negative control, representing the supernatant when the exosomes are extracted.

Expression Of Lnc RNA PCSK2-2:1 In Serum Exosomes

After more than three tests on all serum samples, it was found that the ΔCt of Lnc RNA PCSK2-2:1 in serum exosomes of healthy control was 1.85, and 3.55 in gastric cancer patients. Relative expression of Lnc RNA psck2-2:1 in serum exosomes of gastric cancer patients was significantly lower than that of healthy control (p=0.006) (Figure 2).

Association Between Exo-lncRNA psck2:2-1 Expression And Clinicopathologic Features

Since the relative expression of Lnc RNA psck2:2–1 was significantly decreased in serum exosomes of patients with gastric cancer, we further analyzed whether there was a vague correlation between the expression level of Exo-Lnc RNA psck2:2–1 and the clinicopathological parameters. No correlation was observed between the expression level of Exo-Lnc RNA psck2:2–1 and age (p=0.4501), gender (p=0.3211), and lymph node metastasis (p=0.4879). However, the expression level of Exo-Lnc RNA psck2:2–1 was correlated with tumor size (p=0.0441), tumor stage (p=0.0061), and degree of venous invasion (p=0.0367) (Table 1).

Table 1 Relationship Between Exo-lncRNA PSCK2:2–1 Expression And Clinicopathological Parameters Of Human Gastric Cancer

Characteristics	Total	Mean ± SD	P-Value
Age (years)			
<60	21	3.664 ± 0.4176	0.4501
≥60	42	3.303 ± 0.4226	
Gender			
Male	45	3.736 ± 0.3506	0.3211
Female	18	3.079 ± 0.557	
Diameter (cm)			
<5	40	3.058 ± 0.2381	0.0441
≥5	23	3.995 ± 0.4408	
Lymphatic metastasis			
Negative	37	3.726 ± 0.3506	0.4879
Positive	26	3.333 ± 0.4408	
Tumor staging			
I–II	23	3.021 ± 0.4351	0.0061
III–IV	40	4.193 ± 0.2928	
Venous invasion			
Negative	31	3.004 ± 0.3358	0.0367
Positive	32	3.761 ± 0.3827	

Evaluate Diagnostic Efficacy Via ROC Curve

Due to the differential expression of Exo-Lnc RNA psck2-2:1 in patients with gastric cancer and healthy volunteers, a receiver operating characteristic (ROC) curve was constructed. Comparison to the traditional diagnostic markers (CEA, CA724, and CA199), Exo-Lnc RNA psck2-2:1 showed significant advantages (Figure 3). The Exo-Lnc RNA psck2-2:1 level differentiated patients with gastric cancer from healthy volunteers, the area under the curve (AUC) was 0.896. The optimal cutoff value was 2.39, with a sensitivity of 84% and a specificity of 86.5%. However, the diagnostic efficacy of traditional markers for gastric cancer is not satisfactory, as shown in Table 2. Among them, CA724 is the best diagnostic indicator for gastric cancer, but its diagnostic sensitivity is only 56%.

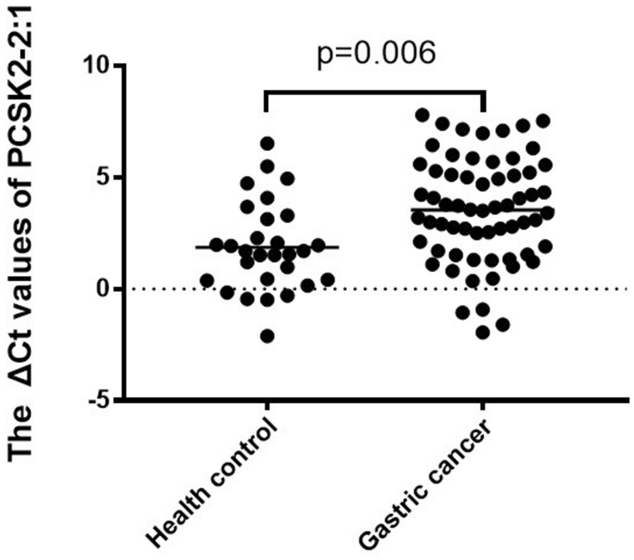

Figure 2 qRT-PCR was used to detect the expression levels of Exo-LncRNA psck2-2:1 in patients with gastric cancer group (n=63) and healthy individuals (n=29), p< 0.05.

Discussion

In this study, we evaluated the expression of serum Exo-Lnc RNA PCSK2-2:1 and its clinical relevance in healthy and gastric cancer patients. We observed that serum exosomes-encapsulated Lnc RNA psck2-2:1 was differentially expressed in healthy and gastric cancer patients (p=0.006), and its expression level was correlated with tumor size (p=0.0441), tumor stage (p=0.0061), and venous invasion (p=0.0367). It is particularly noteworthy that Exo-Lnc RNA psck2-2:1 is very relevant to tumor staging, suggesting it may be involved in the adjuvant diagnosis of early gastric cancer. Using ROC curve to evaluate the diagnostic

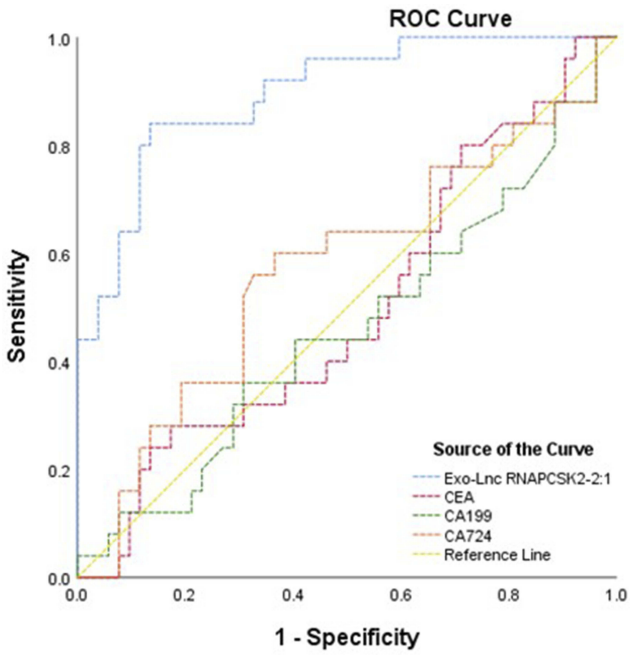

Figure 3 ROC curve was used to evaluate Exo-Lnc RNA psck2-2:1 diagnostic performance.

efficacy, compared to traditional markers, Exo-Lnc RNA psck2-2:1 has higher diagnostic efficiency, which is certainly a gratifying discovery.

According to the literature, more than 50% of the researchers used ultracentrifugation to separate exosomes.[14] The advantage of ultracentrifugation for extracting exosomes is that it is not easy contaminated, but the disadvantages of this method are also obvious, not only time-consuming, but the structure and function damaging, which is not conducive to downstream experimental analysis. For our study, a commercial kit for exosomal extraction was selected, which is simple and time-saving, and does not require special equipment. The results showed successful separation of serum exosomes, which laid the foundation for subsequent experiments.

The early accurate diagnosis of gastric cancer has great socioeconomic significance. Blood tests have unique advantages compared with other invasive methods, easy to obtain, and also can be used for real-time monitoring and even prognosis diagnosis. Exosomes, due to their special structure, can protect nucleic acids and prevent their rapid degradation. The formation of exosomes is closely related to the state of progenitor cells, and the detection of exosome contents is more specific than traditional tumor markers. For example, Cheng et al pointed out that low long noncoding RNA growth arrest-specific transcript 5 expression in the exosomes of lung cancer cells promotes tumor angiogenesis.[15] Wang et al found that there was a positive correlation between the expression of long non-coding RNA HOTAIR in serum exosomes and breast cancer tissues with positive HER2.[16] Li et al also observed that the expression of LncRNA GAS5 in serum exosomes of non-small cell lung cancer was low, but combined with CEA for diagnosis, the AUC was as high as 0.929.[17] Similarly, to previous studies, Exo-Lnc RNA psck2-2:1 is significantly low in serum of patients with gastric cancer and has a certain diagnostic value. The reason why Lnc RNA psck2-2:1 is low in the exosomes of gastric cancer patients needs to be further investigated. Barbagallo et al found that LncRNA UCA1 is highly expressed in tumor tissues but is lower in plasma exosomes. It is possible that LncRNA UCA1 plays an important role in the development of colorectal cancer. Tumor cells restrict their secretion, resulting in differential expression of tissues and serum exosomes.[18] These observations will facilitate further investigation on the possible causes of low expression of Lnc RNA psck2-2:1 in gastric cancer.

Table 2 Sensitivity, Specificity, Accuracy, And AUC Of Exo-Lnc RNA PCSK2-2:1, CEA, CA199, and CA724 In The Diagnosis Of Gastric Cancer

	Sensitivity	Specificity	Accuracy	AUC
Exo-Lnc RNA PCSK2-2:1	0.84(53/63)	0.865(25/29)	0.848	0.896
CEA	0.30(19/63)	0.827(24/29)	0.467	0.498
CA199	0.52(33/63)	0.620(18/29)	0.554	0.541
CA724	0.56(35/63)	0.655(19/29)	0.587	0.570

Despite the aforementioned advantages of our study, it is undeniable that this study has some shortcomings. First of all, the number of samples collected in this study is not sufficient, and more samples are needed to comprehensively evaluate the diagnostic efficacy of Exo-Lnc RNA psck2-2:1. Secondly, when it comes to grouping, benign stomach problems and healthy people are not well distinguished. Finally, due to the difficulty of follow-up studies, relevant data of survival analysis were lacking in this study.

In summary, our data confirmed that the expression of serum Exo-Lnc RNA PCSK2-2:1 was significantly down-regulated in patients with gastric cancer, which indicates that Exo-Lnc RNA PCSK2-2:1 has great potential as an auxiliary diagnostic marker for gastric cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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