Circulating Long RNAs in Serum Extracellular Vesicles: Their Characterization and Potential Application as Biomarkers for Diagnosis of Colorectal Cancer

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Abstract

Background: Long noncoding RNA (lncRNA) and mRNAs are long RNAs (≥200 nucleotides) compared with miRNAs. In blood, long RNAs may be protected by serum extracellular vesicles, such as apoptotic bodies (AB), microvesicles (MV), and exosomes (EXO). They are potential biomarkers for identifying cancer.

Methods: Sera from 76 preoperative colorectal cancer patients, 76 age- and sex-matched healthy subjects, and 20 colorectal adenoma patients without colorectal cancer were collected. We investigated the distribution of long RNAs into the three vesicles. Seventy-nine cancer-related long RNAs were chosen and detected among three subtypes of extracellular vesicles in serum. Most mRNAs and lncRNAs in serum could be used as biomarkers to detect colorectal cancer.

Results: The quantity of long RNA has varying distribution among three subtypes of extracellular vesicles in serum. Most mRNA and lncRNA genes had higher quantity in EXOs than that in ABs and MVs, whereas MVs contain lowest quantity. We investigated 79 long RNAs chosen from The Cancer Genome Atlas and the LncRNA_Disease database in the sera of healthy patients, and those with colorectal cancer. In the training and test sets, the AUCs were 0.936 and 0.877, respectively. The AUC of total serum RNA was lower (0.857) than that of exosomal RNA in the same samples (0.936).

Conclusion: The present study shows that exosomal mRNAs and lncRNAs in serum could be used as biomarkers to detect colorectal cancer.

Impact: Among three types of vesicles in sera, EXOs were the richest reservoir for almost all measured long RNAs. The combination of two mRNAs, KRTAP5-4 and MAGEA3, and one lncRNA, BCAR4, could be potential candidates to detect colorectal cancer.

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Introduction

Cancer has become the leading cause of morbidity and mortality globally. It is estimated that the death toll of cancer will continue to increase in the coming decades. Lack of effective early diagnosis, efficient monitoring, and cancer management are among the main drivers of increased cancer deaths (1). For example, colorectal cancer is the third most common cancer and the third leading cause of cancer-related death in men and women in the United States. Every year, it is estimated that more than 130,000 people will be diagnosed with colorectal cancer, and about 50,000 people will die of the disease (2). Minimally invasive and clinically validated biomarkers that can detect cancer at an early stage and be able to monitor its progression are urgently needed.

RNA plays an important role in carcinogenesis. There are various types of RNA in the human body. Messenger RNA (mRNA, average length 1,000–1,500), microRNA (miRNA, ≤200 nucleotides), and long noncoding RNA (lncRNAs, ≥200 nucleotides) are closely studied due to their association with cancer. mRNAs, such as adenosomatous polyposis coli (APC), and Kirsten rat sarcoma viral oncogene homolog (KRAS) can directly participate in the development of colorectal cancer as tumor suppressor genes and oncogenes (3). As non-coding RNAs, miRNA, and lncRNA can indirectly result in up- or downregulation of targeted mRNAs specific to tumor promoters or inhibitor genes, nearly 100 dysregulated miRNAs have been identified in relation to colorectal cancer, and each is able to affect the expression of more than one targeted mRNA (4). IncRNAs affect cancer through various mechanisms, such as chromatin remodeling, chromatin interaction, competing endogenous RNAs, and natural antisense transcripts. More than a dozen IncRNAs are associated with colorectal cancer (5). The number of colorectal cancer-related lncRNAs is expected to rise as research progresses.

Circulating nucleic acids (CNA) are novel sources used to hunt cancer biomarkers (6, 7). Compared with DNA, RNA directly represents the expression level of certain genes, which might
significantly differ between patients with cancer and their healthy counterparts (8–10). Here, we define mRNA and IncRNA as long RNA. We, and other researchers, have demonstrated that some circulating long RNAs are stable in blood and have diagnostic potential in cancer management (10–15). The diagnostic value of circulating miRNAs has been intensely investigated (16–19). For example, telomerase RNA found in serum extract of breast cancer patients is undetectable in normal subjects (20). Five cancer-related mRNA diagnostic models were validated by qPCR of serum from oral squamous cell carcinoma patients (21). Circulating IncRNA urothelial cancer associated 1 (UCA1 or CIHDR), long stress-induced noncoding transcript 5 (LSINCT-5), phosphatase and tensin homolog pseudogene 1 (PTENP1) and H19 in gastric cancer (10, 22), metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in prostate cancer (23), and hepatocellular carcinoma upregulated IncRNA (HULC) in liver cancer (24) were found to show diagnostic value.

The mechanism of how these circulating RNAs maintain their stability in RNase-rich blood is not clearly understood. They may be protected by extracellular vesicles (19, 25). However, the distribution of long RNA in these vesicles is unknown.

Some studies have focused on identifying circulating long RNAs as cancer biomarkers in serum or plasma. Circulating mRNAs were reported as effective cancer biomarkers in patients with oral cancer and breast cancer (20, 21). However, circulating IncRNAs were reported to be effective biomarkers for patients with gastric, hepatocellular, and prostatic cancer (10, 14, 22, 23). Due to the variability in methodology in these studies, the results of these findings are inconsistent. Further, some techniques even fail to isolate circulating IncRNA from plasma (26). Therefore, in order to find an optimal method to evaluate the potential utility of circulating long RNAs, more research is needed.

In our study, we investigated the distribution of selected mRNAs and IncRNAs in different vesicles in serum. Focusing on exosomes (EXO), which contain the highest quantities of mRNA and IncRNA, we then assessed the diagnostic values of the selected molecules. Through our work, we seek to provide improved diagnostic tools to patients with colorectal cancer.

Materials and Methods

Patient samples and study design

Sera from 76 preoperative colorectal cancer patients, 76 age- and sex-matched healthy subjects, and 20 colorectal adenoma patients without colorectal cancer were collected at the tissue bank of Fudan University Shanghai Cancer Center from 2013 to 2014 (FUSCC). The diagnosis of colorectal cancer was histopathologically confirmed. Tumors were staged according to the tumor–node–metastasis staging system of the American Joint Committee on Cancer (7th edition). Histologic grade was assessed according to the World Health Organization criteria (27).

The study protocol was approved by Ethics Committee of Fudan University Shanghai Cancer Center and conducted in accordance with the tenets of the Declaration of Helsinki. Prior to surgery, 500 μL to 1 mL of whole blood was collected from each subject. The blood samples were centrifuged at 2,000 × g for 10 minutes to extract blood cells from serum. The sera samples were stored at −80°C until analysis.

Paired sera from eight colorectal cancer and eight healthy subjects were chosen to measure the total RNA and RNA in various vesicles. In this set, both total serum RNA and EXO RNA were extracted. Paired sera from 30 colorectal cancer patients and 30 healthy subjects were designated as the training set. Thirty paired sera samples from colorectal cancer and healthy subjects and 20 sera from colorectal adenomas were designated as the test set (Fig. 1).

Biomarker genes selection

We used The Cancer Genome Atlas (TCGA) to select candidate mRNAs for colorectal cancer diagnosis. Normalized RNA sequencing (RNA-Seq) data of colon adenocarcinoma gene
expression [COAD (IlluminaHiSeq)] were downloaded from the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/). Differential analysis of RNA expression in tissue with and without colon cancer was performed based on value of reads per kilobase per million reads. The analysis included exon expression profiles (the illumina Genome Analyzer RNA Sequencing platform) and the respective clinicopathologic information of 329 cases of colon cancer. There are 20,530 genes in total. The version of the data is 2015-02-24, and Dataset Id is TCGA_COAD_exp_HiSeqV2. Forty-one of these 329 cases had cancerous tissue and cancerous adjacent tissue. For these 41 paired samples, the gene expression averages were calculated for each incidence of cancer and adjacent tissue cancer. The 40 genes with the highest ratio of cancer to noncancer were chosen.

Cancer-related lncRNAs were chosen from the LncRNA Disease database (http://www.cuilab.cn/lncrnadisease) with "cancer" as the search term. Then, we checked the results of gained lncRNAs with refseq database of the National Center for Biotechnology Information, and 39 cancer-related lncRNAs were chosen for this study.

RNA extraction, reverse transcription, and qPCR
To investigate the distribution of long RNAs in serum, we isolated different serum vesicles according to size. There are three main diameter class sizes of vesicles in serum: apoptotic bodies (ABs, 800–5,000 nm), microvesicles (MVs, 50–1,000 nm), and EXOs (40–100 nm).

For isolation of AB vesicles, serum samples were thawed at 4°C. Five hundred microliters of each sample was centrifuged at 2,000 × g for 30 minutes, the supernatants was removed, and the AB-containing pellets were washed off and diluted by 250 μL RNase-free water.

To isolate the MV vesicles, 40 μL of serum was added into Trizol LS and extracted along with endogenous genes.

An external reference control, Luciferase mRNA (Promega) was added into Trizol LS and extracted along with endogenous genes. The total volume of each sample in the training set was more than 1,000 μL. Each sample was divided into two portions, each with 500 μL. One portion of serum was used for total RNA extraction; the other portion was for EXO RNA extraction. RNA was isolated from vesicles or whole serum using TRizol LS reagent (Ambion) according to the manufacturer’s protocol. When the aqueous phase was separated during the RNA isolation procedure, coprecipitant GlycoBlue (Ambion) was applied in order to visualize the centrifuged pellet.

Reverse transcription (RT) and qPCR kits were used to evaluate the expression levels of the selected mRNAs and lncRNAs. RT reactions were performed using the PrimeScript RT reagent Kit (Takara) incubated for 15 minutes at 37°C and 5 seconds at 85°C. For real-time PCR, 1 μL of diluted RT product was mixed with 10 μL of 2 × SYBR Premix Ex TaqTM (Takara), 0.6 μL of gene-specific forward and reverse primers (10 μmol/L), and 8.4 μL of nuclelease-free water in a final volume of 20 μL. The primers used in this study are listed in Supplementary Table S1. All reactions were performed using a LightCycler 480 II (Roche) with the following conditions: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. Samples were analyzed in triplicate and included no-template controls. Amplification of the appropriate product was evaluated by melting curve analysis following amplification. The relative expression of each mRNA or lncRNA was calculated using the comparative cycle threshold (CT) (2−ΔΔCT) method with L13 as the endogenous control for data normalization.

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the ΔCT of the test samples. The fold change of the mRNA or lncRNA was calculated using the equation $2^{-\Delta\Delta CT}$.

The evaluation of serous vesicles

Slides were made of the pellet of AB vesicles to observe and measure the sizes and shapes of big vesicles. The slides were fixed by 95% ethanol and stained using 0.5% crystal violet (Shanghai Yeasen Biotechnology Co. Ltd). The diameters of vesicles were calculated using Image-Pro Plus (Media Cybernetics, Inc.).

The MV and EXO vesicular pellets were fixed at 4°C overnight using 4% paraformaldehyde in 0.01 mol/L phosphate buffer pH 7.4. The samples underwent a secondary fix in 1% OsO₄ for 30 minutes. After rinsing with distilled water, the pellets were dehydrated in graded ethanol, stained with 1% uranyl-acetate in 50% ethanol for 30 minutes, and embedded in Taab 812. After overnight polymerization at 60°C and sectioning for transmission electron microscopy (TEM), the ultrathin sections were analyzed with a Hitachi 7100 electron microscope.

The three types of EV, including AB, MV, and EXO, were analyzed using a Nanoparticle Tracking Analyzer (NTA) NS-300 with red laser (638 nm; 40 mW; Nanosight Technology) and Merlin F-033B ASG-camera (Allied Vision Technologies GmbH) to provide data on size distribution and EV concentration. All the samples were diluted 1:100 to 1:1,000 in Deionized Sterile Water before analysis. Triplicate measurements were performed for each sample in which the analysis settings remained unaltered within experiments. Data analysis was performed with NTA 2.3 software (Nanosight Technology). In all cases, the median particle size, SD, size distribution, and particle concentration values were obtained.

Statistical analysis

The Student $t$ test was used to evaluate differences in the expression of the chosen mRNAs or lncRNAs in serum from colorectal cancer patients and healthy subjects. When colorectal cancer, colorectal adenoma, and healthy were compared, one-way ANOVA was used. Bivariate regression analysis was applied to determine the best combination of the selected long RNAs for cancer prediction. ROC curves were constructed, and the AUC was calculated to evaluate the specificity and sensitivity of predicting colorectal cancer patients and healthy controls. We applied 0.632+ bootstrap method to adjust for overfitting of the apparent misclassification error and overestimation of AUCs by the unadjusted estimate (29). The R-package "ROC632" was applied to perform bootstrap analyses (30). The number of bootstrap iterations was set as 100.

All statistical tests were two-sided, and a probability level of $P < 0.05$ was considered statistically significant. Data analysis was performed using IBM SPSS 20.0 software (SPSS, Inc.).

Results

Patient characteristics

The patients’ clinical information of age, gender, grade, stage, location, and tumor size was summarized in Table 1. There was no
significant difference in the combined values of the 3 EXO long RNA of these patient characteristics (Table 1).

Size and morphology of AB, MV, and EXO

The shapes and sizes of three subgroups of extracellular vesicles were evaluated by TEM and NTA. The slides showed that Abs (1–4 μm) were oval or spherical in shape (Fig. 2A). No nucleus was observed in the vesicles in the slides, indicating these big vesicles were cell free. TEM and NTA showed the MVs at a size range of 75 to 465 nm and EXOs at a range of 45 to 205 nm (Fig. 2). MVs and EXOs were both wrinkled, with an oval or spherical shape (Fig. 2).

Evaluation of potential internal controls for the quantification of circulating mRNAs and lncRNAs

All 13 reference genes were measured in total serum RNA. We failed to detect the existence of PBGD, G6PDH, TBP, RPII, and Alb (Supplementary Table S1). Using both geNorm and NormFinder to determine stability, L13 ranked the most suitable reference gene both in whole-serum RNA and exosome RNA (Supplementary Fig. S1).

Distribution of long RNAs in AB, MV, and EXO

A total of 40 colorectal cancer–related mRNAs and 39 cancer–related lncRNAs were detected in the sera of 8 colorectal cancer patients and 8 healthy subjects. Twenty mRNAs and 24 lncRNAs were detected by RT-qPCR (Supplementary Tables S2 and S3). These long RNAs, along with 8 reference genes, were measured in Abs, MVs, and EXOs. The results of RT-qPCR showed that different kinds of RNA have different distributions among these three subtypes of extracellular vesicles (Fig. 3). The expression of most mRNAs and lncRNAs was higher in EXOs than that in Abs or MVs. Compared with MVs, AB vesicles contained higher levels of long RNA. The reference genes

![Distribution of long-RNAs in ABs, MVs, and EXOs. Twenty cancer-related mRNAs, 24 IncRNAs, and reference mRNAs were detected in Abs, MVs, and EXOs using real-time qPCR. The relative quantity of genes was normalized by treating that of EXO as 1. Then, to draw the heat map, the quantity of RNA in AB and MV of each gene was divided by their PCR quantities in EXO.](image-url)
showed higher amounts of RNA than cancer-related mRNAs and lncRNAs (Fig. 3).

Application of long RNAs in EXOs as biomarkers for diagnosis of colorectal cancer

The 20 mRNAs and 24 lncRNAs detected in sera were measured in EXOs of 8 paired samples. Of those, the quantity of 16 mRNAs and 21 lncRNAs were found to be significantly different between EXOs of healthy and colorectal cancer–positive samples (Fig. 1; Supplementary Tables S2 and S3). When the primers of these genes were designed, they were quality tested to ensure 85% to 105% efficiency. The 10 genes with the lowest $P$ values were chosen. These 10 long RNAs were measured in another 60-paired serum of healthy and colorectal cancer, which were further divided into training set and test set (30 paired for each set; Fig. 4).

Bivariate regression analysis was performed on the training set. Stepwise selection model revealed that the combination of Keratin-associated protein 5–4 (KRTAP5-4), Melanoma antigen family A3 (MAGEA3), and Breast cancer anti-estrogen resistance 4 (BCAR4) provided the greatest predictive ability, with an AUC of 0.936 [95% confidence interval (CI), 0.840–0.983; $P<0.0001$]. In the test set, these 3 long RNAs also showed predictive ability with an AUC of 0.877 (95% CI, 0.765–0.948; $P<0.0001$; Figs. 4 and 5).

Comparison of specificity and sensitivity of total serum RNA and exosome RNA for colorectal cancer prediction

The total serum RNA from samples of paired patients in the training set were isolated. KRTAP5-4, MAGEA3, and BCAR4 were measured, and their relative values were calculated using the constants gained in regression analysis. Then, ROC analysis showed the AUC of total serum RNA was 0.857 (95% CI, 0.742–0.935; $P<0.0001$; Fig. 5).
Extracellular vesicles are small, phospholipid-enclosed vesicles released by cells into their environment (24, 25). Three subgroups of extracellular vesicles can be classified according to size by diameter. These include AB (50–5,000 nm), MV (50–1,000 nm), and EXO (40–100 nm; refs. 26, 27). These three types of vesicles are not only distinguishable by size, but by their sources of origin (27, 28). ABs are produced by cells undergoing programmed cell death, MVs are vesicles directly released from cell membranes, and EXOs are intracellular in origin. All types of EVs contain DNA, RNA, lipid content, and protein which can be transferred among cells (24, 25).

Because these three types of vesicles share some physical and biochemical properties, there is no reliable procedure to effectively separate them until now. The most commonly used method for separating and accumulating vesicles is differential centrifugation (27). In our study, NTA showed that MVs and EXOs separated from sera were successfully separated based on their size parameters (Fig. 2). TEM revealed the size and shapes of ABs, MVs, and EXOs (Fig. 2). The thickness of ABs (1–4 μm) is beyond the capability of TEM. Pellet smear and crystal violet staining were performed to confirm the vesicles separated from sera were not cells with nuclei.

It is reasonable to anticipate that different types of vesicles may contain different kinds and amounts of RNAs based on their sources of origin. Using an Agilent 2100 Bioanalyzer, Crescitelli and colleagues reported that ABs, MVs, and EXOs in cell culture medium had distinct RNA profiles (24). In our study, the profiles of four groups of long RNAs in different vesicles were also distinct. Among the three types of vesicles in sera, EXOs were the richest reservoir for almost all measured long RNAs, whereas MV long RNAs were present in the lowest quantities. Due to their intercellular origin, and higher quantities of long RNA, EXOs seem to be the most promising candidate for cancer diagnosis.

Appropriate normalization of RT-PCR data to generate meaningful results is of great importance. There have been several studies (10, 16, 29), including ours (9), that measure the plasma or serum lncRNAs with normalization of endogenous reference genes. β-Actin and GAPDH have been chosen as reference genes in our study. The results of our search for stable reference genes showed that, in patients with and without colorectal cancer, structure-related genes can be detected in both serum and EXOs. In the other three groups, not all genes are found to exist in serum and EXOs. L13 was the most stable endogenous reference gene in our study.

We examined 40 mRNAs and 39 lncRNAs in serum and EXOs. Differential analysis of TCGA data showed that amounts of these RNAs in serum and EXOs were different. The ROC curves of the three long RNAs diagnostic model in exosome of the training set (A) and test set (B). The three long RNAs diagnostic model (C) in serum sample which came from the same cohort of the training set. Adjustment for overoptimism by 0.632 bootstrap method (D).
mRNAs were significantly different between adjacent normal colorectal mucosa and colorectal cancerous tissue. The lncRNAs in this study were all cancer-related. First, we found there were 20 mRNAs and 24 lncRNAs detectable in 8 colorectal cancer sera and 8 healthy sera, and 37 (16 mRNAs and 21 lncRNAs) of them showed significantly different quantity between colorectal cancer group and healthy group. Of these 37 long RNAs, 10 with lowest P value were chosen to be tested in the training set (Supplementary Tables S2 and S3). The results of training set showed that the combination of 2 mRNAs, KRTAP5-4 and MAGEA3, and 1 lncRNA, BCAR4, makes best predictable ability (AUC = 0.936). In test set, they also showed promising result (AUC = 0.877). Our results showed that the quantity of these three genes in sera EXOs of patients with colorectal adenoma was significantly different from those in healthy subjects. This suggests that long RNA in EXOs could be useful in detecting cancer in precancerous or early stages of cancer.

We also compared the specificity and sensitivity of colorectal cancer prediction by total serum RNA and EXO RNA. The results showed that the AUC of total serum RNA was lower (0.857) than that of exosome RNA in the same samples (0.936). The enrichment of long RNAs in EXOs may cause this difference. It is a simpler procedure to isolate RNA from serum than from EXOs. As a result, further investigation is needed to evaluate which method is more practical.

Of the three long RNA biomarkers, MAGEA3 has been reported as a colorectal cancer–related serological biomarker (31). BCAR4 was reported to play a role in breast cancer metastasis (32). KRTAP5-4 was firstly identified in human hair roots (33). Although there are no current data regarding the relation of KRTAP5-4 to cancer, our preliminary qPCR data showed that KRTAP5-4 is expressed in cancerous tissue in the stomach, colon, and liver (raw data not shown).

With the exception of individual genes, we did not expect to see lower quantities of nearly all long RNAs in cancer-positive sera as compared with healthy sera. The mRNAs were chosen from the highest ratio (cancer to healthy) genes in the TCGA database. It seems that these genes also should exist in higher amounts in cancer-positive serum. In our previous study, we also observed this phenomenon in patients with gastric cancer (10). A possible mechanism is the selective releasing of exosomal RNA. It has been reported that exosomes of more aggressive bladder cancer cell lines can release tumor-suppressor miRNA in order to more efficiently invades tissues and metastasize (34). This report indicates that cancer cells are able to prevent cancer-related genes from secreting in order to keep their invasives or metastatic ability. It is therefore possible that certain cancer-related genes may be highly expressed in cancerous tissue, but exist in lower levels in serum.

In summary, our results support evidence for the important role of exosomes in the serum long RNAs and may be valuable as the sources of cancer-detecting biomarkers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Dong, W. Lin, P. Qi, X. Zhou, X. Du
Development of methodology: L. Dong, W. Lin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Lin, P. Qi, X. Wu, D. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Dong, W. Lin, M.-d. Xu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ni, D. Huang, W.-w. Weng, C. Tan
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