Research Article

Cancer Epidemiology, Biomarkers & Prevention

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

Lei Dong[1,2,3], Wanrun Lin[1,2,3], Peng Qi[1,2,3], Mi-die Xu[1,2,3], Xiaoben Wu[4], Shujuan Ni[1,2,3], Dan Huang[1,2,3], Wei-wei Weng[1,2,3], Cong Tan[1,2,3], Weiqi Sheng[1,2,3], Xiaoyan Zhou[1,2,3], and Xiang Du[1,2,3]

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 using qPCR.

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 LncRNADisease 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.
Cancer Epidemiol Biomarkers Prev; 25(7); 1158–66. ©2016 AACR.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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 adenomatous 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 noncoding 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). lncRNAs affect cancer through various mechanism, such as chromatin remodeling, chromatin interaction, competing endogenous RNAs, and natural antisense transcripts. More than a dozen lncRNAs 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

[1]Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai, China. [2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China. [3]Institute of Pathology, Fudan University, Shanghai, China. [4]Clinical Laboratory, Shandong Provincial Hospital, Jinan, Shandong, China.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

L. Dong and W. Lin contributed equally to this article.

Corresponding Author: Xiang Du, Fudan University Shanghai Cancer Center, 270 Dongan Road, Xuhui District, Shanghai 200032, China. Phone: 086-21-64175590; Fax: 086-21-64174774; E-mail: dx2008cn@163.com

doi: 10.1158/1055-9965.EPI-16-0006

©2016 American Association for Cancer Research.

AACR

significantly differ between patients with cancer and their healthy counterparts (8–10). Here, we define mRNA and lncRNA 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 lncRNA urothelial cancer associated 1 (UCA1 or CUDR), 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 lncRNA (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), whereas circulating lncRNAs 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 lncRNA 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 lncRNAs in different vesicles in serum. Focusing on exosomes (EXO), which contain the highest quantities of mRNA and lncRNA, 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

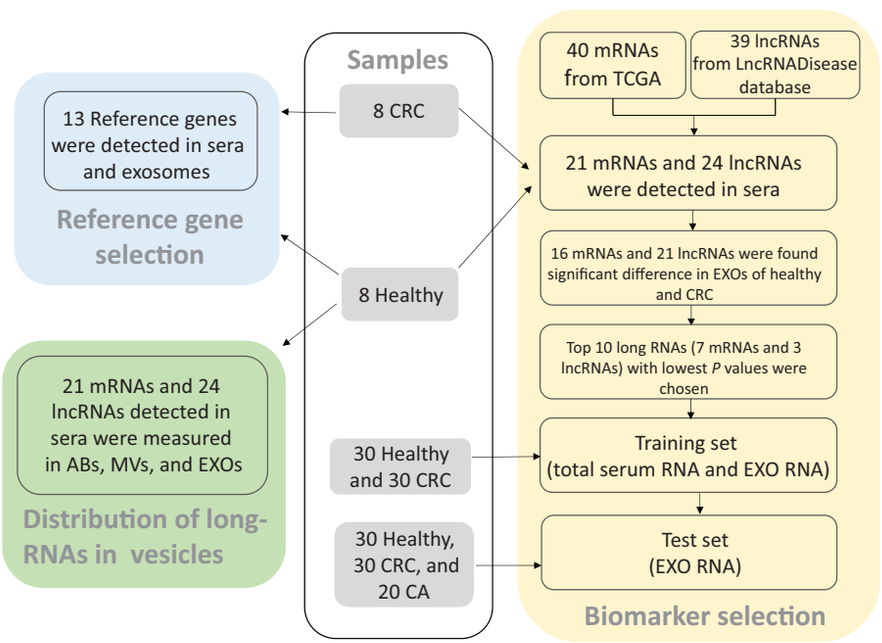

Figure 1.
The work flow chart of selecting colorectal cancer biomarkers from exosome long RNA. CRC, colorectal cancer; CA, colorectal adenoma.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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 LncRNADisease 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 microliter 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, the supernatants obtained from AB isolation were centrifuged at 12,000 × *g* for 60 minutes. They were then transferred to another tube, and the pellet was diluted by 250 μL RNase-free water.

To isolate the EXO vesicles, the supernatants obtained from MV isolation were filtered through a 0.2 μm pore filter (syringe filter; Life Science). The filtered liquid was diluted in 6 mL of 1× RNase-free water to fill the ultracentrifuge tubes (Beckman Coulter), and then centrifuged at 120,000 × *g* for 120 minutes. The pellets were washed off and diluted by 250 μL RNase-free water. All centrifugation steps were performed at 4°C.

Total serum RNA and EXO RNA were extracted from 300 μL serum of the same subject using TRIzol LS reagent (Ambion) and eluted in 20 μL of RNase-free water according to the manufacturer's instructions.

Reference gene identification

To systematically select reference genes which are applicable in total serum RNA and EXO RNA (8 paired colorectal cancer and healthy control samples), 13 common reference candidates were chosen (28). Of our reference candidates, β-actin, L13, and Tub are structure related. HPRT, PBGD, GAPDH, G6PDH, and phospholipase A2 (PLA) are metabolism related. TBP and RNA polymerase II (RPII) are transcription related, whereas albumin (Alb), β2M, and PPIA cannot be clearly put into above categories. The sequences of primers of these reference genes were listed in Supplementary Table S3. The most stable reference genes in total serum RNA and EXO RNA were determined using geNorm and NormFinder which are algorithms for reference gene selection. As 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 nuclease-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) ($2^{-\Delta\Delta CT}$) method with L13 as the endogenous control for data normalization. Samples with a CT > 35 were considered negative. ΔCT was calculated by subtracting the CT values of L13 from the CT values of the chosen mRNA or lncRNA. ΔΔCT was then calculated by subtracting the mean ΔCT of the control samples from

Table 1. Summary of clinical details of subjects used for this study and their correlations with three long RNA panels: KRTAP5-4, MAGEA3, and BCAR4

			P value	
	Training set	**Test set**	**Training**	**Test**
Age				
Mean age in years (range)	62 (45–82)	60.5 (26–83)		
Sex			0.807	0.835
Male, *n* (%)	20 (66.7%)	10 (33.3%)		
Female, *n* (%)	10 (33.3%)	20 (66.7%)		
Tumor location			0.150	0.098
Cecum	2	0		
Ascending colon	6	10		
Transverse colon	0	1		
Descending colon	3	0		
Sigmoid	2	3		
Rectum	17	16		
Tumor size			0.639	0.677
<3 cm	15	6		
≥3 cm	15	24		
Tumor grade			0.473	0.513
1	1	2		
2	26	24		
3	3	4		
Tumor stage			0.306	0.301
I	8	10		
II	7	9		
III	14	10		
IV	1	1		

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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% OsO_4 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

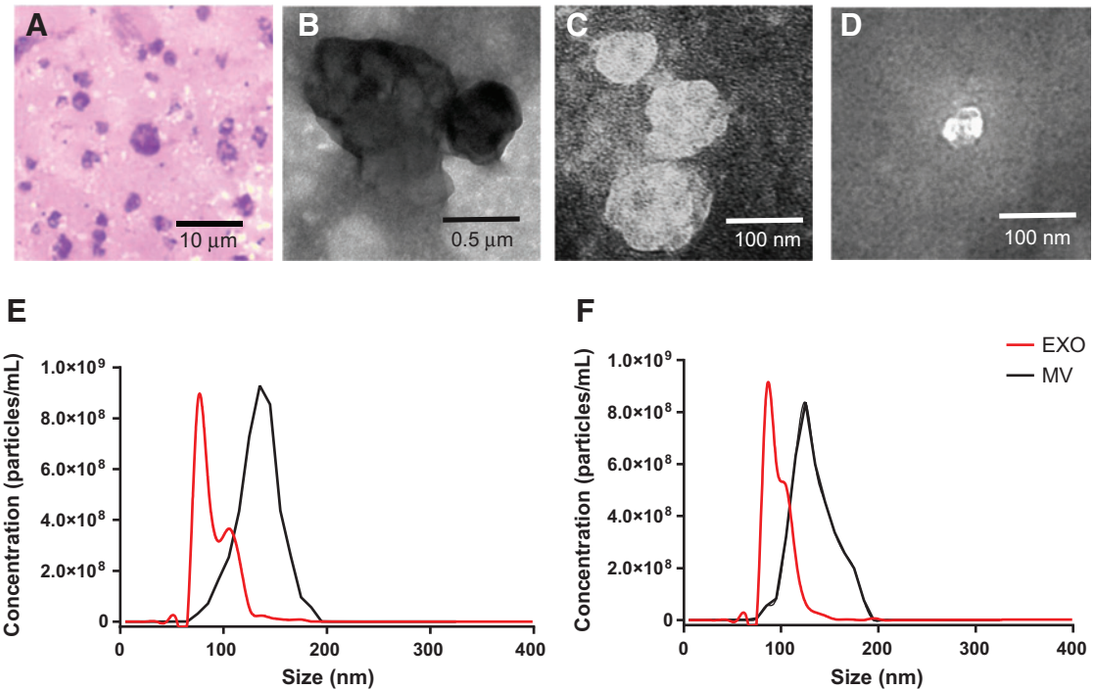

Figure 2.

Size and morphology of ABs, MVs, and EXOs. A, smear slides with crystal violet staining observed under light microscope showed the Abs to be oval or spherical. No nuclei were observed. B–D, microphotographs of TEM showed MVs and EXOs were winkled oval or spherical in shape. NanoSight particle tracking analysis showed that the MVs were in the size range of 75 to 465 nm, and EXOs were in the size range of 45–205 nm. E and F, concentration of MV and EXO vesicles (particles/mL) in healthy controls and patients with colorectal cancer.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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 winkled, 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

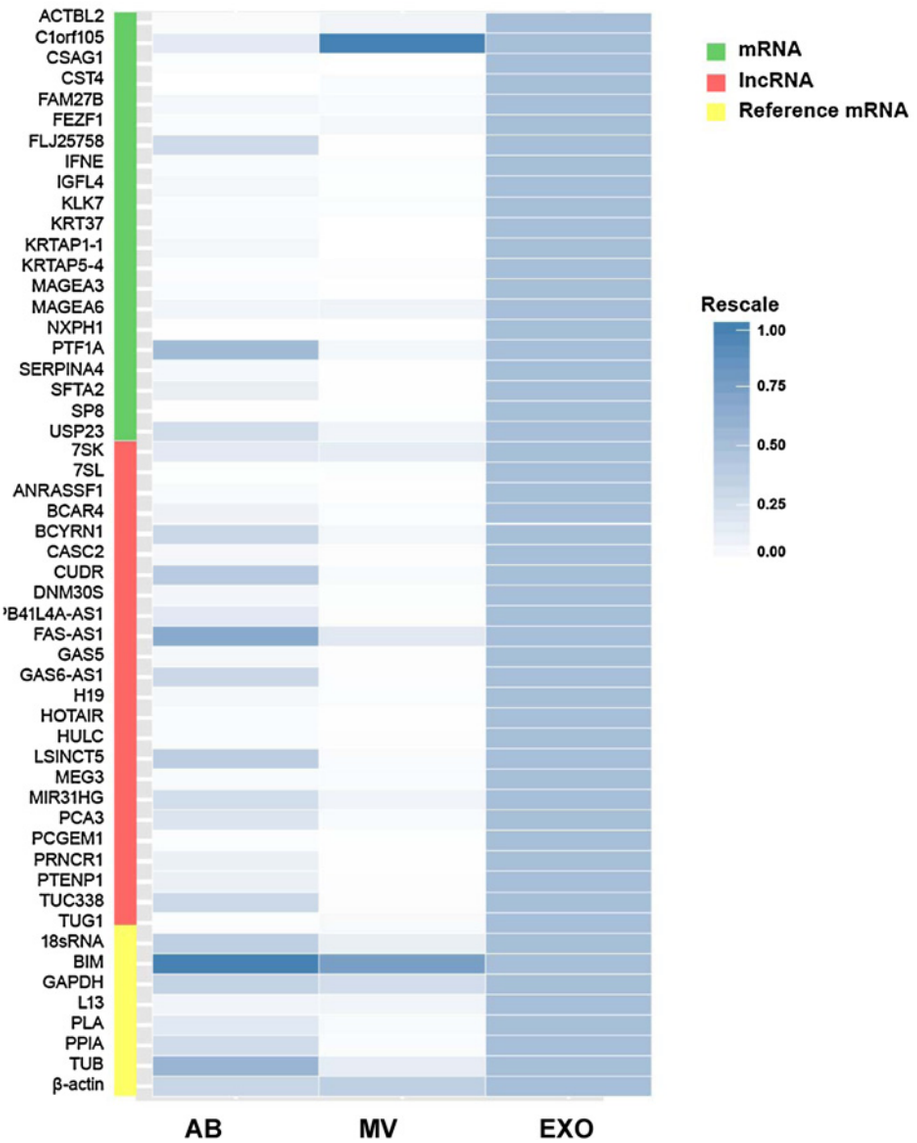

Figure 3.
Distribution of long-RNAs in ABs, MVs, and EXOs. Twenty cancer-related mRNAs, 24 lncRNAs, and reference mRNAs were detected in Abs, MVs, and EXOs using real-time qPCR. The relative quantity of genes was normalized by treating that of EXO as 1. Then, to draw the heat map, the quantity of RNA in AB and MV of each gene was divided by their PCR quantities in EXO.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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). The adjusted AUC calculated by the 0.632+ bootstrap method with 100 replicates was 0.87 (Fig. 5D).

Twenty samples of sera exosome RNA from patients with colorectal adenomas were also tested for the three genes. The quantity of KRTAP5-4 and BCAR4 in exosome RNA was significantly lower for samples with colorectal adenoma than that in controls, but was not significantly different from colorectal cancer. The quantity of MAGEA3 in exosome RNA from colorectal adenoma samples was significantly different from both colorectal cancer and healthy samples (Fig. 4). The quantity of these three long RNAs showed no statistically significant differences between male and female patients. The tumor size (<3 cm and ≥3 cm), tumor grades, and tumor stages also did not associate with the quantity of these three long RNAs (Table 1).

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).

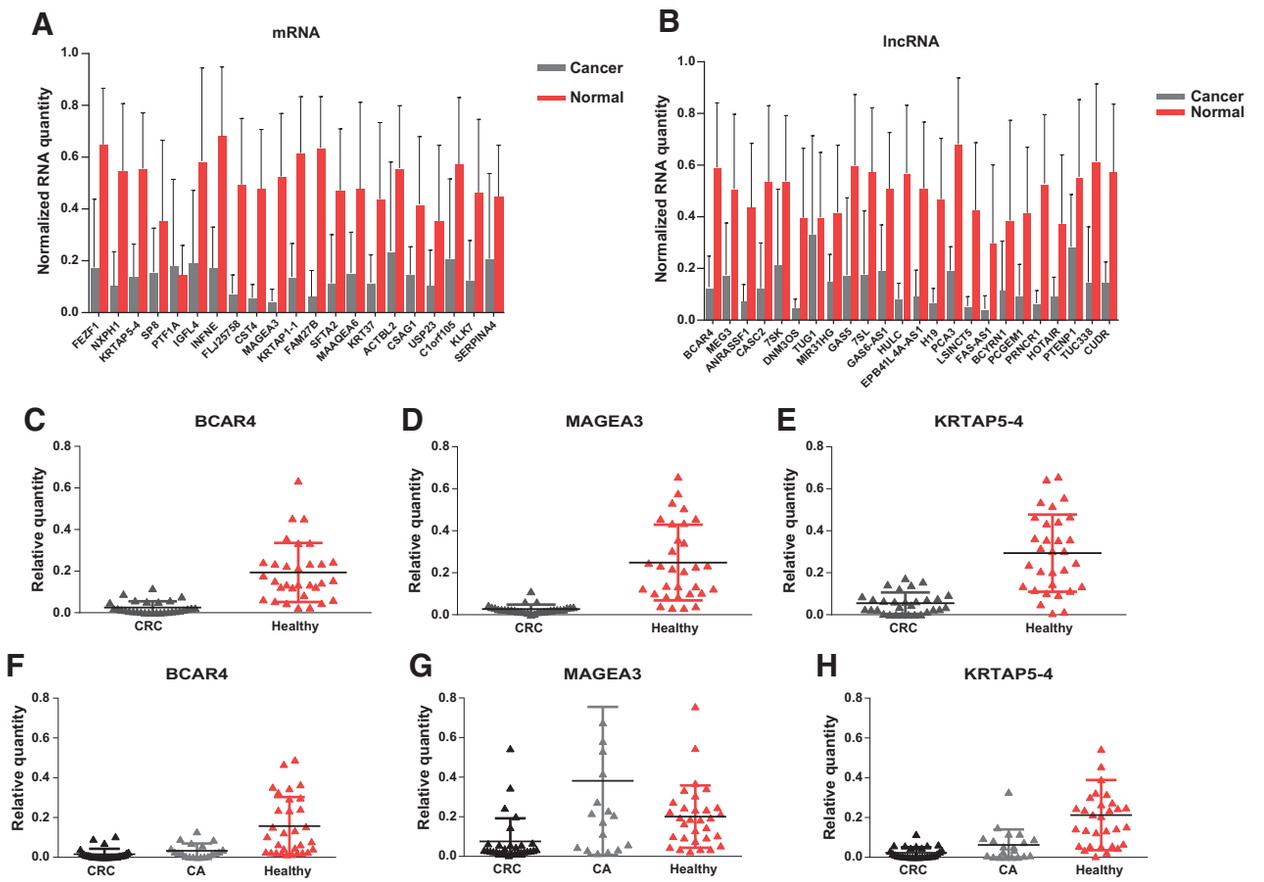

Figure 4.

Histograms of the quantity of cancer-related mRNAs and lncRNAs in sera of colorectal cancer patients and healthy subjects. Data were normalized to make all values of their quantity span from 0 to 1 (A and B). Comparison of the relative quantity of three genes, which were selected as biomarkers of colorectal cancer, in exosome RNA of colorectal cancers (CRCs), colorectal adenomas (CA), and healthy controls (C–H).

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

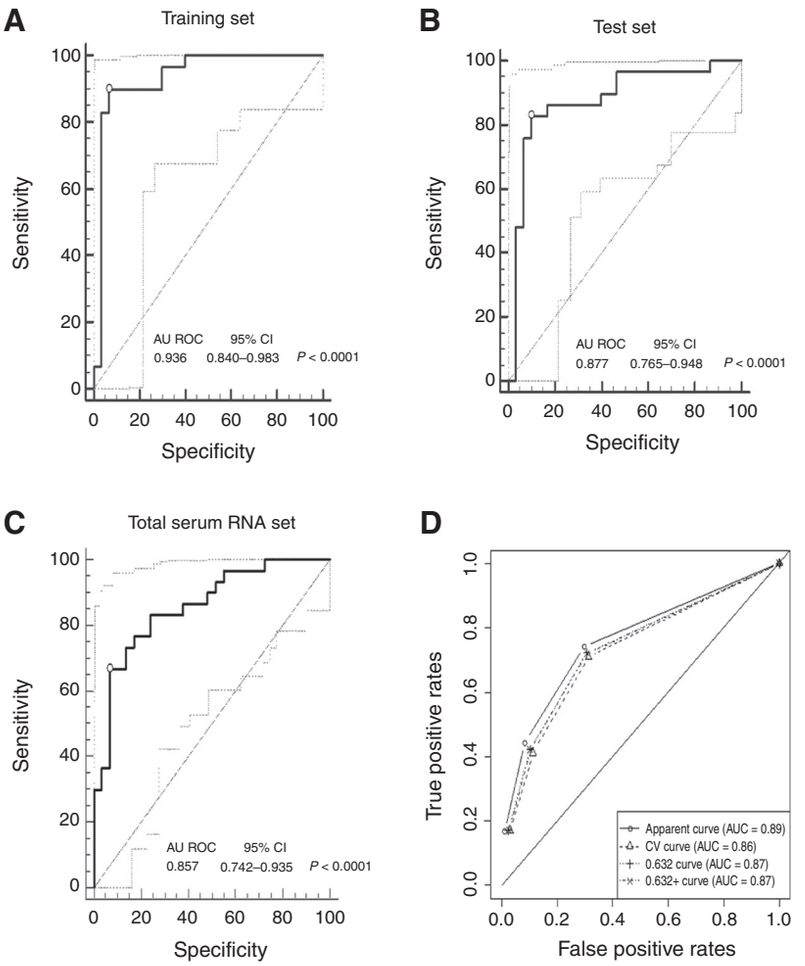

Figure 5.
ROC curves of the three long RNAs diagnostic model in exosome of the training set (A) and test set (B). The three long RNAs diagnostic model (C) in serum sample which came from the same cohort of the training set. Adjustment for overoptimism by 0.632+ bootstrap method (D).

Discussion

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 programed 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 serum RNA measurement. 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

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

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 pervious 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 invade tissues and metastasize (34). This report indicates that cancer cells are able to prevent cancer-related genes from secreting in order to keep their invasive 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. Further work needs to be done to elucidate the mechanism of the exosomal release of RNA.

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.

Authors' Contributions

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
Writing, review, and/or revision of the manuscript: L. Dong, X. Du
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ni, D. Huang, W.-w. Weng, C. Tan
Study supervision: W. Sheng, X. Zhou

Grant Support

This work was supported by National Natural Science Foundation of China (NSFC; 81071791, 81472220), which was received by Dr. Xiang Du.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 29, 2016; revised April 25, 2016; accepted April 26, 2016; published OnlineFirst May 18, 2016.

References

1. Are C, Rajaram S, Are M, Raj H, Anderson BO, Chaluvarya Swamy R, et al. A review of global cancer burden: Trends, challenges, strategies, and a role for surgeons. J Surg Oncol 2013;107:221–6.
2. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. CA Cancer J Clin 2014;64:104–17.
3. Jass JR. Colorectal cancer: A multipathway disease. Crit Rev Oncog 2006;12:273–87.
4. Orang AV, Barzegari A. MicroRNAs in colorectal cancer: From diagnosis to targeted therapy. Asian Pac J Cancer Prev 2014;15:6989–99.
5. Xu MD, Qi P, Du X. Long non-coding RNAs in colorectal cancer: Implications for pathogenesis and clinical application. Mod Pathol 2014;27: 1310–20.
6. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.
7. Bremnes RM, Sirera R, Camps C. Circulating tumour-derived DNA and RNA markers in blood: A tool for early detection, diagnostics, and follow-up? Lung Cancer 2005;49:1–12.
8. Lo YM. Circulating nucleic acids in plasma and serum: An overview. Ann N Y Acad Sci 2001;945:1–7.
9. Kopreski MS, Benko FA, Gocke CD. Circulating RNA as a tumor marker: Detection of 5T4 mRNA in breast and lung cancer patient serum. Ann N Y Acad Sci 2001;945:172–8.
10. 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;137:1128–35.
11. El-Hefnawy T, Raja S, Kelly L, Bigbee WL, Kirkwood JM, Luketich JD, et al. Characterization of amplifiable, circulating RNA in plasma and its potential as a tool for cancer diagnostics. Clin Chem 2004;50:564–73.
12. Isin M, Ozgur E, Cetin G, Erten N, Aktan M, Gezer U, et al. Investigation of circulating lncRNAs in B-cell neoplasms. Clin Chim Acta 2014;431:255–9.
13. Lorenzen JM, Schauerte C, Kielstein JT, Hubner A, Martino F, Fiedler J, et al. Circulating long noncoding RNATapSaki is a predictor of mortality in critically ill patients with acute kidney injury. Clin Chem 2015; 61:191–201.
14. Ronnau CG, Verhaegh GW, Luna-Velez MV, Schalken JA. Noncoding RNAs as novel biomarkers in prostate cancer. BioMed Res Int 2014;2014:591703.
15. Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem 2002;48:1647–53.
16. Ferracin M, Veronese A, Negrini M. Micromarkers: miRNAs in cancer diagnosis and prognosis. Expert Rev Mol Diagn 2010;10:297–308.
17. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg 2010;251:499–505.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024

18. Ma R, Jiang T, Kang X. Circulating microRNAs in cancer: Origin, function and application. J Exp Clin Cancer Res 2012;31:38.
19. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513–8.
20. Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, et al. Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin Cancer Res 2000;6:3823–6.
21. Li Y, Elashoff D, Oh M, Sinha U, St John MA, Zhou X, et al. Serum circulating human mRNA profiling and its utility for oral cancer detection. J Clin Oncol 2006;24:1754–60.
22. Zhou X, Yin C, Dang Y, Ye F, Zhang G. Identification of the long non-coding RNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer. Sci Rep 2015;5:11516.
23. Ren S, Wang F, Shen J, Sun Y, Xu W, Lu J, et al. Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. Eur J Cancer 2013;49:2949–59.
24. Xie H, Ma H, Zhou D. Plasma HULC as a promising novel biomarker for the detection of hepatocellular carcinoma. BioMed Res Int 2013;2013:136106.
25. Revenfeld AL, Baek R, Nielsen MH, Stensballe A, Varming K, Jorgensen M. Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. Clin Ther 2014;36:830–46.
26. Svoboda M, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, et al. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. Carcinogenesis 2014;35:1510–5.
27. Hamilton SR, Nakamura S, Bosman FT, Quirke P, Boffetta P, Riboli E, et al. In: F. T. Bosman FC, R.H. Hruban, N.D. Theise, editors. WHO classification of tumours of the digestive system. 4th ed. Lyon: IARC. p.134–46.
28. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004;313:856–62.
29. Efron B TR. Improvements on cross-validation: The .632+ bootstrap method. J Am Stat Assoc 1997;92:548–60.
30. Foucher Y. Construction of diagnostic or prognostic scoring system and internal validation of its discriminative capacities based on ROC curve and 0.632+ boostrap resampling 2013 2013-12-27. Available from: https://cran.r-project.org/web/packages/ROC632/index.html
31. Shantha Kumara HM, Grieco MJ, Caballero OL, Su T, Ahmed A, Ritter E, et al. MAGE-A3 is highly expressed in a subset of colorectal cancer patients. Cancer Immun 2012;12:16.
32. Godinho M, Meijer D, Setyono-Han B, Dorssers LC, van Agthoven T. Characterization of BCAR4, a novel oncogene causing endocrine resistance in human breast cancer cells. J Cell Physiol 2011;226:1741–9.
33. Yahagi S, Shibuya K, Obayashi I, Masaki H, Kurata Y, Kudoh J, et al. Identification of two novel clusters of ultrahigh-sulfur keratin-associated protein genes on human chromosome 11. Biochem Biophys Res Commun 2004;318:655–64.
34. Ostenfeld MS, Jeppesen DK, Laurberg JR, Boysen AT, Bramsen JB, Primdal-Bengtson B, et al. Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic properties. Cancer Res 2014;74:5758–71.

Downloaded from http://aacrjournals.org/cebp/article-pdf/25/7/1158/2281435/1158.pdf by guest on 25 July 2024