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Two Novel Long Noncoding RNAs – RP11-296E3.2 and LEF1-AS1can – Separately Serve as Diagnostic and Prognostic Bio-Markers of Metastasis in Colorectal Cancer

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Background: Late diagnosis and metastasis are leading causes of the high mortality of colorectal cancer (CRC). Long noncoding RNAs (lncRNAs) have been reported to play a critical role in the development and progression of CRC. This study aimed to explore the clinical significance of 2 novel lncRNAs – RP11-296E3.2 and LEF1-AS1 – including their expression pattern, as well as diagnostic and prognostic values, for metastatic CRC patients.

Material/Methods: lncRNAs expression was examined in tissues (91 cases) and plasma (60 cases) from CRC patients by real-time quantitative PCR (qRT-PCR), and the correlations between its expression and clinicopathological features and diagnosis values in metastasis were analyzed. TCGA datasets were further used to analyze their utility in prediction of overall survival (OS) and disease-free survival (DFS). ATP-based tumor chemosensitivity assay (ATP-TCA) was used to evaluated tumor chemoresistance.

Results: Compared with adjacent normal tissues, RP11-296E3.2 was significantly downregulated while LEF1-AS1 was significantly upregulated in cancer tissues (p=0.0143, p=0.0322, respectively). High levels of RP11-296E3.2 and LEF1-AS1 in tissues and plasma were correlated with tumor metastasis (p=0.0488, p=0.0252 in tissues, p=0.0331, p=0.1862 in plasma, respectively). Further analysis showed that RP11-296E3.2 sensitivity and specificity in diagnosis of CRC metastasis is better than CEA in plasma (0.690 and 0.621, and 0.621 and 0.500, respectively), and the OS of metastatic CRC patients with higher LEF1-AS1 expression levels in tissues was short (log-rank p<0.05).

Conclusions: Our findings suggest that RP11-296E3.2 and LEF1-AS1 could separately serve as potential novel diagnosis and prognostic markers for CRC metastasis.

MeSH Keywords: Biological Markers • Colorectal Neoplasms • Lymphatic Metastasis • RNA, Long Noncoding

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Background

Colorectal cancer (CRC) is a highly lethal cancer with increasing incidence and mortality in China. The high mortality rate is mainly due to its late diagnosis and metastasis [1,2]. It was reported that the 5-year survival rate of patients diagnosed at the early stage was about 90%, but for those patients with late diagnosis, the survival rate dropped sharply to only 5–10% [3], indicating that the treatment can be more effective if CRC is detected early [4.5]. To improve the early diagnosis rate and reduce the high metastasis rate of CRC, it is critical to screen novel bio-makers for predicting CRC diagnosis and metastasis [6].

The Human Genome Project found that only 1–2% of the DNA sequences in the human genome is transcribed to produce mRNAs, and 70–90% of RNAs are transcribed into noncoding RNAs (ncRNAs) [7]. Among ncRNAs, long noncoding RNAs (lncRNAs) are longer than 200 nucleotides in length and have no potential to code proteins [8]. lncRNA has been studied extensively, and increasing evidence suggests that dysregulated lncRNAs are associated with tumorigenesis, metastasis, and drug resistance [9,10]. For example, HOX antisense intergenic RNA (HOTAIR), Metastasis-associated-in-lung-adenocarcinoma-transcript-1 (MALAT1), and H19 were found to be upregulated and promote tumor invasion and metastasis by regulating various genes in multiple tumors [11]. Hua Shen et al. [12] previously identified several differentially expressed lncRNAs in CRC tissues from patients with metastasis compared with those without metastasis from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). We verified those lncRNAs by testing clinical patients' tissues and plasma, and found that the expression of RP11-296E3.2 and LEF1-AS1 were significantly associated with CRC patient metastasis and recurrence. RP11-296E3.2 (ENST00000623685.1_1) is a novel lncRNA located at chr1: 99125944-99126615. To date, few studies have reported the physiological and pathological function of RP11-296E3.2. LEF1-AS1 (lymphoid enhancer-binding factor 1 antisense RNA 1, ENSG00000232021), located at chr4: 109088681-109097586 and was reported to be significantly upregulated and to promote GBM cell malignancy via ERK and Akt/mTOR signaling pathway [13]. However, the relationships between these 2 lncRNAs and clinicopathological features of CRC patients are still unknown.

In this study, we examined the levels of RP11-296E3.2 and LEF1-AS1 in the tissues and plasma of CRC. Further analysis focused on their diagnostic potential for predicting CRC progression and metastasis, as well as the correlations between their expression and chemoresistance. Our study demonstrated that LEF1-AS1 was upregulated in CRC cancerous tissues compared with paired noncancerous tissues, while RP11-296E3.2 was downregulated, and both of them were significantly correlated with metastasis of CRC and chemoresistance status, suggesting their potential as diagnostic markers and metastasis predictors for CRC.

Material and Methods

Human CRC samples

Before surgery and any other treatment, plasma was collected from CRC patient (n=60, 2 patients' clinical information was incomplete) and matched healthy volunteers (n=34), then stored at –80°C within 2 h. Tumor tissues and paired adjacent non-tumor tissues from 91 histopathologically diagnosed CRC patients without preoperative chemotherapy or radiotherapy were collected at the Huzhou First People's Hospital from December 2016 to February 2018. Upon removal, the respective tissues were frozen immediately with liquid nitrogen immediately after colorectal cancer resection and stored at –80°C. Patient clinical information was summarized at the same time. Tumor stages were determined according to the American Joint Committee on Cancer tumor node metastasis (TNM) staging criteria.

The study was reviewed and approved by the Ethics Committee of Huzhou First People's Hospital and was conducted according to the Ethical Guidelines for Human Genome/Gene Research issued by the Chinese government.

RNA extraction and quality control

Tissue was dissociated to single-cell suspensions using the gentleMACS Dissociator (MACS, Germany). Total RNA was extracted from CRC and volunteer plasma, CRC, and paired adjacent non-tumor (normal) tissue suspensions using Trizol reagent (Invitrogen, Carlsbad, CA, USA), respectively. RNA integrity was assessed using standard denaturing agarose gel electrophoresis. Total RNA concentration and purity of each sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

RT-qPCR for CRC tissues

Total RNA (0.5 μg) was reverse transcribed using the PrimeScript® RT Master Mix (Perfect Real-Time) (TAKARA, Kusatsu, Japan). qRT-PCR was performed for cDNA using the StepOneTM Real-Time PCR System (Life Technologies, Gaithersburg, MD, USA) and the TB Green® premix Ex TaqTM assay (TAKARA). The PCR program was as follows: pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 1 min.

The primers used for qRT-PCR detection of RP11-296E3.2 and LEF1-AS1 were as follows:
RP11-296E3.2: Forward, 5'-CCAAGACCCAAGGACAACT-3',
Reverse, 5'-GAAGGATGGGCAGGAGATG-3';
LEF1-AS1: Forward, 5'-AAGGACGAGAGAAAAGCAC-3',
Reverse, 5'-CACACAAAGGGGAAGACC-3';
GAPDH: Forward, 5'-GCACCGTCAAGGCTGAGAAC-3',

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Reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

ATP-based tumor chemosensitivity assay (ATP-TCA)

ATP-TCA kit, oxaliplatin (L-OHP), irinotecan, and 5-fluorouracil (5-FU) were purchased from Huzhou Haichuang Biotechnology. Tumor tissues were immediately sent to the laboratory under sterile conditions. The tumor tissues were then cut into small pieces (1.0 mm^3) and digested by enzyme for 1.5–3.0 h at 37°C. Then, 4 clinical first-line drug combinations (5-FU+L-OHP, 5-FU+ irinotecan, L-OHP+ irinotecan, and 5-FU+L-OHP+ irinotecan) were diluted to 5 test drug concentrations (TDC) (200%, 100%, 50%, 25%, and 12.5%) and added to 10-μL cell suspension. The concentration of 100% TDC was determined by peak plasma concentration (PPC) under the conventional clinical dose according to the manufacturer's protocol [14]. Three days later, ATP extraction agent (TCE) was added and the fluorescence reader was used to test the ATP fluorescence intensity of the cell suspension at 560 nm.

The inhibition rate (%) of the 5 TDCs can be calculated by the following equation: Inhibition rate (%)=[1–(X–MI)/(M0–MI)]×100%
Index=500–5 TDCs inhibition rate
X: The fluorescence intensities of cells with drug treatment.
M0: The fluorescence intensities of cells without drug treatment.
MI: The fluorescence intensities of medium without cells and drugs.

It was considered drug-resistant if IC90 >100% TDC, IC50 >25% TDC, and Index >300.

Statistical analysis

All experimental data were analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 16.0 software system (IBM Corp., Armonk, NY, USA). The chi-squared test was applied to analyze the differential expression of RP11-296E3.2 and LEF1-AS1 between tumor and adjacent non-tumor tissues, CRC patients' and health volunteers' plasma, and subgroups classified by clinicopathological parameters, respectively. Overall survival (OS) rates and disease-free survival time (DFS) were plotted with the Kaplan-Meier method, and the log-rank test was used to analyze survival curves. $p<0.05$ was considered to be significant.

Results

Validation of the expression of lncRNA candidates in CRC tissues and plasma

We examined the expression level of RP11-296E3.2 and LEF1-AS1 levels in 91 CRC tissue samples and 60 plasma samples. As shown in Figure 1A and 1B, RP11-296E3.2 was downregulated in 78.7% (70/89) of CRC tissues compared with the corresponding non-tumor tissues ($p=0.0143$), The positive rate of LEF1-AS1 was 23.1% (21/91) in tissues. LEF1-AS1 expression was upregulated in tissues ($p=0.0322$). RP11-296E3.2 and LEF1-AS1 were both slightly down- or upregulated in plasma compared with healthy volunteers, and the difference was not statistically significant ($p=0.2765$ and $p=0.1132$, respectively) (Figure 1C, 1D).

lncRNAs are significantly associated with tumor metastasis

Compared with CRC tissues and plasma from patients without metastasis, RP11-296E3.2 was upregulated in metastasis patients' samples ($p=0.0488$ and $p=0.0331$, respectively), and LEF1-AS1 was upregulated in tissues but was not different in plasma ($p=0.0252$ and $p=0.1862$, respectively) (Supplementary Figure 1A–1D). Next, we analyzed the correlations between the expression of lncRNAs and clinicopathological features of CRC patients. As shown in the Table 1, decreased expression of RP11-296E3.2 in CRC was significantly correlated with age ($p=0.016$), lymph node metastasis (p=0.005), and E-cadherin (E-cad) levels (p=0.030) (Table 1). The expression of RP11-296E3.2 in plasma was also correlated with E-cad levels ($p=0.016$) (Supplementary Table 1). We did not find any correlations between RP11-296E3.2 and the other clinicopathological features. Immunohistochemical staining (IHC) showed that E-cad was significantly underexpressed in the CRC tissue that expressed high level of RP11-296E3.2, and the morphology of mesentery also had changed (Supplementary Figure 1E).

The expression of LEF1-AS1 in CRC tissues was significantly correlated with lymph node metastasis ($p=0.022$) and Ki67 expression ($p=0.008$), one of the clinical diagnostic markers indicating cell proliferation viability. There were no significant correlations between LEF1-AS1 and other clinicopathological features, including age, sex, and metastasis markers like carcinoembryonic antigen (CEA), CA199, E-cad, and CD44-V6. In addition, the expression of LEF1-AS1 in plasma was correlated with carcinoembryonic antigen (CEA) levels ($p=0.009$) (Supplementary Table 1).

Potential diagnostic and prognostic values of lncRNAs in CRC metastasis

To evaluate the potential diagnostic value of metastasis, an ROC curve was plotted for lncRNAs levels in CRC tissues and plasma. In CRC tissues, the area under the ROC curve (AUC) of RP11-296E3.2 was 0.654 ($p=0.012$), and the sensitivity and specificity were 0.674 and 0.609, respectively (Figure 2A). In plasma, the AUC of RP11-296E3.2 was 0.663 ($p=0.032$), and the sensitivity and specificity was better than that of CEA (0.690 and 0.621, 0.621 and 0.500, respectively) (Figure 2B). When the expression level of RP11-296E3.2 was combined with CEA level (AUC=0.660, $p=0.038$), the AUC increased to 0.722 ($p=0.004$),

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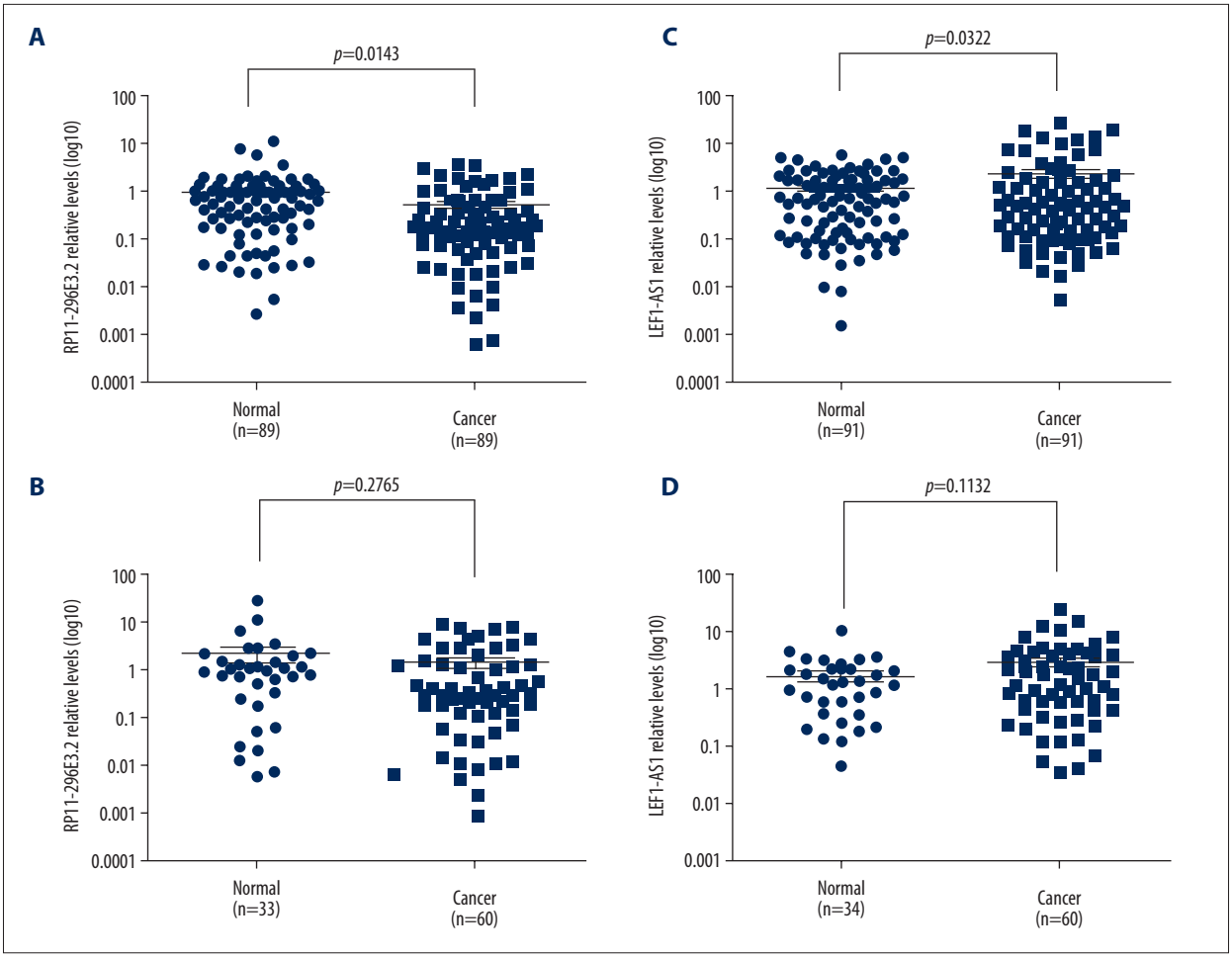

Figure 1. RP11-296E3.2 and LEF1-AS1 expression levels in CRC tissues and plasma. (**A, C**) RP11-296E3.2 and LEF1-AS1 expression levels in CRC cancer tissues and paired adjacent non-tumor tissues. (**B, D**) RP11-296E3.2 and LEF1-AS1 expression levels in CRC patients' and health volunteers' plasma. The expression is shown as the $2^{-\Delta\Delta Ct}$.

and the sensitivity and specificity were 0.724 and 0.714, respectively (Figure 2C), suggesting RP11-296E3.2 may have utility in CRC metastasis prediction and diagnosis. However, the AUC of LEF1-AS1 was 0.613 (p=0.067) in tissues and 0.602 (p=0.184) in plasma, indicating LEF1-AS1 was unable to distinguish patients with metastasis from others (Figure 2A, 2B).

We performed Kaplan-Meier analysis with log-rank test to determine the prognostic potential for lncRNAs in CRC patients using GEPIA software. The OS and DFS of CRC patients with higher LEF1-AS1 expression levels were significantly shorter (log-rank p<0.01) (Figure 2D, 2E). RP11-296E3.2 expression in CRC tissues was not significantly correlated with OS and DFS (Supplementary Figure 2). We assessed the prognostic potential for LEF1-AS1 in metastatic CRC patients using the TCGA database (TCGA, Nature 2012). As shown in Figure 2F, the OS of metastatic CRC patients with higher LEF1-AS1 expression levels was shorter (log-rank p<0.05), suggesting LEF1-AS1 may be a prognostic marker for metastatic CRC patients.

High RP11-296E3.2 was significantly correlated with drug resistance

Extensive studies have proved that metastasis plays a major role in cancer multiple drug resistance (MDR) [15]. Therefore, we further explored the relationships between MDR and lncRNAs expression in 20 patients' tissues using an ATP-based tumor chemosensitivity assay (ATP-TCA). As shown in Table 2, RP11-296E3.2 displayed a statistically significant relationship with colorectal cancer MDR (p=0.03). The chemosensitivity profiles of CRC patients are shown in Table 3, and the type of drug resistance combination was 5-FU+L-OHP.

Discussion

The metastasis of CRC can occur in the early stage, and the 5-year survival rate of patients with metastasis is less than 40% [16]. Early diagnosis, metastasis monitoring, and guided

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Table 1. Correlation between LncRNAs expression and clinicopathological features in CRC tissues.

Clinicopathological factor	Total	RP11-296E3.2		χ^2	*P* value	Total	LEF1-AS1		χ^2	*P* value
		Positive	Negative				Positive	Negative		
Sex										
Male	55	11	44	0.005	0.945	55	11	44	0.742	0.820
Female	34	7	27			36	10	26		
Age										
<60 years	37	3	34	5.762	**0.016***	38	10	28	0.386	0.535
≥60 years	52	15	37			53	11	42		
Tumor size										
<5 cm	48	10	38	0.024	0.877	50	11	39	0.072	0.788
≥5 cm	41	8	33			41	10	31		
Lymph node metastasis										
Presence	43	14	29	7.844	**0.005****	45	15	30	5.275	**0.022***
Absence	46	4	42			46	6	40		
CEA										
Poor	19	4	15	0.010	0.919	21	4	17	0.169	0.681
Well/moderate	70	14	56			70	17	56		
Ca199										
Poor	60	12	48	0.006	0.940	61	12	49	1.208	0.172
Well/moderate	29	6	23			30	9	21		
E-cad										
Poor	37	13	24	4.698	**0.030***	39	10	29	0.159	0.690
Well/moderate	37	5	32			37	11	26		
CD44-V6										
Poor	60	14	46	0.019	0.889	60	18	42	0.799	0.371
Well/moderate	16	4	12			16	3	13		
Ki67										
≥0.7	55	10	45	0.372	0.542	57	8	49	7.026	**0.008****
<0.7	34	8	26			34	13	21		

* *P* value less than 0.05; ** *P* value less than 0.01.

treatment for CRC at the early stage would have many social benefits [17]. Recent studies have found that lncRNAs participate in various physiological and pathological processes of cancer, such as cell proliferation, invasion, and drug resistance [18–20]. In this study, we confirmed that 2 novel lncRNAs – RP11-296E3.2 and LEF1-AS1 – were differentially expressed in patients with CRC and was closely associated with tumor metastasis. Further analysis showed that RP11-296E3.2 sensitivity and specificity in diagnosis of CRC metastasis is better than that of CEA, and LEF1-AS1 is a good potential prognosis maker for metastatic CRC patients. In addition, high RP11-296E3.2 expression indicated postoperative drug resistance in CRC patients.

lncRNA can affect tumor invasion and metastasis *by* complex mechanisms, including dysregulated expression levels and regulation of target genes, and thus are considered as the next generation of potential prognosis markers. For example, lncRNA/TP73-AS1 was found to function as a competitive endogenous RNA to sponge miR-194, promoting colorectal cancer cell

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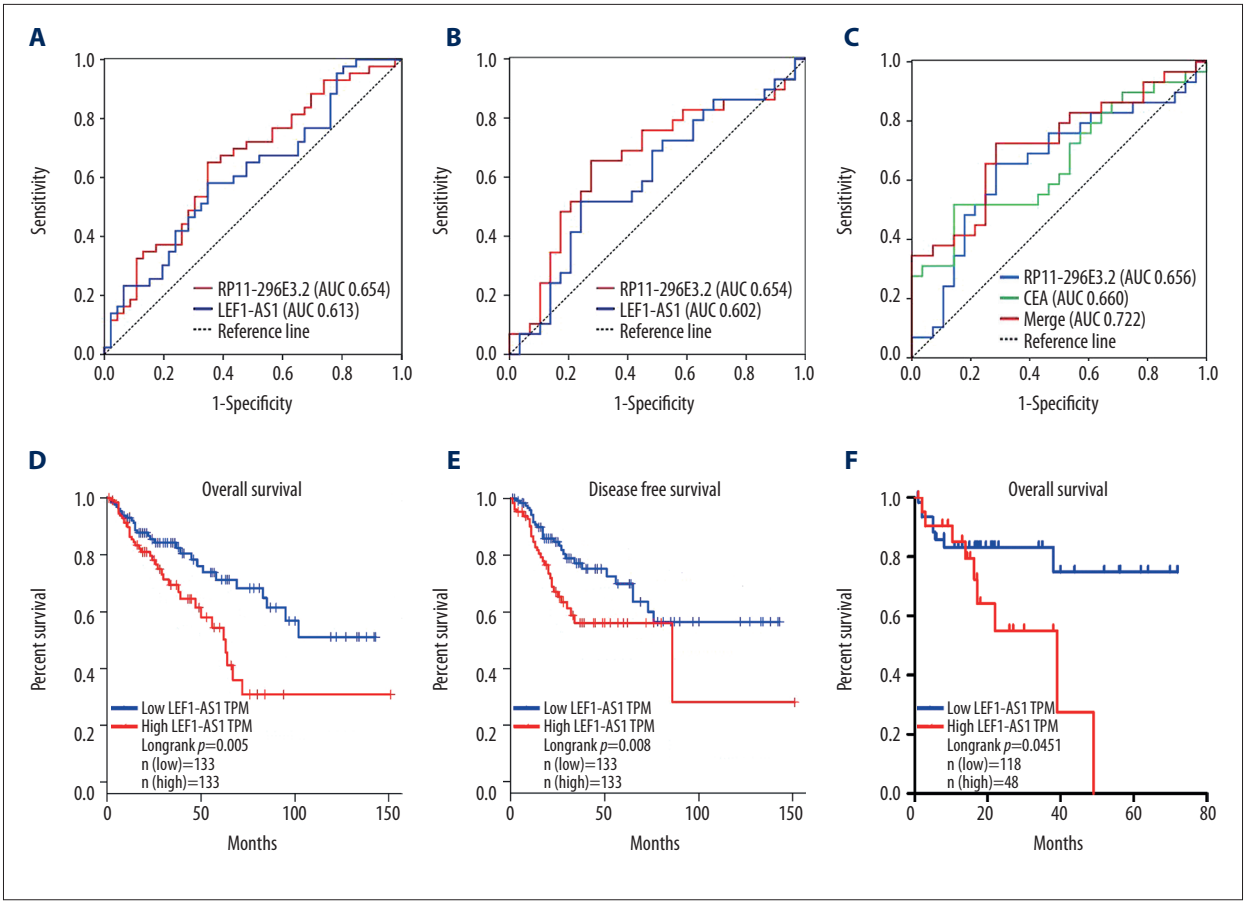

Figure 2. Potential diagnostic and prognostic values of lncRNAs in CRC metastasis. (**A**) The ROC curves of tissues RP11-296E3.2 and LEF1-AS1 for CRC patients with metastasis. (**B, C**) The ROC curves of plasma RP11-296E3.2, LEF1-AS1, and CEA for CRC patients with metastasis. (**D, E**) RNA-seq data from the TCGA database showed that the OS and DFS of patients with high LEF1-AS1 expression level were short. (**F**) The OS of metastasis CRC patients with high LEF1-AS1expression was short.

Table 2. Correlation of LncRNAs expression with MDR.

	n	RP11-296E3.2		χ^2	*P* value	LEF1-AS1		χ^2	*P* value
		High	Low			High	Low		
Drug resistance	10	4	6	5.00	0.03*	4	6	3.81	0.12
No drug resistance	10	0	10			1	9		

* *P* value less than 0.05.

proliferation, migration, and invasion [18]. LINC00961 was reported to serve as a prognostic indicator and a potential therapeutic target for glioma patients [21]. SNHG20 was identified as an independent unfavorable prognostic factor of osteosarcoma patients, modulating tumor cell migration and invasion [22].

In our study, we found the level of RP11-296E3.2 in CRC tissues without metastasis was lower than in adjacent normal tissues, suggesting that it may act as a tumor suppressor. When compared with CRC without metastasis, RP11-296E3.2 showed higher levels in cases with metastasis, suggesting it may participate in metastasis through some other signal pathway and have a double-edged effect on tumors. The double-edged function of genes has been reported in several studies. For example, DEAD-box RNA helicase 3 (DDX3) either activates Wnt signaling to promote lung cancer progression or acts as a lung cancer suppressor through the MDM2/Slug/E-cadherin pathway [23]. The nuclear lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is located in the transcriptional regulatory region of the adjacent gene. It can promote or suppress breast cancer metastasis by activating or inactivating the adjacent pro-metastatic transcription factor

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Table 3. Chemosensitivity profiles of CRC patients.

Patient number	IC50	IC90	Index	Drug-resistant (yes/no)	Drug-resistant combination
1	216.16	67.11	301	Yes	5-FU+L-OHP
2	214.00	19.93	155	No	
3	218.17	34.54	184	No	
4	185.41	45.86	201	No	
5	269.95	127.97	311	Yes	5-FU+L-OHP
6	236.46	136.10	359	Yes	5-FU+L-OHP
7	324.40	174.94	367	Yes	5-FU+L-OHP
8	213.65	28.88	182	No	
9	218.17	340.54	205	No	
10	201.83	12.34	167	No	
11	574.10	318.95	410	Yes	5-FU+L-OHP
12	123.64	20.37	143	No	
13	190.54	47.00	204	No	
14	258.17	91.26	309	Yes	5-FU+L-OHP
15	368.48	204.71	428	Yes	5-FU+L-OHP
16	232.13	88.80	257	No	
17	452.04	251.13	386	Yes	5-FU+L-OHP
18	898.41	499.12	426	Yes	5-FU+L-OHP
19	317.46	103.95	308	Yes	5-FU+L-OHP
20	213.14	48.40	223	No	

TEAD [24]. Therefore, we speculate that the dual role of RP11-296E3.2 in CRC proliferation and metastasis might be correlated with differences in time and space. Further research is needed to explain the underlying mechanism.

CEA has been used as a serum biomarker for CRC diagnosis and prognosis for several years, but its sensitivity and specificity in screening for CRC metastasis in a serum is limited [25] and its efficacy in predicting prognosis and monitoring CRC patients remains controversial [26]. In our study, we found that RP11-296E3.2 had better sensitivity and specificity (0.674 and 0.609, respectively) in diagnosis of CRC metastasis compared with CEA (0.621 and 0.500, respectively) in plasma. When the expression level of plasma RP11-296E3.2 was combined with CEA level, the AUC increased to 0.722, indicating that RP11-296E3.2 is complementary to CEA as a prognostic indicator in metastasis CRC.

LEF1-AS1 was upregulated in colorectal cancer tissues and the overexpression of LEF1-AS1 was also correlated with lymph node metastasis. Since patients were retrospectively included between December 2016 and February 2018 in our study and the follow-up is still ongoing, prognostic data are unavailable. Alternatively, we downloaded TCGA data to analyze the predictive function of RP11-296E3.2 and LEF1-AS1 in tumor prognosis. We found that the OS of metastatic CRC patients with higher LEF1-AS1 expression levels was shorter than that of patients with lower LEF1-AS1 expression levels. In glioblastoma (GBM), Wang et al. proved that LEF1-AS1 expression was significantly upregulated and promoted GBM cell malignancy via ERK and Akt/mTOR signaling pathway [13]. Our study and the report from Wang et al. both support that high level of LEF1-AS1 expression in tumor tissues indicates poor prognosis and may be a pivotal biomarker for these diseases. The instability of lncRNA in the blood [27] may be the major reason why RP11-296E3.2 and LEF1-AS1 perform differently in plasma *vs.* tissues.

In terms of tumor chemoresistance, lncRNAs have been reported to be associated with the progression of drug resistance. For example, increased lncRNA EBIC expression in ovarian cancer can improve cisplatin resistance though regulating the Wnt/β-catenin signaling pathway [28]. lncRNA/CASC9 regulates the MDR1 gene by binding to EZH2 and promoting doxorubicin-resistance in breast cancer [29]. In our study, we explored the relationships between MDR and expression of RP11-296E3.2 and LEF1-AS1 in 20 patients

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using an ATP-based tumor chemosensitivity assay (ATP-TCA). The high level of ATP released shows good cell viability, indicating tumor cells were drug-resistant. We found that a high level of RP11-296E3.2 expression was significantly correlated with colorectal cancer cell MDR (p=0.03 and p=0.05, respectively).

Conclusions

We showed for the first time that 2 novel lncRNAs expressed in CRC were associated with tumor metastasis. When compared with LEF1-AS1 levels in tissues, RP11-296E3.2 showed higher sensitivity and specificity in diagnosis of CRC metastasis. When combined with CEA level in plasma, the diagnostic ability of RP11-296E3.2 is better. The high level of LEF1-AS1 expression in CRC tissues indicated poor prognosis and short survival time of metastatic CRC patients and may be a pivotal prognostic marker for those diseases. In additional, the molecular mechanism underlying the close relationship between CRC metastasis and LEF1-AS1 and RP11-296E3.2 expression and needs further clarification.

Conflict of interest

None.

Supplementary Data

Supplementary Table 1. Correlation between lncRNAs expression and clinicopathological features in CRC plasma.

Clinicopathological factor	Total	RP11-296E3.2		χ^2	*P* value	LEF1-AS1		χ^2	*P* value
		Positive	Negative			Positive	Negative		
Sex									
Male	37	11	26	1.856	0.173	20	17	0.222	0.637
Female	21	10	11			10	11		
Age									
<60 years	26	12	14	2.019	0.155	14	12	0.085	0.771
≥60 years	32	9	23			16	16		
Tumor size									
<5 cm	33	12	21	0.056	0.813	18	15	0.115	0.734
≥5 cm	24	8	16			12	12		
Lymph node metastasis									
Presence	29	14	15	3.658	**0.056**	17	12	1.105	0.293
Absence	29	7	22			13	16		
CEA									
Poor	13	4	9	0.215	0.643	2	9	6.893	**0.009****
Well/moderate	45	17	28			28	17		
Ca199									
Poor	53	20	33	0.622	0.430	27	25	0.119	0.730
Well/moderate	5	1	4			3	2		
E-cad									
Poor	31	16	15	5.858	**0.016***	15	16	0.234	0.628
Well/moderate	18	3	15			10	8		
CD44-V6									
Poor	40	17	23	1.273	0.259	20	20	0.091	0.763
Well/moderate	9	2	7			5	4		
Ki67									
≥0.7	34	11	23	0.528	0.467	20	14	1.658	0.198
<0.7	24	10	14			10	14		

* *P* value less than 0.05; ** *P* value less than 0.01.

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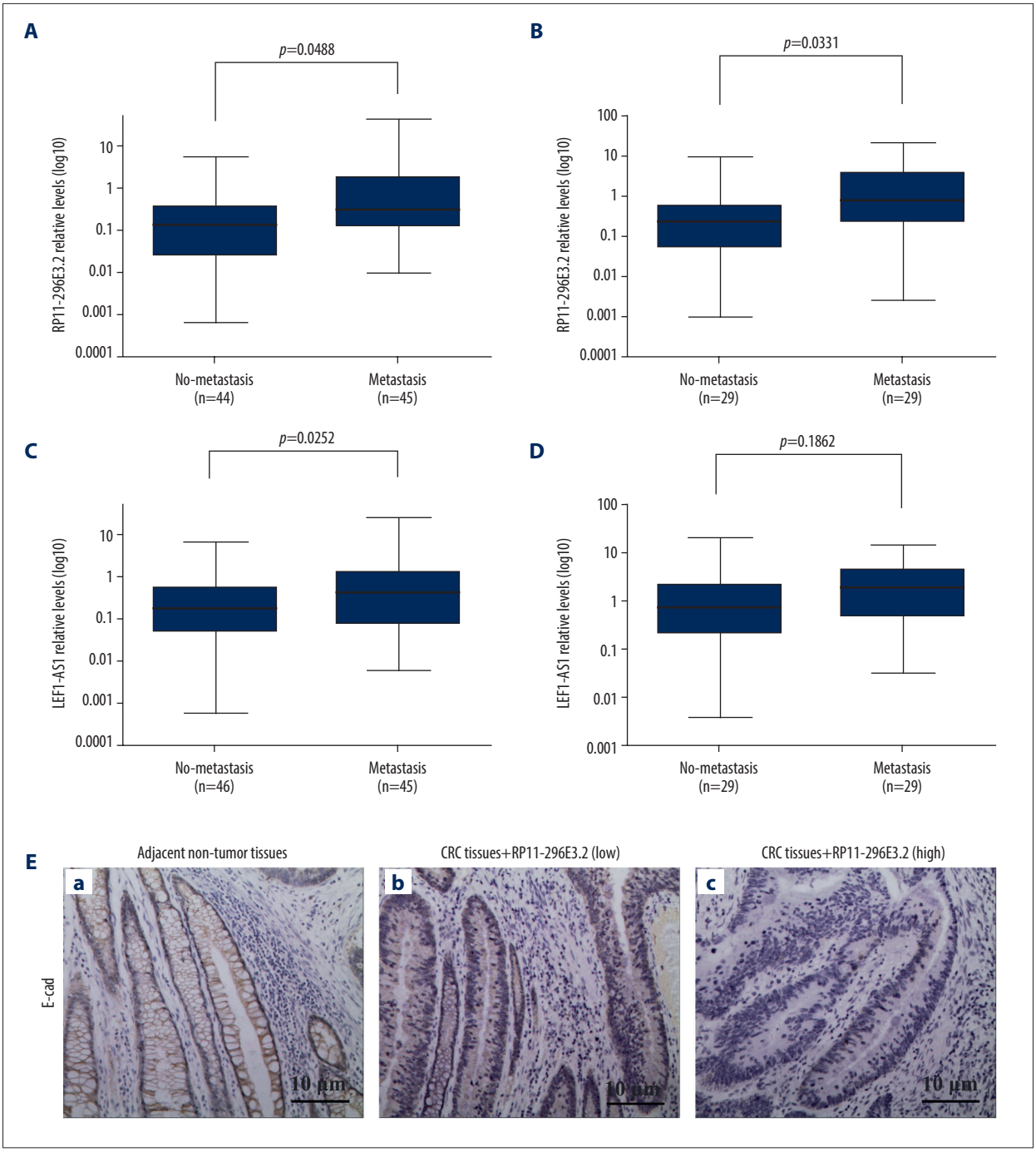

Supplementary Figure 1. RP11-296E3.2 and LEF1-AS1 expression are closely associated with CRC metastasis. (**A–D**) RP11-296E3.2 and LEF1-AS1 expression levels in CRC cancer tissues and plasma with metastasis and paired non-metastasis tumor tissues. The expression is shown as the $2^{-\Delta\Delta Ct}$ value. (**E**) IHC stain showed the E-cad expression and morphology of mesentery changes in CRC tissues with different RP11-296E3.2 expressions.

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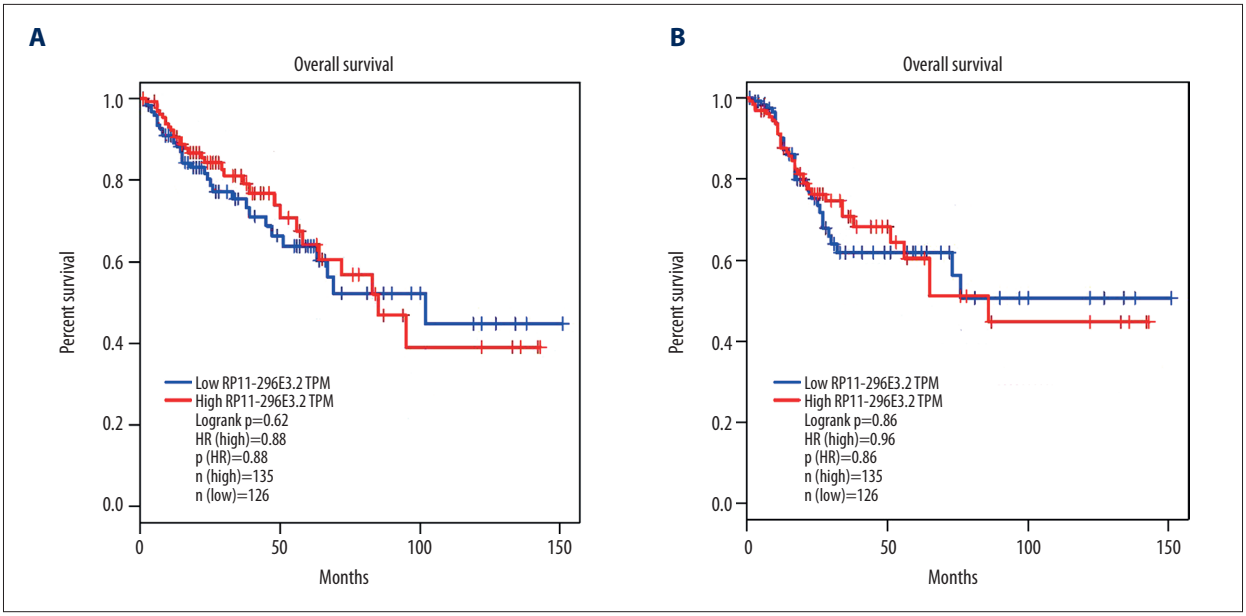

Supplementary Figure 2. Kaplan-Meier analysis for the OS and DFS of CRC patients with different expression levels of RP11-296E3.2. (**A, B**) RP11-296E3.2 expression in CRC tissues showed no significant correlations with OS or DFS.

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