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Differentially expressed long non-coding RNAs and the prognostic potential in colorectal cancer

Q. LI[1,‡], Y. DAI[2,‡], F. WANG[2], S. HOU[3,*]

[1]Department of Clinical Laboratory, The First Hospital of Zibo City, Zibo 255200, Shandong, China; [2]Department of General Surgery, Zoucheng People's Hospital, Zoucheng 273500, Shandong, China; [3]Department of Pediatric Surgery, Zoucheng People's Hospital, Zoucheng 273500, Shandong, China

**Correspondence: housu5981@126.com*
‡Contributed equally to this work.

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Colorectal cancer (CRC) is a disease with high incidence, especially in developed countries. Long non-coding RNAs (lncRNAs) are new research hotspots for their vital roles in regulating gene expression. This study aims to investigate the prognostic value of lncRNAs in CRC patients.

A total of 21 cancer-related lncRNAs were detected by PCR array to reveal their expression changes in CRC tissue. A 120-week-long follow-up was performed in 30 CRC patients to analyze the relationship between lncRNA levels and CRC prognosis.

Most of the 21 lncRNAs were differentially expressed in CRC tissue compared to the adjacent normal tissue, among which seven lncRNAs were significantly changed: AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 and PVT1 were up-regulated, and ADAMTS9-AS2 was down-regulated in CRC tissue samples. No obvious correlation was found between lncRNA levels and the age, gender, tumor size or TNM stage of these patients. Log-rank test indicated that higher levels of AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 or PVT1 and lower level of ADAMTS9-AS2 might predict the poor prognosis of CRC patients.

This study suggests the potential value of the seven lncRNAs in the prognosis of CRC, providing reference information for future research on CRC prognostic and treatment strategy.

Key words: colorectal cancer (CRC), long non-coding RNAs, prognosis, differential expression

Colorectal cancer (CRC) is the cancer developed in the colon or rectum. It has a high incidence in developed countries and has become the third most common cancer and the third leading cause of cancer death in the United States [1]. Patients with CRC suffer from symptoms like hematochezia, changes in the bowel movements and weight loss. Risk factors include unhealthy lifestyle such as smoking and alcohol, older age and inherited genetic disorders [2, 3]. Treatments of CRC involve the combination of surgery, radiation therapy, chemotherapy and targeted therapy. For example, colonoscopic polypectomy may help to prevent death from CRC [4], and screening with flexible sigmoidoscopy is capable of decreasing incidence of CRC [5]. Various screening strategies are adopted by European countries [6]. Although the death rate has declined in the past decade due to the improvement of screening and standard treatment, the frequency of relapses and the poor prognosis still greatly impact the outcome of treating CRC. Thus effective prevention and treatment of CRC is not an easy task.

Numerous studies intensively focused on the pathology of CRC and have revealed the central role of Wnt signaling in the etiology of CRC [7]. Tumor suppressor adenomatous polyposis coli (APC) protein also possesses a significant position that may cause CRC. Mutation in *APC* gene leads to uncontrolled accumulation of β-catenin, which activates the expression of proto-oncogenes [8]. In addition to *APC*, mutations in *TP53*, *SMAD4*, *SOX9*, as well as the abnormal DNA copy number, promoter methylation and microsatellite instability have also been uncovered in CRC patients with the help of genome-scale analyses [9].

PCR array is a high throughput method to detect gene expression conveniently and reliably, which has contributed

to the revelation of key factors in CRC, such as interleukins [10] and DEAH box polypeptide 32 [11]. However, few studies refered to a wide-range expression profile of long non-coding RNAs (lncRNAs) in CRC. lncRNAs are non-coding RNAs longer than 200 nt, participating in diverse cellular activities as key regulators of gene expression. Some lncRNAs, such as ADAMTS9-AS2, are tumor suppressors [12], while some may facilitate tumor development and progression, among which MALAT1 and AFAP1-AS1 have been reported as therapeutic targets for cancers [13, 14]. A recent study found that 761 lncRNAs were hypermethylated in CRC tissue samples [15], further emphasizing the role of lncRNAs in CRC development. Still, more expression profiles and the application value of lncRNAs in CRC need to be investigated.

This study aimed at revealing the role of lncRNAs in the prognosis of CRC. We selected 21 cancer-related lncRNAs that might be involved in cancer development according to existed researches (Supplementary Table), and detected their expression in CRC tissue samples compared to normal samples by means of PCR array. Seven of these lncRNAs were identified to be up- or down-regulated in CRC. A 120-week-long follow-up was performed on 30 CRC patients to investigate the possible prognostic role of the seven lncRNAs in CRC. These results will provide proof for the application of lncRNAs in CRC prognosis.

Materials and methods

Clinical samples. A total of 15 female and 15 male patients (Table 1) diagnosed with CRC were selected from the patients that underwent radical surgical resection during 2010 to 2013. None of the patients had accepted chemotherapy or radiotherapy before the surgery. The cancer tissue and adjacent normal tissue samples were obtained during the surgery. All patients were informed with the experiment before sampling. The tissue samples were frozen in liquid nitrogen immediately after the sampling procedure, and stored at -80°C until RNA extraction. The cancer tissue samples were pathologically examined and staged according to the 7th version of the American Joint Committee on Cancer (AJCC) cancer staging system. After the surgery, a follow-up of 120 weeks was performed on each patient via reexamination or phone call. This study was approved by a local ethics committee. Three of the 30 patients were randomly selected and their tissue samples were used in PCR array.

RNA extraction and reverse transcription. Total RNAs from tissue samples were extracted with Trizol (Invitrogen, Carlsbad, CA) and purified with PureLink® RNA Mini Kit (Thermo Scientific, Carlsbad, CA) according to the manufacturers' instructions. The quality and quantity of RNAs were examined by agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific). First-strand complementary DNAs (cDNAs) were synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

PCR array. Three of the 30 patients were randomly selected and their tissue samples were used in PCR array after RNA extraction and reverse transcription. The cDNA samples were used for PCR array by TaqMan® Gene Expression Assays (Applied Biosystems, Carlsbad, CA) with specific primers for 21 lncRNAs according to the manufacturer's instruction. The assay was performed on QuantStudio™ 6 Flex Realtime PCR System (Applied Biosystems). GAPDH was used as an internal control.

qPCR. The expressions of seven up- or down-regulated lncRNAs in each tissue sample of 30 patients were examined on QuantStudio™ 6 Flex Realtime PCR System using the specific primers for these lncRNAs (Table 2). Detection was repeated in triplicate. Data were analyzed by $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal control.

Statistical analysis. Data were represented as the mean ± standard deviation and analyzed with SPSS 20 (IBM, New York, NY). Comparison between groups was performed by *t* test, and categorical data were analyzed by Fisher's exact test. In the survival curve assay, loss of follow-up and the

Table 1. Age, gender, tumor size and TNM stage information of the 30 colorectal patients used in this study.

Variables		Number (%)
Age (years)	< 60	18 (60)
	≥ 60	12 (40)
Gender	Female	15 (50)
	Male	15 (50)
Tumor size	Rectum	16 (53.3)
	Colon	14 (46.7)
TNM stage	II	20 (66.7)
	III	10 (33.3)

TNM, tumor node metastasis.

Table 2. Specific primers of seven lncRNAs and GAPDH used in qPCR.

Primer	Sequence (5' to 3')
AFAP1-AS1	(Fw) GGAGTGACGGCATCCAACTC
	(Rv) GTCATCCCTGTCCCTGGTTC
BCAR4	(Fw) CTTGCAGAAAACCACGAGGC
	(Rv) CAGCGAGGTGCTAGCGATTA
H19	(Fw) GGAGACTAGGCCAGGTCTC
	(Rv) GCCCATGGTGTTCAAGAAGGC
HOXA-AS2	(Fw) GGCGTTTCCTTTTCCCACAG
	(Rv) GTCAACGGATTTGGTCTGTATT
MALAT1	(Fw) CAGTGGGGAACTCTGACTCG
	(Rv) GTGCCTGGTGCTCTCTTACC
PVT1	(Fw) TGCTCTAGAATCTGATGCACGTTCCACC
	(Rv) CCGGAATTCCTTAATTCTCCAATCTCAAAATAC
ADAMTS9-AS2	(Fw) GACCCTCTTCCAGAAGGCAC
	(Rv) GGACAAGCGAAGGACATCC
GAPDH	(Fw) ATCCATCCATAATGGCTTCC
	(Rv) AGTCTTCTGGGTGGCAGTGAT

survival when the follow-up ended were considered as censored data. Survival curves were analyzed by log-rank test or Cox's proportional hazards regression model. PCR array data were analyzed by DataAssist™ Software (Applied Biosystems) using the $2^{-\Delta\Delta Ct}$ method. Fold change was calculated as the normalized lncRNA expression in the cancer tissue samples divided the normalized expression in the normal tissue samples. In data analysis, $P < 0.05$ was considered as statistically significant.

Results

Fold changes of lncRNAs in CRC tissue. Based on the PCR array results, the expression levels of 21 lncRNAs in the cancer tissue samples of three patients were compared to their expression in the corresponding adjacent normal tissue after normalized by GAPDH. The heat map showed that HNF1A-AS1, ADAMTS9-AS2, BANCR and TUSC7 might possess of higher levels in normal tissue samples and 14 lncRNAs were relatively up-regulated in cancer tissue samples (Figure 1). It should be noticed that differences among the three patients could be observed, since the expression of some lncRNAs like ACTA2-AS1 and CBR3-AS1 were obviously down-regulated in the adjacent normal tissue sample of patient No. 1 while almost unchanged in patient No. 2 compared to cancer tissue samples.

Data analysis calculated the fold changes of each lncRNA in cancer tissue compared to adjacent normal tissue samples of the three patients (Table 3). The expression level change of more than two folds was considered as up- or down-regulated lncRNAs, thus seven of the lncRNAs were filtered out, all of which possessed significant expression changes between cancer and adjacent normal tissue samples ($P < 0.05$). Among them, AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 and PVT1 were significantly up-regulated, and ADADMTS9-AS2 was significantly down-regulated in cancer tissue samples. The histograms in Figure 2 showed the expression level comparison of the seven lncRNAs more directly. These results implied that the seven lncRNAs found by PCR array might be involved in the progression or development of CRC.

Prognostic potential of seven lncRNAs in CRC. Before the survival curve assay, we performed χ^2 test on the relationship between lncRNA levels and the age, gender, tumor size or

Table 3. Fold changes of 21 lncRNAs detected by qPCR array.

Symbol	Fold change	95% Cl	*P* value
ACTA2-AS1	1.271	(0.96, 1.58)	0.117998
BLACAT1	1.169	(0.92, 1.42)	0.219244
BANCR	0.6717	(0.58, 0.77)	0.005683*
HNF1A-AS1	0.8078	(0.65, 0.96)	0.097723
TUSC7	0.7768	(0.69, 0.86)	0.013389*
ADAMTS9-AS2	0.4416	(0.33, 0.56)	0.012486*
AFAP1-AS1	2.4529	(2.14, 2.77)	0.00062*
BCAR4	4.0113	(3.48, 4.55)	0.000015*
CCAT1	1.3478	(1.23, 1.47)	0.002568*
CCAT2	1.1599	(0.99, 1.33)	0.115921
CBR3-AS1	1.1336	(0.86, 1.41)	0.380373
HAND2-AS1	1.5859	(1.19, 1.99)	0.027724*
JADRR	1.1604	(1.03, 1.30)	0.066514
CRNDE	1.8058	(1.58, 2.03)	0.000446*
H19	2.1165	(1.72, 2.52)	0.0004*
HEIH	1.3849	(1.22, 1.55)	0.004886*
SNHG16	1.0711	(0.90, 1.24)	0.44561
HOXA-AS2	2.2759	(1.97, 2.58)	0.000175*
HOTAIR	1.7209	(1.45, 1.99)	0.00232*
MALAT1	7.6984	(3.74, 11.66)	0.003436*
PVT1	4.4358	(1.21, 7.66)	0.01295*
GAPDH	0.9997	(0.93, 1.07)	0.998402

* $P < 0.05$. lncRNAs with a change fold larger/lower than 2 are in bold.

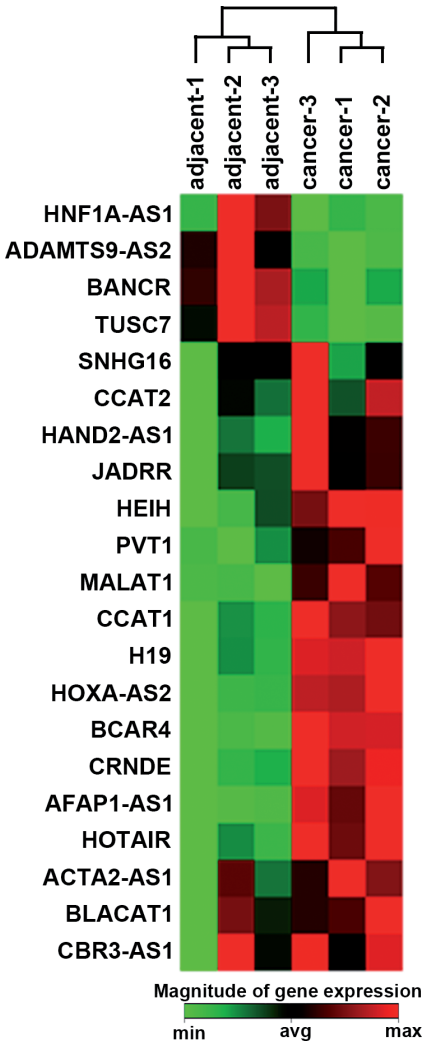

Figure 1 Heat map of 21 lncRNAs expression levels in cancer and adjacent normal tissue samples of CRC patients (n = 3). Adjacent-1 and cancer-1 samples are from the same patient, and so on. Expression level from low to high is represented as green to red.

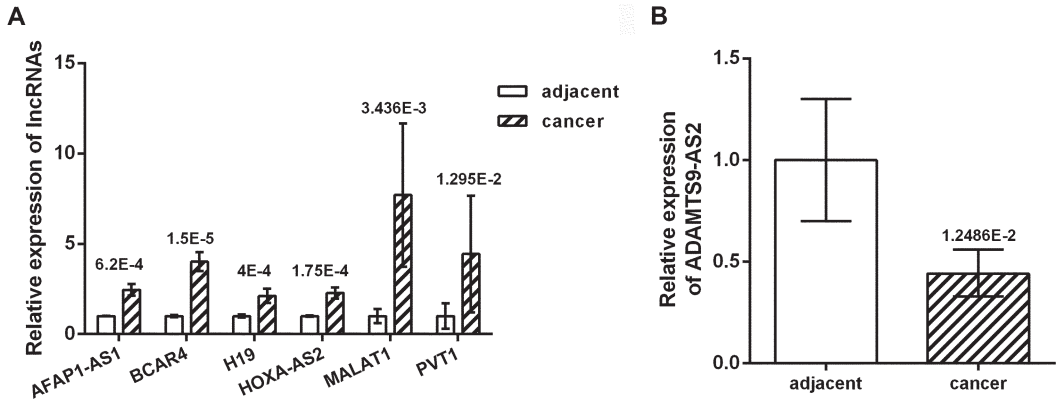

Figure 2. Histogram showing the significantly up-regulated and down-regulated lncRNAs in CRC tissue samples compared to adjacent normal tissue samples based on PCR array results (n = 3). (A) lncRNAs that are up-regulated in CRC tissue samples. (B) lncRNA ADAMTS9-AS2 is down-regulated in CRC tissue samples. *P* values are indicated above each comparison.

TNM stage of the 30 patients, and no obvious association was found ($P > 0.05$, Table 4). When the 30 patients were divided into two groups – 15 patients with high AFAP1-AS1 levels and 15 patients with lower AFAP1-AS1 levels – the survival curve assay indicated that the patients with higher AFAP1-AS1 levels had a relatively shorter survival time than patients in the other group ($P = 0.0103$, Figure 3 and Table 5). Similar results were also detected from BCAR4, H19, HOXA-AS2, MALAT1 and PVT1. However, patients with higher ADAMTS9-AS2 levels had a relatively longer survival time ($P = 0.0046$). Thus lncRNA AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 and PVT1 might be correlated with a worse prognosis and ADAMTS9-AS2 might indicate a better prognosis for CRC patients.

Discussion

As hotspots of gene regulation research, lncRNAs and their pivotal position in cancer modulation have been gradually revealed. This study examined the expression profile of 21 cancer-related lncRNAs in cancer tissue samples of

Table 4. Association between lncRNA levels and variables of patients (n = 30).

		Age (years)		Gender		Tumor site		TNM stage	
		< 60	≥ 60	Female	Male	Rectum	Colon	II	III
AFAP-AS1	High (n = 15)	8	7	6	9	7	8	10	5
	Low (n = 15)	10	5	9	6	9	6	10	5
	P value	0.7104		0.4661		0.7152		1.0000	
BCAR4	High (n = 15)	9	6	7	8	9	6	9	6
	Low (n = 15)	10	5	8	7	6	9	11	4
	P value	1.0000		1.0000		0.4661		0.6999	
H19	High (n = 15)	8	7	8	7	9	6	12	3
	Low (n = 15)	10	5	7	8	7	8	8	7
	P value	0.7104		1.0000		0.7152		0.2451	
HOXA-AS2	High (n = 15)	9	6	6	9	9	6	9	6
	Low (n = 15)	9	6	9	6	8	7	10	5
	P value	1.0000		0.4661		1.0000		1.0000	
MALAT1	High (n = 15)	10	5	7	8	10	5	11	4
	Low (n = 15)	8	7	8	7	7	8	9	6
	P value	0.7104		1.0000		0.4621		0.6999	
PVT1	High (n = 15)	9	6	6	9	9	6	11	4
	Low (n = 15)	9	6	9	6	7	8	9	6
	P value	1.0000		0.4661		0.7152		0.6999	
ADAMTS-AS2	High (n = 15)	10	5	8	7	7	8	10	5
	Low (n = 15)	8	7	7	8	9	6	10	5
	P value	0.7104		1.0000		0.7152		1.0000	

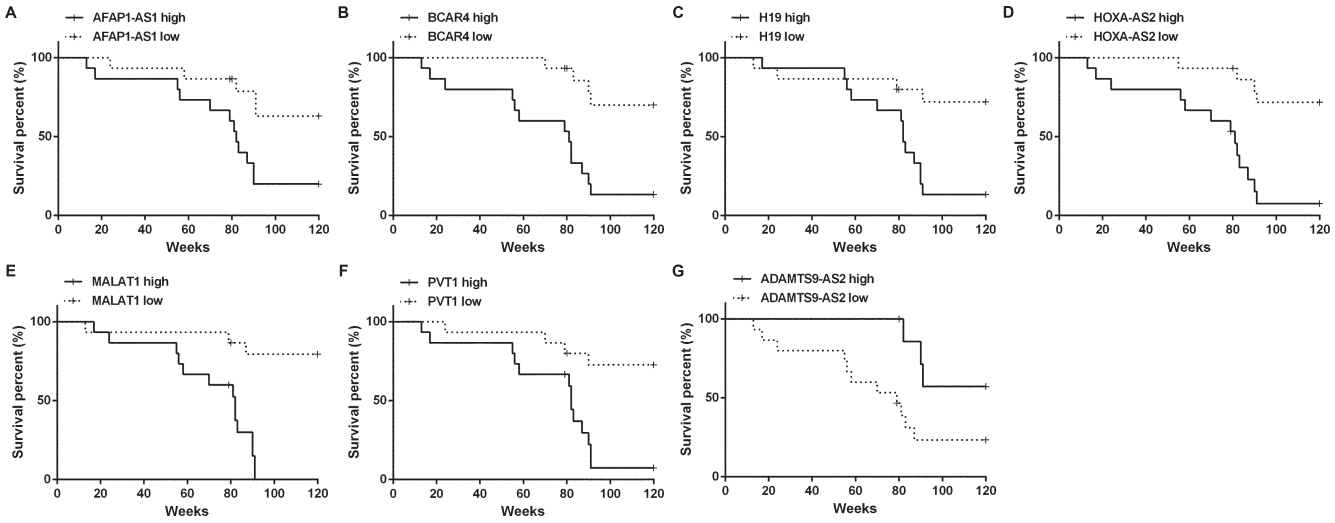

Figure 3. Survival curves of seven lncRNAs and the survival percent of colorectal patients (n = 30). + indicates censored data due to loss of follow-up and the survival when the 120-week-long follow-up ended. For each survival curve assay, the 30 patients are divided into two groups according to the higher or lower AFAP1-AS1 (A), BCAR4 (B), H19 (C), HOXA-AS2 (D), MALAT1 (E), PVT1 (F) or ADAMTS9-AS2 (G) levels.

CRC patients and screened seven lncRNAs that had significantly aberrant expression between cancer and normal tissue samples. The follow-up and survival curve assay of these seven lncRNAs implied their possible relationship with CRC prognosis.

Among the 21 lncRNAs detected by PCR array, some were up-regulated and some were down-regulated to some extent compared to their expressions in normal tissue samples. In consistent with existed studies of gastric cancer and hepatocellular carcinoma, that HNF1A-AS1 and TUSC7 were down-regulated and acted as tumor suppressors [16, 17], this study also detected lower expression of the two lncRNAs in CRC samples. In malignant melanoma, BANCR was up-regulated and promoted tumor growth [18], whereas it was inhibited in CRC samples. We conjectured this discrepancy may generate from individual difference or specific regulatory mechanisms of lncRNAs in different cancer cells.

By setting the expression change threshold to 2 folds, we screened seven lncRNAs that had significantly up- or down-regulated expression in CRC tissue samples. Among the seven lncRNAs, AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 and PVT1 were up-regulated, while ADAMTS9-AS2 was down-regulated. AFAP1-AS1 has been found hypomethylated in Barrett's esophagus and esophageal adenocarcinoma, which led to its overexpression [19]. BCAR4 has been discovered overexpressing in breast cancer [20]. Overexpression of H19 was found in gastric cancer, breast cancer and bladder cancer, enhancing carcinogenesis and cancer metastasis [21-23]. Similarly, HOXA-AS2, MALAT1 and PVT1 were all reported to be up-regulated in cancer cells and promoted cancer progression [24-26]. As for ADAMTS9-AS2, it was revealed to be a cancer suppressor factor that lower expressed in glioma cells, with its overexpression inhibiting glioma cell migration [12]. Together with the findings of this study, it could be deduced that the seven lncRNAs have relatively conserved expression pattern in various cancers including CRC. Their aberrant levels in CRC tissue compared to normal tissue may imply the involvement of these lncRNAs in the development and progression of CRC, which needs to be investigated in further research.

An important finding of this study is the relationship between the seven lncRNAs and the prognosis of CRC. CRC patients with lower AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 or PVT1 levels, or a higher ADAMTS9-AS2 level tended to show better prognosis. Some of these lncRNAs have been investigated in cancers to of possess potential prognostic values. For example, the low ADAMTS9-AS2 expression was a predictor for poor survival in glioma [12]. Poor prognosis in renal cell carcinoma, nasopharyngeal carcinoma, non-small-cell lung cancer and cervical cancer has also been found related to high expression of H19 [27], AFAP1-AS1 [28, 29] and MALAT1 [30]. Similar to the former studies, higher AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 or PVT1 levels and lower ADAMTS9-AS2 level may also predict a poor prognosis of CRC.

Table 5. Log-rank test results of survival curves.

lncRNA	*P* value	χ^2 value
AFAP1-AS1	0.0103	6.574
BCAR4	0.0005	12.29
H19	0.0038	8.354
HOXA-AS2	0.0001	14.51
MALAT1	< 0.0001	16.53
PVT1	0.0011	10.60
ADAMTS9-AS2	0.0046	8.039

Existed prognostic markers for CRC included miRNAs, some genome signatures like microsatellite instability and gene copy number, as well as other factors like CD133 [31-34]. The seven lncRNAs that were up- or down-regulated in CRC tissue sample may have great potential in CRC prognosis. Our future research will concentrate on exploring more accurate prognostic methods for CRC, possibly via combining multiple lncRNAs. Moreover, the detailed functions and regulatory mechanism of these lncRNAs in CRC are worth discussing, which may provide more useful informations for controlling CRC and other cancers.

In summary, this study revealed seven lncRNAs with aberrant expression in CRC tissue samples: AFAP1-AS1, BCAR4, H19, HOXA-AS2, MALAT1 and PVT1 were up-regulated, and ADAMTS9-AS2 was down-regulated. The expression of the seven lncRNAs was associated with the prognosis of CRC patients. These results provided potential prognostic alternatives for the prediction of CRC outcome and the exploration of prognostic strategies.

Supplementary information is available in the online version of the paper.

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Supplementary Table: The 21 lncRNAs in this study and their related diseases in literature.

lncRNA	Disease in literature
HNF1A-AS1	lung adenocarcinoma [1], esophageal adenocarcinoma [2], gastric cancer [3]
ADAMTS9-AS2	glioma [4], head and neck cancer [5]
BANCR	CRC [6], hepatocellular carcinoma [7], melanoma [8]
TUSC7	non-small cell lung cancer [9], gastric cancer [10]
SNHG16	thyroid cancer [11]
CCAT2	cervical squamous cell cancer [12], breast cancer [13]
HAND2-AS1	endometrial hyperplasia [14]
JADRR	breast cancer [15]
HEIH	hepatocellular carcinoma [16]
PVT1	cervical carcinoma [17], pancreatic cancer [18]
MALAT1	CRC [19], pancreatic cancer [20]
CCAT1	CRC [21], hepatocellular carcinoma [21]
H19	breast cancer [22], bladder cancer [23], gastric cancer [24]
HOXA-AS2	gastric cancer [25], leukemia [26]
BCAR4	breast cancer [27]
CRNDE	CRC [28], glioma [29]
AFAP1-AS1	nasopharyngeal carcinoma [30], non-small cell lung cancer [31]
HOTAIR	gallbladder cancer [32], urothelial cancer [33]
ACTA2-AS1	lung adenocarcinoma [34]
BLACAT1	bladder cancer [35], gastric cancer [36]
CBR3-AS1	prostate cancer [37], esophageal squamous carcinoma [38]

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