Evaluation of the Association Between *CRNDE* Plasma Expression Level, *KRAS, NRAS*, and *BRAF* Variants in Patients with advanced CRC

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ABSTRACT

Colorectal cancer is one of the most common types of cancer. *CRNDE* is a novel-defined *lncRNA*. We evaluated the possibility of using *CRDNE* circulating *lncRNA* as a noninvasive biomarker. Also, we examined the effect of circulating *CRNDE lncRNA* on the pathogenesis of CRC and its association with the ***KRAS***, ***NRAS***, and ***BRAF*** somatic variants commonly observed in CRC. In this study, we enrolled plasma and FFPE tissue samples of 50 advanced CRC patients and plasma samples of 31 individuals in the control group of similar ages. Then, we performed plasma extraction, total RNA isolation, cDNA synthesis, and circulating *lncRNA* expression analysis, respectively. Also, we carried out ***KRAS, NRAS***, and ***BRAF*** somatic variant analysis from FFPE tissues. Our results showed that the expression level of *CRNDE* (p= 0.002) were significantly upregulated in the CRC when it was compared to with the control group. The calculated area under the curve of the receiver operating characteristic was 0.70. We found a statistically significant difference between the ***KRAS*** somatic variants and the circulating *CRNDE lncRNA* (p= 0.031). This study demonstrated that *CRNDE* circulating *lncRNAs* may be used as a potential non-invasive biomarker in CRC. In our study, it was determined that there is a significant relationship between the frequently observed ***KRAS*** somatic variants and *CRNDE* in advanced-stage CRC cases. To the best of our knowledge, this is the first study to show the association of *CRNDE* circulating *lncRNA* with advanced CRC. Moreover, it is the first study to show a relationship between the ***KRAS*** somatic variants between circulating *CRNDE* lncRNA in advanced CRC.

Keywords: *CRNDE*, Circulating *lncRNA*, ***KRAS***, CRC, RT-PCR

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world, also is the second leading cause of cancer-related death.[1,2] CRC is a multifactorial disease, the development of which is accompanied by environmental and genetic factors. Geographical and ethnic differences are effective in the development of CRC.[3] It has a higher incidence in men than in women, and the incidence rate in the most developed countries is 3-4 times higher than in developing countries.[4] Approximately 60-65% of cases occur sporadically, and 90% are 50 years of age or older at the time of diagnosis. The localization of CRCs is as follows; approximately 45-60% in the rectosigmoid colon, 15-30% in the ascending colon and cecum, 5-10% in the transverse colon, and 10-15% in the descending colon.[3] CRC develops through a series of events that lead to the transformation of normal mucosa into adenoma and then carcinoma. This gradual process in colorectal cancer formation is defined as the "Adenoma-Carcinoma Sequence".[5]

One of the signaling pathways that are effective in the pathogenesis of CRC is the RAS-RAF-MAPK pathway. Variants in the *KRAS* gene are an important factor in the transition from the early adenoma stage to the late adenoma stage in sporadic CRCs.

One of the most common single-gene changes observed at this stage are variants in the *KRAS* gene. It has been suggested that activated, *KRAS* may play an important role in the transition from adenoma to carcinoma through the activation of down-regulated target genes. *KRAS*, a 21 kDa membrane-bound protein involved in signal transduction, is activated in response to extracellular signals. The modified protein remains in an active form continuously due to impaired GTPase activity. More than 90% of the activating variants are located in codons 12 and 13 of exon 2 of the *KRAS* gene. Variants at codon 12 show a more oncogenic phenotype than variants at codon 13. Codon 13 variants are thought to play a more active role in the adenoma-carcinoma transition. It has been reported that codon 12 variants cause local invasion and metastasis of tumor cells. RAS activation affects multiple cellular pathways: growth, differentiation, survival, apoptosis, cytoskeleton organization, cell motility, and proliferation.[6] *NRAS* is a member of the ras family, and mutations in exons 2-4 are observed in 3-5% of CRCs. *BRAF* is from the RAF gene family and is the downstream effector of *KRAS*. It has been reported that mutations in the *BRAF* gene are observed in approximately 10% of CRC adenocarcinomas.[7]

Long non-coding RNAs (*lncRNA*) are heterogeneous ncRNAs ranging in size from 200 bp to 100 kbp. *LncRNAs* are involved in the regulation of numerous biological processes, including the cell cycle, apoptosis, histone modifications, chromosome imprinting, and cell differentiation.[8] *CRNDE* (Colorectal Neoplasia Differentially Expressed) is a *lncRNA* localized next to the IRX5 gene on chromosome 16q12. *CRNDE* has been identified as an oncogene in some solid cancers, including CRC, glioma, breast cancer, and hepatic carcinoma. It has been notified to be associated with promoting cell proliferation, migration, and invasion in tumor cells.[9]

Metastases are present at the time of the first diagnosis in 20-25% of patients with colon cancer and 18% of patients with rectal cancer.[1] However, the clinical significance and molecular mechanisms of *lncRNA*s in the progression of CRC are not fully understood. For this reason, every study aimed at elucidating the CRC mechanism gains importance. We aimed to examine the relationship between plasma *CRNDE* expression level and *KRAS*, *NRAS*, and *BRAF* somatic variants in patients with advanced CRC in this study.

MATERIALS and METHODS

Study Population

Plasma and FFPE tissue samples of 50 advanced stage (stage III and IV) patients who were followed up with a diagnosis of colon or rectum cancer in the Department of Medical Oncology between February 2007 and April 2021 were included in this study. Plasma samples of 31 individuals were included in the study as a control group. Patients in the control group were selected from a colonoscopy cohort. Patients did not undergo colonoscopy for study purposes. Ethics committee approval was obtained for the study with the number 2019-349. Informed consent was obtained from each participant in this study. This study was conducted by the principles of the Helsinki Declaration.

Plasma and Total RNA Isolation

Peripheral blood samples were collected from each participant in the study. Blood samples were centrifuged within 2 h after collection at 3000 rpm for 15 min at +4^{0}C, followed by 3000 rpm for 10 min at +4^{0}C, and plasma was separated. The supernatant plasma was stored at –80^{0}C until analysis. Total RNA was isolated from 700 μl plasma by Trizol reagent according to the protocol of the manufacturer's instructions (A.B.T. Blood/Tissue RNA Purification Kit for Leukemia, Atlas Biotechnology Laboratory Materials Industry and Trade Ltd. Şti, Ankara, Turkiye) and stored at –80^{0}C until analysis. The purity and concentration of RNA were assessed using the Qubit device (Qubit 3 Fluorometer, Invitrogen, by Thermo Fisher Scientific, Malaysia).

cDNA Synthesis from Total RNA

Total RNA was reversely transcribed and cDNAs were synthesized using a reverse transcription kit (RevertAid H Minus First Strand cDNA Synthesis Kit; Thermo Fisher Scientific Baltics UAB, Vil-

Table 1. Primer sequences for amplification of *CRNDE lncRNA* and *Beta-actin*

Gene	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')
CRNDE	TTTCCGGAGTAGAGCCCTTG	CTCCTCCTTCCAATAGCCAGT
BETA-ACTIN	GCCAACTTGTCCTTACCCAGA	AGGAACAGAGACCTGACCCC

nius, Lithuania) according to the manufacturer's instructions. Then, incubation period (including 5 min at 65^{0}C), cDNA was synthesized with the following program of 5 min at 25^{0}C, 60 min at 42^{0}C, and 5 min at 70^{0}C. cDNA Synthesis reactions were completed in a Thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Singapore)

Circulating CRNDE lncRNA Expression Analysis

RT-PCR was performed using CFX96 real-time PCR systems (BIO-RAD, C1000 Touch Thermal Cycler, Bio-Rad Laboratories, Inc, California, USA). SYBR Green Master Mix (Thermo Scientific™ Maxima SYBR Green/ROX qPCR Master Mix (×2), Thermo Fisher Scientific Inc, California, USA) was carried out for PCR amplification. The real-time PCR amplification mix was incubated at 95^{0}C for 10 min for Taq activation, followed by 95^{0}C for 15 sec for denaturation and 60^{0}C for 40 sec annealing and extension during 40 cycles. The β-*actin* gene was used as an endogenous control. The *CRNDE lncRNA* and β-*actin* were investigated using a qRT-PCR technique by *lncRNA*-specific oligonucleotide primers (Table 1). The specificity of the primer was confirmed by melting curve analysis. Each sample was evaluated for *CRNDE* circulating *lncRNA* and β-*actin* housekeeping gene, and each was studied in duplicate and the mean Ct (threshold cycle) data for patients and control group were realized using cycle threshold settings. A comparative CT method (2-ΔΔCt) was used to calculate *CRNDE lncRNA* expression in plasma of CRC patients samples normalized to β-*actin* expression and relative to healthy controls.

DNA Isolation from FFPE Tissue

DNA isolation was carried out from paraffin tumor tissue sections (FFPE) taken from patients (QIAamp DNA FFPE Tissue Kit-50 Test, QIAGEN, Hilden, Germany)

K*RAS, NRAS, and, BRAF Somatic Variant Analysis from FFPE Tissues*

KRAS, *NRAS*, and *BRAF* somatic variants were evaluated using commercial kits EntroGen RAS Mutation Screening Panel Real-Time PCR(EntroGen) and EntroGen *BRAF* Mutation Screening Panel Real-Time PCR (Entrogen).The concentrations of DNA samples used for the study were set between 10-30 and 10-20 ng for *KRAS*/*NRAS*, and *BRAF*, respectively. Positive and Negative Control was also used in each reaction. Real-Time PCR program for *KRAS*/*NRAS* variants 95^{0}C 10 min, 95^{0}C 15 sec and 60^{0}C 60 sec 40 cyles and for *BRAF* variants 95^{0}C 10 min, 95^{0}C 15 sec and 60^{0}C 30 sec 40 cycles applied to be. *GAPDH* was used as an endogenous control for all somatic variants.

Statistical Analysis

All data were statistically analyzed and using the Statistical Package for the Social Sciences (SPSS) 21.0 software (SPSS Inc., Chicago, IL). Summary values for qualitative variables are shown as frequency and percentage, and for quantitative variables as mean±standard deviation or median (Q1-Q3). The conformity of quantitative variables to normal distribution was evaluated with the Shapiro-Wilk test. The comparison of the two groups was made with the t-test for normally distributed variable and with the Mann-Whitney U-test for non-distributed variables. The relationship between qualitative variables was evaluated with Chi-square analysis. Areas under the curve (AUC), sensitivity, and specificity values of IncRNA expression, which are recommended to be used in distinguishing between patient and control groups, were evaluated with Receiver Operating Characteristic (ROC) analysis. P< 0.05 was considered significant. This work received ethics committee approval (Eskişehir Osmangazi University Non-

Table 2. Clinical characteristics of patients with colorectal cancer and of control group

Variable	Parameter	Patient Group n (%)	Control Group (n) (%)	Mean±SD	Median (25%-75%)	p
Gender	Male	34 (68%)	16 (51.6%)			
	Female	16 (32%)	15 (48.4%)			
Age (Years)	≤ 50	6 (12%)	4 (12.9%)			
	> 50	44 (88%)	27 (87.1%)			
Diagnosis age (Years)	≤ 50	7 (14%)				
	> 50	43 (86%)				
TNM stage	III	6 (12%)				
	IV	44 (88%)				
Tumor grade	Middle differentiation	45 (90%)		18.09±20.99	18.50 (4.74±32.32)	
	Well differentiation	4 (8%)		-2.21±29.03	-4.92 (-21.59±13.01)	0.023***
	Poor differentiation	1 (2%)				
Histological type	Adenocarcinoma	47 (94%)				
	Adenocarcinoma with a mucinous component	2 (4%)				
	Stone ring component adenocarcinoma	1 (2%)				
Diagnosis	Colon	29 (58%)				
	Rectum	21 (42%)				
Metastasis site	Lung	20				
	Liver	25				
	Lymph Node	17				
	Bone	6				
	Peritonitis	3				
	Karsinomatoza	1				
	Brain	9				
	Metastasis	0				
Lymphovascular Invasion (LVI)	Presence	26 (52%)				
	Absence	21 (42%)				
	Unknown	3 (6%)				
Perineural Invasion (PNI)	Presence	14 (28%)				
	Absence	33 (66%)				
	Unknown	3 (6%)				
Localization of the primary tumor	Right colon	13 (26%)		3.84±26.14	10.38(-9.14-21.80)	0.009**
	Left colon	37 (74%)		20.17±19.58	19.64(4.47±32.73)	
Primary tumor operation	Yes	38 (76%)				
	No	12 (26%)				
Tumor size	≤ 5 cm	46 (92%)		18.15±19.72	17.68 (4.13±32.32)	0.010**
	> 5 cm	4 (8%)		-6.22±36.10	-6.09 (-21.59±21.81)	
Response to treatment	Complete Response	------				
	Partial Response	42 (84%)				
	Stable Disease	5 (10)				
	Progress	2 (4%)				
	Unknown	1 (2%)				
Neoadjuvant Therapy		No	21 (42%)	14.01±29.92	13.01 (1.31±32.70)	0.571**
		Yes	29 (58%)	17.24±20.79	18.19 (4.98±32.11)	
Survive	Alive	45 (90%)				
	Death	5 (10%)				

The relationship between CRNDE expression level and clinical parameters is shown. as median and percentile (Q1-Q3).
SD: Standart Deviation. * p< 0.05 was considered significant , ** Student's t test, *** Mann-Whitney U test

Table 3. Frequencies of common somatic variants in the *KRAS, NRAS*, and *BRAF* genes

***KRAS-NRAS-BRAF* somatic**	**n**	**%**
***KRAS* mutations total**	17	34
Codon 12-13	11	22
Codon 61	3	6
Codon 146	3	6
***NRAS* mutations total**	6	12
Codon 61	6	
***BRAF* mutations total**	3	6
Codon 600 (V600D)	3	
Negative	24	48

Interventional Research Ethics Committee; Date: December 10, 2019; No= 2019-349). A signed and written informed consent form was obtained from the individuals in the patient and control groups.

RESULTS

Association between Plasma Expression Levels of CRNDE lncRNA and Clinicopathological Characteristics

We have analyzed the relationship between expression levels of *CRNDE lncRNA* and clinicopathological features.

When we evaluated circulating *CRNDE lncRNA* in terms of clinical parameters, it was found that there was a correlation between tumor differentiation (p= 0.023), primary tumor location (p= 0.009), and tumor size (p= 0.010) with *CRNDE lncRNA* expression level. No significant correlation was found between *CRNDE lncRNA* expression and neoadjuvant therapy (p= 0.571) (Table 2).

Association between Plasma Expression Levels of CRNDE lncRNA and the most common somatic variants in CRC

The clinical characteristics of the cases are summarized in Table 2. The presence of *KRAS*, *NRAS*, and *BRAF* somatic mutations was evaluated in the advanced stage patient group with CRC. *KRAS* variant was seen in 34% of the cases, *NRAS* variant in 12%, *BRAF* variant in 6% of the cases, and no somatic change was observed in 48% (Table 3). *KRAS* variant was observed most frequently in codon 12-13 and codon 61 in 50 cases in the advanced stage group. When the relationship between the *CRNDE* circulating *lncRNA* expression level and the frequently observed somatic variants in the *KRAS*, *NRAS*, and *BRAF* genes was evaluated in the study, a statistically significant difference was found between the *KRAS* somatic variants and the *CRNDE lncRNA* (p= 0.031) (Table 4).

Evaluation of CRNDE circulating lncRNA Expression Level in Patients with advanced CRC

The difference in expression between advanced-stage patients with CRC and the control group was investigated. The data obtained as a result of the RT-PCR expression analysis were statistically evaluated with the Mann- Whitney U- test. As a result of the analysis, a statistically significant difference was found between the *CRNDE* circulating *lncRNA* between the patient and control groups (p= 0.002) (Figure 1) (Table 5).

ROC and AUC Analyzes of Circulating CRNDE lncRNA

ROC and AUC analyses were performed in terms of *CRNDE* circulating *lncRNA* in the advanced CRC patients (n= 50) and control (n= 31) groups.

Table 4. The relationship between the presence of *KRAS* somatic variant and *CRNDE* lncRNA

	KRAS variant positive (n= 17)		**KRAS variant negative (n= 33)**		
Circulating lncRNA	**Mean**	**Standard deviation**	**Mean**	**Standart deviation**	**p**
CRNDE	8.9010	9.7280	19.6629	20.4280	0.031*

Analyzed by Student's t test. * p< 0,05 was considered significant

Table 5. Comparison of patient and control group expression levels is shown as median and percentile (Q1-Q3)

	Control Group		Patient Group							
Circulating lncRNA	Mean±SD	Median (25%-75%)	Mean±SD	Median (25%-75%)	P	Sensitivity	Specificity	AUC	95% CI	p
CRNDE	2.80±21.40	0.00 (0.00-9.26)	15.89±22.47	17.39 (3.55-32.11)	0.002*	81.5	64.5	0.70	0.586-0.814	0.002*

Analyzed by Mann-Whitney U test. ROC and AUC values of circulating lncRNA genes examined in CRC patient and control groups.
*CI: Confidence interval; AUC: Area Under the Curve; SD: Standart Deviation; * p< 0.05 was considered significant.*

According to these analyzes, the sensitivity and specificity values of the test were calculated to use the relevant circulating *CRNDE lncRNA* as a biomarker (Table 5) (Figure 2).

DISCUSSION

CRC is one of the most common types of cancer. The majority of cases are metastatic at the time of diagnosis. One of the effective pathways in metastasis formation in CRC is the RAS/RAF/MAPK pathway. *KRAS*, *NRAS* and *BRAF* genes are among the genes that are effective in the pathogenesis of CRC in this pathway. There are studies investigating the effect of circulating *lncRNA*s in the etiopathogenesis of CRC.[10] In our study, we aimed to examine the relationship between variations in *KRAS*, *NRAS*, and *BRAF* genes and *CRNDE* circulating *lncRNA*. Thus, we believe that we will contribute to elucidating the molecular mechanism of CRC etiopathogenesis.

CRNDE has been identified as a *lncRNA* overexpressed in colorectal cancers.[11] It has been shown to promote proliferation, migration, and invasion. It has been shown to exhibit a high expression profile, especially in cancer types such as CRC, gastric cancer[12], glioma[13], and hepatocellular carcinoma[14]. We investigated the relationship between plasma *CRNDE* expression level and *KRAS*, *NRAS*, and *BRAF* somatic variants in patients with advanced CRC in this study.

As a result of the meta-analysis by Wang et al., in breast, colorectal, gastric, glioma, liver, lung, multiple myeloma, and pancreatic several human cancers, it was reported that *CRNDE* exhibits an upregulated expression profile. In addition, it was observed that *CRNDE* acts as an oncogene, and its overexpression profile reduces overall survival and has poor prognostic effects in these studies.[11]

In some studies with CRC cancer cells and tumor tissue samples, the *CRNDE* was found to be overexpressed and, it was shown that *CRNDE* inhibited Wnt/β-catenin signaling by promoting the expression of miR-181a-5p in the development of CRC. Besides, it has been suggested that *CRNDE* plays an important role in the CRC signaling pathway in CRC tissues and may be an independent clinical marker in the prognosis and treatment of CRC.[15]

Liu et al. showed that *CRNDE* was highly expressed in both sample groups as a result of their analysis of exosomes in serum samples of 148 CRC cases and tumor tissue samples of 142 CRC cases. Exosomal *CRNDE* has been reported to be associated with aggressive tumor behavior and poor prognosis.[10] They observed that ovarian expression of *CRNDE* was associated with tumor size, regional lymph node metastasis, and distant metastasis. They indicated that high expression of *CRNDE* was positively correlated with IRX5 mRNA in CRC tissues. They suggested that *CRNDE* may play an important role in the development and progression of CRC and can be used as a biomarker.[16]

James de Bony et al. found 282 associated loci in their microarray study with 566 patients with CRC. After that, they evaluated the possible functions of these genes in the effective signaling pathways (WNT/β-catenin, TGF-β, and EMT) in CRC. lncmulti *POTEM-2* was found to be upregulated in TP53-mutant tumors but downregulated in *KRAS*-mutant tumors. They also showed that lnc *ITGB8-4* was highly down-regulated and *lncRNA HAGLR* was highly up-regulated when a *BRAF* mutation was present.[17]

In another study, the role of *IncRNA SLCO4A1-AS1* was investigated in colorectal cancer. Researchers showed that *SLCO4A1-AS1* acts as an oncogene, promoting the development of CRC, and is asso-

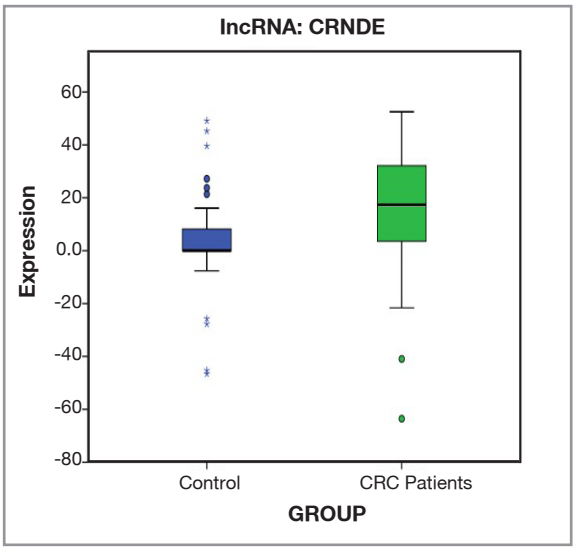

Figure 1. Differential expression of plasma CRNDE lncRNA. The mean expression levels of CRNDE lncRNA in CRC plasma samples compared with the control group plasma samples. Relative gene expression was calculated using the 2-ΔΔCt method

CRC: Colorectal cancer; lncRNA: long non-coding RNA

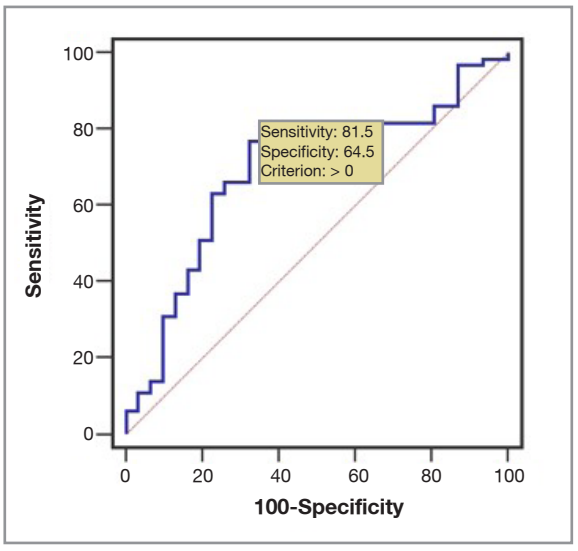

Figure 2. Receiver operating characteristic (ROC) curves for evaluating the diagnostic power of lncRNA CRNDE. ROC curve and the area under the curve were calculated to determine the sensitivity and specificity CRC patients as a clinical biomarker

ciated with the EGFR/MAPK pathway. They suggested *SLCO4A1-AS1* may become a novel biomarker or therapeutic target for colorectal cancer in the future.[18]

In present study, we have determined circulating *CRNDE lncRNA* differentially expressed in plasma of the CRC patient group. It was upregulated by an average of 0.008-fold in the CRC patient group with a statistically significant difference in the patient group (p= 0.002). In parallel with other studies, our data showed a strong correlation between the expression level of *CRNDE* and tumor size, tumor differentiation, and primary tumor location. Our data are compatible with the literature and support the knowledge that circulating *CRNDE* is may be effective in processes such as proliferation, migration, invasion, and suppression of apoptosis. Our results support the circulating *CRNDE lncRNA* may be used as a noninvasive prognostic biomarker and a potential clinical target for therapy in CRC patients.

We investigated the most common *KRAS*, *NRAS*, and *BRAF* somatic variants with advanced CRC. Among the *KRAS* variants, the frequency of codons 12-13 and 61 variants was higher. A statistically significant difference was found between circulating *CRNDE lncRNA* plasma expression level and *KRAS* somatic variants (p= 0.031). These findings support the hypothesis that circulating *lncRNA*s may act on somatic variants of *KRAS* and have a role in the development of tumorigenesis.

In conclusion, in our study, it was determined that there is a significant relationship between the frequently observed *KRAS* somatic variants and *CRNDE* in advanced-stage CRC cases. The findings revealed that there may be a relationship between somatic variants commonly observed in CRC and circulating *lncRNA*s. This suggests that circulating *lncRNA*s may be effective on somatic variants. Our results support the circulating *CRNDE lncRNA* may be used as a noninvasive prognostic biomarker and a potential clinical target for therapy in CRC patients.

We think that our results will contribute to the literature. To the best of our knowledge, this is the first study to show the association of *CRNDE* circulating *lncRNA* with advanced CRC. Moreover, it is the first study to show a relationship between the KRAS somatic variants between circulating *CRNDE lncRNA* in advanced CRC. More studies are needed to understand the molecular mechanisms of circulating *lncRNA*s.

Limitations of the study: There are some limitations of our study. The limitations of the study are the relatively small population of the patient group studied, and the absence of all gene analyses in terms of only common variants in the *KRAS*, *NRAS*, and *BRAF* genes.

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