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Original Article

Combined identification of long non-coding RNA CCAT1 and HOTAIR in serum as an effective screening for colorectal carcinoma

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Abstract: Long non-coding RNAs (lncRNAs) CCAT1 and HOTAIR have been shown to play an important regulatory role in cancer biology, and CCAT1 and HOTAIR are upregulated in several cancers, however, its value in the diagnosis of colorectal cancer (CRC) is unclear. Therefore, the aim of this study is to evaluate the clinical significance of plasma CCAT1 and HOTAIR as a biomarker in the screening of CRC. In our study, we found that the levels of HOTAIR ($P < 0.05$) and CCAT1 ($P < 0.05$) were significantly higher in plasma of CRC patients than that of the healthy control. Moreover, the levels of lincRNA-p21 ($P < 0.05$) were obviously decreased in plasma of CRC patients as compared to those of healthy control. There was highly correlated for CCAT1 ($R = 0.752$, mean differences = -0.06 ± 1.20), HOTAIR ($R = 0.739$, mean differences = -0.26 ± 0.76) and lincRNA-p21 ($R = 0.848$, mean differences = -0.41 ± 0.89) in plasma and serum. By receiver operating characteristic curve (ROC) analysis, plasma CCAT1 provided the higher diagnostic performance for detection of CRC (the area under the ROC curve (AUC), 0.836; $P < 0.001$; sensitivity, 75.7%; specificity, 85.3%). Moreover, CCAT1 combining with HOTAIR could provide a more effective diagnosis performance (AUC, 0.954, $P < 0.001$, sensitivity, 84.3%; specificity, 80.2%). Most importantly, this combination was effective to detect CRC at an early stage (85%). In conclusion, our results demonstrated that increased plasma HOTAIR and CCAT1 could be used as a predictive biomarker for CRC screening, and that combination of HOTAIR and CCAT1 had a higher positive diagnostic rate of CRC than HOTAIR or CCAT1 alone.

Keywords: Colorectal carcinoma, long non-coding RNA, CCAT1, HOTAIR, tumor biomarker

Introduction

Colorectal cancer (CRC) is the third most common cancer and accounts for approximately 8%, more than 600,000 deaths of all cancer deaths, meanwhile around 1.2 million new cancer cases are diagnosed each year in the worldwide [1, 2]. In China, the incidence and death rates of CRC have been rapidly increasing over the last few years, and the rates are much higher than the worldwide average [3]. In recent years, there are mounting progresses in clinical treatment for CRC, but the overall survival time of CRC patients have not improved dramatically. An important reason for that is the lack of molecular biomarkers. Recent biomarkers such as human cartilage glycoprotein 39 (YKL-40) [4], carbohydrate antigen 72-4 (CA72-4) [5] and carcino-embryonic antigen (CEA) [6] are the classic tumor markers commonly used in the management of patients with CRC. However, these tumor markers have limited utility in the early detection of CRC due to lack of sufficiently high diagnostic sensitivity and specificity. Therefore, the significance of exploration of new biomarkers with high sensitivity and specificity in early detection of CRC should be emphasized.

Eukaryotic genomes encode numerous long non-coding RNAs (also known as lncRNAs), which are defined as endogenous cellular RNAs with length longer than 200 nucleotides, but lack open reading frames of significant length [7]. Within 4 years, the number of identified lncRNA genes increase more than 8000, and

Table 1. Summary of CRC-related LncRNAs in human

Symbol	Expression levels	Chromosome Location	Start	End	Species
H19	undifferentiated	Chr 11	2016406	2019065	Human
HOTAIR	upregulated	Chr 12	54356096	54362515	Human
MALAT1	undifferentiated	Chr 11	65265233	65273940	Human
MEG3	undifferentiated	Chr 14	101292445	101327363	Human
CCAT1	upregulated	Chr 8	128219629	128231724	Human
CRNDE	upregulated	Chr 16	54952775	54963079	Human
HULC	upregulated	Chr 6	8652442	8654079	Human
KCNQ1OT1	upregulated	Chr 11	2661768	2721228	Human
lincRNA-p21	downregulated	Chr 17	29057474	29078961	Human
NPTN-IT1	downregulated	Chr 15	73859365	73861635	Human
SNHG16	downregulated	Chr 17	76557764	76565348	Human
ATB	upregulated	N/A	N/A	N/A	Human
LSINCT5	upregulated	N/A	N/A	N/A	Human

estimates suggest the number of human lncRNAs ranging from 10,000 to 20,000 [8, 9]. Although the function of most lncRNAs are still unknown, increasing numbers and accumulating evidences indicate that lncRNAs have been correlated to cancer development, invasion and metastasis in the malignant cell, including CRC [10]. Mounting evidences have showed that circulating lncRNA in plasma, serum or urine has been an emerging field for noninvasive diagnostic applications [7, 11]. For example, plasma lncRNA-POU3F3 can serve as a potential biomarker for diagnosis of esophageal squamous cell carcinoma (ESCC), in particular for early tumor screening [7]. LncRNA-MALAT1 has been found to be significantly upregulated in plasma and urine of prostate cancer patients and can be used to discriminate cancer patients from healthy controls [11, 12]. In gastric cancer patients, the levels of lncRNA-AA174084 in plasma are markedly lower on day 15 after surgery than preoperative level [13]. A similar study has found that plasma lncRNA-H19 levels are significantly higher in gastric cancer patients than in healthy controls and reduced in postoperative patients [14]. However, for all we know, no literature has been reported regarding the circulating lncRNAs for early screening of CRC patients.

LncRNA colon cancer-associated transcript 1 (CCAT1) is a recently discovered 2628 nucleotide lncRNA, which is located in the chromosome 8q24.21 and described as a "hot spot" with many genetic alternations in colon cancer [15, 16]. Previous studies show that CCAT1 is significantly upregulated in tumor tissues of colon cancer patients compared with the healthy human tissues and promotes colon cancer cell proliferation and invasion [16, 17]. In this study, by using lncRNA and disease database (http://210.73.221.6/lncrnadisease), other 12 lncRNAs, including H19, HOTAIR, MALAT1, MEG3, CRNDE, HULC, KCNQ1OT1, lincRNA-p21, NPTN-IT1, SNHG16, ATB and LSINCT5, were previously reported abnormal expression and selected as candidate diagnostic biomakers in colorectal cancer [18-30]. We were examined the expression levels of lncRNAs in plasma and serum, and their potential use as tumor biomarkers for CRC detection were evaluated. We hypothesized that these CRC-related lncRNAs might be released into the circulation during CRC initiation and could be utilized to detect and screen CRC.

Materials and methods

Patients and specimens

Thirty-two CRC tissues and matched adjacent non-tumor tissues were collected from the Second and Third Affiliated Hospital of Xinjiang Medical University between Jan 2012 and June 2014. All patients recruited in this study were not subjected to preoperative radiotherapy or chemotherapy and diagnosed with CRC based on histopathological evaluation. All collected tissue samples were immediately stored at liquid nitrogen until use. Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics

Table 2. The PCR primers used in this study

Symbol	Forward (5'-3')	Reverse (5'-3')
H19	CTCCACGACTCTGTTTCC	CTCCACGACTCTGTTTCC
HOTAIR	AATAGACATAGGAGAACACTT	AATCTTAATAGCAGGAGGAA
MALAT1	CCGCTGCTATTAGAATGC	CTTCAACAATCACTACTCCAA
MEG3	TGGCATAGAGGAGGTGAT	AGACAAGTAAGACAAGCAAGA
CCAT1	CATTGGGAAAGGTGCCGAGA	ACGCTTAGCCATACAGAGCC
CRNDE	TGGATGCTGTCAGCTAGTTCAC	TTCCAGTGGCATCCTCCTTATC
HULC	TCAGAGTTCCTGCATGGTCTGGTTC	GTTCCTGCATGGTCTGGTTCTCGTG
KCNQ1OT1	GCATATCTGTCTTCCGTAT	CCTCTTCCTTCGTTCAAT
lincRNA-p21	CCCGGGCTTGTCTTTTGTT	GAGTGGGTGGCTCACTCTTCTG
NPTN-IT1	GACCGGTGCGAAGTGGCTTC	GTTCCCGACGGACCTTGCGAG
SNHG16	GGCCGATGAGATGTGTAT	CCTGATGTGAGCGTAGCTGT
ATB	CCGATGGATGCGTGAGCTTT	GAGAGTGTAGATGCTGCAG
LSINCT5	TAGACAACTTACTTAACCTCAT	TCCTTATCCACCTTATCCA
GAPDH	ATGGTGAAGGTCGGTGTGA	CCATGTAGTTGAGGTCAATGAG

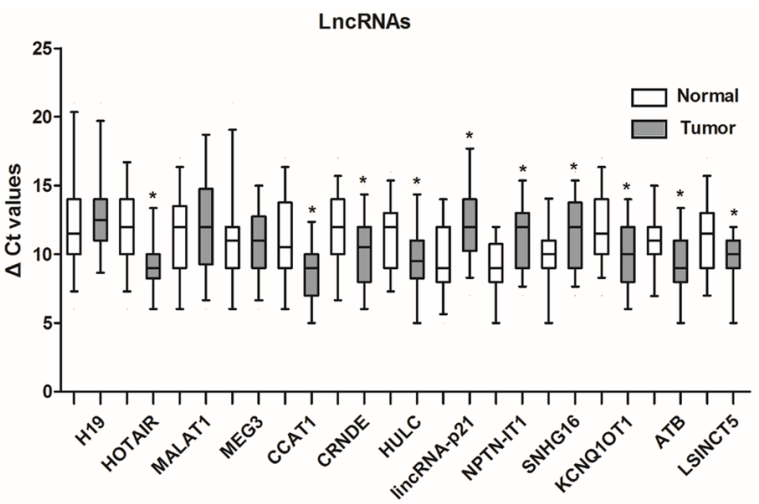

Figure 1. Identification of tumor tissues-enriched lncRNA implicated in CRC patients. 13 CRC-related lncRNAs are filtered from lncRNA and disease database. Real-time PCR analysis is performed to determine the expression levels of lncRNAs in 32 pairs of CRC tumor tissues and corresponding non-tumourous specimens. ΔCt method is used to calculate lncRNA expression, which is normalized to GAPDH, and smaller ΔCt value indicated higher expression. Values are expressed as mean ± SD, $^{*}P < 0.05$ versus non-tumourous group.

Committee of the Xinjiang Medical University, China.

Real-time PCR

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reaction mixture (20 μl) containing 2 μg of total RNA was reversely transcribed to cDNA by using PrimeScript RT-polymerase (Takara, Dalian, China). Quantitative PCR was performed on the cDNA using specific primers (Sangon, Shanghai, China). The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). Glyceraldehyde-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the expression levels of lncRNAs were calculated using ΔCt method, where $\Delta Ct = Ct_{target}-Ct_{reference}$, smaller ΔCt value indicates higher expression. Relative expression of lncRNAs was calculated using $2^{-\Delta\Delta Ct}$ method normalized to endogenous control, with $\Delta Ct = Ct_{target}-Ct_{reference}$, $-\Delta\Delta Ct = -(\text{sample } \Delta Ct - \text{control } \Delta Ct)$. All the primers used in the present study were listed in **Table 1**.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data were analyzed using independent two-tailed t test. Categorical data were analyzed using the two-

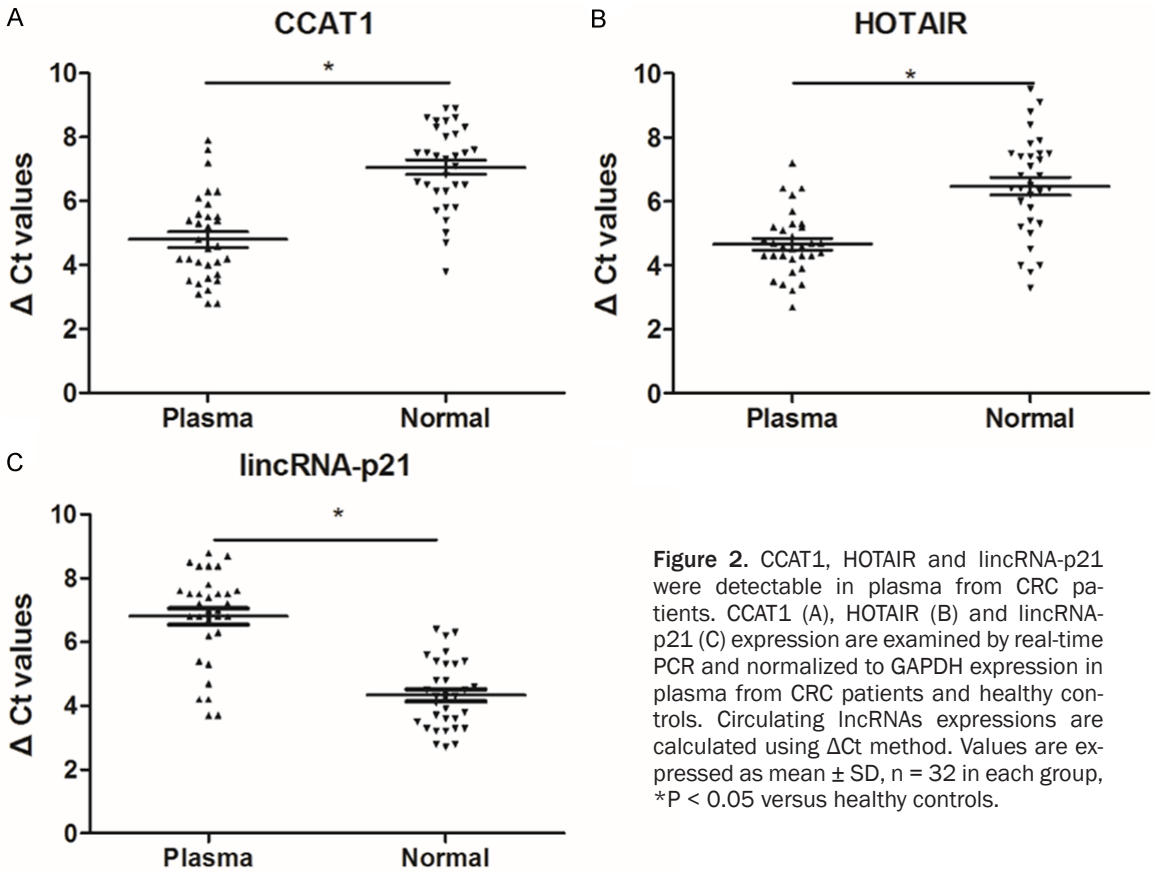

Figure 2. CCAT1, HOTAIR and lincRNA-p21 were detectable in plasma from CRC patients. CCAT1 (A), HOTAIR (B) and lincRNA-p21 (C) expression are examined by real-time PCR and normalized to GAPDH expression in plasma from CRC patients and healthy controls. Circulating lncRNAs expressions are calculated using ΔCt method. Values are expressed as mean ± SD, n = 32 in each group, *P < 0.05 versus healthy controls.

side chi-square test. Overall survival was estimated by using Kaplan-Meier method, and univariate analysis was conducted by log-rank test. The Cox proportional hazards model was used in the multivariate analysis. Values of $P < 0.05$ were considered statistically significant.

Results

Identification of tumor tissues-enriched lncRNA implicated in CRC patients

Based on previous research, by using lncRNA and disease database, 13 lncRNAs, including CCAT1, H19, HOTAIR, MALAT1, MEG3, CRNDE, HULC, KCNQ1OT1, lincRNA-p21, NPTN-IT1, SNHG16, ATB and LSINCT5, were previously reported abnormal expression and selected as candidate diagnostic biomakers in CRC (**Table 2**). To further validated lncRNAs levels in CRC, the real-time PCR analysis was performed to determine the expression level of lncRNAs in 32 pairs of human CRC tissues and corresponding non-tumourous specimens. The results showed that 7 lncRNAs (HOTAIR, CCAT1, CRNDE, HULC, KCNQ1OT1, ATB and LSINCT5) were significantly higher in most of CRC tumor tissues than that observed in pair-matched adjacent non-tumourous tissues (**Figure 1**). Moreover, among them, lincRNA-p21, NPTN-IT1 and SNHG16 were obviously down-regulated in most of CRC tumor tissues as compared to those of non-tumourous group. However, the levels of H19, MALAT1 and MEG3 did not show any significantly different between CRC tumor tissues and corresponding non-tumourous specimens (**Figure 1**).

HOTAIR, CCAT1 and lincRNA-p21 were detectable in plasma

To explore whether these CRC-related lncRNAs could reach the circulation at levels sufficient to be detectable, real-time PCR analysis was performed to determine the expression levels of HOTAIR, CCAT1 and lincRNA-p21 in 32 pairs of CRC tumor tissues and corresponding non-tumourous specimens. As shown in **Figure 2A** and **2B**, the levels of HOTAIR ($P < 0.05$) and CCAT1 ($P < 0.05$) were significantly higher in plasma of CRC patients than that of the healthy control. Moreover, the levels of lincRNA-p21 (P

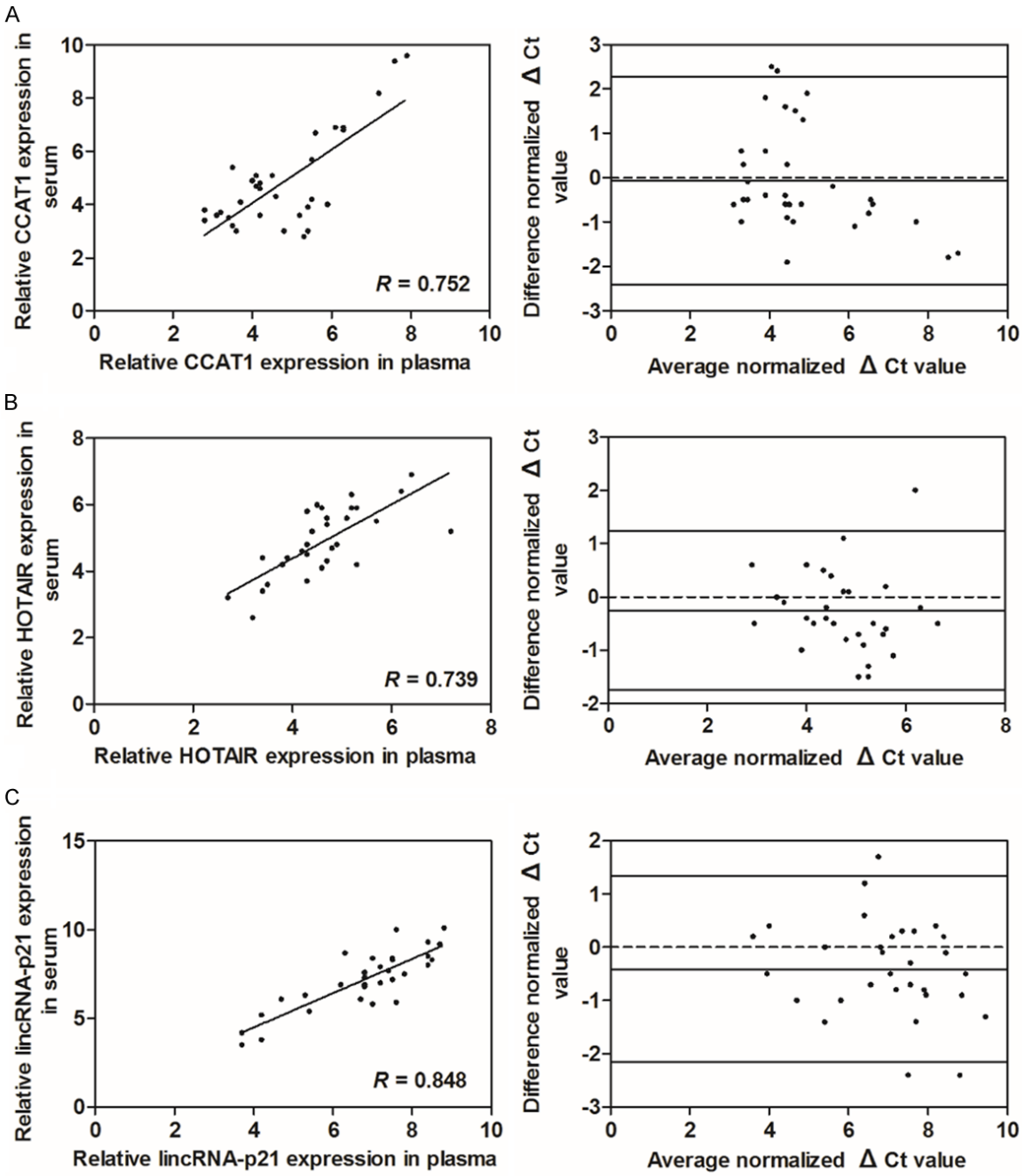

Figure 3. Correlation of lncRNAs levels between plasma and serum in CRC patients. Linear correlation plot of CCAT1 (A), HOTAIR (B) and lincRNA-p21 (C) in plasma and serum (left). There is a high correlation comparing the indicated lncRNAs levels between plasma and serum. Bland-Altman plot of the difference between plasma and serum CRC-related lncRNAs level versus their average. Horizontal solid lines in the middle represent the mean difference. Upper and lower solid lines represent the limits of agreement (95% confidence intervals) (right).

< 0.05) were obviously decreased in plasma of CRC patients as compared to those of healthy control (**Figure 2C**). These results indicated that CRC-related lncRNAs could enter into the circulation, and their differentiated expression in plasma could be used as diagnostic biomarkers for CRC.

Correlation of lncRNAs levels between plasma and serum in CRC patients

To test whether there was a relationship of lncRNAs levels between plasma and serum in CRC patients, CCAT1, HOTAIR and lincRNA-p21 were measured in plasma and serum from the

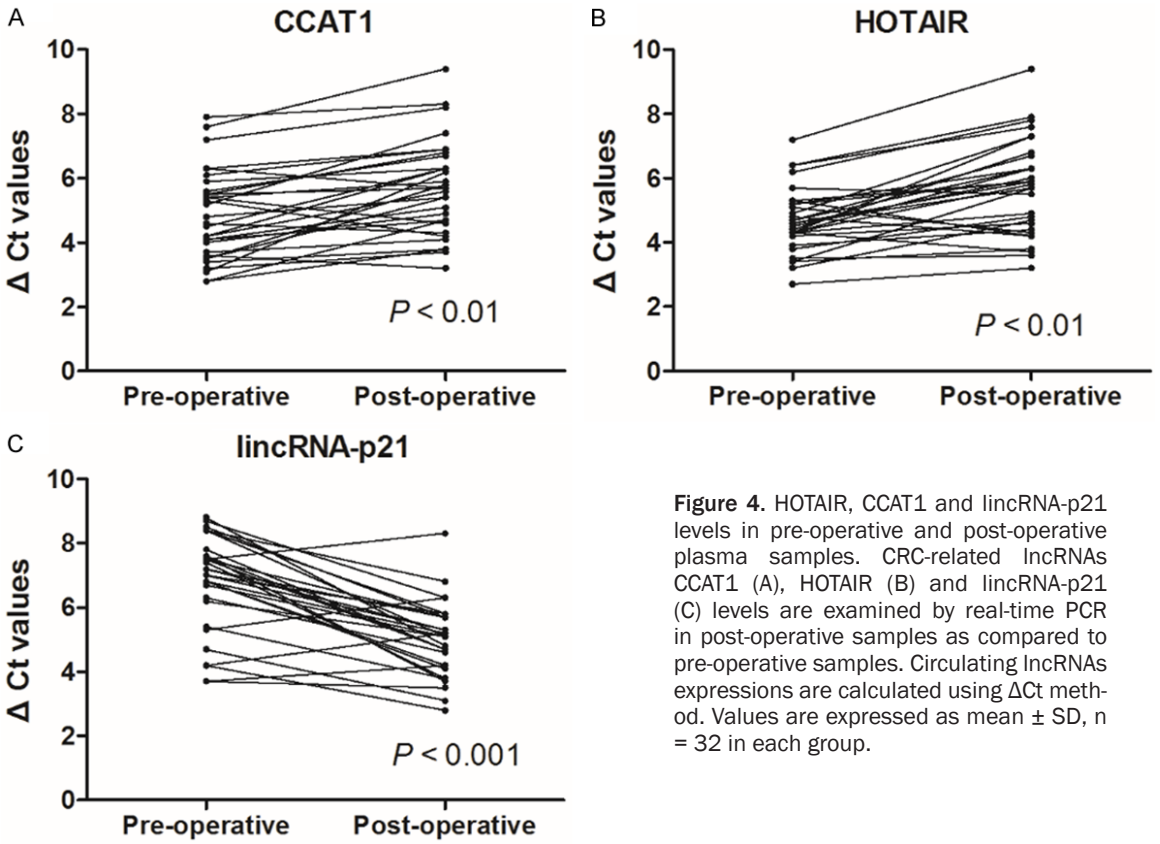

Figure 4. HOTAIR, CCAT1 and lincRNA-p21 levels in pre-operative and post-operative plasma samples. CRC-related lncRNAs CCAT1 (A), HOTAIR (B) and lincRNA-p21 (C) levels are examined by real-time PCR in post-operative samples as compared to pre-operative samples. Circulating lncRNAs expressions are calculated using ΔCt method. Values are expressed as mean ± SD, n = 32 in each group.

same individuals. As shown in **Figure 3A-C**, there was highly correlated for CCAT1 (R = 0.752, mean differences = -0.06 ± 1.20), HOTAIR (R = 0.739, mean differences = -0.26 ± 0.76) and lincRNA-p21 (R = 0.848, mean differences = -0.41 ± 0.89) in plasma and serum.

HOTAIR, CCAT1 and lincRNA-p21 levels in pre-operative and post-operative plasma samples

Since circulating lncRNAs were primarily released or leaked from tumor cells, they would revert to normal after the tumor has been resected. In the present study, the CCAT1, HOTAIR and lincRNA-p21 were carried out to investigate the differences in CRC-related lncRNAs in plasma pro-operative and 14 days post-operative. As expected, serum levels of CCAT1 and HOTAIR were significantly decreased after surgical treatment as compared to pre-operative (**Figure 4A** and **4B**), and lincRNA-p21 was significantly increased after surgical treatment as compared to pre-operative (**Figure 4C**).

Evaluation of HOTAIR, CCAT1 or lincRNA-p21 in plasma as predictive CRC-related biomarkers

To investigate the characteristics of HOTAIR, CCAT1 or lincRNA-p21 as potential biomarkers for CRC, receiver operating characteristics (ROC) curves and the area under the ROC curves (AUC) were performed on data from all subjects, including 32 CRC patients and 32 healthy donors. The ROC curves illustrated strong separation between the CRC patients and control group, with an AUC of 0.836 (95% CI: 0.739-0.934; P < 0.001) for CCAT1, 0.777 (95% CI: 0.663-0.891; P < 0.001) for HOTAIR and 0.886 (95% CI: 0.802-0.970; P < 0.001) for lincRNA-p21 (**Figure 5A-C**). Intriguingly, there is increasing evidence showing that combination several tumor markers could improve diagnostic accuracy. In the present study, we determined whether the combination of HOTAIR and CCAT1 could provide a more effective screening for CRC. The results indicated that combination of HOTAIR and CCAT1 yielded an AUC of 0.954 (95% CI: 0.903-1.000; P < 0.001), which was significantly improved as compared to HOTAIR (AUC = 0.777) or CCAT1 (AUC = 0.836) alone (**Figure 5D**). Another aim of this work was to diagnose CRC patients at as early stage as possible. Therefore, the diagnostic positivity rate between different clinical stages was then investigated. As shown in **Table 3**, the diagnostic positivity rate for CRC in stage I/II for HOTAIR,

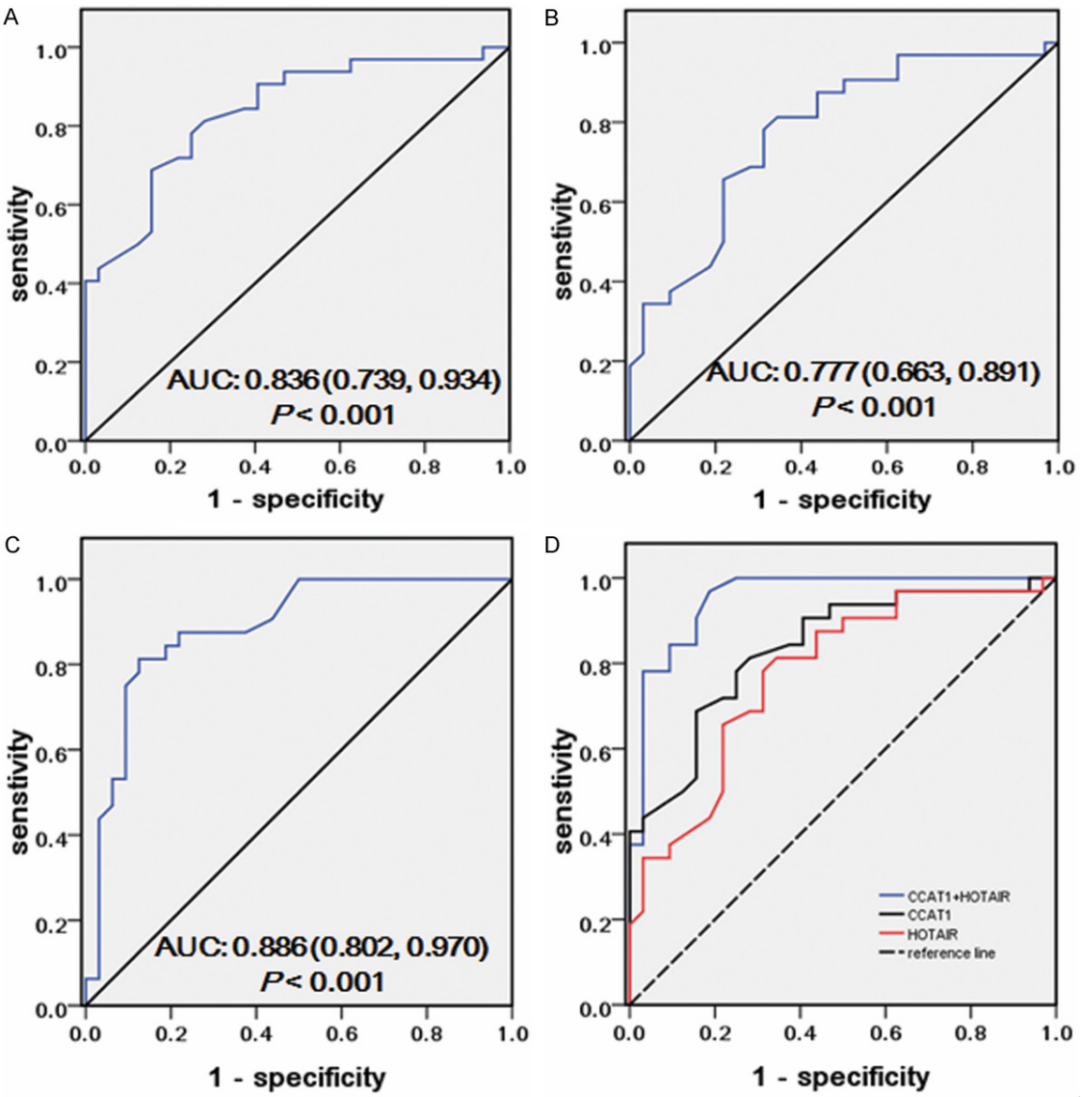

Figure 5. Evaluation of HOTAIR, CCAT1 or lincRNA-p21 in plasma as a predictive CRC-related biomarker. Receiver operating characteristics (ROC) curves are drawn with the data of plasma lncRNAs, CCAT1 (A), HOTAIR (B) and lincRNA-p21 (C), from 32 CRC patients and 32 healthy controls. ROC corves of a combination of HOTAIR and CCAT1 to discriminate CRC from healthy controls (D).

CCAT1 or HOTAIR combined with CCAT1 was 25%, 40%, and 85%, respectively.

Discussion

Recent genome-wide studies have indicated that the mammalian genome is abundantly transcribed and that at least 80% of this transcription is exclusively associated with lncRNAs [31]. So far, numerous studies have indicated that lncRNAs play an important role in tumor occurrence, invasion and metastasis by regulating gene expression as well as signaling pathways [32]. Emerging data strongly implicates that lncRNAs in plasma or serum may be utilized as a tool for cancer diagnosis [7]. However, we found that the effect of lncRNAs in the early diagnosis and prediction of CRC had not been investigated thoroughly. Therefore, we tried to investigate the role of CCAT1 and HOTAIR in the screening of CRC.

In our work, the initial CRC-related lncRNAs screening was performed based on different

Table 3. Performance of CCAT1, HOTAIR and both CCAT1 and HOTAIR in the detection of different clinical stage CRC

	Clinical stage		
	I/II	III/IV	Total
CCAT1	40.0% (8/20)	66.7.0% (8/12)	50.0% (16/32)
HOTAIR	25.0% (5/20)	58.3% (7/12)	37.5% (12/32)
CCAT1 + HOTAIR	85.0% (17/20)	91.7% (11/12)	87.5% (28/32)
P value			
Combination vs. CCAT1	< 0.001	0.042	< 0.001
Combination vs. HOTAIR	< 0.001	0.018	< 0.001

expression profiling between tumor tissues and corresponding non-tumourous specimens, and all lncRNAs of interest were then subjected to real-time PCR validation. The results demonstrated that the levels of CCAT1 and HOTAIR were significantly higher in plasma from CRC patients compared with healthy controls. We used the ROC curve to analyze the diagnostic value of plasma CCAT1 and HOTAIR. The results showed that the individual AUC of CCAT1 and HOTAIR for the diagnosis of CRC were about 0.836 and 0.777, respectively, Moreover, the combination of CCAT1 and HOTAIR could provide a more effective screening for CRC. The results indicated that combination of CCAT1 and HOTAIR yielded an AUC of 0.954, which was significantly improved as compared to CCAT1 (AUC = 0.836) or HOTAIR (AUC = 0.777) alone. These results indicated that CRC-related lncRNAs could be released into the circulation and that their different expression profiles in plasma could be used as diagnostic markers for ESCC. Intriguingly, CCAT1 combining with HOTAIR could provide a more powerful differential diagnosis between CRC patients and healthy controls than use of POU3F3 or SCCA alone. Some classic tumor biomarkers have limited utility in the early detection of CRC [4-6], however, the levels of lncRNA in plasma appear to play an important role in the early screening of CRC. In our study, the results indicated that plasma CCAT1 was more effective than HOTAIR for early detection of CRC (40% vs. 25%), and the combination of CCAT1 and HOTAIR could significantly provide the positivity rate (85%) for CRC screening.

As we know, early discovery, early diagnosis, and early treatment could greatly increase the survival rate of cancer patients. Biomarkers in body fluid have a potential capacity to detect cancers in early stage [32]. Measurements obtained from plasma and serum were strongly correlated for CCAT1 and HOTAIR. The results suggested that circulating lncRNAs were acceptable for CRC screening. HOTAIR as a candidate lncRNA has been shown to play an important modulatory role in the development and progression of cancer, and as a biomarker has been applied to screen gastric cancer [33] and breast cancer [34]. The circulating HOTAIR shows a 2.15-fold change in breast cancer patients compared with healthy controls ($P < 0.0001$, AUC = 0.786), and the optimal cutoff value for diagnosis was 0.30 with sensitivity of 80.0% and specificity of 68.3% [34]. In our study, the levels of HOTAIR showed a 4-fold change in plasma from CRC patients compared with healthy controls ($P < 0.001$, AUC = 0.777). Moreover, plasma level of HOTAIR could discriminate CRC from normal controls with 67.5% sensitivity and 89.9% specificity, and the sensitivity and specificity of CCAT1 for CRC diagnosis were 75.7% and 85.3% respectively. The sensitivity and specificity of combination (SCCA + POU3F3) group for distinguishing CRC from healthy controls were 84.3% and 80.2% respectively.

In conclusion, our results demonstrated that increased plasma HOTAIR and CCAT1 could be used as a predictive biomarker for CRC screening, and that combination of HOTAIR and CCAT1 had a higher positive diagnostic rate of CRC than HOTAIR or CCAT1 alone.

Disclosure of conflict of interest

None.

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