


## RESEARCH ARTICLE

# LncRNAs *GACAT3* and *LINC00152* regulate each other through miR-103 and are associated with clinicopathological characteristics in colorectal cancer

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## Abstract

**Background:** Long non-coding RNAs (lncRNAs) perform pivotal regulatory roles in tumor development. Our previous work revealed that the lncRNA *gastric cancer-associated transcript 3* (*GACAT3*) was significantly overexpressed and associated with tumor size and metastasis in gastric cancer.

**Methods:** Total RNAs were extracted from colorectal cancer (CRC) and reverse transcribed, and then quantitative real-time PCR (qRT-PCR) was conducted. Cell counting was performed to assess the effect of *GACAT3* on CRC cell line proliferation. Bioinformatics prediction, dual luciferase assay, miRNA mimics, siRNAs, and transfection experiments were applied to determine whether *GACAT3* and *LINC00152* are reciprocally regulated by miR-103. The relationship between their expression levels and clinicopathological factors of patients was explored. A receiver operating characteristic (ROC) curve was used to assess the potential diagnostic value of *GACAT3* and *LINC00152*.

**Results:** *GACAT3* was identified to be highly expressed in CRC tissues and associated with cell proliferation. Furthermore, we demonstrated that *GACAT3* acted as a competing endogenous RNA of *LINC00152* and they were both regulated by miR-103. Moreover, analysis of clinicopathological characteristics revealed that *GACAT3* and *LINC00152* were positively correlated with the depth of invasion, TNM stage, lymph node metastasis, and CA19-9 level. Importantly, a combination of *GACAT3* and *LINC00152* showed a superior diagnostic capacity compared with the use of the two molecules alone.

**Conclusion:** Our work shows that *GACAT3* and *LINC00152* are both overexpressed in CRC and they act as a ceRNA network. Therefore, our data suggest that *GACAT3* and *LINC00152* may be a promising potential diagnostic biomarker for CRC.

## KEYWORDS

colorectal cancer, competing endogenous RNA, *GACAT3*, *LINC00152*, lncRNA

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## 1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide and the third leading cause of cancer death in both sexes,<sup>1</sup> generally due to the late diagnosis and recurrence or metastasis of tumor cells.<sup>2</sup> In China, CRC is the fifth leading cause of cancer-related morbidity and mortality with a 5-year survival rate of only 31%.<sup>3,4</sup> Therefore, exploring the key molecules involved in colon cancer progression and demonstrating their functional mechanisms would provide potential diagnostic biomarkers and have a significant impact for CRC therapy.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides without protein-coding ability. lncRNAs are reported to play crucial roles in fundamental biological processes through regulating gene expression.<sup>5</sup> Recently, growing evidence indicates that aberrantly expressed lncRNAs not only have roles in tumorigenesis but also could be potential diagnostic biomarkers,<sup>6</sup> such as *H19* in non-small cell lung cancer,<sup>7</sup> *metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)* in osteosarcoma cells,<sup>8</sup> and *prostate cancer antigen 3 (PCA3)* in prostate cancer.<sup>9</sup> One representative function of lncRNA is that it acts as a microRNA (miRNA) sponge to regulate the stability and/or translation efficiency of other RNAs, and such a lncRNA is called a competing endogenous RNA (ceRNA).<sup>10</sup> Increasing evidence has revealed that lncRNAs act as ceRNAs and possess significant regulatory effects on carcinogenesis and tumor development.<sup>11</sup> For example, *LINC00152* eliminates miR-139-5p-mediated inhibition of NOTCH1 by competitively binding to miR-139-5p, promoting colon cancer cell proliferation and metastasis.<sup>12</sup> *MALAT1* regulates HDAC4-mediated proliferation and apoptosis by acting as a decoy of miR-140-5p in osteosarcoma cells.<sup>8</sup> In previous study, we have identified *GACAT3* as a novel lncRNA in gastric cancer tissues based on a

lncRNA microarray<sup>13,14</sup> and found it to be positively correlated with tumor size, distant metastasis, and tumor-node-metastasis (TNM) stage. Recent research identified *GACAT3* as a downstream target of the IL-6/STAT3 signaling pathway and a ceRNA of *high mobility group protein A1 (HMGA1)* that promotes gastric cancer cell proliferation.<sup>15,16</sup> Moreover, further studies have shown that *GACAT3* participates in the development of breast cancer, non-small cell lung cancer, and glioma.<sup>17-19</sup> Thus, *GACAT3* might have fundamental roles and serve as a potential biomarker in various types of cancer. However, the role of *GACAT3* in colon cancer remains unclear.

In the present study, we demonstrate that *GACAT3* is overexpressed in CRC and plays a role in regulating colon cancer cell growth. Furthermore, *GACAT3* and *LINC00152* are positively correlated and act as ceRNAs, and their expression is associated with clinicopathological characteristics of CRC.

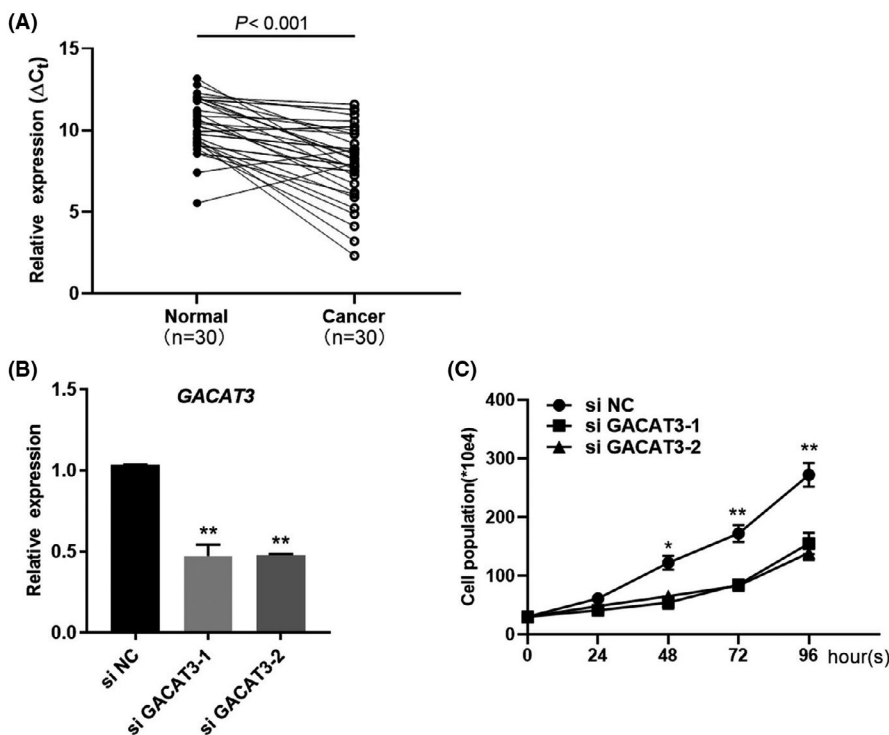
## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

HCT-116 human CRC cells were cultured in RPMI 1640 medium (Corning) supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin-streptomycin (Sigma-Aldrich).

### 2.2 | RNA extraction and reverse transcription

Total RNAs were extracted from cultured cells or tissues using TRIzol reagent (Invitrogen). For RNA extraction from formalin-fixed



**FIGURE 1** *GACAT3* is overexpressed in CRC and promotes cell growth. A, *GACAT3* expression in CRC tissues of cohort 1. The  $\Delta C_t$  value by subtracting the  $\beta$ -actin  $C_t$  value from the *GACAT3*  $C_t$  value was used. Higher gene expression was indicated by a smaller  $\Delta C_t$  value. B, Knockdown of *GACAT3* by gene-specific siRNAs was validated in human colon cancer cell line HCT116. *GACAT3* expression was determined by qRT-PCR. C, Effects of *GACAT3* on cell proliferation. Cell numbers were counted on days 0, 1, 2, 3, and 4 after *GACAT3* knockdown. Data represent the mean  $\pm$  SD of three independent experiments. \* $P < .05$ ; \*\* $P < .01$

and paraffin-embedded CRC tissues, EZNA<sup>®</sup> FFPE RNA Kit (OMEGA) was used. The extracted RNAs were reverse transcribed into cDNAs using HiFiScript 1st strand cDNA Synthesis Kit (CWBI0).

## 2.3 | Quantitative real-time PCR

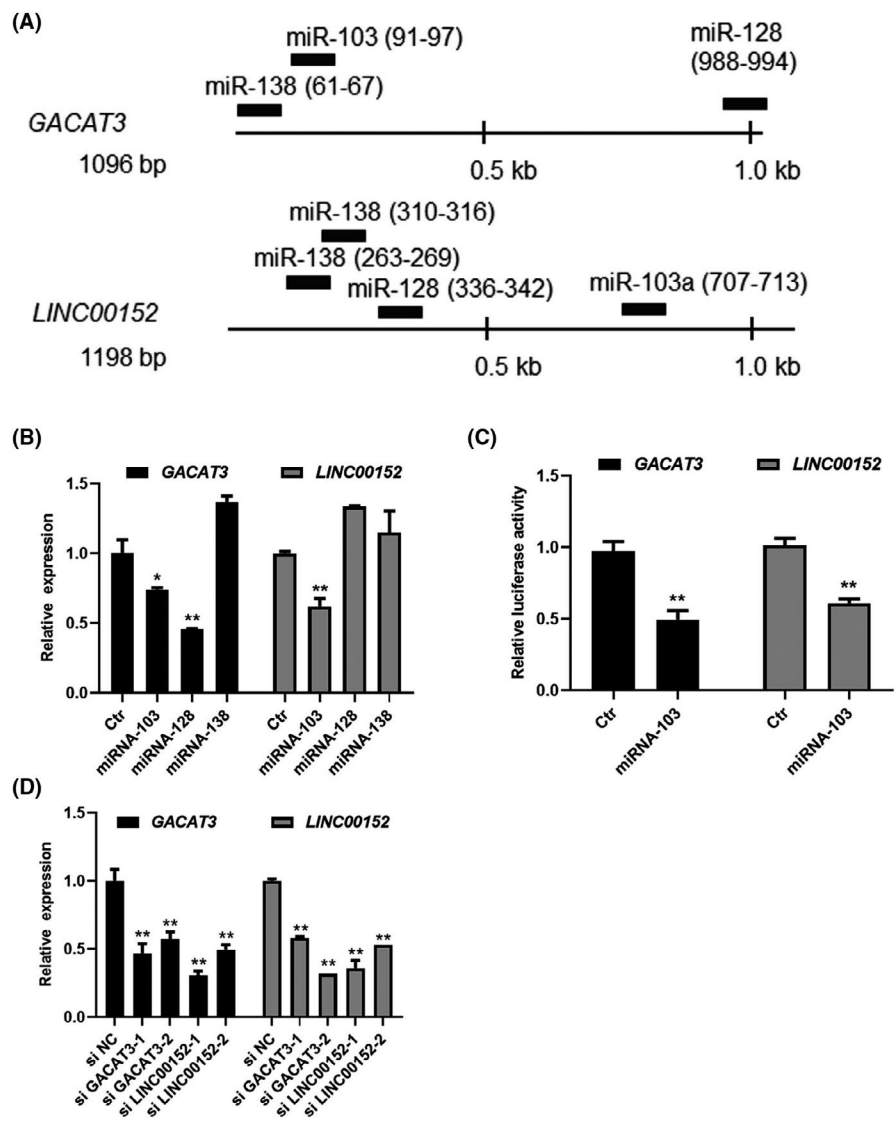
QRT-PCR was performed using the Light Cycler 480 SYBR Green I Master (Roche). The PCR conditions were 95°C for 5 minutes, 95°C for 10 seconds, 56°C for 20 seconds, and 72°C for 30 seconds with the latter three steps repeated for 45 cycles. The primer sequences of *LINC00152* were 5'-CACCAGCATCTTTCCAACC-3' (forward primer) and 5'-AAGGCCGACTCTCCTACACA-3' (reverse primer). The primer sequences of *GACAT3* and  $\beta$ -actin were as described in a previous publication.<sup>16</sup> The relative expression was shown as  $\Delta C_t$  value by subtracting the  $\beta$ -actin  $C_t$  value from the *LINC00152*  $C_t$  value.<sup>20</sup> Higher gene expression was indicated by a smaller  $\Delta C_t$  value.

## 2.4 | miRNA mimics, siRNAs, and transfection experiments

The mimics of miR-103, miR-128, and miR-138, as well as the non-targeting negative control were purchased from GenePharma. Lipofectamine 2000 (Invitrogen) was used for transfection. Two *LINC00152* siRNAs with sequences of siLINC00152-1:5-CAUUUGGUCUUCUAUGAACATT-3 and siLINC00152-2:5-GCUCUAUGACACACUUGAUTT-3 were used for the knockdown experiment. The *GACAT3* siRNAs were previously described<sup>16</sup> and knockdown experiments were performed using transfection reagent RNAiMAX (Invitrogen).

## 2.5 | Dual luciferase assay

The *LINC00152* cDNA sequence (1198 bp, ENSG00000222041) was synthesized and inserted into pGL3 to construct pGL3-LINC00152 by Novobio Biotechnology. pGL3-GACAT3 was described previously.<sup>15</sup> Dual luciferase assay was conducted according to the protocol from



**FIGURE 2** Reciprocal regulation between *GACAT3* and *LINC00152* through miR-103. A, *GACAT3* and *LINC00152* share common binding sites of three miRNAs. Common miRNA binding sites were predicted by microT and miRcode. B, *GACAT3* and *LINC00152* regulation by the miRNAs. The miRNA mimics of miR-103, miR-128, and miR-138 were applied to HCT116, and then *GACAT3* and *LINC00152* expression was determined by qRT-PCR. C, Direct regulation of *GACAT3* and *LINC00152* by miR-103 shown by a dual luciferase reporter assay. Mimics of miR-103 were applied after the transfection of plasmid pGL3-GACAT3 or pGL3-LINC00152 into 293T cells, and luciferase assay was performed. D, *GACAT3* and *LINC00152* have the reciprocal regulatory effects. Knockdown was performed by specific siRNAs of *GACAT3* and *LINC00152*, and then their expression was determined by qRT-PCR. Data represent the mean  $\pm$  SD of three independent experiments. \* $P < .05$ ; \*\* $P < .01$ .

the manufacturer (Promega). In brief, a constructed pGL3 reporter vector was co-transfected into 293T cells with the reference vector pRL-SV40 by X-tremeGENETM HP DNA (Roche). Then, the miRNA mimics was applied 48 hours post-transfection. The activities of firefly luciferase were normalized to that of Renilla luciferase.

## 2.6 | Subject recruitment and data collection

Sample from two clinical patient cohorts was collected at the Changhai Hospital (Shanghai, China) from 2011 to 2015 as described previously.<sup>20</sup> In brief, a total of 30 fresh CRC tissues and the adjacent colorectal mucosa were obtained to be as cohort 1. Cohort 2 consisted of 406 formalin-fixed and paraffin-embedded CRC biopsies. The clinicopathological information was collected, including sex, age, body mass index (BMI), morphological classification (ulcerative type and mass type), depth of invasion (Tis, T1, T2, T3, and T4), differentiation (poor and intermediate/well), intestinal lymph nodes (0, 1-3, and  $\geq 4$ ), lymph node metastasis (negative and positive), TNM (I-II and III-IV), carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA19-9). The above research was approved by the Human Ethics Committee of Chancel Hospital, Second Military Medical University and performed according to Declaration of Helsinki principles. Each patient had been informed with the written informed consent.

## 2.7 | Statistical analysis

Continuous variables (age, BMI, CEA, CA19-9,  $GACAT3 \Delta C_t$ , and  $LINC00152 \Delta C_t$ ) were described as mean  $\pm$  standard deviation (SD) or median values and quartiles depending on whether the data had a normal distribution. Student's *t* tests were used to analyze the continuous variables between two groups. Categorical variables were presented as proportions, and chi-square test or Fisher's exact test was used for the comparisons between groups.

The correlation between the expression of lncRNAs and the clinicopathologic data was analyzed using the Pearson or Spearman rank correlation methods. Receiver operating characteristic (ROC) curves were performed to evaluate the diagnostic value of lncRNAs. The graphs were created using GraphPad Prism 6. Statistical analyses were performed using SPSS 18.0 statistical software. A statistically significant difference in all comparisons was defined as *P*-value  $< .05$ .

## 3 | RESULTS

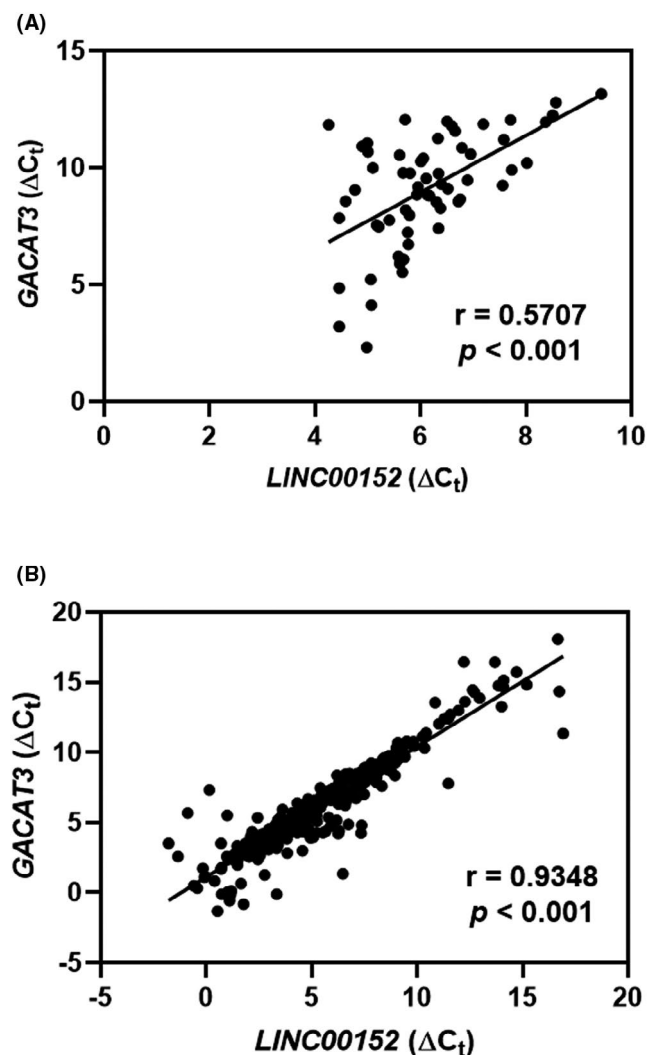
### 3.1 | GACAT3 promotes cell growth and is overexpressed in CRC

To determine the expression of *GACAT3*, qRT-PCR was performed using samples from CRC tissues of cohort 1 as described in Materials and Methods. As shown in Figure 1A, *GACAT3* expression was

significant ( $P < .001$ ) higher in CRC tissues than in the adjacent normal tissues based on the statistical analysis. To uncover the role of *GACAT3* overexpression, knockdown experiment was performed in the human CRC HCT-116 cell line, where the expression of *GACAT3* was decreased approximately 2.2-fold by two independent sets of specific siRNAs (Figure 1B). As a result, both *GACAT3* siRNAs inhibited the growth of CRC cells (Figure 1C).

### 3.2 | GACAT3 and LINC00152 are reciprocally regulated by miR-103

lncRNAs could regulate each other as ceRNA through the same miRNAs.<sup>10</sup> We therefore asked whether *GACAT3* and *LINC00152* act as ceRNAs. The entire mRNA sequences of *GACAT3* and *LINC00152* were obtained from the NCBI database, and common



**FIGURE 3** *GACAT3* and *LINC00152* are positively correlated in CRC. A, Correlation between *GACAT3* expression and *LINC00152* expression in cohort 1. B, Correlation between *GACAT3* expression and *LINC00152* expression in cohort 2. The  $\Delta C_t$  value was calculated by subtracting the  $\beta$ -actin  $C_t$  value from the *GACAT3* or *LINC00152*  $C_t$  value

putative miRNA binding sites were scanned using microT and miR-code. As shown in Figure 2A, miR-103, miR-128, and miR-138 had putative binding sites in both the *GACAT3* and *LINC00152* genes. Next, to verify the miRNA binding sites, miRNA mimics were synthesized and applied to the human CRC cells. The mimics of miR-103 significantly decreased expression of both *GACAT3* (1.4-fold) and *LINC00152* (1.6-fold) (Figure 2B). The downregulation of *LINC00152* by miR-103 was also reported by a previous study.<sup>21</sup> Although miR-128 decreased *GACAT3* expression, it did not show any effects on the expression of *LINC00152*, while miR138 had no effects on either lncRNA. To further identify the direct regulatory role of miR-103 on both lncRNAs, a dual luciferase activity assay

was performed and miR-103 decreased the luciferase expression driven by either *GACAT3* or *LINC00152* (Figure 2C) suggesting that *GACAT3* and *LINC00152* are both regulated by miR-103.

We next evaluated whether *GACAT3* and *LINC00152* had the reciprocal regulatory effects. First, the expression of *GACAT3* or *LINC00152* was knocked down by specific siRNAs and identified through the qRT-PCR (Figure 2D). Under *GACAT3* knockdown, *LINC00152* expression was decreased 1.7-fold (si*GACAT3*-1) and 2.2-fold (si*GACAT3*-2) (Figure 2D). Similarly, *GACAT3* expression was downregulated 2.3-fold (si*LINC00152*-1) and 2-fold (si*LINC00152*-2) in cells treated with *LINC00152* siRNAs (Figure 2D). These results strongly indicated that *GACAT3* and *LINC00152* had

**TABLE 1** Associations between clinicopathological features and levels of *GACAT3* and *LINC00152* in 406 CRC patients

Variables	GACAT3		P-value	LINC00152		P-value
	Low (n = 203)	High (n = 203)		Low (n = 203)	High (n = 203)	
Gender						
Male	121	113	.325	131	106	.012*
Female	79	90		72	97	
Age (y)	59.5 ± 13.9	59.15 ± 12.5	.742	59.9 ± 13.5	58.6 ± 12.9	.302
BMI (kg/m <sup>2</sup> )	22.9 ± 3.2	22.8 ± 3.3	.733	22.8 ± 3.2	23.0 ± 3.2	.604
Morphological classification						
Ulcerative type	125	134	.405	127	132	.586
Mass type	66	59		65	60	
Depth of invasion						
Tis	18	5	.016**	19	4	.018**
T1	10	7		7	10	
T2	38	43		35	46	
T3	60	81		70	71	
T4	73	62		69	66	
Differentiation						
Poor	12	14	.782	13	13	.92
Intermediate/Well	167	174		167	174	
Intestinal lymph nodes						
0	106	112	.916	107	113	.989
1-3	46	51		48	49	
≥4	34	33		33	34	
Lymph node metastasis						
Negative	120	99	<.001***	118	101	.0038**
Positive	58	104		63	104	
TNM						
I-II	134	100	<.001***	135	99	<.001***
III-IV	69	103		68	104	
CEA	10.6 (6.7-14.4)	15.9 (6.0-25.9)	.324	10.8 (6.6-15.0)	16.9 (6.8-3.9)	.27
CA19-9	16.6 (14.2-19.0)	33.7 (18.1-43.3)	.0143*	20.2 (15.6-24.9)	37.0 (21.2-52.8)	.047*

Abbreviations: BMI, Body mass index; CA19-9, Carbohydrate antigen 19-9; CEA, Carcinoembryonic antigen; TNM, Tumor-node-metastasis.

\**P* < .05.

\*\**P* < .01.

\*\*\**P* < .001.

a reciprocal regulatory association and acted as ceRNAs of each other in CRC cells.

### 3.3 | GACAT3 and LINC00152 are positively correlated in CRC

To evaluate the relationship between *GACAT3* and *LINC00152* in clinical CRC, two cohorts of samples were used. We first assessed their expression in 30 fresh CRC samples (cohort 1) and observed that *GACAT3* expression was positively correlated with *LINC00152* expression ( $r = 0.5707$ ,  $P < .001$ ) (Figure 3A). Moreover, a positive correlation between the expression of *GACAT3* and *LINC00152* was observed in the 406 CRC samples embedded in paraffin in cohort 2 ( $r = 0.9348$ ,  $P < .001$ ) (Figure 3B).

### 3.4 | GACAT3 and LINC00152 are associated with depth of invasion, lymph node metastasis, and TNM stage

To explore the relationship of *GACAT3* and/or *LINC00152* expression with the clinicopathological characteristics, cohort 2 was divided into *GACAT3*-low and -high or *LINC00152*-low and -high groups. Both *GACAT3* and *LINC00152* were found to be associated with the depth of invasion, lymph node metastasis, TNM, and CA19-9 level (Table 1). Furthermore, significantly higher expression of *GACAT3* and *LINC00152* was observed in the T3-T4 group than in the Tis & T1-T2 group ( $P = .0029$  and  $P = .0458$ , respectively; Table 2). Moreover, the levels of *GACAT3* and *LINC00152* were higher in TNM stage III-IV tissues than in stage I-II tissues ( $P < .001$  for both; Table 2). Similarly, higher levels of *GACAT3* and *LINC00152* were positively related to lymph node metastasis ( $P < .001$  for both; Table 2).

### 3.5 | Potential diagnostic values of GACAT3 and LINC00152

We next explored the potential diagnostic value of *GACAT3* and *LINC00152*. The ROC curve analysis was conducted, and the area under ROC curve was 0.8183, 0.7533, and 0.8411 for *GACAT3*, *LINC00152*, and their combination, respectively, in cohort 1 (Figure 4A). Moreover, the area under ROC curve was 0.6238, 0.6284, and 0.6675 for *GACAT3*, *LINC00152*, and their combination, respectively, in cohort 2 (Figure 4B). The combined use of *GACAT3* and *LINC00152* slightly increased the diagnostic value.

## 4 | DISCUSSION

Increasing evidence has revealed that lncRNAs perform important regulatory roles in gene expression and contribute to human diseases,

**TABLE 2** Comparisons of *GACAT3* and *LINC00152* expression ( $\Delta C_t$ ) in groups stratified by depth of invasion, lymph node metastasis, and TNM stage

Variables	GACAT3 ( $\Delta C_t$ )	P-value	LINC00152 ( $\Delta C_t$ )	P-value
Depth of invasion				
Tis & T1-T3 (n = 128)	5.92 ± 3.09	.0029**	5.15 ± 3.14	.0458*
T3-T4 (n = 271)	5.03 ± 2.50		4.54 ± 2.50	
TNM				
I-II (n = 234)	6.17 ± 3.25	<.001***	5.42 ± 3.27	<.001***
III-IV (n = 172)	4.95 ± 2.74		4.15 ± 2.73	
Lymph node metastasis				
Negative (n = 219)	5.85 ± 3.02	<.001***	5.07 ± 3.05	<.001***
Positive (n = 162)	4.77 ± 2.52		4.17 ± 2.69	

\* $P < .05$ .

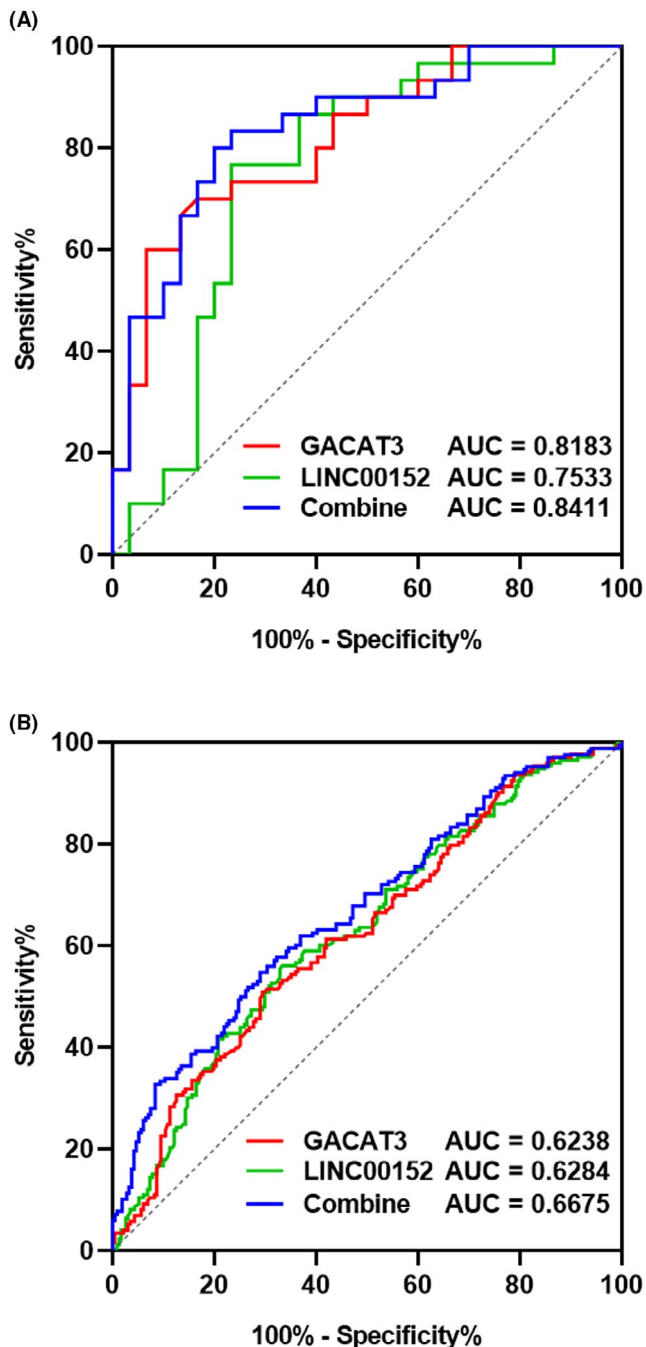
\*\* $P < .01$ .

\*\*\* $P < .001$ .

including tumor development.<sup>22-24</sup> One of the key mechanisms is that lncRNAs act as miRNA sponges, building a ceRNA network to regulate key signaling pathways in oncogenesis. For example, *HOX* transcript antisense RNA (*HOTAIR*) promotes renal cell carcinoma proliferation via miR-217/HIF-1 $\alpha$ /AXL signaling,<sup>25</sup> and long non-coding RNA *XIST* promotes colon cancer development through regulating Wnt/ $\beta$ -catenin signaling pathway by competitively binding to miR-34a.<sup>26</sup> Our previous work revealed that *GACAT3* is significantly overexpressed in gastric cancer and associated with tumor size and metastasis.<sup>16</sup> Moreover, *GACAT3* promotes cell proliferation through inhibiting the expression of cell cycle-related genes.<sup>15</sup> Furthermore, *GACAT3* was reported to be regulated by several miRNAs including miR-128.<sup>15</sup> Recently, high expression level of *LINC00152* has been detected in human malignant tumors of the digestive system.<sup>27</sup> *LINC00152* can specifically recognize the EGFR-binding site and activate the PI3K/AKT signaling pathway to promote the proliferation of gastric cancer cells.<sup>28</sup> In the present study, although several common miRNA binding sites were discovered in both *GACAT3* and *LINC00152* sequences, only miR-103 was found to inhibit the expression of both lncRNAs suggesting that *GACAT3* might have an oncogenic role by acting as the ceRNA of *LINC00152* via competitive binding to miR-103. A previous study found that miR-138 could downregulate *GACAT3* in gastric cancer cells<sup>15</sup>; however, this inhibition was not observed in CRC cells in the present study suggesting a cancer type-specific regulatory role of miR-138.

*GACAT3* is a lncRNA recently identified in gastric cancer, and studies have suggested that it plays oncogenic roles and is overexpressed in many types of human tumor tissues.<sup>18,29</sup> Here, we report that *GACAT3* was highly expressed in CRC tissues and associated with cell proliferation. Importantly, analysis of clinicopathological





**FIGURE 4** ROC analysis of the expression of *GACAT3*, *LINC00152*, and their combination for diagnosis and prognosis of CRC. A, ROC analysis for CRC diagnosis in cohort 1. B, ROC analysis of the colon cancer prognostic value in TNM staging in cohort 2. ROC, receive operating curve; AUC, area under the curve

characteristics revealed that *GACAT3* expression was positively associated with the depth of invasion, TNM stage, lymph node metastasis, and CA19-9 level, which is in line with previous discoveries that *GACAT3* is a potential diagnostic biomarker of gastric cancer,<sup>14,30</sup> non-small cell lung cancer,<sup>17</sup> and glioma,<sup>29</sup> suggesting a common fundamental regulatory role of *GACAT3* in tumor development.

CRC currently ranks third in tumor incidence rates, which may bring a huge economic burden on patients. Although great

advancement in CRC treatment has been seen, the survival rate of CRC patients remains low, which may be related to the lack of a specific and efficient screening method for early stage disease. Some molecular biomarkers, such as CEA and CA19-9, are widely used in clinical medicine. Nonetheless, the specificity and sensitivity of these conventional molecular biomarkers are dissatisfactory for clinical diagnosis and prognosis. Increasing evidence shows that lncRNAs are potential biomarkers in many types of human malignant tumors, including *H19*,<sup>20</sup> *HOTAIR*,<sup>25</sup> *GACAT3*,<sup>16</sup> and *LINC00152*.<sup>27</sup> In the present study, we demonstrated that the levels of *GACAT3* and *LINC00152* were significantly correlated to the depth of invasion, lymph node metastasis, TNM stage, and CA19-9 level, which are known prognostic features of colon cancer.<sup>31-33</sup> Furthermore, the diagnostic accuracy of *GACAT3* and *LINC00152* combined is higher than that of either one alone.

In summary, our work shows that *GACAT3* and *LINC00152* are both overexpressed in CRC and they act as a ceRNA network. The levels of both lncRNAs are correlated with the depth of invasion, lymph node metastasis, TNM stage, and CA19-9 level. A combination of *GACAT3* and *LINC00152* showed a superior diagnostic capacity compared with the use of the two molecules alone. Therefore, our data suggest that *GACAT3* and *LINC00152* may be a promising biomarker for CRC.

#### ACKNOWLEDGMENTS

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