

# Long non-coding RNA 00152 functions as a competing endogenous RNA to regulate NRP1 expression by sponging with miRNA-206 in colorectal cancer

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**Abstract.** Colorectal cancer (CRC) is the third most common type of cancer; however, the molecular mechanisms underlying colorectal tumor metastasis and growth remain elusive. Recently, accumulating evidence has indicated that long non-coding RNAs (lncRNAs) play a critical role in CRC progression and metastasis; however, the biological role and clinical significance of lncRNA 00152 (lnc00152) in CRC remains largely unknown. Thus, in this study, lnc00152 expression was measured in 80 human CRC tissue samples, 40 non-cancerous tissue samples, and 3 CRC cell lines (SW480, SW620 and LoVo) using RT-qPCR. We examined the effects of lnc00152 on CRC cells following transfection with lnc00152 overexpression plasmid or respective siRNA *in vitro* and *in vivo*. Luciferase assays revealed the mechanism driving competitive endogenous RNA (ceRNA). We identified that lnc00152 was aberrantly overexpressed in colorectal tumors and cancer cells and that lnc00152 was modulated by miRNA-206. lnc00152 overexpression enhanced the proliferative and invasive ability of CRC cells *in vitro*, promoted tumor growth *in vivo*, and was associated with the shorter overall survival of patients with CRC. In addition, lnc00152 overexpression promoted epithelial-mesenchymal transition (EMT) and increased neuropilin-1 (NRP1) expression in the CRC cells. By contrast, lnc00152 silencing exerted a counteractive effect. Collectively, these findings demonstrate the critical role of lnc00152 in tumor growth and progression in CRC,

and identify a novel therapeutic target associated with CRC development and progression.

## Introduction

Colorectal cancer (CRC), one of the most common types of cancer, is associated with a high mortality rate worldwide (1). While the diagnosis of and treatment strategies for CRC have improved, the overall survival of patients with CRC remains unsatisfactory (2). Current CRC treatment includes neoadjuvant radiotherapy and chemotherapy (3), surgical therapy (4,5) and immunization therapy (6,7). In the majority of cases, however, CRC is diagnosed at an advanced pathological stage before any symptoms appear, resulting in poor survival rates. Recent studies have indicated that long non-coding RNAs (lncRNAs) are critical factors in regulating the development, differentiation and apoptosis (8-10) of cancer cells. lncRNAs are also associated with tumor progression and metastasis (11). In CRC specifically, lncRNA could inhibit tumor growth by deactivating Akt signaling (12). The aberrant expression of lncRNAs is associated with a poor survival (13), lymph node metastasis (12) and the modulation of epithelial-mesenchymal transition (EMT) in CRC (14). Therefore, lncRNAs may function as novel therapeutic targets and molecular markers for the diagnosis of CRC. Several studies have identified molecular markers affecting CRC dynamics (15-17). For instance, miRNA-126 expression has been shown to exert a critical effect on CRC pathogenesis (18). However, further clarifications of the systemic correlation between miRNAs and lncRNAs are required.

The lncRNA-miRNA complex forms a regulatory network in non-small cell lung cancer A549 cells (19), indicating that lncRNAs are involved in controlling tumor progression by interacting with miRNAs. Additional molecules, such as the orphan nuclear receptors, Nur77, RAR $\gamma$  and WNT, signal the inhibitors, OVOL2, Myb-like SWIRM and MPN domains 1 (MYSM1), which influence the invasion, metastasis and progression of CRC (14,20-22). Some bioactive enzymes, such as serum  $\beta$ -glucuronidase, are also considered potential markers of CRC (23). Neuropilin-1 (NRP1) is a transmembrane glycoprotein that can function as an oncogene by participating

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in the development and progression of various types of cancer (24,25), including CRC (26). Numerous biomarkers have been identified in CRC cells; however, there is still a need for further clarification of the mechanisms driving the regulation between biomarkers and tumor progression and metastasis.

In this study, we found that lncRNA 00152 (lnc00152) was overexpressed in CRC tumors and cancer cells when compared to adjacent non-tumor tissues. lnc00152 is an important factor affecting cell proliferation and invasion *in vitro* and tumor growth *in vivo*. The regulation of lnc00152 altered NRPI expression and affected EMT by modulating N-cadherin and E-cadherin expression. Finally, we found that the effects of lnc00152 were regulated by miRNA-206. Our findings indicate that lnc00152 may function as a therapeutic target against CRC.

## Materials and methods

**Ethics statements.** The use of human samples was approved by the Ethics Committee Guangzhou First People's Hospital and all patients involved in the study provided written informed consent. The use of animals in this study was approved by the Ethics Committee of the Laboratory Animal Center of South China University of Technology.

**Patients and tissue samples.** This study was conducted using a total of 80 paraffin-embedded CRC samples and 40 adjacent non-tumor tissue samples. All the samples were histopathologically and clinically diagnosed at Guangzhou First People's Hospital from 2010 to 2016. None of the recruited participants had received adjunctive treatment prior to surgery. The histological characterization and clinicopathological staging of the samples were determined according to the Union for International Cancer Control (UICC). The clinicopathological information was available for all samples (Table I). The CRC specimens and the matched adjacent non-cancerous tissues were frozen and stored in liquid nitrogen until further use. The overall survival (OS) was defined as the period between diagnosis and either death or the last follow-up. Follow-up ranged from 0 to 78 months. The Kaplan-Meier overall survival curve was obtained according to lnc00152 expression and the cumulative survival ratio was calculated at different time-points during follow-up.

**Cell culture.** We obtained the CRC cell lines LoVo, SW480 and SW620 from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were grown at 37°C with 5% CO<sub>2</sub> in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

**RNA extraction and reverse transcription (real-time) quantitative PCR (RT-qPCR).** RT-qPCR was used to determine the expression of lnc00152. We extracted total RNA from the tissues and cultured cells using TRIzol reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions. Subsequently, 2 µg of the extracted total RNA was subjected to reverse transcription (RT) using a reverse transcription kit (Cat. no. 639505; Takara, Tokyo, Japan). In order

to perform real-time (quantitative) PCR, we used the One Step SYBR-Green® (Tokyo, Japan) PrimeScript™ RT-PCR kit (Cat. no. RR064A; Takara). For real-time fluorescence detection we used the ABI7300 real-time PCR thermal cycle instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The 20 µl total reaction mixture contained 20 ng cDNA, 10 µl of 2X SYBR-Green master mix, 200 nM of forward and reverse primers and PCR-grade water. The PCR cycling conditions were set to 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, and 55°C for 30 sec. A melting curve analysis verified the specificity of each primer after PCR to ensure amplification specificity. Relative gene expression was calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (27), and we normalized the results to the expression of 18s rRNA. For miRNA-206 expression detection, we validated human U6 as the normalizer. The sequences of the primers used for PCR are presented in Table II.

**Transfection with small interfering RNA (siRNA) targeting lnc00152.** We selected the site in the β-catenin mRNA sequence as a siRNA target. Three targeted siRNAs against lnc00152 (lnc00152-siRNAs) were created and the sequences were as follows: siRNA1, GGGAAATAAATGACTGGAT; siRNA2, GGAGATGAAACAGGAAGCT; and siRNA3, GGGAAATGGAGGGAAATAAA. All the siRNAs, as well as the negative control siRNA were synthesized and purified by RiboBio Biotech Corp. (Guangzhou, China). The siRNA plasmids were transfected into the SW620 cells with Lipofectamine 2000 (Invitrogen) at a working concentration of 200 nM and incubated for 24 h. After the incubation period, the cells were harvested for use in RT-qPCR analysis.

**lnc00152 overexpression, plasmid construction and transfection.** The full-length lnc00152 was synthesized by Shanghai Sangon Biotech Corp. (Shanghai, China) and cloned into pLV4 plasmids (Promega Corp., Madison, WI, USA). Empty pLV4 plasmids, without the insertion, served as the negative control. Cell transfection was conducted with Lipofectamine 2000 reagent according to the manufacturer's instructions. Further analyses were conducted at 24 h following transfection.

**Cell Counting kit-8 (CCK-8) assay.** A Cell Counting kit-8 assay ('CCK-8', KeyGen Biotech, Nanjing, China) was used to evaluate cell viability at 12, 24, 36, 48, 60 and 72 h of culture. Briefly, both the control and infected cells were seeded at a density of 2x10<sup>3</sup> cells/well into a 96-well plate, to which 10 µl of the 10% CCK-8 solution was subsequently added. The plate was incubated at 37°C for 4 h in a 5% CO<sub>2</sub> incubator. A microplate reader (Spectramax M5; Molecular Devices Corp., San Jose, CA, USA) was used to measure the product absorbance at 490 nM, and the results are reported as follows: (OD value of sample - OD value of blank)/(OD value of blank). Data were collected from 3 independent experiments.

**Transwell invasion assay.** A Transwell invasion assay was used to evaluate the invasive potential of the transfected cells. We used Matrigel invasion chambers (Corning Inc., Corning, NY, USA) with 8-µm pores (BD Biosciences, San Jose, CA, USA). In brief, a concentration of 2x10<sup>4</sup> cells/100 µl was re-suspended in DMEM without fetal bovine serum (FBS) then subsequently seeded on top of a Matrigel-coated Transwell. A total

Table I. Detailed clinical information of the patients with colorectal cancer.

Clinical characteristics	Patients		
	No. of patients	lncRNA 00152 expression	P-value
Age (years)			
≥60	60	288.76±150.94	0.968
<60	20	321.38±129.96	
Sex			
Male	43	295.75±152.82	0.563
Female	37	298.27±139.44	
Pathological grade			
I-II	37	222.71±83.22	<b>0.008</b>
III-IV	43	360.77±158.31	
Tumor invasion			
T <sub>1</sub> -T <sub>2</sub>	19	253.38±111.75	0.383
T <sub>3</sub> -T <sub>4</sub>	61	316.08±150.66	
Lymph-node metastasis			
N <sub>0</sub>	39	227.72±84.06	<b>0.004</b>
N <sub>1</sub> -N <sub>2</sub>	41	362.73±161.88	
Distant metastasis			
M <sub>0</sub>	60	279.70±144.43	0.429
M <sub>1</sub>	20	348.55±141.26	
Vascular invasion			
No	65	290.74±155.12	0.142
Yes	15	323.67±95.55	

Numbers in bold indicate statistical significance (P<0.05).

of 600  $\mu$ l of DMEM containing 10% FBS was added to the lower chambers. Following a 24-h incubation period, the filters separating the upper and lower chamber were washed twice with PBS, fixed by methanol and stained with crystal violet at room temperature for 10 min (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). We then counted the number of stained cells under a light microscope (Nikon Eclipse E200; Nikon, Tokyo, Japan; magnification, x100). The area of each membrane was, on average, 5 visual fields. This experiment was repeated independently, in triplicate.

**Establishment of tumor xenograft models.** We purchased 12 athymic nude mice (6 males and 6 females; weighing 18-20 g, 4 weeks old) from Beijing Slac Laboratory Animal Co. Ltd. (Beijing, China), which we housed in high-efficiency particulate air-filtered cages in a pathogen-free facility. The housing environment was maintained at 25±2°C, 45-55% humidity, and a standard 12-h dark/12-h light cycle, and we fed mice an autoclaved diet with free access to water. We cleaned and sterilized the inoculation area (right upper limb) with ethanol and iodine solutions before subcutaneously injecting the SW620 cells (1x10<sup>6</sup>/ml) transfected with pLV-NC or pLV-lnc00152 into the mice (n=6 mice per group). Tumor

Table II. Sequences of primers used in RT-qPCR.

Gene name	Primer sequence (5'-3')
lnc00152	F: CCACCAGCCTCTCCTTGAAT R: GGCTGAGTCGTGATTTTCGGT
E-cadherin	F: TGCCGCCATCGCTTACACCATC R: GGTCAGCAGCTTGAACCACCAG
N-cadherin	F: CCCACAGCTCCACCATATGACTC R: CCTGCTCACCACCCTACTTGAG
Human U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
NRP1	F: GAGGCATGAAGGCAGACAGAG R: GAGGCATGAAGGCAGACAGAG
18s rRNA	F: CCTGGATACCGCAGCTAGGA R: GCGGCGCAATACGAATGCCCC
miRNA-206	F: ACACTCCAGCTGGGTGGAATGTAAG GAAGTGTG R: GCAGGGTCCGAGGTATTCG

volumes (0.5 x length x width<sup>2</sup>) were measured on days 10, 14, 18, 22 and 26 following implantation. Of note, the largest tumor diameter of a single tumor in our study was 18 mm and the largest volume was 570.317 mm<sup>3</sup>. No mouse developed multiple tumors. After 4 weeks, the mice were sacrificed by CO<sub>2</sub> inhalation (20% of the chamber volume was displaced per minute by the flow of CO<sub>2</sub>). Tumor tissues were excised, then fixed in a 4% paraformaldehyde solution for further analysis. All the animal experiments were performed in the Animal Laboratory Center of Guangzhou First Hospital.

**Western blot analysis.** For protein extraction, the cultured cells were lysed in a cell lysis buffer containing 140 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA and 1X protease inhibitor. The tumor tissues were homogenized in lysis buffer (pH 7.5) containing 300 mM NaCl, 50 mM Tris-HCl, 0.5% Triton X-100 and 1X protease inhibitor, and then incubated at 4°C for 30 min. The cell and tissue lysates were centrifuged at 3,000 x g at 4°C for 15 min. We used the BCA method to determine the protein concentration (Sangon, Shanghai, China). Subsequently, 10  $\mu$ g aliquots of the cell and tissue lysates were loaded onto each lane of a 10% polyacrylamide gel, then blotted onto polyvinylidene difluoride (PVDF) membranes. After blocking with a PBST containing 5% non-fat dry milk, the membranes were incubated with antibodies against NRP1 (1:500; Cat. no. ab81321; Abcam, Cambridge, MA, USA), N-cadherin (1:500; Cat. no. ab18203; Abcam), E-cadherin (1:500; Cat. no. cst9961, 1:1,000; Cell Signaling Technology, Danvers, MA, USA) and GAPDH (1:1,000; Cat. no. 8245; Abcam). The membranes were then washed with TBST 3 times, then incubated them with peroxidase-linked anti rabbit IgG secondary antibody (Cat. no. A16096; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. These proteins were visualized by using an ECL western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA). This experiment was repeated in triplicate.

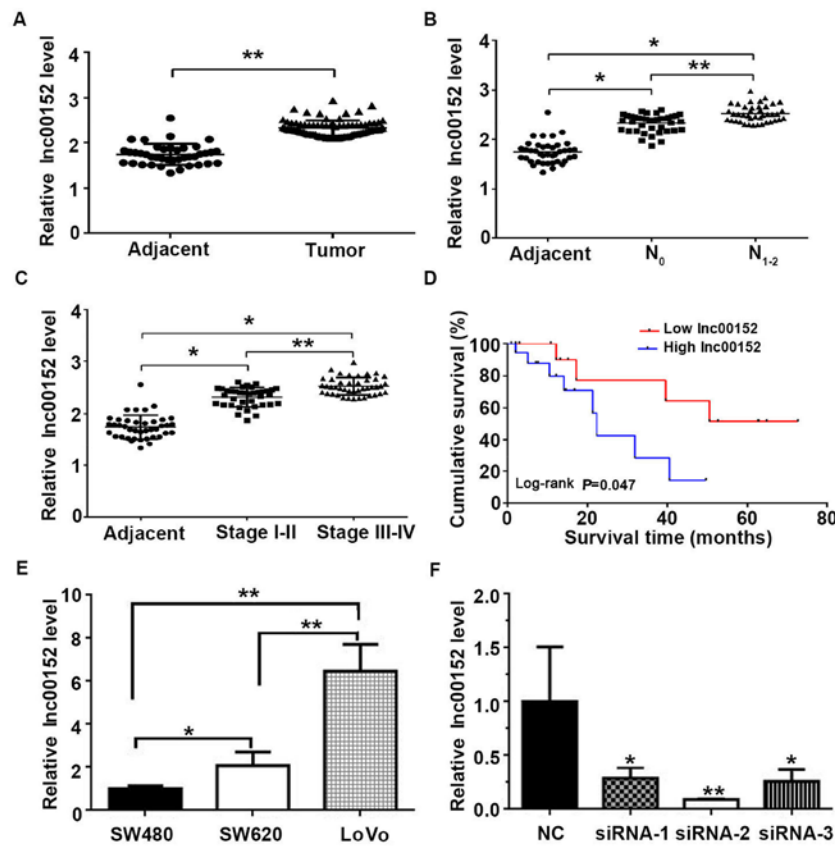


Figure 1. Relative lnc00152 expression in colorectal tumor tissues, cancer cells and its clinical significance. (A) Relative expression of lnc00152 expression was examined by RT-qPCR and normalized to 18s rRNA. lnc00152 expression was higher in the tumor tissues (n=80) than in the adjacent non-tumor tissues (n=40). (B) The association between lnc00152 expression and lymph-node metastasis indicated that tumors with lymph-node metastasis had higher expression levels of lnc00152. (C) Tumors at an advanced pathological stage also exhibited higher levels of lnc00152. (D) Kaplan-Meier overall survival curves display higher lnc00152 levels in patients with colorectal cancer (CRC) with lower survival times (log-rank test,  $P=0.047$ ). (E) Relative lnc00152 expression in CRC cell lines (SW480, SW620 and LoVo) was determined by RT-qPCR; differential expression levels of lnc00152 were observed in the 3 CRC cell lines. (F) siRNAs targeting lnc00152 effectively downregulated the lnc00152 levels in the SW620 cells. \* $P<0.05$ , \*\* $P<0.01$  vs. NC group or as indicated.

independently. Visualization of proteins and band intensity was determined using ImageJ software version 14.8 (NIH, MD, USA). The gray level was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Hematoxylin and eosin (H&E) staining.** The tumor xenograft sections were deparaffinized in xylene and dehydrated in alcohol. The sections were then stained in Harris' hematoxylin solution for 8 min before bluing in 0.2% ammonia water or saturated lithium carbonate solution for 1 min. After rinsing in 95% alcohol, the sections were counterstained in eosin-phloxine solution for 1 min. Finally, we mounted sections with a xylene-based medium and visualized the slides with a Nikon ECLIPSE 90i (Nikon; magnification,  $\times 100$ ). Two pathologists evaluated the images in a blinded manner.

**Immunohistochemistry.** The tumor xenograft sections were washed in PBS and blocked for 60 min in 0.3% Triton X-100 and PBS with 5% bovine serum albumin, before being incubated overnight at 4°C with anti-E-cadherin (1:50; Cat. no. 9961) or anti-N-cadherin (1:50; Cat. no. 4061) antibodies (Cell Signaling Technology). We then applied HRP-conjugated secondary antibody (1:2,000; Cat. no. 7074; Abcam, Cambridge, UK) to the slides before a 1 h room temperature incubation. To develop color on the slide, we added diaminobenzidine (DAB)/H<sub>2</sub>O<sub>2</sub>

before the slides were visualized with a Nikon ECLIPSE 90i microscope (Nikon; magnification,  $\times 100$ ). As mentioned above, two pathologists evaluated the images in a blinded manner.

**Site-directed mutagenesis and dual-luciferase reporter assay.** We used a SQE-PCR to perform site mutation on lnc00152 and then ligated the lnc00152 insert into a psiCHECK<sup>TM</sup>-2 vector (Promega Corp.). Three fragments were cloned by PCR using the following primers, including Psi-152F, CCGCTC GAGCATCAT TGGGAATGGAGGGAAAT; Psi-152R, ATTTGCGGCCGCTT CTGTTTTCTTTAGTTTGTCTT; Mut-152F1, GTTTCAAAT TGGAGCCTTCGACAAGCGGTGCCTGAGC; Mut-152R1, CACCGCTTGTCTGAAGGCTCCAATTTGAACTTAAAA AGC; Mut-152F2, GCCTCCATCCGAGCCTT CACCTCCGTC TGCATCCCTCG; and Mut-152R2, AGACGGAGGTGAAGG CTCGGATGGAGGCTGGCAAGTTTC. Human 293T cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were co-transfected with 150 ng of miRNA-206 mimics, miRNA NC mimics, miRNA-206 inhibitors and miRNA-NC inhibitor (RiboBio). Subsequently, 50 ng of wild-type lnc00152 or lnc00152 mutant fluorescent vector were co-transfected with the psiCHECK<sup>TM</sup>-2 vector into the human 293T cells using Lipofectamine 2000 (Invitrogen). We used miRNA-NC as a negative control and repeated the transfections in triplicate. Luciferase assay was conducted at

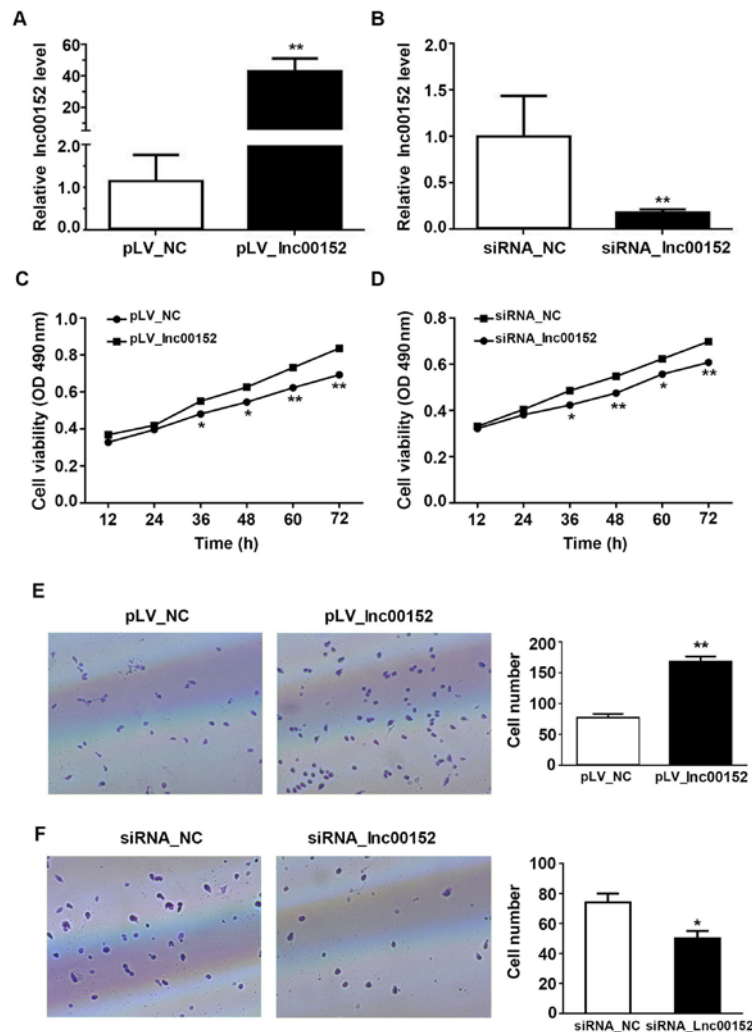


Figure 2. A high Inc00152 expression enhances colorectal cancer (CRC) cell viability and invasion. (A) SW620 cells were transfected with pLV\_NC and pLV\_Inc00152. The efficiency of the Inc00152 overexpression plasmid was evaluated by RT-qPCR. (B) The SW620 cells were transfected with Inc00152 siRNA-2 or siRNA-NC. The efficiency of the siRNA-Inc00152 construct was evaluated by RT-qPCR. (C) A CCK-8 assay was used to examine changes in cell viability. Inc00152 overexpression enhanced SW620 cell viability. (D) A CCK-8 assay was used to examine changes in cell viability. Inc00152 silencing impaired SW620 cell viability. (E) A Transwell invasion assay was used to examine changes in cell invasiveness. Inc00152 overexpression promoted the invasive ability of the SW620 cells. (F) Inc00152 silencing suppressed SW620 cell invasion. \* $P < 0.05$ , \*\* $P < 0.01$  vs. pLV\_NC.

48 h following transfection, and we normalized the relative luciferase activity using the luciferase assay kit (Promega Corp.) at 48 h after transfection. Finally, to elucidate the interaction between Inc00152 and miRNA-206, we expressed miRNA inhibitor and mimics into the 293T cells and subsequently applied qPCR analysis for miRNA-206 and Inc00152 expression at 48 h following transfection.

**Statistical analysis.** Summarized data are presented as the means  $\pm$  SEM. We performed all statistical analyses using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). A Chi-square test was used to analyze the association between Inc00152 expression and the patient clinicopathological characteristics. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. Comparisons between 2 groups for statistical significance were carried out with two-tailed paired Student's *t*-tests. For analyses involving multiple sample groups, statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons. Statistical significance was set at a *P*-value  $< 0.05$ .

## Results

*High expression of Inc00152 is associated with low survival rates of patients with CRC.* In order to determine the potential role of Inc00152 in CRC, we first compared Inc00152 expression levels between CRC tissues and adjacent non-tumor tissues. The Inc00152 expression level was significantly higher in the CRC tissues compared with the adjacent non-tumor tissues (Fig. 1A,  $P < 0.01$ ). In terms of lymph-node metastasis, the Inc00152 expression level was higher in the  $N_{1-2}$  grade tumors than in the  $N_0$  grade tumors (Fig. 1B,  $P < 0.01$ ). Furthermore, both the  $N_{1-2}$  and  $N_0$  grade tumors exhibited significantly higher levels of Inc00152 than the adjacent non-tumor tissues (Fig. 1B,  $P < 0.05$ ). Similarly, the Inc00152 level was associated with the pathological grade of colorectal tumors. The malignancy of CRC was associated with the level of Inc00152 expression, with significantly higher levels observed in stage III-IV than in stage I-II tumors (Fig. 1C,  $P < 0.01$ ). Furthermore, the Inc00152 levels were associated with the overall survival, with significantly shorter survival times observed in patients with CRC

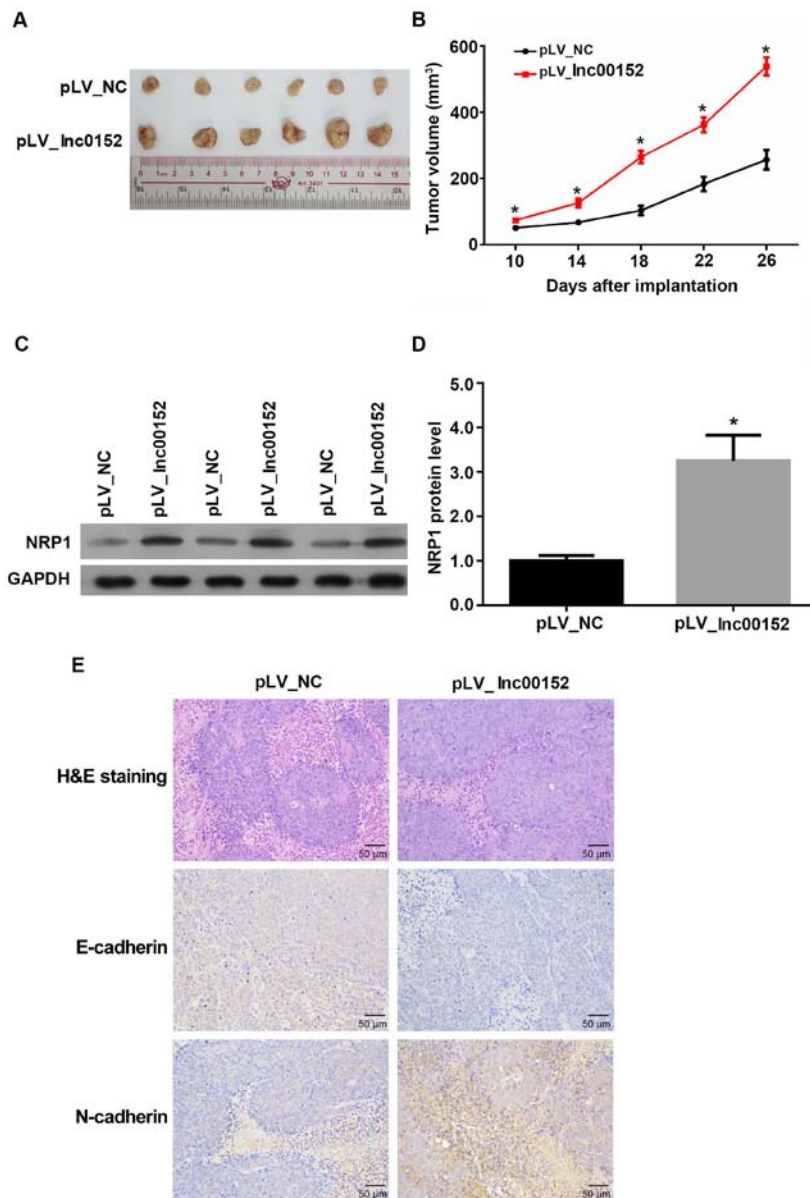


Figure 3. lnc00152 overexpression promotes tumor growth *in vivo*. (A) The tumors were separated from nude mice following the injection of SW620 cells transfected with pLV\_NC or pLV\_lnc00152 (n=6 per group). (B) Tumor volume curves were measured on days 10, 14, 18, 22 and 26 following implantation. Tumor volume significantly increased in the lnc00152-overexpressing (pLV\_lnc00152 transfection) group. (C and D) Western blot analysis of NRP1 expression in tissues of resected tumors. lnc00152 overexpression led to an increased NRP1 level. (E) Results of immunohistochemistry indicated that lnc00152 overexpression downregulated E-cadherin expression and upregulated N-cadherin expression. Upper panels, H&E staining; lower panels, immunostaining of E-cadherin and N-cadherin. Scale bar, 50 μm. \*P<0.05.

with higher lnc00152 levels (Fig. 1D and Table I, log-rank test, P=0.047). Neither age nor sex were significantly associated with lnc00152 expression (P=0.968 and P=0.563, respectively); however, lnc00152 expression was significantly associated with the pathological grade (P=0.008) and lymph-node metastasis (P=0.004). The human CRC cells (SW480, SW620 and LoVo) also expressed lnc00152, with the highest expression observed in the LoVo cells (Fig. 1E). These results suggest that lnc00152 overexpression is an indicator of CRC in human tissues and cells, and it may be useful in the prognosis of CRC.

*lnc00152 enhances the proliferative and invasive ability of the CRC cells in vitro.* To elucidate the mechanisms through which lnc00152 regulates CRC growth, we controlled the expression level of lnc00152 in the CRC cancer cell line, SW620. lnc00152

expression was significantly decreased in the siRNA-transfected cells, with an optimal silencing effect observed with siRNA-2 (P<0.01, Figs. 1F and 2B). Additionally, we established that the expression of lnc00152 in the SW620 cells was significantly higher in the cells transfected with the lnc00152 overexpression vector compared with the pLV-NC-transfected cells (Fig. 2A, P<0.01). The overexpression of lnc00152 enhanced the viability of the SW620 cells at 36, 48, 60 and 72 h following transfection (Fig. 2C). The silencing of lnc00152, however, significantly decreased cellular proliferation (Fig. 2D) at 36 (P<0.05), 48 (P<0.01), 60 (P<0.05), and 72 h (P<0.01) following transfection, but not at 12 or 24 h. From the Transwell assay evaluating the cell invasive potential, we found that the overexpression of lnc00152 enhanced the invasive ability of the SW620 cells (Fig. 2E, P<0.01). Conversely, lnc00152 silencing decreased the



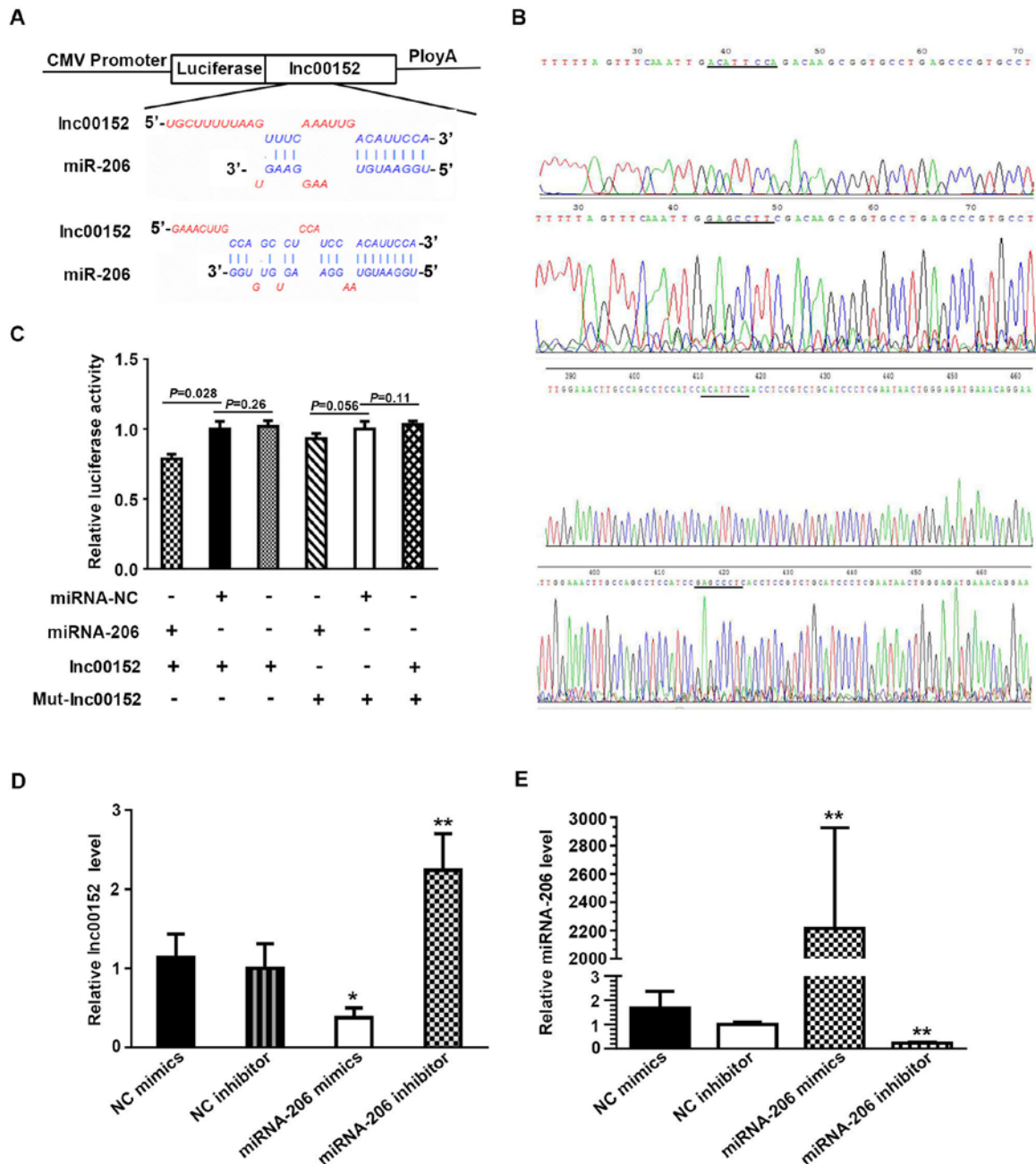


Figure 4. Effects of Inc00152 on colorectal cancer cells are regulated by miRNA-206. (A) Schematic diagram showing the interaction between Inc00152 and miRNA-206. (B) Sequence of mutant Inc00152. (C) Luciferase assay showing the specific interaction between Inc00152 and miRNA-206. (D) The regulation of miRNA-206 altered the Inc00152 level. (E) The miRNA-206 expression level was affected by transfection with miRNA-206 mimics and inhibitor. \* $P<0.05$ , \*\* $P<0.01$ .

number of invading cells relative to those transfected with the control siRNA (siRNA\_NC) (Fig. 2F,  $P<0.05$ ). Consequently, our findings indicate that Inc00152 enhances the proliferative and invasive ability of CRC cells.

**Inc00152 overexpression enhances colorectal tumor growth in vivo.** To determine whether Inc00152 regulates tumor growth *in vivo*, we xenografted Inc00152 stably expressing SW620 cells into nude mice. The volumes of tumors derived SW620 cells overexpressing Inc00152 were significantly greater than those of tumors derived from the pLV-NC-transfected control cells from days 10 to 26 following implantation (Fig. 3A and B,  $P<0.05$ ). Additionally, the protein levels of NRP1 were increased in tumors derived from the Inc00152-overexpressing cells (Fig. 3C

and D,  $P<0.05$ ). Furthermore, Inc00152 overexpression also promoted EMT in the SW620-derived tumors by decreasing the level of E-cadherin, while increasing N-cadherin expression (Fig. 3E). H&E staining also confirmed the cellular alteration in Inc00152-overexpressing colorectal tumors, which exhibited typical morphological characteristics of more malignant tumors (large cell morphology, large cell nucleus and malformed nuclei). Collectively, these data indicate that Inc00152 overexpression promotes tumor growth and EMT *in vivo*.

**miRNA-206 is a target gene of Inc00152.** A dual luciferase assay verified the target gene of miRNA-206 as Inc00152. Plasmid construction and sequence data and the sequence of mutant Inc00152 are described in Fig. 4A and B. When the

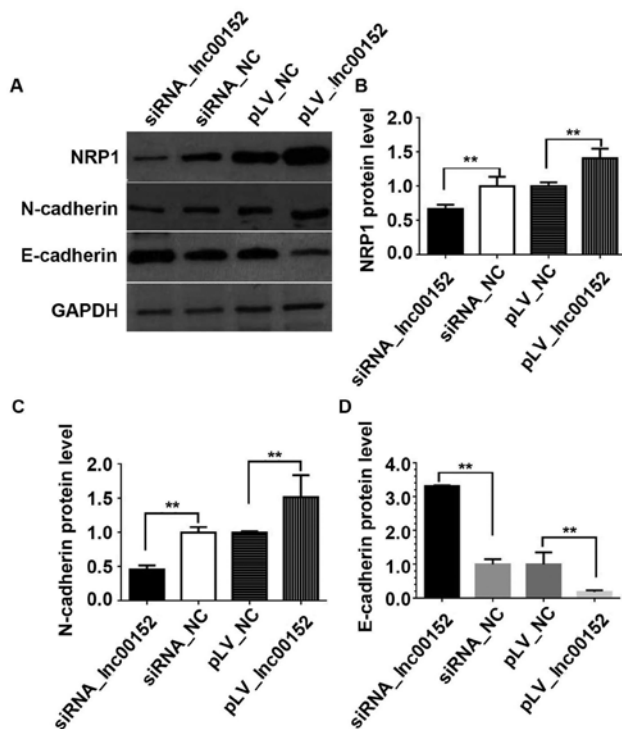


Figure 5. Regulation of lnc00152 affects NRP1 expression and epithelial-mesenchymal transition in colorectal cancer (CRC) cells. (A) Western blot analysis of NRP1, E-cadherin and N-cadherin expression following the transfection of human 293T cells transfected with siRNA\_NC, siRNA\_lnc00152, pLV\_NC, or pLV\_lnc00152. The regulation of lnc00152 expression altered the expression level of (B) NRP1, (C) N-cadherin, and (D) E-cadherin. \*\* $P < 0.01$ .

SW620 cells were transfected with the lnc00152 overexpression vector together with miRNA-NC, the relative luciferase activity did not differ significantly from that in the cells transfected with the lnc00152 overexpression vector only (Fig. 4C,  $P = 0.26 > 0.1$ ). By contrast, the relative luciferase activity decreased when the cells were transfected with the lnc00152 overexpression vector and the miRNA-206 mimic (Fig. 4C,  $P = 0.028 < 0.05$ ), demonstrating a direct interaction between lnc00152 and miRNA-206. Co-transfection with mutated lnc00152 (Mut-lnc00152) and miRNA-206 did not result in reduced relative luciferase activity (Fig. 4C,  $P > 0.05$ ), indicating that mutated lnc00152 disrupted the interaction between lnc00152 and miRNA-206. In addition, the lnc00152 level was lower in the cells transfected with miRNA-206 mimics compared to those transfected with NC mimics (Fig. 4D,  $P < 0.05$ ), while the lnc00152 level increased significantly following incubation with the miRNA-206 inhibitor compared to the NC inhibitor (Fig. 4D,  $P < 0.01$ ). In the presence of miRNA-206 mimics, the miRNA-206 level increased significantly (Fig. 4E,  $P < 0.01$ ), while it decreased significantly following incubation with miRNA-206 inhibitor (Fig. 4E,  $P < 0.01$ ). This displayed the specific efficiency of miRNA-206 mimics and inhibitor; collectively, these data indicate that miRNA-206 can directly bind to lnc00152 through miRNA recognition sites.

*lnc00152 regulates the miRNA-206 target gene, NRP1.* Yin *et al* (28) previously demonstrated that miRNA-206

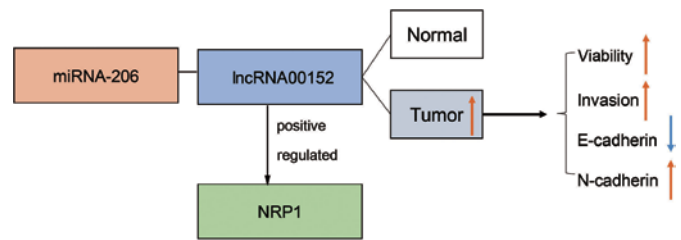


Figure 6. A schematic model demonstrating the mechanisms through which lnc00152 functions as a competing endogenous RNA (ceRNA) to regulate NRP1 expression by sponging miRNA-206 in colorectal cancer (CRC). lnc00152 was highly expressed in CRC tumor tissues compared with adjacent normal tissues and promoted CRC cell viability and invasion by competitively binding miRNA-206 upregulating NRP1, and subsequently up-regulating N-cadherin, while downregulating E-cadherin.

significantly inhibited the luciferase activity of the RLuc-NRP1 3'-UTR reporter, confirming that NRP1 is a target gene of miR-206. Thus, one of our aims in this study was to clarify whether lnc00152 regulates NRP1 in CRC cells. Our results indicated that lnc00152 silencing downregulated NRP1 expression, while lnc00152 overexpression elevated NRP1 expression (Fig. 5A and B,  $P < 0.01$ ). Additionally, we validated the effects of lnc00152 expression on EMT in CRC cells. lnc00152 silencing downregulated N-cadherin expression, whereas it upregulated E-cadherin expression (Fig. 5A-D,  $P < 0.01$ ). By contrast, lnc00152 overexpression increased N-cadherin expression, while it decreased E-cadherin expression (Fig. 5A-D,  $P < 0.01$ ). Therefore, lnc00152 regulates NRP1 expression and additionally affects EMT in CRC cells.

*The mechanisms through which lnc00152 regulates NRP1 in colorectal cancer.* From the above-mentioned results, we drew a schematic model to demonstrate the possible mechanisms through which lnc00152 regulates NRP1 (Fig. 6). Compared with the adjacent normal tissues, lnc00152 was highly expressed in CRC tumor tissues and promoted CRC cell viability and invasion by competitively binding miRNA-206, upregulating NRP1, and subsequently upregulating N-cadherin, while downregulating E-cadherin.

## Discussion

In this study, we found that lnc00152 was expressed in colorectal tumor tissues and the CRC cell lines, SW480, LoVo and SW620. The regulation of lnc00152 also affected CRC cell viability, invasion and EMT *in vitro*, as well as tumor growth *in vivo*. This process was associated with NRP1, a miRNA-206 target gene, and miRNA-206 was associated with lnc00152 expression.

lncRNAs play critical roles in cancer development and progression (29). Recent studies have indicated that lncRNAs can regulate both the migration and invasiveness of CRC. For example, the downregulation of lncRNA H19 has been shown to inhibit CRC cell migration and metastasis (30). This is consistent with our findings in that the silencing of lnc00152 decreased the proliferative and invasive ability of CRC cells. lncRNAs have also been reported to suppress EMT in CRC (11). EMT is a hallmark in tumor progression (31).



Our findings further validated this observation by showing that the downregulation of lnc00152 reduced N-cadherin and increased E-cadherin expression, while lnc00152 overexpression had the opposite effect. This is consistent with the role of other lncRNAs in the CRC cell lines LoVo and HCT116 (11).

Furthermore, we found that overexpression of lnc00152 may increase the malignant potential of CRC cells. This has also been observed in a previous study which showed that the upregulation of lncRNA BANCR was associated with metastasis and the poor survival of patients with CRC (12). In this study, patients with a higher level of lnc00152 had a shorter survival time, displaying an association between the lnc00152 level and CRC prognosis. This finding, too, is supported by the findings of other studies that have demonstrated an unfavorable prognosis of patients with CRC with a lncRNA expression (32-34). In our tumor xenograft experiment, lnc00152 overexpression promoted tumor growth, which demonstrates that lnc00152 plays an essential role in regulating the malignancy of CRC. Importantly, as lnc00152 silencing appears to have an anti-tumor effect, it may function as a therapeutic target in the treatment of CRC, and may thus aid in the treatment and prognosis of CRC.

Some miRNAs have been observed to be dysregulated in CRC tissues (35). The function of miRNAs in CRC involves controlling EMT (36) and affecting cell proliferation, migration and invasion (37), similar to the function of lncRNAs in CRC. In ovarian cancer, the miRNA-lncRNA signature is important in patient survival (38). The miRNA-lncRNA complex is also involved in the transformation process of gastric cancer initiation to malignancy (39). Accordingly, lncRNA may interact with miRNAs to regulate colorectal tumor malignancy. Previous studies have reported that lnc00152 is implicated in CRC by regulating cell cycle, apoptosis, cell motility and EMT (40,41). In addition, lnc00152 has been demonstrated to promote oncogenesis via several mechanisms, including serving as a sponge for miR-4767 and miR-205 (42,43) and a partner for EZH2 (41). In this study, we observed the interaction of lnc00152 with miRNA-206 with a dual luciferase assay. This observation and other reports of miR-206 inhibiting tumor invasion and migration in CRC (44), highlight an important association between miRNA-206 and lnc00152 in CRC.

Our experiments have additionally located the mutation site on lnc00152 that interacts with miRNA-206. NRPI is the target gene of miRNA-206 (28), and our data demonstrated that regulating the lnc00152 levels altered NRPI expression. NRPI is a co-receptor for vascular endothelial growth factor (VEGF), and the blockaded of NRPI suppresses tumor growth by inhibiting angiogenesis or by directly inhibiting tumor cell proliferation (45). In CRC, NRPI is associated with liver metastasis (46), and, importantly, EMT in CRC is dependent on NRPI (26). Future studies, therefore, are warranted in order to focus on the molecular mechanisms that underlie the synergic effects of miRNA-206 and lnc00152 on CRC progression and on the signaling pathway of the NRPI-associated modulation of CRC metastasis.

In conclusion, the results of the present study identify lnc00152 as a competing endogenous RNA that positively regulates NRPI expression by sponging miRNA-206 (Fig. 6). Given its critical role in CRC, it may be an effective therapeutic target in CRC prognosis and treatment.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

ZPC, JCW and QW conceived and designed the research and drafted the manuscript. PY, WLL and JBZ conducted the experiments. FH, HCC and HH performed the data analysis. JC substantially contributed to the design and conception of the study, and critically reviewed and revised the manuscript, and approved the final version to be published. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The use of human samples was approved by the Ethics Committee Guangzhou First People's Hospital, Guangzhou, China and all patients involved in the study provided written informed consent. The use of animals in this study was approved by the Ethics Committee of the Laboratory Animal Center of South China University of Technology, Guangzhou, China.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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