Biological functions and clinical significance of the newly identified long non-coding RNA RP1-85F18.6 in colorectal cancer

YESHUO MA[1], YIFEI CHEN[2], CHANGWEI LIN[3] and GUI HU[3]

[1]Department of Cardiology, The Third Xiangya Hospital of Central South University;
[2]Department of Otolaryngology-Head Neck Surgery, The Fourth Hospital of Changsha (The Changsha Affiliated Hospital of Hunan Normal University), Hunan Normal University;
[3]Department of Gastrointestinal Surgery, The Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, P.R. China

Received March 31, 2018; Accepted September 7, 2018

DOI: 10.3892/or.2018.6694

Abstract. The biological functions of long non-coding RNAs (lncRNAs) in cancer have not been fully elucidated. The present study demonstrated that the expression of a newly identified lncRNA, RP1-85F18.6, was upregulated in colorectal cancer (CRC) tissues and cell lines. Knockdown of lncRNA RP1-85F18.6 served a key role in tumor inhibition, reduced cell proliferation and invasion, disrupted the cell cycle, and increased apoptosis and pyroptosis of CRC cells. Conversely, overexpression of lncRNA RP1-85F18.6 exerted the opposite effects. Furthermore, silencing lncRNA RP1-85F18.6 decreased ΔNp63 expression at both the mRNA and protein levels. Furthermore, co-transfection with ΔNp63 siRNA and lncRNA RP1-85F18.6-expressing vector attenuated the tumor-promoting effects of lncRNA RP1-85F18.6 overexpression. The expression levels of lncRNA RP1-85F18.6, ΔNp63 and gasdermin D (GSDMD) were revealed to be associated with lymph node and distant metastases in patients with CRC, and therefore may serve as predictors in CRC. The findings of the present study suggested that lncRNA RP1-85F18.6 may trigger CRC cell proliferation, invasion and cell cycle disruption, and suppress apoptosis and pyroptosis of CRC cells through regulating ΔNp63 expression. Therefore, lncRNA RP1-85F18.6 and ΔNp63 may be considered unfavorable biomarkers, whereas GSDMD may be a favorable biomarker in CRC; these markers may prove valuable in the future diagnosis and prognosis of CRC.

Correspondence to: Professor Gui Hu, Department of Gastrointestinal Surgery, The Third Xiangya Hospital of Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, P.R. China
E-mail: hugui22@qq.com

Abbreviations: lncRNA, long non-coding RNA; CRC, colorectal cancer; GSDMD, gasdermin D; ncRNA, non-protein-coding ribonucleic acid; NCT, non-cancerous tissue; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LDH, lactate dehydrogenase; ROC curve, receiver operating characteristic curve; AUC, area under the ROC curve; CEA, carcinoembryonic antigen

Key words: long non-coding RNA, ΔNp63, colorectal cancer, pyroptosis, apoptosis, gasdermin D

Introduction

Colorectal cancer (CRC) is the second most prevalent type of cancer and the third leading cause of cancer-associated mortality worldwide (1). Despite significant advances in the diagnosis and treatment of CRC, the overall survival rate of CRC remains unsatisfactory (2,3). Therefore, it is necessary to identify the molecular mechanisms underlying the occurrence and development of CRC, in order to develop more effective diagnostic and therapeutic methods.

Genomic studies have confirmed that only 2% of human gene transcripts encode proteins, whereas numerous transcripts encode non-coding ribonucleic acids (ncRNAs) (4). Long ncRNAs (lncRNAs) are a subtype of ncRNAs >200 nucleotides long, which lack protein-coding ability. Although the exact functions of lncRNAs remain unclear, they appear to regulate numerous biological behaviors through epigenetic regulation, transcription and post-transcriptional processing (5). Accumulating evidence has indicated that the aberrant expression of lncRNAs may regulate cancer cell proliferation, migration, invasion, apoptosis and metastasis (6,7).

p63 is a member of the p53 family, which is highly homologous and structurally similar to p53 (8). p63 has two isoforms, TAp63 and ΔNp63, which differ at the N-terminal. TAp63 contains an N-terminal transactivation domain, whereas ΔNp63 lacks this domain (9). These two isoforms perform different functions during tumorigenesis. TAp63 acts as a tumor suppressor, similar to p53, and induces cell cycle arrest and cell apoptosis (10). Conversely, ΔNp63 acts as an oncogene, facilitating proliferation, survival, invasion, metastasis and chemoresistance, and reducing apoptosis of various cancer

cells (11-13). Numerous studies have revealed that lncRNAs mediate the biological functions of CRC cells through modulating p53 expression (14-16). However, the number of studies on lncRNAs regulating CRC tumorigenesis by targeting p63 is limited. The present study aimed to determine whether there is a newly identified lncRNA targeting ΔNp63, and to investigate the biological functions of this newly identified lncRNA in the proliferation, cell cycle progression, invasion, apoptosis and pyroptosis of CRC cells, in the aim of providing a novel target for the treatment of patients with CRC.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of The Third Xiangya Hospital of Central South University (Changsha, China). The study protocol conformed to the principles outlined in the Helsinki Declaration and to local legislation. Informed consent was obtained from all of the participants.

Patient specimens. A total of 34 pairs of human primary CRC tissues and corresponding non-cancerous tissues (NCTs) were collected from patients who had undergone tumor resection at the Department of Gastrointestinal Surgery, The Third Xiangya Hospital of Central South University between October 2014 and June 2015. The specimens were immediately frozen in liquid nitrogen following surgical resection and maintained at -80˚C. The clinical characteristics of all patients, including age, tumor site, tumor differentiation, stage, lymph node metastasis and distant metastasis, are summarized in Table I. Cancer staging was performed according to the 7th TNM classification by the American Joint Committee on Cancer (17). None of the patients underwent radiotherapy or chemotherapy prior to surgery.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was extracted from CRC tissues/NCTs, or fresh cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RT of 3 μg total RNA was performed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using the SYBR-Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and was run using a thermal cycler (Bio-Rad Laboratories, Inc.). The thermal cycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles at 95˚C for 30 sec, 50˚C for 30 sec and 72˚C for 30 sec, and a final extension step at 72˚C for 10 min. GAPDH was used for normalization. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18). The primers used in the present study are listed in Table II.

Cell culture. The NCM460 human colorectal epithelial cell line, and the SW480, SW620 and HCT116 CRC cell lines were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) (HyClone; GE Healthcare, Logan, UT, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell transfection. LncRNA RP1-85F18.6 small interfering (si)RNA, ΔNp63 siRNA and negative control (NC) siRNA were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences were as follows: lncRNA RP1-85F18.6 siRNA, 5'-GACTCCGCCGTGAACCCT TCA-3'; ΔNp63 siRNA, 5'-ACAAUGCCCAGACUCAAU UUU-3'. A scramble siRNA (siN05815122147) was used as the NC siRNA. Once the SW620 cells reached 70% confluence, the cells were transfected for 48 h at 37˚C with lncRNA RP1-85F18.6 siRNA, ΔNp63 siRNA or NC siRNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The siRNAs were diluted to a final concentration of 60 nM for transfection. In addition, the entire sequence of human lncRNA RP1-85F18.6 was amplified from SW620 cells using PCR and cloned into the pcDNA3.1 vector. The negative control empty vector, which was purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China), and the lncRNA RP1-85F18.6 plasmid were transfected into SW620 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The plasmid was diluted to a final concentration of 2 μg/ml for transfection.

Cell proliferation assay. Transfected SW620 cells were cultured in 96-well plates and incubated for 24, 48 and 72 h. Optical density values were measured using the MTT Cell Proliferation and Cytotoxicity Assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocol. Cell proliferation was calculated using the absorbance values, which were measured at 490 nm at each time point.

Flow cytometric analysis of apoptosis. Cell apoptosis was estimated using flow cytometric analysis with the Apoptosis Detection kit (Nanjing KeyGen BioTech Co., Ltd.), according to the manufacturer's protocol. After transfection for 48 h, the cells were washed with ice-cold PBS and resuspended with binding buffer. Subsequently, the cells were incubated with propidium iodide (PI) and Annexin V at room temperature for 15 min in the dark. The cells were then resuspended with PBS and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (v7.6.2, FlowJo; LLC, Ashland, OR, USA).

Cell cycle analysis. Cell cycle analysis was performed as previously described (19). Briefly, after transfection for 48 h, the cells were washed with PBS and fixed with ice-cold 70% ethanol at 4˚C overnight. Fixed cells were washed with PBS and incubated with PI and RNase, which were obtained from the Cell Cycle Detection kit (Nanjing KeyGen BioTech Co., Ltd.), for 30 min at room temperature in the dark. Subsequently, the incubated cells were analyzed by flow cytometry (BD Biosciences) and FlowJo software (v7.6.2, FlowJo; LLC).

Transwell assay. Cellular invasion was evaluated using Transwell migration chambers precoated with a layer of Matrigel®. This assay was performed as previously described at 48 h post-transfection (20). Briefly, cells suspended in 100 μl medium without FBS were seeded in the upper chamber (10^4 cells/well). To the lower chamber, 600 μl medium

Table I. Association of clinical and pathological characteristics with lncRNA RP1-85F18.6, ΔNp63 and GSDMD mRNA expression.

		lncRNA RP1-85F18.6			ΔNp63			GSDMD		
Clinicopathological features	Cases	Low[a] (n=17)	High[a] (n=17)	P-value	Low[a] (n=17)	High[a] (n=17)	P-value	Low[a] (n=17)	High[a] (n=17)	P-value
Age (years)	34	56.36±15.17	58.55±10.31	0.905	58.11±12.10	56.27±13.16	0.918	57.68±11.25	56.89±12.39	0.962
Tumor site										
Left colon	9	5	4	0.774	4	5	0.257	5	4	0.856
Right colon	5	3	2		1	4		2	3	
Rectum	20	9	11		12	8		10	10	
Tumor differentiation										
Well	6	2	4	0.634	4	2	0.533	2	4	0.285
Moderate	23	12	11		10	13		11	12	
Poor	5	3	2		3	2		4	1	
Stage										
I	5	2	3	0.726	3	2	0.362	3	2	0.905
II	9	4	5		3	6		5	4	
III	16	8	8		10	6		7	9	
IV	4	3	1		1	3		2	2	
Lymph node metastasis										
N0	16	11	5	0.034[b]	12	4	0.022[b]	3	13	0.003[b]
N1	10	5	5		3	7		8	2	
N2	8	1	7		2	6		6	2	
Distant metastasis										
M0	30	17	13	0.033[b]	17	13	0.033[b]	13	17	0.033[b]
M1	4	0	4		0	4		4	0	

[a]Low and high expression groups were determined according to the median value of lncRNA RP1-85F18.6, ΔNp63 and GSDMD mRNA in the 34 tumor tissue specimens. [b]Statistical significance ($P<0.05$). GSDMD, gasdermin D; lncRNA, long non-coding RNA.

Table II. Reverse transcription-quantitative polymerase chain reaction primers used in this study.

Gene name	Primer sequence (5'-3')
lncRNA RP1-85F18.6	Forward: GGCTCTTTGCTCACATCG
	Reverse: AAGGAAACCACAGGCTCA
ΔNp63	Forward: GAAGAAAGGACAGCAGCAT TGA
	Reverse: GGGACTGGTGGACGAGGAG
TAp63	Forward: TGTATCCGCATGCAGGACT
	Reverse: CTGTGTTATAGGGACTGGTG GAC
GSDMD	Forward: GTGTGTCAACCTGTCTATCA AGG
	Reverse: CATGGCATCGTAGAAGTGGAAG
GAPDH	Forward: ACCACAGTCCATGCCATCAC
	Reverse: TCCACCACCCTGTTGCTGTA

GSDMD, gasdermin D; lncRNA, long non-coding RNA.

supplemented with 20% FBS was added. After 24 h incubation at 37˚C, cells on the top of the membrane were removed with cotton swabs. The cells that invaded through the membrane were washed with PBS, fixed in 4% methanol for 20 min and stained with 0.1% crystal violet for 10 min at room temperature. The number of invasive cells was counted in five randomly selected fields under a light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Protein extraction and western blotting. Protein extraction and western blotting were performed as previously described (21). Briefly, proteins were extracted from tissues and cells using radioimmunoprecipitation assay lysis buffer containing 1% 100 mM phenylmethylsulfonyl fluoride (both from Beyotime Institute of Biotechnology). Protein concentration was examined using the bicinchoninic acid method (Nanjing KeyGen Biotech Co., Ltd.). Subsequently, proteins (20 μg) were separated by 10% SDS-PAGE and were transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% non-fat milk for 2 h at room temperature, and were incubated with the following primary antibodies at 4˚C overnight: Anti-β-actin (1:2,000, cat. no. 20536-1-AP) and anti-gasdermin D (GSDMD; 1:1,000, cat. no. 20770-1-AP) (both from Wuhan Sanying Biotechnology, Wuhan, China); anti-ΔNp63 (1:500, cat. no. 619001) and anti-TAp63 (1:500, cat. no. 618901) (both from BioLegend, Inc., San Diego, CA, USA). After washing with PBS-0.1% Tween, the membranes were incubated with anti-rabbit immunoglobulin G secondary antibody (1:5,000, cat. no. SA00001-2; Wuhan Sanying Biotechnology) for 1 h at room temperature. The images were obtained using Advansta WesternBright enhanced chemiluminescence (Advansta lnc., Menlo Park, CA, USA) and the ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc.).

Lactate dehydrogenase (LDH) release assay. After transfection for 48 h, the supernatants of transfected cells were collected to measure LDH release using LDH Cytotoxicity Assay kit, according to the manufacturer's protocol (Beyotime Institute of Biotechnology). Data were collected based on the absorbance at 490 nm, which was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis. All experiments were repeated three times and data are expressed as the means ± standard deviation. All statistical calculations were performed using GraphPad Prism version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS (PASW Statistics) version 20 (IBM Corporation, Armonk, NY, USA). The Mann-Whitney U test was used to compare differences between two groups. One-way analysis of variance followed by the Student-Newman-Keul's test was used to compare multiple groups. The correlation between two factors was determined using the Spearman's rank correlation test. The sensitivity and specificity in CRC tissues were calculated using the Youden's index. The area under the receiver operating characteristic (ROC) curve (AUC) was also estimated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

p63-associated lncRNA identification. To identify novel lncRNAs targeting p63, a microarray analysis was performed in our previous study, and thousands of lncRNAs and mRNAs were differentially expressed between CRC tissues and NCTs (22). Subsequently, LncTar, which is an efficient tool for predicting RNA targets of lncRNAs that is provided by Cui Lab from the Department of Biomedical Informatics, Peking University Health Science Center (http://www.cuilab.cn/lnctar) (23), was used. The Gibbs chemical bond free energy between tumor protein 63 (TP63; NM_003722.4) and candidate lncRNAs was determined. Using a value of <0.05 as the threshold, lncRNAs below that threshold were considered to have a lower Gibbs chemical bond free energy. A total of 24 lncRNAs were predicted to target TP63; among those, six were upregulated and 18 were downregulated in a CRC lncRNA microarray. The top five upregulated TP63-associated lncRNAs are listed in Table III.

Expression of a newly identified lncRNA is upregulated in CRC tissues. To further verify the lncRNAs that are associated with p63 in tumor samples, RT-qPCR was performed to assess the expression levels of upregulated lncRNAs in 10 pairs of matched CRC tissues and NCTs. Among these lncRNAs, lncRNA RP1-85F18.6 (ENST00000415054) was the most markedly increased in CRC tissues (data not shown). Therefore, lncRNA RP1-85F18.6 was selected for subsequent experiments. Compared with the NCTs, lncRNA RP1-85F18.6 expression was increased in 24 tumor tissues (70.6%, $P<0.05$), out of the 34 pairs of matched CRC tissues and NCTs (Fig. 1A). These results provided further evidence to suggest that lncRNA RP1-85F18.6 was increased in CRC tissues. The results of RT-qPCR also demonstrated that ΔNp63 expression was increased in 22 tumor tissues (64.8%, $P<0.05$), and TAp63 expression was reduced in 22 tumor

Table III. Top five upregulated tumor protein 63-associated long non-coding RNAs.

Sequence name	ndG	Fold change
ENSG00000232754|ENST00000415054	-0.0889	13.3513184
ENSG00000257453|ENST00000552367	-0.0823	4.7515723
ENSG00000259933|ENST00000563284	-0.0746	6.7542724
ENSG00000259479|ENST00000564140	-0.0712	5.0080102
ENSG00000204787|ENST00000447469	-0.0698	10.0279087

dG, delta of Gibbs chemical bond free energy.

tissues (64.8%, P<0.05) (Fig. 1B and C). Similarly, the protein expression levels of ΔNp63 were obviously increased in CRC tissues (Fig. 1D), whereas the protein expression levels of TAp63 were reduced (Fig. 1D). These results were consistent with the findings of previous studies (13,21). Furthermore, lncRNA RP1-85F18.6 was positively correlated with ΔNp63 expression (r=0.678, P<0.001), whereas the correlation with TAp63 expression was negative (r=-0.371, P=0.0308) (Fig. 1E and F). These findings indicated that lncRNA RP1-85F18.6 may be correlated with TP63, and may facilitate the progression of CRC.

Expression of lncRNA RP1-85F18.6, ΔNp63 and TAp63 in CRC cells. To assess the expression of lncRNA RP1-85F18.6, ΔNp63 and TAp63 in CRC cells, their expression was examined in various CRC cell lines (SW480, SW620 and HCT116) compared with in the NCM460 normal human colorectal epithelial cell line, using RT-qPCR and western blotting. Consistent with the results of CRC tumor samples, the mRNA expression levels of lncRNA RP1-85F18.6 and ΔNp63 were markedly upregulated, whereas those of TAp63 were downregulated in the SW480 and SW620 cell lines (Fig. 1G); the protein expression of ΔNp63 and TAp63 was altered accordingly (Fig. 1H). These results further confirmed that lncRNA RP1-85F18.6 and TP63 may serve key roles in triggering the tumorigenic process in CRC. In addition, the differences in the expression of lncRNA RP1-85F18.6, ΔNp63 and TAp63 were most significant in the SW620 cell line; therefore, SW620 cells were selected for subsequent experimentation.

lncRNA RP1-85F18.6 acts as an oncogene in CRC cells. To evaluate the biological role of lncRNA RP1-85F18.6 in CRC tumorigenesis, lncRNA RP1-85F18.6 was silenced by transfecting SW620 cells with lncRNA RP1-85F18.6 siRNA or NC siRNA (Fig. 2A). The Matrigel-coated Transwell assay revealed that silencing lncRNA RP1-85F18.6 inhibited invasion of CRC cells compared with in the NC group (215 vs. 272, P<0.05) (Fig. 2B). This finding suggested that lncRNA RP1-85F18.6 may promote the invasive phenotype of CRC cells. To investigate whether lncRNA RP1-85F18.6 regulated the proliferation of CRC cells, the MTT assay was performed. As shown in Fig. 2C, the proliferative ability of lncRNA RP1-85F18.6 siRNA-transfected SW620 cells was significantly reduced. Subsequently, cell cycle analysis revealed that SW620 cells exhibited a shortened S phase (11.9 vs. 22.0%, P<0.05) and were arrested at the G_2 phase (17.8 vs. 7.6%, P<0.05) following transfection with lncRNA RP1-85F18.6 siRNA compared with in the NC group (Fig. 2D). Furthermore, the apoptotic rate of SW620 cells was upregulated after silencing lncRNA RP1-85F18.6 (13.38 vs. 4.81%, P<0.05) compared with in the NC group (Fig. 2E). These results suggested that the reduced cell proliferation may be attributed to disrupted cell cycle progression and increased apoptosis.

Furthermore, LDH release was markedly elevated following inhibition of lncRNA RP1-85F18.6, thus indicating rupture of the plasma membrane and pyroptosis (Fig. 2F). This result suggested that the reduced proliferation may also be caused by cell pyroptosis. GSDMD is a member of the gasdermin family, which has been confirmed to be the main effector molecule for pyroptosis (24,25). In various CRC cells, the expression of GSDMD was revealed to be downregulated, both at the mRNA and protein levels (Fig. 2G and H). Furthermore, it was demonstrated that, although silencing lncRNA RP1-85F18.6 did not alter the full-length protein expression of GSDMD, it increased GSDMD-N domain cleavage, as demonstrated by western blotting (Fig. 3A). Following cleavage from the full-length GSDMD, the GSDMD-N domain acts as the executioner of pyroptosis, perforating the cell membrane, and causing cell swelling and lysis (26).

lncRNA RP1-85F18.6 acts as an oncogene in CRC cells through regulating ΔNp63. To further investigate the underlying mechanisms by which lncRNA RP1-85F18.6 mediates the biological functions of CRC cells, the present study examined whether lncRNA RP1-85F18.6 regulates the expression of ΔNp63 and TAp63. lncRNA RP1-85F18.6 was silenced in SW620 cells, and the RT-qPCR and western blotting results revealed that the expression of ΔNp63 was reduced at both the mRNA and protein levels (Fig. 3A and B). Unexpectedly, there were no significant alterations in the expression of TAp63 at the mRNA or protein levels (Fig. 3A and B).

Subsequently, SW620 cells were transfected with lncRNA RP1-85F18.6-expressing vector, or were co-transfected with lncRNA RP1-85F18.6-expressing vector and the ΔNp63 siRNA (Fig. 3C-G), which was revealed to decrease ΔNp63 expression (Fig. 4). The expression of lncRNA RP1-85F18.6 was markedly upregulated following transfection with lncRNA RP1-85F18.6-expressing vector (Fig. 3C). Compared with the NC group, overexpression of lncRNA RP1-85F18.6 increased the percentage of cells in S phase and decreased the percentage

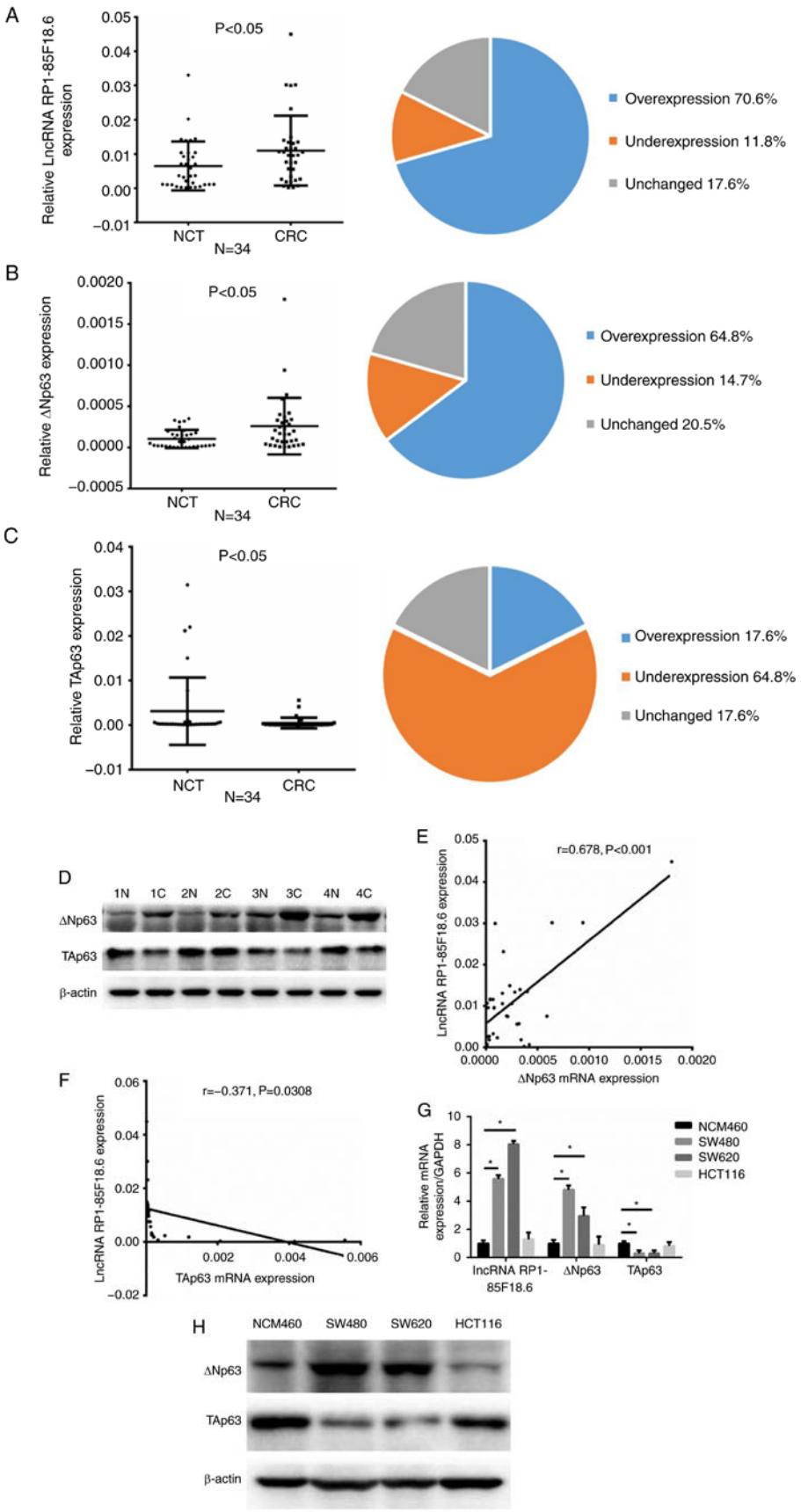

Figure 1. Expression of lncRNA RP1-85F18.6, ΔNp63 and TAp63 in CRC tissues and cell lines. (A-C) Expression of lncRNA RP1-85F18.6, ΔNp63 and TAp63 in CRC tissues, as assessed by RT-qPCR (n=34, P<0.05). The mean expression levels are shown in the left scatter diagram and the expression distributions are summarized in the right pie chart. GAPDH was used for normalization. (D) Representative results of increased ΔNp63 expression and reduced TAp63 expression in CRC tissues, as assessed by western blotting (N, NCT; C, CRC). (E and F) lncRNA RP1-85F18.6 was positively correlated with ΔNp63 and negatively correlated with TAp63 in CRC samples. (G and H) Expression levels of lncRNA RP1-85F18.6, ΔNp63 and TAp63 in various cell lines were evaluated by RT-qPCR and western blotting; n=3, *P<0.05. CRC, colorectal cancer; lncRNA, long non-coding RNA; NCT, non-cancerous tissue; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

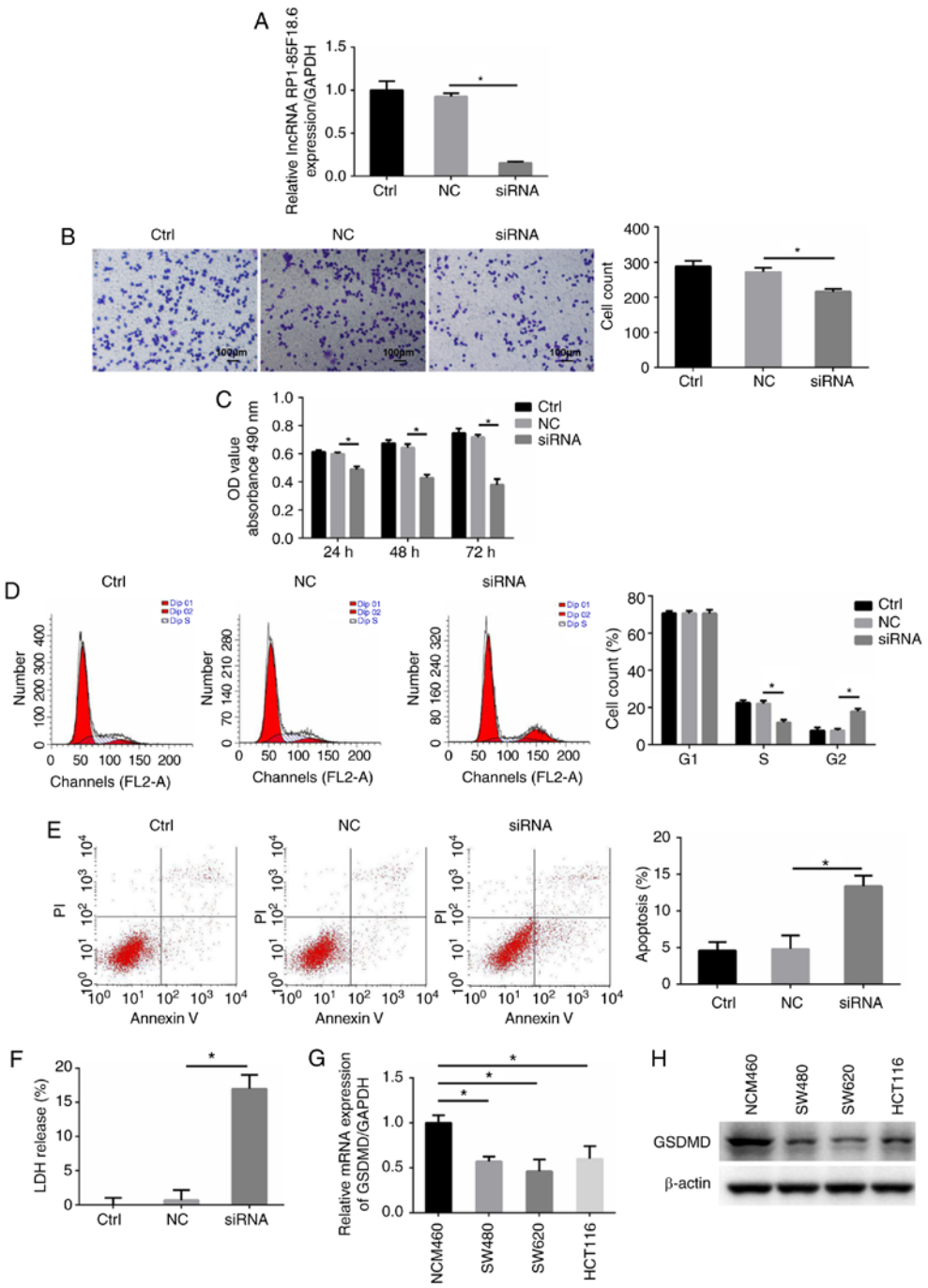

Figure 2. Biological functions of lncRNA RP1-85F18.6 in CRC cells. The CRC cells were transfected with lncRNA RP1-85F18.6 siRNA or NC siRNA. (A) Post-transfection with lncRNA RP1-85F18.6 siRNA, the expression levels of lncRNA RP1-85F18.6 were assessed by RT-qPCR. (B) Matrigel-coated Transwell assay was used to determine the invasive ability of cells; magnification, x200. (C) Proliferative ability was assessed by the MTT assay. (D and E) Cell cycle distribution and apoptosis were determined by flow cytometric analysis. (F) LDH release was measured by colorimetric assay. (G and H) mRNA and protein expression levels of GSDMD in various cell lines were evaluated by RT-qPCR and western blotting, respectively. n=3, *P<0.05. CRC, colorectal cancer; GSDMD, gasdermin D; LDH, lactate dehydrogenase; lncRNA, long non-coding RNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

of cells in G_2 phase (Fig. 3D). In addition, increased lncRNA RP1-85F18.6 expression promoted cell proliferation (Fig. 3E) and invasion (Fig. 3F), and inhibited apoptosis (Fig. 3G) and cleavage of the GSDMD-N domain (Fig. 3H) in SW620 cells. However, co-transfection with lncRNA RP1-85F18.6-expressing vector and ΔNp63 siRNA reversed these tumor-promoting effects. These data suggested that lncRNA RP1-85F18.6 may induce the proliferation, invasion and cell cycle disruption, and inhibit apoptosis and pyroptosis of CRC cells through regulating ΔNp63.

Association of lncRNA RP1-85F18.6, ΔNp63 and GSDMD expression with clinicopathological parameters. To establish the association of GSDMD with CRC, the mRNA and protein expression levels of GSDMD were detected in CRC tissues. The mRNA expression levels of GSDMD

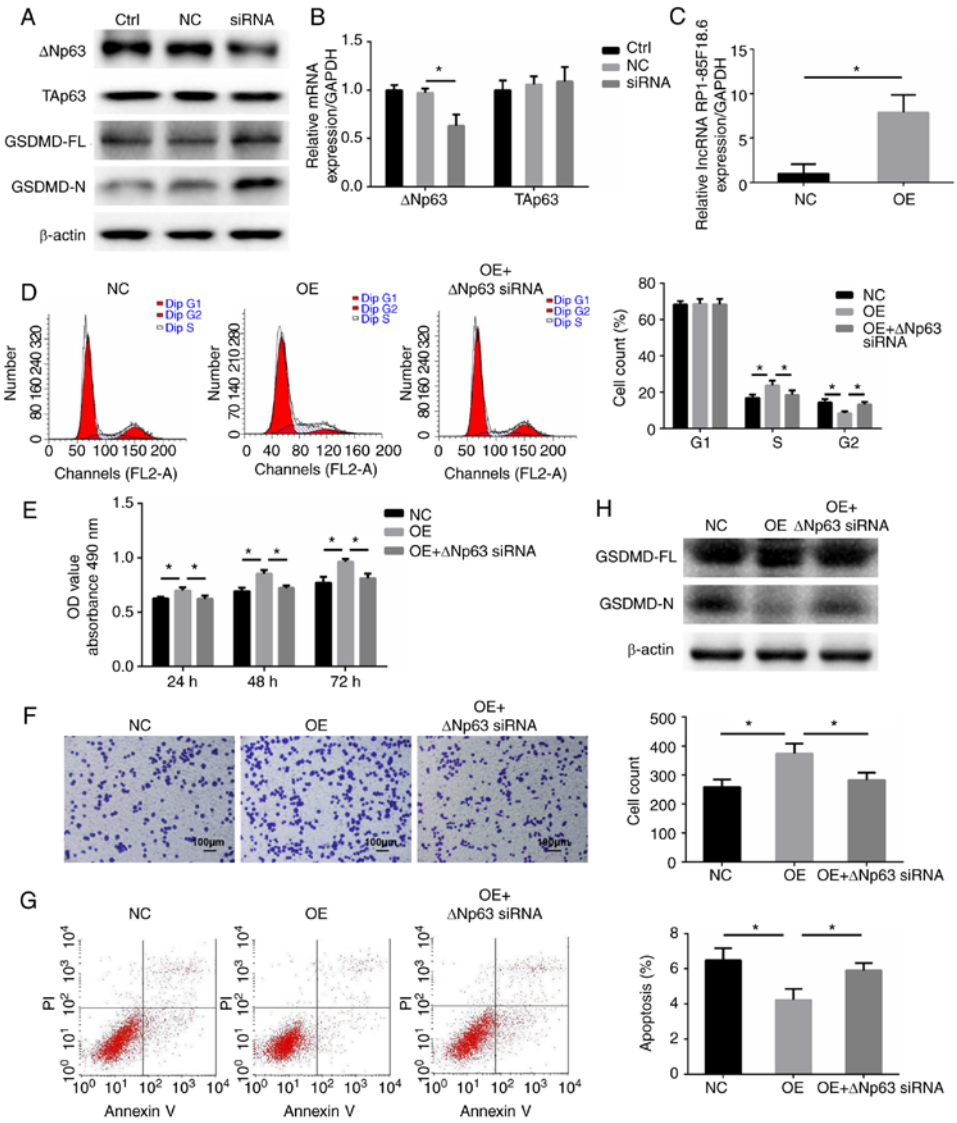

Figure 3. lncRNA RP1-85F18.6 mediates biological functions of CRC cells through regulating ΔNp63. (A) Western blotting was performed to evaluate ΔNp63, TAp63, GSDMD-FL and GSDMD-N expression after knockdown of lncRNA RP1-85F18.6. (B) Following transfection with lncRNA RP1-85F18.6 siRNA, the expression levels of ΔNp63 and TAp63 were assessed by RT-qPCR. CRC cells were transfected with empty vector or lncRNA RP1-85F18.6-expressing vector, or were co-transfected with lncRNA RP1-85F18.6-expressing vector and ΔNp63 siRNA. (C) Following transfection with the lncRNA RP1-85F18.6-expressing vector, the expression of lncRNA RP1-85F18.6 was assessed by RT-qPCR. (D) Cell cycle distribution was determined by flow cytometry. (E) Proliferative ability of CRC cells was evaluated by the MTT assay. (F) Matrigel-coated Transwell assay was used to determine invasive ability of cells; magnification, x200. (G) Flow cytometric analysis was applied to evaluate cell apoptosis. (H) Protein expression levels of GSDMD-FL and GSDMD-N were determined by western blotting. n=3, *P<0.05. CRC, colorectal cancer; GSDMD, gasdermin D; GSDMD-FL, GSDMD-full length; GSDMD-N, GSDMD-N domain; lncRNA, long non-coding RNA; NC, negative control; OD, optical density; OE, overexpression of lncRNA RP1-85F18.6; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

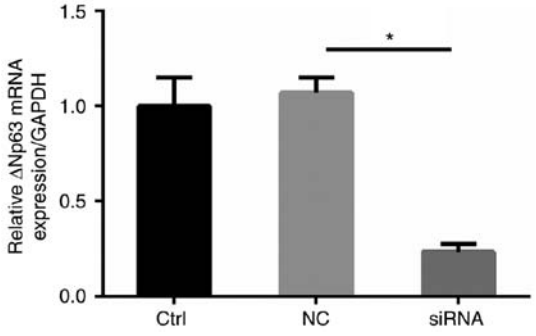

Figure 4. ΔNp63 expression was knocked down by siRNA in colorectal cancer cells. mRNA expression levels were determined using reverse transcription-quantitative polymerase chain reaction. n=3, *P<0.05. NC, negative control; siRNA, small interfering RNA.

were reduced in 26 tumor tissues (76.4%, P<0.05) (Fig. 5A). Consistently, the protein expression levels of GSDMD were markedly decreased in CRC tissues (Fig. 5B). Furthermore, the association of lncRNA RP1-85F18.6, ΔNp63 and GSDMD expression with the clinicopathological characteristics of patients with CRC was evaluated. As shown in Table I, lncRNA RP1-85F18.6 and ΔNp63 expression was positively correlated with lymph node and distant metastases (P<0.05). Conversely, an inverse association was determined between GSDMD expression and lymph node and distant metastases (P<0.05). In addition, there were no statistically significant associations between lncRNA RP1-85F18.6, ΔNp63 or GSDMD expression, and other clinicopathological parameters (P>0.05).

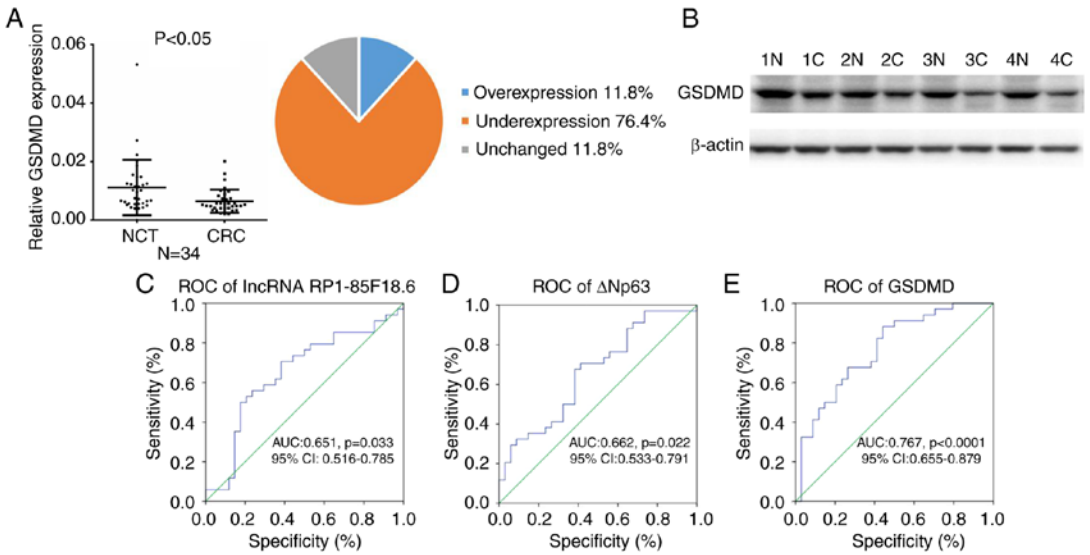

Figure 5. Association between lncRNA RP1-85F18.6, ΔNp63 and GSDMD expression, and clinicopathological characteristics of patients with CRC. (A) Expression of GSDMD in CRC tissues, as assessed by reverse transcription-quantitative polymerase chain reaction (n=34, P<0.05). The mean expression of GSDMD is shown in the left scatter diagram and the expression distribution is summarized in the right pie chart. GAPDH was used for normalization. (B) Representative results of reduced expression of GSDMD in CRC tissues from four cases, as assessed by western blotting (N, NCT; C, CRC). (C-E) ROC curve analysis of the expression levels of lncRNA RP1-85F18.6, ΔNp63 and GSDMD for discriminating CRC from NCT samples. AUC, area under the ROC curve; CRC, colorectal cancer; GSDMD, gasdermin D; lncRNA, long non-coding RNA; NCT, non-cancerous tissue; ROC, receiver operating characteristic.

The present study also demonstrated that lncRNA RP1-85F18.6, ΔNp63 and GSDMD were suitable predictors of CRC through a ROC curve analysis. The AUC of lncRNA RP1-85F18.6 for CRC was 0.651 [95% confidence interval (CI): 0.516-0.785; P=0.033], with 55.9% sensitivity and 76.5% specificity (Fig. 5C). The AUC of ΔNp63 for CRC was 0.662 [95% CI: 0.533-0.791; P=0.022], with 70.6% sensitivity and 58.8% specificity (Fig. 5D). The AUC of GSDMD for CRC was 0.767 [95% CI: 0.655-0.879; P<0.0001], with 88.2% sensitivity and 55.9% specificity (Fig. 5E). These results suggested that lncRNA RP1-85F18.6, ΔNp63 and GSDMD may be implicated in CRC tumorigenesis and metastasis, and may serve as potential prognostic biomarkers of CRC in the future.

Discussion

Initially, lncRNAs were considered to be 'transcriptional noise'; however, over the past few years, research has indicated that lncRNAs may act as oncogenes or tumor suppressor genes, and exert a multitude of biological effects on various types of cancer, including CRC (20,27,28). Accumulating evidence has indicated that lncRNAs regulate the proliferation, migration, invasion, apoptosis, pyroptosis and metastasis of cancer cells during tumorigenesis (6,7).

In the present study, the expression levels of the newly identified lncRNA RP1-85F18.6 were upregulated in CRC tissues and cell lines, thus indicating that it may have a key role in CRC tumorigenesis. Subsequently, the biological role of lncRNA RP1-85F18.6 was evaluated in CRC cells, and the underlying molecular mechanisms were explored. The results demonstrated that lncRNA RP1-85F18.6 triggered the proliferation, invasion and cell cycle disruption, and suppressed apoptosis and pyroptosis of CRC cells. In addition, it was revealed that knockdown of lncRNA RP1-85F18.6 decreased ΔNp63 expression without affecting TAp63 expression. Furthermore, the results demonstrated that the tumor-promoting effects of lncRNA RP1-85F18.6 overexpression were reversed by knockdown of ΔNp63. It is well known that p53 serves as a tumor suppressor in CRC cells through the regulation of various target genes (29). ΔNp63, which is a member of the p53 family, acts as an oncogene (30,31); specifically, ΔNp63 acts as a transcriptional repressor and oncoprotein through opposing the activity of p53 (32,33). Previous studies have reported that ΔNp63 facilitates transition through the cell cycle, proliferation, migration, invasion and metastasis of cancer cells, whereas it inhibits apoptosis, through stimulating the expression of several target genes (12,13,34). These biological functions of ΔNp63 are in accordance with those of lncRNA RP1-85F18.6, which further supports the present evidence. These findings indicated that the tumor-promoting effects of lncRNA RP1-85F18.6 on CRC cells are partially mediated through the ΔNp63 signaling pathway.

Based on the results obtained over the last few years, lncRNAs appear to regulate gene expression at epigenetic, transcriptional or post-transcriptional levels (5). In the present study, knockdown of lncRNA RP1-85F18.6 reduced ΔNp63 expression at both the mRNA and protein levels, thus suggesting that lncRNA RP1-85F18.6 regulates ΔNp63 partly through transcriptional regulation. Previous research revealed that lncRNA PURPL regulates p53 expression and stability through association with MYB Binding Protein 1a, a protein that binds to and stabilizes p53 (16). It may be hypothesized that lncRNA RP1-85F18.6 regulates ΔNp63 by a certain gene that promotes ΔNp63 stability; we hope to elucidate this in future studies.

In the present study, silencing lncRNA RP1-85F18.6 also induced pyroptosis of CRC cells through cleavage of GSDMD. To the best of our knowledge, there are few studies focusing on the role of non-protein-coding genes in pyroptosis (7). In

addition to apoptosis, pyroptosis is another type of programmed cell death, which is characterized by plasma membrane rupture. Recently, Shi *et al* redefined pyroptosis as gasdermin-mediated programmed necrosis (26). Furthermore, Wang *et al* revealed that chemotherapeutic drugs induce pyroptosis of cancer cells through cleavage of gasdermin E (35). In the present study, cleavage of GSDMD, which was induced by silencing lncRNA RP1-85F18.6, promoted the pyroptosis of CRC cells, thus suggesting that GSDMD may represent a novel focus in CRC research.

Carcinoembryonic antigen (CEA) is the most widely used molecular marker of CRC, which has been proven to be a valuable tool for the diagnosis of CRC (36). Previous research demonstrated that the sensitivity of CEA is 46.59% and its specificity is 80% (37). In the present study, the sensitivity and specificity values of lncRNA RP1-85F18.6 were 55.9 and 76.5%, respectively, which are comparable with those of CEA. These findings suggested that lncRNA RP1-85F18.6 may be a valuable prognostic and diagnostic biomarker for CRC in the future.

In conclusion, to the best of our knowledge, the present study is the first to report that lncRNA RP1-85F18.6 expression may be increased in CRC tissues and cell lines. This lncRNA was revealed to promote proliferation, invasion and cell cycle disruption, whereas it inhibited the apoptosis and pyroptosis of CRC cells through inducing ΔNp63. In addition, the present findings indicated that lncRNA RP1-85F18.6, ΔNp63 and GSDMD may prove to be valuable prognostic and diagnostic biomarkers for early-stage CRC in the future.

Acknowledgements

Not applicable.

Funding

The present study was supported by the New Xiangya Talent Project of The Third Xiangya Hospital of Central South Univ ersity (grant no. JY201508).

Availability of data and materials

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

Author's contributions

YM performed the experiments and wrote the manuscript; YC statistically analyzed the data; CL collected the tissues and analyzed the clinical characteristics of all patients; GH designed the experiments, analyzed the ata, supervised the experiments and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Third Xiangya Hospital of Central South University (No. 2014-S009), and patients provided written informed consent.

Patient consent for publication

The patients provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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