Involvement of The Oncogenic Small Nucleolar RNA SNORA24 In Regulation of P⁵³ Stability In Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is a common malignant cancer worldwide. Although the molecular mechanism of CRC carcinogenesis has been studied extensively, the details remain unclear. Small nucleolar RNAs (snoRNAs) have recently been reported to have essential functions in carcinogenesis, although their roles in CRC pathogenesis are largely unknown. In this study, we found that the H/ACA snoRNA SNORA24 was up-regulated in various cancers, including CRC. SNORA24 expression was significantly associated with age and history of colon polyps in CRC patient cohorts, with high expression associated with a decreased 5-year overall survival. Our results indicated that the oncogenic function of SNORA24 is mediated by promoting G1/S phase transformation, cell proliferation, colony formation and growth of xenograft tumors. Furthermore, SNORA24 knockdown induced massive apoptosis. RNA-sequencing and Gene Ontology (GO) enrichment analyses were performed to explore its downstream targets. Finally, we confirmed that SNORA24 regulates p53 protein stability in a proteasomal degradation pathway. Our study clarifies the oncogenic role of SNORA24 in CRC and advance the current model of the role of the p53 pathway in this process.

Introduction

Colorectal cancer (CRC) is a common malignancy associated with high mortality worldwide. Based on the GLOBOCAN 2020 database (Sung et al. 2021), CRC is the second most common cancer among females and the third most common among males, with mortality rates of 9.5% and 9.3%, respectively. In terms of epidemiology, the incidence of CRC shows geographical variations because of multiple factors such as ethnicity, economy, environment and lifestyle. In 2020, CRC was the second most common cancer in China after lung cancer, and cancer-related death caused by CRC ranked fifth for both sexes (Cao et al. 2020). Cytotoxic chemotherapy, radiotherapy and surgical intervention remain the primary treatments for CRC, although several new therapies have been developed (Ciombor et al. 2015; Jiao et al. 2020; Xie et al. 2020). Although these advances have significantly improved outcomes of CRC patients, the 5-year survival rate for patients with metastasis is only 12% (Messersmith et al. 2019). Fecal occult blood tests and colonoscopy are the preferred methods for early screening, while more effective methods are urgently needed to relieve the increasing CRC burden.

Small nucleolar RNAs (snoRNA) are a class of small noncoding RNAs (60–300 nucleotides), predominantly located in nucleoli. In mammals, snoRNAs are generally encoded in the introns of host genes. Two major classes of snoRNAs guide different post-transcriptional modifications of the target RNAs. C/D box snoRNAs are involved in 2'-O-methylation and H/ACA box snoRNAs are involved in pseudouridylation. The common assumption that snoRNAs function as house-keeping genes in ribosome biosynthesis has been challenged and snoRNAs have emerged as regulators with diverse functions. (Kawaji et al. 2008; Soulé et al. 2020; Bortolin-Cavaillé et al. 2012; Jinn et al. 2015; Lafaille et al. 2019; Michel et al. 2011; Chu et al. 2012; Williams et al. 2012). SnoRNAs function as either cancer suppressors or oncogenes via regulation of proliferation, apoptosis, migration or invasion (Williams et al. 2012). Several snoRNAs are reported to be dysregulated in CRC and target vital cancer-related signaling

pathways (Yoshida et al. 2017; Okugawa et al. 2017; Yang et al. 2017; Zhang et al. 2020; He et al. 2020; Fang et al. 2017; Huang et al. 2020). SNORD126 and ACA11 activate the PI3K-AKT pathway in human hepatocellular carcinoma (HCC) cells (Fang et al. 2017; Wu et al. 2017). SNORD50A and SNORD50B function as tumor suppressors by binding to K-Ras and their deletion in KRAS-mutant cells enhanced carcinogenesis (Siprashvili et al. 2016). Similarly, SNORA18L5 is reported to regulate expression of tumor suppressor p53 protein, leading to an increased risk of HCC (Cao et al. 2018). Moreover, snoRNAs are implicated as potential biomarkers and therapeutic targets for CRC (Zhang et al. 2019).

In humans, p53 is a multifunctional tumor suppressor protein encoded by the *TP53* gene (Kubbutat et al. 1998). It plays a vital role in the regulation of DNA repair, apoptosis and cell cycle progression to maintain cellular homeostasis. Dysfunction of p53 leads to uncontrolled cell proliferation, and more than 50% of cancers are associated with p53 mutations. Naturally synthesized p53 is unstable and the degradation process is precisely regulated (Kubbutat et al. 1998; Chao et al. 2015; Xu et al. 2021; Asher et al. 2005; Asher et al. 2002). Dysregulation of p53 stability seriously disrupts the p53 transcriptional network, which is thought to be primarily based on protein-protein interactions. Recent studies have demonstrated that snoRNAs are an essential component of the p53 network. For example, H/ACA snoRNA derived miR-605 interrupts the p53-MDM2 interaction, leading to rapid accumulation of p53, while SNORD28 derived sno-miR-28 represses p53 stabilization through TAF9B (Cao et al. 2018; Scott et al. 2009; Xiao et al. 2011; Yu et al. 2015). Thus, elucidation of the biological functions of snoRNAs is a vital issue for elucidation of the molecular mechanism of p53 activity in carcinogenesis.

SNORA24 is a H/ACA snoRNA hosted in the long noncoding RNA, SNHG8. McMahon et al. showed that SNORA24 is down-regulated in HCC and functions as a tumor suppressor (McMahon et al. 2019). In the present study, we investigated the biological functions of SNORA24 in CRC. Our data indicated that SNORA24 expression is up-regulated in various cancers including CRC. Analysis of the datasets from the SnoRNA in Cancers (SNORic) database and The Cancer Genome Atlas (TCGA) indicated that SNORA24 is as an independent risk factor for survival of CRC patients, with high levels predictive of a poorer prognosis. In further investigations, we demonstrated that SNORA24 overexpression promoted cell proliferation and growth of xenograft tumors via regulation of cell cycle progression. Our data also following suggested that SNORA24 is involved in regulation of p53 protein stability, indicating that SNORA24 acts as an oncogene in CRC in a p53-dependent manner.

Methods And Materials

Patients and samples

Fresh-frozen carcinoma tissues and adjacent normal tissues from CRC patients were obtained from Liaoning Cancer Hospital (Shenyang, China). Written informed consent was obtained from patients and the study protocol was approved by the Ethics Committee on Human Investigation of the Liaoning Cancer Hospital. Clinical details of the participants are shown in **Table S1**.

Cell culture

CRC cell lines (HCT116^{p53+/+}, HCT116^{p53-/-}, SW620 and HT29) were purchased from GeneChem (Shanghai, China), the human normal colon epithelial cell line (FHC) was purchased from ATCC (Manassas, USA), the cervical cancer cell line (HeLa) and the breast cancer cell line (MCF-7) were purchased from the Chinese National Infrastructure of Cell Line Resource (NICR) (Beijing, China). HCT116, SW620 and HeLa cells were cultured in RPMI-1640 medium (HyClone, Logan, USA). HT29 and MCF7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (HyClone, USA). Both media were supplemented with streptomycin (100 µg/ml), penicillin (100 units/ml) and 10% fetal bovine serum (FBS; ExCell Bio, China). FHC cells were cultured in complete DMEM: F-12 medium (ATCC, USA) according to the instructions. All cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

RNA extraction from cells or tissues

Tissues were homogenized in 1 mL TRIzol reagent (Sigma, USA) and total RNA was extracted according to the manufacturer's instructions. To extract cellular RNA, $3 \cdot 10^5$ – $5 \cdot 10^5$ cells were homogenized in 1 mL TRIzol reagent. PARIS™ kits (ThermoFisher, USA, Cat. No. AM1921) were used to isolate nuclear and cytoplasmic fractions from whole cells, and RNA was extracted from each fraction. Total RNA (1 µg) from each sample was reverse transcribed into cDNAs using PrimeScriptRT reagent kit with gDNA Eraser (TaKaRa, Japan).

Quantitative real-time PCR (qRT-PCR)

Gene expression was measured by qRT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) and performed on a CFX96™ real-time PCR system (Bio-Rad, USA). All PCR reactions were performed in triplicate under the following conditions: (1) initial denaturation at 95°C for 2 min; (2) denaturation at 95°C for 5 s; (3) anneal at 63°C (SNORA24, U6 and SNHG8) or 57°C (p53 and GAPDH) for 30 s; 40 cycles from (2) to (3). U6 and GAPDH were used as normalization controls, and the relative expression of target genes was calculated by the $2^{-\Delta\Delta C_t}$ method. Sequences of the primers used for qRT-PCR are shown in **Table S2**.

Lentivirus infection

Lentiviruses (GV235) expressing SNORA24 (LV-SNORA24) or control oligonucleotide sequences (LV-control) were constructed by Shanghai JiKai Gene Medicine Technology Co., Ltd. (China). Lentiviruses (GV112) expressing shRNA sequences targeting SNORA24 (LV-SNORA24-KD) or negative control sequences (LV-NC) were purchased from the same company. Oligonucleotide sequences cloned into the vector were as follows:

Control (5'–3'): TTCTCCGAACGTGTCACGT;

SNORA24(5'–3'): (Agel restriction endonuclease site) ACCGGTCTCCATGTATCTTTGGGACCTGTCAGCCGTGGCAGTCTCCCTTCCTAGCCATGGAAGAGCATATCCTTGTTTATTGGCAAAGCTGTCACCATTTAATTGGTA

TCAGATTCTGACTTGCACAAGTAACATTCTTTTTTGAATTC

(EcoRI restriction endonuclease site).

ShRNA negative control (5'–3'): TTCTCCGAACGTGTCACGT;

ShRNA targeting SNORA24 (5'–3'): TATCTTTGGGACCTGTCAG.

Cells were cultured in 12-well plates ($1.5 \cdot 10^5$ cells/well) for 24 h before infection with lentiviruses at a multiplicity of infection (MOI) of 10–20. 72 h later infection, cells were employed in further investigations or cultured in selection medium containing puromycin (2 µg/mL). Stably infected cells were maintained in medium containing puromycin (0.67 µg/mL).

Flow cytometry

Cell apoptosis was measured by flow cytometry (ACEA Bio, USA) using Annexin V-FITC apoptosis detection kits (Dojindo, Japan); 10 000 cells were analyzed for each sample and in triplicates for each group.

For cell cycle analysis, cells were fixed with 70% (v/v) ethanol at -20°C for approximately 24 h. Before flow cytometry detection, the fixed cells were washed twice with phosphate buffer solution (PBS), then stained with propidium iodide solution; 20 000 cells were analyzed for each sample and in triplicates for each group.

Cell proliferation assay

Cells were seeded in 96-well plates (2 000 cells/well) and proliferation was measured using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). The absorbance in each well was measured at 450 nm on a microplate reader (Sunrise, Tecan, Switzerland); five replicates were included for each group.

Colony formation assay

Cells were cultured in 6-well plates (500–1 000 cells/well) for 12–14 days and the medium was refreshed every 4 days. Finally, the colonies were fixed in anhydrous methanol for 30 min at room temperature, stained with Giemsa for 30 min, and colonies were counted using Image J software.

EdU incorporation assay

HCT116 cells stably infected with LV-SNORA24 or LV-control were seeded on coverslips in 6-well plates ($3 \cdot 10^5$ cells/well). After 24 h, the cells were incubated with 10 µM EdU for 2 h at 37°C in a humidified incubator. EdU was stained with Alexa Fluor 488 according to the manufacturer's instructions (Beyotime, China). The images were recorded by laser confocal microscopy imaging system NIKON TI2-E and CRESTIPTICS X-LIGHT V3 (Nikon, Japan).

Western blotting

Cellular protein extraction and western blotting were performed as previously described (Shen et al. 2018). The following antibodies were used in this study: anti-PARP (Cell Signaling Technology (CST), Massachusetts, USA; Cat. No. 9542S, 1:1 000), anti-caspase3 (CST, USA; Cat. No. 9662S, 1:1 000), anti- β -actin (Proteintech, Chicago, USA; Cat. No. 60008-1-Ig, 1:2 000), anti-p53 (Santa Cruz, California, USA; Cat. No. sc-126, 1:1 000), anti-p21 (Santa Cruz, USA; Cat. No. sc-6246, 1:500), anti-CDK2 (Abcam, Cambridge, UK; Cat. No. ab32147, 1:1 000), anti-p-CDK2 (T160) (Abcam, UK; Cat. No. ab194868, 1:1 000), anti-cyclin E (Santa Cruz, USA; Cat. No. sc-248, 1:1 000), mouse horseradish peroxidase (HRP)-conjugated IgG (KPL, Gaithersburg, USA; Cat. No. 074-1806, 1:5 000), rabbit HRP-conjugated IgG (KPL, USA; Cat. No. 074-1516, 1:5 000). Protein bands were scanned using the Image Quant LAS 500 system (GE Healthcare, Chicago, USA).

Mouse tumor xenograft model

Female BABL/c nude mice (aged 4 – 5 weeks) were purchased from Vital River company (Beijing, China) and randomly divided into two groups: LV-SNORA24 group and LV-control group (n = 6 per group). HT29 and SW620 cells stably infected with LV-SNORA24 or LV-control were injected subcutaneously behind the thigh (approximately $1 \cdot 10^7$ cells/mouse). Mice were euthanized after continuous observation for 28 – 30 days. Tumor volume and weight were measured as described previously (Liu et al. 2020). The study protocol was approved by the Ethics Committee of the Academy of Military Medical Science (AMMS, Beijing, China).

Hematoxylin and eosin (H&E) and immunohistochemistry (IHC)

Paraffin-embedded xenograft tumor tissues were sliced into 3 μ m sections. Histological analyses of the tumors were measured by staining with H&E and IHC.

The paraffin-embedded slides were first deparaffinized with xylene and then dehydrated with a graded series of alcohols, stained with H&E for histological analyses. Heat-mediated antigen retrieval was used in IHC assay, Ki-67 was stained with anti-Ki-67 (Abcam, UK; Cat. No. ab15580, 1:2 000) in conjunction with HRP-conjugated antibodies (ZSGB-BIO, China, Cat. No. PV-9001), and visualized with 3,3'-diaminobenzidine (DAB) substrate.

Bioinformatics database

Data for CRC patients were obtained from TCGA (<https://portal.gdc.cancer.gov/>). Expression profiles of SNORA24 and the data about 5-year overall survival of patients were obtained from the SNORic database (<http://bioinfo.life.hust.edu.cn/SNORic>). Data about the expression of SNORA24 in colorectal adenoma were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The secondary molecular structure of SNORA24 was predicted by RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). Analysis of homology was performed using the UCSC database (<https://genome.ucsc.edu>). Analysis of data from RNA-sequencing using Metascape (<https://metascape.org>).

RNA-sequencing

HCT116 cells stably expressing LV-SNORA24 or LV-control were employed for RNA-sequencing (triplicates in each group) by LC-BIOTECHNOLOGIES CO., LTD. (Hangzhou, China) using an Illumina X10 sequencer. Differentially expressed genes were identified based on the following criterion: $P < 0.05$, $\log_2(\text{FC}) > 1$ or $\log_2(\text{FC}) < -1$. The data were analyzed by Gene Ontology (GO) enrichment and protein-protein interaction prediction.

Cycloheximide (CHX) exposure

HCT116 and HT29 cells expressing LV-SNORA24 or LV-control were seeded in 6-well plate (5×10^5 cells/well). 24 hours later, cells were treated with 100 $\mu\text{g/mL}$ CHX or dimethyl sulfoxide (DMSO) for 0, 1, 2, 4, 8 and 12 h.

Statistical analysis

Data were presented as the mean $\pm$ standard deviation (SD), all experiments were performed in triplicate or as three independent experiments. All qRT-PCR, CCK-8 assay, colony formation assay and flow cytometric data were analyzed by two-tailed Student's *t*-test or two-way ANOVA. Data from CRC patient cohorts were analyzed by Chi-square (χ^2) test, the Multiple Kaplan–Meier Plotter (KM-Plotter) and Cox proportional hazards (Coxph) regression model. $P < 0.05$ was set as the threshold for statistical significance.

Results

SNORA24 was up-regulated in various cancers and high levels of SNORA24 were indicated a poorer prognosis in CRC patients

SNORA24 is down-regulated in HCC and acts as a tumor suppressor [34]. However, our analysis of dataset GSE20916 from the GEO indicated that SNORA24 is significantly up-regulated in CRC ($P = 0.0004$). Further searches of the SNORic database revealed that SNORA24 is up-regulated in various cancers, but down-regulated in liver hepatocellular carcinoma (LIHC) (Fig. 1a), and not changed in kidney and thyroid cancer (**Fig. S1a**). Through qRT-PCR analysis, we confirmed that SNORA24 was up-regulated in clinical CRC tissues compared with adjacent normal tissues ($P_{\text{unpaired}} < 0.0001$, $P_{\text{paired}} = 0.0026$), while there were no significant differences between groups of different stages and grades among the patient cohort in this study. The receiver operating characteristic (ROC) curve analysis confirmed SNORA24 levels significantly discriminated tumor tissue from adjacent normal tissues ($P = 0.0046$), implicating SNORA24 as a diagnostic biomarker of CRC (Fig. 1b, **Fig. S1b**). Although SNHG8 was also up-regulated in CRC tissues (**Fig. S1c**) (Zhen et al. 2019), there was no correlation with the expression of SNORA24.

To evaluate the hypothesis that SNORA24 expression impacts on the prognosis of CRC patients, we screened “SNORA24” in SNORic and KM-Plotter datasets. We found that high expression of SNORA24

decreased the 5-year overall survival of patients with colon adenocarcinoma (COAD) and kidney cancer ($P = 0.026$), while the opposite trend was observed in LIHC, but without statistical significance (Fig. 1c, **Fig. S 1d**). To further validate this association, we analyzed the correlation between SNORA24 expression and clinicopathological parameters of CRC patient data downloaded from TCGA. SNORA24 was found to be significantly associated with age and history of colon polyps (Table 1). Strikingly, we found that SNORA24 was also up-regulated in colorectal adenoma biopsy tissue ($P < 0.0001$), which is widely recognized as a precancerous lesion in CRC (**Fig. S2, Table S3**). Furthermore, univariate and multivariate analysis using the Coxph regression model indicated that, similar to TNM stage and age, SNORA24 is as an independent prognostic risk factors for survival of CRC patients ($P_{univariate} = 0.045$, $P_{multivariate} = 0.025$) (Fig. 1d-e, Table 1). Thus, our findings demonstrated that SNORA24 is differentially up-regulated in CRC tissues and in precancerous CRC biopsy tissues. Furthermore, we identified high SNORA24 expression as a negative prognostic marker in CRC.

Table 1
Correlations between SNORA24 expression and clinicopathological parameters

Characteristic	Case	SNORA24 expression, N (%) #		χ^2	P
		Low	High		
Sex				0.335	0.562
Female	206	100 (48.54)	106 (51.46)		
Male	224	115 (51.34)	109 (48.66)		
Age (yr)				4.174	0.041*
≤ 70	230	125 (54.35)	105 (45.65)		
> 70	198	88 (44.44)	110 (55.56)		
TNM Stage (AJCC)				1.185	0.276
Stage I/II	238	114 (47.90)	124 (52.10)		
Stage III/IV	186	99 (53.23)	87 (46.77)		
Pathological T category				0.015	0.902
T1/T2	81	41 (50.62)	40 (49.38)		
T3/T4	349	174 (49.86)	175 (50.14)		
Lymph node metastasis				2.757	0.097
Negative	249	116 (46.59)	133 (53.41)		
Positive	181	99 (54.70)	82 (45.30)		
Distant metastasis				4.962	0.084
Negative	310	153 (49.35)	157 (50.65)		
Positive	64	28 (43.75)	36 (56.25)		
Lymphatic invasion				2.428	0.119
Negative	236	127 (53.81)	109 (46.19)		
Positive	151	69 (45.70)	82 (54.30)		
Venous invasion				0.131	0.717
Negative	278	145 (52.16)	133 (47.84)		
Positive	94	47 (50.00)	47 (50.00)		

Abbreviations: AJCC, American Joint Committee on cancer; #Cut-off threshold of SNORA24 expression is median value in all patients in this cohort; *p < 0.05.

Characteristic	Case	SNORA24 expression, N (%) #		χ^2	P
		Low	High		
History of colon polyps				6.918	0.009*
Negative	239	129 (53.97)	110 (46.03)		
Positive	122	48 (39.34)	74 (60.66)		
Abbreviations: AJCC, American Joint Committee on cancer; #Cut-off threshold of SNORA24 expression is median value in all patients in this cohort; *p < 0.05.					

SNORA24 strongly enhanced CRC cell survival in vitro

We hypothesized that SNORA24 might be characterized as an oncogene during CRC carcinogenesis. We predicted its secondary structure using the RNAfold WebServer and analyzed its homology using the UCSC database (**Fig. S3a-b**). To validate our hypothesis, we first examined the cellular distribution of SNORA24 and confirmed that it dominantly located in nuclei (**Fig. S3c**). Analysis of the abundance of SNORA24 was measured in three CRC cell lines (HCT116, HT29 and SW620) and normal colorectal epithelial cells (FHC) revealed that SNORA24 was not consistently up-regulated in all CRC cells versus FHC (**Fig. S3d**). Lentivirus-mediated knockdown of SNORA24 in CRC cells dramatically suppressed cell proliferation, decreased colony formation, and induced massive apoptosis, indicated by the detection of cleaved PARP and caspase3 (**Fig. 2**). In accordance with this, we also observed remarkable inhibition of survival after SNORA24 knockdown in HeLa and MCF-7 cells (**Fig. S4**).

To verify its function, we generated cells stably expressing SNORA24. As expected, SNORA24 overexpression in CRC cells strongly promoted cell proliferation and colony formation (**Fig. 3a-c**). SNORA24 overexpression induced cell cycle progression by promoting G1/S phase transition, thereby decreasing the proportion of cells in G1 phase, while increasing the proportion in the S and G2/M phases (**Fig. 3d**). In EdU incorporation assays, we demonstrated that SNORA24 overexpression led to a higher proportion of EdU⁺ cells (SNORA24 59.39% ± 13.34% versus control 41.19% ± 5.81%, $P = 0.0033$), indicating that SNORA24 increases cell proliferation by promoting DNA synthesis (**Fig. 3e**). We also showed that SNORA24 overexpression promoted proliferation and colony formation in HeLa cells and MCF-7 cells (**Fig. S4**). These results suggested that SNORA24 supports cell survival through regulation of the cell cycle and proliferation; however, its effects may be tissue-type dependent.

SNORA24 promoted growth of CRC xenografts in vivo

To explore the effects of SNORA24 in vivo, we used cells stably infected with lentiviruses LV-SNORA24 or LV-control in a mouse tumor xenograft model. Through a 30-day observation, we found that SNORA24 overexpression enhanced the growth of xenograft tumors derived from HT29 cells. The tumor volume in the LV-SNORA24 group was increased 1.94–2.39-fold ($P = 0.0045$), and the tumor weight was increased by approximately 2-folds ($P = 0.0264$). Ki-67 represents a biomarker of cell viability in cancer. Ki-67

staining indicated that SNORA24 overexpression increased the percentage of proliferative cells in the xenografted tumor ($P < 0.0001$) (Fig. 4a-g). Similarly, we found that SNORA24 overexpression enhanced the growth of xenograft tumors derived from SW620 cells. The tumor weight in the LV-SNORA24 group increased 2.8-fold ($P = 0.0361$), and the tumor volume increased 4.68-fold in the LV-SNORA24 group ($P = 0.0227$). SNORA24 overexpression also increased the percentage of Ki-67⁺ proliferative cells in the xenografted tumor ($P = 0.0002$) (Fig. 4h-n). The findings suggested that SNORA24 acts as an oncogene both in vitro and in vivo.

SNORA24 targeted the p53 pathway to regulate CRC cell survival

To explore downstream targets of SNORA24 involved in its functional network, we performed RNA-sequencing analysis of HCT116 cells infected with LV-SNORA24 or LV-control. We identified a total of 343 differentially expressed genes (104 up-regulated and 239 down-regulated) (Fig. 5a-b, **Table S4**). GO analysis using Metascape website indicated that SNORA24 is involved in diverse cellular biological processes, including nuclear receptors meta-pathway, extracellular matrix organization, p53 downstream pathway, regulation of cell adhesion, regulation of protein kinase activity, etc. The data from RNA-sequencing showed that p53 downstream pathway was significantly enriched in cells infected with LV-SNORA24 with several targets of p53 down-regulated (Fig. 5c-d). Protein-protein interaction network was predicted using Metascape (**Fig. S5a**).

p53 is one of the most important tumor suppressors and it acts as a key factor during G1/S phase transition via the p53-p21 signaling pathway. We identified p53 pathway as significantly enriched in relation to SNORA24. SNORA24 overexpression decreased p53 and p21 protein in HT29 cells and HCT116 cells, and induced changes in the downstream targets that function as core components in G1/S checkpoint regulation (Fig. 5e) (Hume et al. 2020).

We next investigated the hypothesis that SNORA24 targets p53 to regulate proliferation in CRC using HCT116^{p53+/+} (wtp53) and HCT116^{p53-/-} (p53 deletion) cells. CCK-8 and colony formation assays revealed that p53 deletion abolished the ability of SNORA24 to promote cell proliferation, attenuated its ability to enhance colony formation (Fig. 5f-h). In accordance with this phenotype, SNORA24 overexpression decreased p21 levels in HCT116^{p53+/+} cells, but had no obvious impact on HCT116^{p53-/-} cells (**Fig. S5b**). In addition, SNORA24 knockdown in HCT116^{p53-/-} cells failed to induce apoptosis as dramatically as in HCT116^{p53+/+} cells (Fig. 5i-j). These observations indicated that the effects of SNORA24 are dependent on the p53-p21 signaling pathway.

SNORA24 negatively regulated p53 stability in a proteasomal degradation pathway

We further explored the mechanism by which p53 levels were reduced by SNORA24 overexpression. Using cells infected with LV-SNORA24 or LV-control, we first verified that SNORA24 overexpression had no impact on p53 mRNA levels (Fig. 6a). Since p53 protein is prone to hydrolytic degradation, we analyzed

the effect of SNORA24 on p53 protein stability. As shown in Fig. 6b-c, SNORA24 overexpression promoted degradation of p53 protein. As far as we know, proteasome-mediated degradation is the most essential pathway for regulation of p53 stability (Kubbutat et al. 1998; Chao et al. 2015; Xu et al. 2021). To confirm the role of this pathway in the mechanism by which SNORA24 regulates p53 stability, cells stably expressing SNORA24 were treated with MG132 proteasome inhibitor. Evaluation of p53 expression in HCT116 and HT29 cells showed that MG132 treatment rescued the inhibitory ability of SNORA24 to induce a reduction in p53 protein levels in HCT116 cells (partly rescued p53 in HT29 cells) (Fig. 6d). As shown in Fig. 6e, we proposed that SNORA24 regulates p53 protein stability via a classical proteasomal degradation pathway, and that SNORA24 overexpression promoted G1/S phase transition by enhancing p53 degradation, ultimately leading to a rapid cell proliferation.

Discussion

Dysregulated snoRNAs are recognized as promising biomarkers for cancers, but only a few of them are well characterized in carcinogenesis. In the present study, we investigated the biological effects of SNORA24 in CRC. We showed that SNORA24 expression is up-regulated in various cancers. SNORA24 high levels were found to be associated with a poorer prognosis in CRC patients. Our studies showed that SNORA24 was involved in regulation of cell proliferation and tumor growth by cell cycle progression. In further investigations, we found that SNORA24 regulated cell survival via a p53-dependent mechanism. Thus, we provide compelling evidence in support of an oncogenic role for SNORA24 in CRC carcinogenesis, and propose a model of the mechanism by which snoRNA functions via the p53 pathway.

High throughput sequencing technology is an effective method to screen for cancer-related genes and a crucial source of transcriptomic information that provides the opportunity to identify valuable biomarkers of cancers. MicroRNAs that are differentially expressed in tumors have long been considered as potential cancer biomarkers (Jeffrey et al. 2008) and cancer-related snoRNAs have recently become a new focus of research in this field (Huang et al. 2020; Zhang et al. 2019; Koduru et al. 2017). For example, snoRNA U50 acts as a tumor suppressor and is down-regulated in breast and prostate cancers (Dong et al. 2009; Dong et al. 2008). SNORD76 and SNORA7B are up-regulated in HCC and breast cancer, respectively, and both are associated with poorer survival (Sun et al. 2019; Wu et al. 2018). SNORNA21, SNORD42, SNORD14E, and SNORD16 are among the snoRNAs up-regulated in CRC and predict a poor prognosis, while SNORD123, U70c, and ACA59B are down-regulated (Yoshida et al. 2017; Okugawa et al. 2017; He et al. 2020; Huang et al. 2020; Ferreira et al. 2012). In the present study, we showed that SNORA24 expression is dysregulated in various cancers, and that high SNORA24 levels significantly discriminated CRC tissue from adjacent normal tissue. Furthermore, SNORA24 levels were found to be associated with age and history of colon polyps in CRC patient cohorts, with high levels identified as an independent prognostic risk factor for survival of CRC patients. These findings implicate SNORA24 as a potential prognostic biomarker for CRC.

CRC generally develops in the colorectal adenoma-carcinoma sequence, which is affected by various factors such as age, heredity, chronic disease history and lifestyle (Mármol et al. 2017). Genetic alterations have vital impacts on colorectal malignant transformation. Mutation-induced dysfunction of tumor suppressor genes, oncogenes or DNA repair-related genes are commonly associated with CRC carcinogenesis (Mármol et al. 2017; Arvelo et al. 2015; Bogaert et al. 2014). The role of ncRNAs in CRC carcinogenesis has become an important issue. Yang et al. (2017) analyzed snoRNA expression profiles in CRC, ulcerative colitis and healthy control patients and found that SNORA15, SNORA41 and SNORD33 were all up-regulated in both ulcerative colitis and CRC patients compared to healthy control. In the present study, we found that SNORA24 was significantly associated with age and history of colon polyps in CRC patients. Interestingly, data from GEO database revealed that SNORA24 was also up-regulated in colorectal adenoma biopsies as well as CRC tissues. Our in vitro and in vivo studies indicated that SNORA24 acts as an oncogene in CRC and might promote cell malignant transformation at the beginning of the pathological process. Thus, SNORA24 is implicated as a novel diagnostic biomarker for early.

Under various conditions such as genetic, oxidative and, metabolic stresses, p53 function protects cells from uncontrolled proliferation. In this study, we found that SNORA24 regulated CRC cell proliferation and survival in a p53-dependent manner. We showed that SNORA24 reduced p53 protein levels by enhancing its degradation, with no impact on mRNA levels. The activity of p53 in cells is regulated via a feedback loop governed dominantly by p53 protein stability. The ubiquitin-dependent 26S proteasome-mediated degradation is a well-established molecular mechanism by which p53 stability is regulated. E3 ubiquitin-ligase mediated ubiquitination of p53 is an essential step in its degradation by the proteasome (Chao et al. 2015; Xu et al. 2021). During this process, MDM2, binds to the N-terminus of p53, allowing ubiquitination at lysine residues, followed by 26S proteasome-mediated degradation (Kubbutat et al. 1998; Chao et al. 2015; Xu et al. 2021). In addition, Asher et al. proposed a ubiquitin-independent pathway for p53 proteasomal degradation (Asher et al. 2002) in which NQO1 binds to p53 to protect it from 20S proteasome-mediated degradation. However, effect of snoRNAs in the regulation of p53 stability remains to be fully elucidated. SnoRNAs mi-605, sno-miR-28 and SNORA18L5 are involved in regulation of p53 stability via MDM2-mediated ubiquitination (Cao et al. 2018; Scott et al. 2009; Xiao et al. 2011; Yu et al. 2015). In our study, SNORA24 overexpression reduced p53 protein expression; and the inhibitory effect of SNORA24 on p53 protein was abolished by a proteasome inhibitor. These findings confirmed that SNORA24 regulates p53 stability in a ubiquitin-independent manner.

SNORA24-guided pseudouridine modifications of rRNA (ψ 609 and ψ 863) play an essential role in aa-tRNA selection and translational accuracy (McMahon et al. 2019). In this study, we did not investigate whether overexpression or knockdown of SNORA24 expression caused miscoding of p53 protein (or other carcinogenesis-related proteins). Other limitations of our study should also be noted. We did not explore the mechanism underlying the role of SNORA24 in other cancers. In addition, further clinical investigations are required to validate SNORA24 as a diagnosis or prognostic biomarker for CRC.

In summary, the present study demonstrates that SNORA24 is dysregulated in various cancers and provides evidence of the value of SNORA24 expression as an independent risk factor for survival of CRC

patients, with high levels predicting a poor prognosis in CRC. We also confirmed that SNORA24 acts as an oncogene in the pathological process of CRC, possibly in the early stage. Strikingly, we revealed that SNORA24 regulates cell proliferation via a p53 protein-dependent mechanism. We also show that SNORA24 regulates p53 protein stability via a classical proteasomal degradation pathway. Our findings indicate the potential of SNORA24 as a biomarker and therapeutic target in CRC.

Statements And Declarations

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Author contributions

Study concept and design (Zhidong Wang, Xiaofei Zheng); experimental operation (Liping Shen, Chuxian Lin, Wenqing Lu, Yujv Huang); analysis of data (Liping Shen, Junyan He, Qi Wang); drafting of the manuscript (Liping Shen, Zhidong Wang).

Consent for publication

All of the authors have written informed consent.

Competing interests

The authors declare no competing financial interests.

Data availability statements

All data is available in the main text and supplementary materials. All models, nucleotide sequences, vectors or plasmids used in the study are available from the corresponding author upon reasonable request.

Ethical approval and consent to participate

Written informed consent was obtained from participants. Clinical samples used in this study have been reviewed and approved by the Ethics Committee on Human Investigation of the Liaoning Cancer Hospital (20190970). Animal experiments were approved by the Ethics Committee of AMMS (IACUC-DWZX-2020-549, Beijing, China).

Code availability

Not applicable.

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Figures

Figure 1

SNORA24 was up-regulated in various cancers and high levels of SNORA24 were indicated a poorer prognosis in CRC patients

a. SNORA24 was up-regulated in various cancers. SNORA24 expression in CRC tissues based on analysis of GEO dataset (GSE20916, two-tailed t-test, mean $\pm$ SD, $N_{\text{normal}} = 44$, $N_{\text{CRC}} = 101$). SNORA24 expression in bladder urothelial carcinoma (BLCA, $N = 413$), breast invasive carcinoma (BRCA, $N = 181$), esophageal carcinoma (ESCA, $N = 195$), head and neck squamous cell carcinoma (HNSC, $N = 567$), lung adenocarcinoma (LUAD, $N = 559$), lung squamous cell carcinoma (LUSC, $N = 521$), prostate adenocarcinoma (PRAD, $N = 535$), stomach adenocarcinoma (STAD, $N = 446$), uterine corpus endometrial carcinoma (UCEC, $N = 571$), hepatocellular carcinoma (LIHC, $N = 422$), cholangiocarcinoma (CHOL, $N = 45$) and cutaneous melanoma (SKCM, $N = 449$), etc. (tumor tissues versus normal tissues), the data was downloaded from SNORic database.

b. SNORA24 was up-regulated in CRC tissues. SNORA24 expression in CRC tissues and adjacent normal mucosa tissues was detected by qRT-PCR, the data was analyzed in both unpaired (left, $N_{\text{adjacent}} = 47$, $N_{\text{tumor}} = 47$) and paired (middle, $N = 38$) tissues (Two-tailed t-test, mean $\pm$ SD). SNORA24 expression in CRC tissues and adjacent normal mucosa tissues was measured by receiver operating characteristic (ROC) curve analysis (right).

c. High SNORA24 expression was associated with a poorer prognosis of colon adenocarcinoma (COAD) patients. Multiple Kaplan-Meier Plotter (KM-Plotter) datasets about COAD was downloaded from SNORic database. 5-year overall survival of COAD patients were analyzed in SNORA24 high-expression group and SNORA24 low-expression group ($N = 434$).

d-e. High SNORA24 expression was an independent prognostic risk factors for survival of CRC patients. The data about CRC patient cohorts was downloaded from TCGA, Cox proportional hazards regression model (Coxph) via univariate (D) and multivariate (E) analysis was performed to measure its risk for survival ($N = 430$, χ^2 test).

Figure 2

SNORA24 knockdown induced massive apoptosis and suppressed survival of CRC cells

a. SNORA24 knockdown by shRNA interference. HCT116, HT29 and SW620 cells were infected with lentivirus LV-SNORA24-KD or LV-NC. 72 h after infection, SNORA24 expression was tested by qRT-PCR (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).

b. SNORA24 knockdown suppressed cell proliferation. The above infected cells were performed to CCK-8 assay, cells viability was measured on 0, 1, 2, 3, 4 days respectively. The absorbance values at 450 nm were analyzed (Two-way ANOVA, mean $\pm$ SD, N = 5).

c. SNORA24 knockdown decreased colony formation. The infected cells were cultured for 12-14 days, the colonies were counted with Image J software and the fractions of colony formation were compared between LV-SNORA24-KD group and LV-NC group (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).

d. SNORA24 knockdown induced massive apoptosis. SNORA24 knockdown in HCT116, HT29 and SW620 cells using LV-SNORA24-KD or LV-NC, cells were employed to apoptosis analysis in 72 h after infection (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3). Expression of PARP/cleaved PARP and caspase3/cleaved caspase3 were detected by western blotting.

*****P* < 0.01, ****P* < 0.001.**

Figure 3

SNORA24 overexpression strongly enhanced survival of CRC cells

a. SNORA24 expression in lentiviruses infected cells. HCT116, HT29 and SW620 cells were infected with lentivirus LV-SNORA24 or LV-control. 72 h after infection, SNORA24 expression was measured by qRT-PCR (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).

- b.** SNORA24 overexpression promoted cell proliferation. The above infected cells were performed to CCK-8 assay, cell viability was measured on 0, 1, 2, 3, 4 days (Two-way ANOVA, mean $\pm$ SD, N = 5).
- c.** SNORA24 overexpression increased colony formation. Cells infected with LV-SNORA24 or LV-control were employed to colony formation assay as previously described, the fractions of colony formation were compared were analyzed in LV-SNORA24 group versus LV-control group (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).
- d.** SNORA24 overexpression changed the distribution of cell cycle. In 72 h after infection, cells were harvested and performed to cell cycle analysis, the percentage of cells in each phase were analyzed in LV-SNORA24 group versus LV-control group (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).
- e.** SNORA24 overexpression enhanced proliferative DNA synthesis. HCT116 cells stably expressing LV-SNORA24 or LV-control employed to EDU incorporation assay. About 200 cells were randomly analyzed in each group, EDU-Alexa Fluor 488 positive cells were statistically calculated (Two-tailed Student's *t*-test, mean $\pm$ SD).

P* < 0.05, *P* < 0.01, ****P* < 0.001, n.s. means no significance.

Figure 4

SNORA24 overexpression promoted growth of CRC xenografts in vivo

HT29 cells and SW620 cells stably infected with LV-SNORA24 or LV-control were used in xenografts assay. BABL/c nude mice were injected subcutaneously behind the thigh, 6 mice in each group with a continuous observation for 28-30 days.

a-g. Xenografts assay using HT29 cells. (A-B) Mice were euthanized and subcutaneous tumors were removed after a 30-day observation. (C) Analysis of SNORA24 expression by qRT-PCR (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 6). (D) Analysis of mice weight (Two-way ANOVA, mean $\pm$ SD, N = 6). (E) Analysis of tumor volume. Tumor volume was measured every 4 days (Two-way ANOVA, mean $\pm$ SD, n = 6). (F) Analysis of tumor weight (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 6). G. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Xenograft tumor tissue sections were employed to H&E staining and Ki-67 staining via IHC. Cells were counted by Image J software. Ki-67⁺ cells were statistically analyzed in LV-SNORA24 group versus LV-control group (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 6).

h-n. Xenografts assay using SW620 cells. (H-I) Mice were euthanized after a 28-day observation. (J) Analysis of SNORA24 expression by qRT-PCR (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 6). (K) Analysis of mice weight (Two-way ANOVA, mean $\pm$ SD, N = 6). Analysis of tumor volume (L) and tumor weight (M) (Two-way ANOVA, mean $\pm$ SD, N = 6). (N) H&E staining and IHC. Histological analyses via HE staining, the portion of Ki-67⁺ cells were analyzed in the two groups (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 6).

P* < 0.05, *P* < 0.01, ****P* < 0.001, n.s. means no significance.

Figure 5

SNORA24 targeted p53 pathway to regulate CRC cell survival

a-d. Bioinformatic analysis of downstream targets of SNORA24. **(a-b)** HCT116 cells overexpressing SNORA24 were performed to RNA-sequencing, differentially expressed genes were screened following the criterion: $\log_2(\text{FC}) > 1$ or $\log_2(\text{FC}) < -1$, p value < 0.05 . **(c)** Gene Ontology (GO) enrichment in relation to

SNORA24. Data from RNA-sequencing was analyzed in Metascape. The top 20 enriched signaling clusters in relation to SNORA24 were listed. (d) Downstream targets of p53 in relation to SNORA24.

e. SNORA24 overexpression reduced the expression of p53 and p21 protein. HT29 cells and HCT116 cells were infected with LV-SNORA24 or LV-control, cells were harvested in 72 h after infection. Key components in p53 pathway including p53, p21 and downstream targets including p-CDK2(T160), CDK2, Cyclin E, etc. were detected by western blotting.

f-g. Deletion of p53 attenuated the effect of SNORA24 on colony formation. HCT116^{p53+/+} (wild-type p53, wtp53) and HCT116^{p53-/-} (p53 deletion) cells were infected with LV-SNORA24 or LV-control. 72 h after infection, cells were employed to colony formation assay. The fractions of colony formation were analyzed: LV-SNORA24 versus LV-control in HCT116^{p53+/+} cells; LV-SNORA24 versus LV-control in HCT116^{p53-/-} cells; LV-SNORA24 in HCT116^{p53+/+} cells versus LV-SNORA24 in HCT116^{p53-/-} cells. Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3. **P* < 0.05, ****P* < 0.001.

h. P53 deletion abolished the effect of SNORA24 on cell proliferation. The above infected cells were performed to CCK-8 assay in 72 h after infection. Cell viability was analyzed in LV-SNORA24 group and LV-control group (Two-way ANOVA, mean $\pm$ SD, N = 5). #*P*_{p53 deletion} < 0.05, ****P*_{wtp53} < 0.001.

i-j. P53 deletion attenuated the effect of SNORA24 on apoptosis. HCT116^{p53+/+} and HCT116^{p53-/-} cells were infected with LV-SNORA24-KD or LV-NC. 72 h after infection, apoptotic cells were analyzed via flow cytometry assay (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3, ****P* < 0.001). HCT116^{p53+/+} and HCT116^{p53-/-} infected with LV-SNORA24-KD or LV-NC were employed to analysis of PARP/ cleaved PARP via western blotting.

Figure 6

SNORA24 promoted ubiquitin-independent degradation of p53 protein

a. SNORA24 overexpression had no impact on p53 mRNA levels. HT29 cells and HCT116 cells infected with LV-SNORA24 and LV-control were harvested for RNA extraction in 72 h after infection. P53 mRNA

expression was measured by qRT-PCR and result was normalized to GAPDH (Two-tailed Student's *t*-test, mean $\pm$ SD, N = 3).

b-c. SNORA24 overexpression promoted the degradation of p53 protein. HT29 cells and HCT116 cells stably expressing LV-SNORA24 or LV-control were exposure to 100 μ g/mL cycloheximide (CHX) or dimethyl sulfoxide (DMSO) for 0, 1, 2, 4, 8 or 12 h. P53 protein was detected by western blotting and the bands were measured by Image J software.

d. MG132 rescued p53 expression in cells overexpressing SNORA24. HCT116 cells and HT29 cells stably expressing LV-SNORA24 or LV-control were exposure to MG132 for 6 h, p53 protein expression was detected by western blotting.

e. Schematic diagram of SNORA24 in regulation of cell proliferation.

Supplementary Files

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