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Research Paper

SNORA21 – An Oncogenic Small Nucleolar RNA, with a Prognostic Biomarker Potential in Human Colorectal Cancer

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ABSTRACT

Background: Emerging evidence indicates that small nucleolar RNAs (snoRNAs) play a central role in oncogenesis. Herein, we systematically evaluated expression profiles of snoRNAs in colorectal cancer (CRC) and investigated their clinical and functional role in this malignancy.
Methods: We compared expression levels of snoRNAs between cancer and normal tissues using publicly available datasets and identified the most differentially expressed and commonly upregulated snoRNAs in CRC. These results were examined in 489 colorectal tissues to assess their clinical significance, followed by a series of *in vitro* and *in vivo* experiments to evaluate the functional role of candidate snoRNAs.
Results: Using multiple RNA profiling datasets, we identified consistent overexpression of SNORA21 in CRC. In the clinical validation cohorts, the expression level of SNORA21 was upregulated in colorectal adenomas and cancers. Furthermore, elevated SNORA21 emerged as an independent factor for predicting poor survival. Both *in vitro* and *in vivo* experiments revealed that CRISPR/Cas9-mediated inhibition of SNORA21 expression resulted in decreased cell proliferation and invasion through modulation of multiple cancer related pathways.
Conclusions: We systematically identified SNORA21 as a key oncogenic snoRNA in CRC, which plays an important role in cancer progression, and might serve as an important prognostic biomarker in CRC.

1. Introduction

Colorectal cancer (CRC) is a common malignancy, and remains the second leading cause of cancer-related deaths in the Western countries (Siegel et al., 2015). Although cancer-related mortality associated with CRC have declined over the past 2 decades due to advances in cancer prevention, early detection and treatment (Siegel et al., 2015), the five-year relative survival rates from this disease still remain poor (Maeda et al., 2009; Siegel et al., 2015; Van Cutsem et al., 2006; Yoo et al., 2006). In order to improve overall prognosis of CRC patients, more individualized treatments are necessary, which rely on a further understanding of the complex molecular mechanisms underlying its pathogenesis.

Emerging evidence indicates that stepwise accumulation of genetic and epigenetic alterations drives CRC progression (Goel and Boland, 2010; Ogino et al., 2011; Pritchard and Grady, 2011). For instance, mutational activation of genes such as *KRAS* and *BRAF* facilitate tumor formation and progression through gain of function events (Ogino et al., 2011; Pritchard and Grady, 2011). Likewise, epigenetic alterations, including DNA methylation, histone modifications, and dysregulated expression of non-coding RNAs (ncRNAs) are frequently being recognized as pathogenic mechanisms in CRC (Goel and Boland, 2010). The current consensus is that epigenetic alterations in CRC

Abbreviations: CRC, colorectal cancer; ncRNAs, non-coding RNAs; miRNAs, microRNAs; lncRNAs, long non-coding RNAs; rRNAs, ribosomal RNAs; snoRNAs, small nucleolar RNAs; snoRNPs, small nucleolar ribosonucleoproteins; FFPE, formalin-fixed paraffin-embedded; NM, normal mucosa; LM, liver metastasis; pCRC, primary colorectal cancer; TNM, The tumor-node-metastasis; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval; ROC, Receiver operating characteristic; AUC, area under the curve; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9; sgRNA, single guide RNA; FDR, false discovery rate; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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occur early in neoplastic cascade, and perhaps manifest more frequently than genetic alterations – making them attractive candidates for exploitation as cancer biomarkers (Eddy, 2001; Goel and Boland, 2010). Over the past two decades, ncRNAs, particularly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have been recognized as key molecular regulators in oncogenesis. In this very context, more recent evidence indicates that another class of ncRNAs, called 'small nucleolar RNAs or snoRNAs', sized 60–300 bp, may play a critical role in multiple human cancers. Traditionally, snoRNAs have been considered to act as "housekeeping genes" because they are known to facilitate the modification, maturation, and stabilization of pre-ribosomal RNAs (rRNAs) by inducing 2′-*O*-methylation or pseudouridylation of specific rRNA sites by utilizing help from small nucleolar ribosonucleoproteins (snoRNPs) (Mannoor et al., 2012). In 2002, the first report for dysregulated expression of snoRNAs in cancer revealed H5sn2 expression to be significantly downregulated in meningiomas (Chang et al., 2002; Esteller, 2011). More recently, work from our laboratory and others demonstrated that SNORA42 acts as an oncogene in lung cancer and CRC (Mannoor et al., 2014; Mei et al., 2012; Okugawa et al., 2017). On similar lines, SNORD50 appears to play a tumor suppressive role in prostate and breast cancer (Dong et al., 2009, 2008) through modulation of Ras-ERK1/ERK2 signaling via direct biding to the KRAS protein (Siprashvili et al., 2016). In addition, several high-throughput RNA-sequencing and microarray-based analyses have identified several snoRNAs to be deregulated in a number of cancers, suggesting their potential role in oncogenesis. However, considering their small size and stability, snoRNAs are also gaining momentum as plausible disease biomarkers and therapeutic targets (Gao et al., 2015; Liao et al., 2010; Mannoor et al., 2014; Muller et al., 2015; Ravo et al., 2015; Xu et al., 2016).

Herein, we performed a systematic and comprehensive analysis for snoRNAs expression profiles across multiple CRC datasets, and using a series of bioinformatics analysis, identified key snoRNAs involved in oncogenesis. Among these, we identified SNORA21 as a potential oncogenic snoRNA in CRC. We further evaluated its clinical significance in CRC by analyzing clinical specimens from multiple independent patient cohorts, followed by interrogation and confirmation of its oncogenic potential in a series of *in vitro* and *in vivo* experiments. Based on the clinical validation and functional analysis of oncogenic roles, our data suggest SNORA21 to be an oncogenic snoRNA in CRC pathogenesis, could provide potential biomarkers for prognostication of CRC and prediction of metastasis, which has important clinical significance in terms of better management for the patients suffering from CRC.

2. Materials and Methods

2.1. Samples and Study Design

This study analyzed 489 tissue specimens, which comprised of 30 pairs of matched fresh frozen CRC and adjacent normal mucosa (NM), and 429 formalin-fixed paraffin-embedded tissues; 318 primary CRC (pCRC) tissues and 41 corresponding NM, 30 adenoma tissues and 20 pCRC and paired liver metastasis (LM) tissues. These tissues came from patients enrolled at the Okayama University Hospital, Tokushima University Hospital in Japan and Shanghai Tenth People's Hospital in China. Further information on patient demographics and clinicopathological characteristics is provided in the online Supplementary Material and Methods and Supplementary Table 1. Written informed consent was obtained from all patients, and the Institutional Review Boards of all participating institutions approved the study.

2.2. cDNA Generation and qRT-PCR Analysis

Total RNA was extracted from fresh frozen and FFPE specimens using the miRNeasy Mini Kit and miRNeasy FFPE Kit (Qiagen, Hilden, Germany), respectively. Careful macro-dissection of FFPE slides allowed RNA extraction from >80% tumor. The expression of snoRNAs was analyzed by custom TaqMan assays (Applied Biosystems, Foster City, CA, US), using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). U6 snRNA (Applied Biosystems, Cat# 4427975) was used as an endogenous control for data normalization. The expression levels of snoRNAs were determined using the $2^{-\Delta Ct}$ method.

2.3. Cell Lines

Human CRC cell lines SW620, DLD-1, HCT116, SW48, SW480, and CaCo2 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, US). These cell lines are routinely tested and authenticated for various genetic and epigenetic markers every six months. All CRC cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco, Carlsbad, CA, US) containing 10% fetal bovine serum (FBS; Gibco), and 1% penicillin-streptomycin (Gibco) at 37 °C in humidified atmosphere with 5% CO_2.

2.4. Establishment of a SNORA21 Knock-Down Cell Line by CRISPR/Cas9 System

SNORA21 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Applied Biological Materials, Richmond, BC, Canada, Cat# K2214505) was used to inhibit SNORA21 expression in CRC cell lines. Single guide RNA (sgRNA) CRISPR/Cas9 target sequences of SNORA21 and recommended scrambled control (Applied Biological Materials, Cat# K011) are shown in Online Supplementary Table 2. The CRISPR/Cas9 transfection was conducted as described in the online Supplementary Materials and Methods.

2.5. Cell Proliferation, Colony Formation, Apoptosis, Cell Cycle and Invasion Assays

Cell proliferation, colony formation, apoptosis, cell cycle, and invasion assays were performed following the confirmation of downregulation of SNORA21 expression in cell lines. The details of these assays are provided in the online Supplementary Materials and Methods.

2.6. Microarray Analysis

1 μg of total RNA extracted from SW48 cells (control and CRISPR/Cas9-mediated SNORA21 inhibition (CRISPR-SNORA21)) were subjected to gene expression analysis using GeneChip ® Human Gene 2.0ST array assay (Affymetrix, Santa Clara, CA, US). The details of these assays are provided in the online Supplementary Materials and Methods.

2.7. Animal Experiments

Five week-old male athymic nude mice (Harlan Laboratories, Houston, TX, US) were housed under controlled light conditions and were fed ad libitum. Xenograft tumors were generated by subcutaneous injection of 1×10^6 cells. Tumor volume was calculated using the following formula: $(\pi/6) \times (\text{length} \times \text{width} \times \text{height})$. Six mice were used in each scrambled control and CRISPR-SNORA21 group, and subcutaneous tumors were monitored for 42 days following injection. Further information is provided in the online Supplementary Material and Methods.

2.8. Statistical Analysis

Results are expressed as means ± SE, and all statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013). Further information is provided in the online Supplementary Materials and Methods.

3. Results

3.1. SNORA21 Expression is Frequently Dysregulated in Human Colorectal Cancers

We first analyzed snoRNA expression levels in CRC and NM from a publicly available Gene expression Omnibus (GEO) dataset (GEO: GSE76713) (Damas et al., 2016), which included most of known snoRNAs. To identify candidate snoRNAs, we selected differentially expressed snoRNAs that met following criteria; adjusted P value <0.05 and an absolute log fold change >1.00. This analysis identified 28 upregulated snoRNAs in cancerous tissues vis-à-vis NM (Fig. 1a). Subsequently, to identify the most pertinent and differentially expressed snoRNA candidates in CRC, we next performed validation of results in the TCGA dataset. Considering that the length of snoRNAs is between 60 and 300 bp, not all snoRNA can be identified using conventional RNA sequencing or small RNA sequencing approaches independently. Therefore, it is possible that other differentially expressed snoRNAs may exist in CRCs. Nevertheless, we focused on the snoRNAs which are consistently differentially expressed in the datasets which contain annotated snoRNAs. Based upon these analyses, we identified a panel of 4 snoRNAs, which included two upregulated and two downregulated snoRNAs in CRC tissues (Fig. 1a). Interestingly, SNORA21 was the only snoRNA which was dysregulated in both cohorts (Fig. 1b).

Based upon the expression levels in the GEO: GSE76713 dataset (probe ID; ucscGeneNc_uc002hpz_1), SNORA21 expression levels were more than threefold higher in CRC vs. NM ($P < 0.001$; Fig. 1c). The receiver operating characteristic (ROC) curve analysis demonstrated that the expression of SNORA21 successfully discriminated CRC from NM ($P < 0.001$ and an area under the curve (AUC) value = 0.93, Fig. 1c). Similarly, in the TCGA dataset, SNORA21 expression was significantly upregulated in CRC compared to NM, and was able to robustly discriminate cancer tissues vs. NM ($P < 0.001$ and AUC = 0.93; Fig. 1d). Furthermore, we validated the expression of SNORA21 in CRC using another GEO dataset, GEO: GSE21510 (Tsukamoto et al., 2011) ($P < 0.001$ and AUC = 0.99; Fig. 1e), which convincingly highlighted its diagnostic potential in CRC.

3.2. SNORA21 is a Potential Diagnostic Biomarker in Colorectal Neoplasia

To further validate the observation that SNORA21 is upregulated in colorectal neoplasia (adenomas and cancers), we first analyzed the expression levels of SNORA21 in a subset of 30 fresh frozen CRCs along with the matched pairs of NM samples. As expected, SNORA21 expression was significantly higher in CRC tissues compared to NM tissues ($P < 0.001$, Wilcoxon signed-rank test; Fig. 2a). The ROC curve analysis indicated that the expression level of SNORA21 could robustly discriminate CRC tissues from NM ($P = 0.001$ and AUC = 0.74; the online Supplementary Fig. 1a). While the RNA quality was well preserved in fresh frozen samples, we were curious to examine whether we can analyze SNORA21 expression from FFPE tissue-derived RNA samples, as such tissues can be easily obtained from pathology archives as well as offer a more practical approach in routine clinical practice. We analyzed SNORA21 expression in 27 pairs of CRC and adjacent NM derived from macrodissected FFPE samples. Consistent with the results in fresh frozen samples, SNORA21 expression was significantly upregulated in FFPE CRC tissues vs. NM tissues ($P = 0.004$, Wilcoxon signed-rank test; the online Supplementary Fig. 1b). To confirm these data, and to examine whether SNORA21 upregulation may occur even in premalignant adenomatous polyps, we assessed its expression in a larger group of CRCs ($n = 127$) and adenomas ($n = 30$). We once again validated that SNORA21 expression was higher not only in CRC, but was also significantly upregulated in adenoma samples ($P = 0.003$ and <0.001, respectively; Steel-Dwass test; Fig. 2b). The corresponding ROC curves revealed that the SNORA21 expression was able to discriminate both CRC and adenoma from NM tissues (the online Supplementary Fig. 1c), indicating that SNORA21 can be used for diagnosis of colorectal neoplasia.

3.3. SNORA21 is a Potential Prognostic Biomarker in Colorectal Neoplasia

Next, we assessed the association between SNORA21 expression and various clinicopathological characteristics in a group of 127 CRC patient samples and 41 NM in the validation cohort 1, using the cut-off criteria based on 95th percentile of SNORA21 expression in NM. In brief, low SNORA21 expression was categorized as the lower 95th percentile and high SNORA21 expression was classified as the upper 95th percentile of SNORA21 expression in NM (Fig. 2b). These high and low cut-off thresholds were used for both independent validation cohorts. The expression of SNORA21 was positively associated with older age ($P = 0.009$) and degree of tumor invasion ($P = 0.009$; Table 1).

Kaplan-Meier survival analysis showed that high levels of SNORA21 expression resulted in worse overall survival (OS) ($P = 0.021$; Fig. 2c). To evaluate whether the prognostic value of SNORA21 was independent of other risk factors associated with the clinical outcomes in CRC, univariate and multivariate analyses were performed using the Cox proportional hazard models. The results of the univariate analysis demonstrated that high SNORA21 expression was significantly associated with poor OS (hazard ratio [HR] 2.20; 95% confidence interval [CI], 1.14 to 4.21; $P = 0.018$; Table 3). The multivariate analysis revealed that when expression levels of SNORA21, lymph node metastasis, distant metastasis, and histology were added to the analysis, high SNORA21 expression emerged as an independent prognostic factor for OS (HR = 1.97; 95% CI, 1.01 to 3.85; $P = 0.048$; Table 3).

To further investigate the associations of SNORA21 expression with clinicopathological features in CRC patients, we evaluated its clinical significance in an independent, second validation cohort of patients (cohort 2). In the cohort 2, high SNORA21 expression was also significantly associated with advanced TNM stage and distant metastasis ($P = 0.009$ and 0.04, respectively; Table 2), while there was a statistical trend observed for lymph node metastasis and vascular invasion ($P = 0.11$ and 0.16, respectively; Table 2). In addition, CRC patients with high SNORA21 expression had a shorter time of survival compared to those with low expression ($P = 0.007$; Fig. 2d). The univariate analysis revealed that high SNORA21 expression was associated with poor OS (HR = 2.37; 95% CI, 1.24 to 4.54; $P = 0.01$; Table 3). Likewise, the multivariate analysis elucidated that when expression levels of SNORA21, lymph node metastasis, distant metastasis, venous invasion and histology were included in the analysis, high SNORA21 expression in CRC emerged as an independent prognostic factor for OS (HR = 2.0; 95% CI, 1.00 to 3.99; $P = 0.049$; Table 3). Collectively, these results suggest that high SNORA21 expression contributes to poor prognosis in CRC patients, may play an oncogenic role in CRC.

3.4. High SNORA21 Expression is Associated with Distant Metastasis in Colorectal Cancer

In view of its prognostic significance and association with advanced disease, we assessed whether SNORA21 is involved in distant metastasis. We examined its expression in the validation cohort 2 by categorizing into three groups: CRC without metastasis, CRC with single organ metastasis (Stage IVA), and CRCs with multiple organ metastases (Stage IVB). Intriguingly, although no difference was observed between CRC patients with single versus multiple organ metastases, SNORA21 expression was significantly upregulated in Stage IVB patients vs. those without metastasis ($P = 0.004$, Steel-Dwass test; Fig. 2e). These results suggest that high SNORA21 expression in the primary CRC site may contribute to multiple organ distant metastases. We also compared

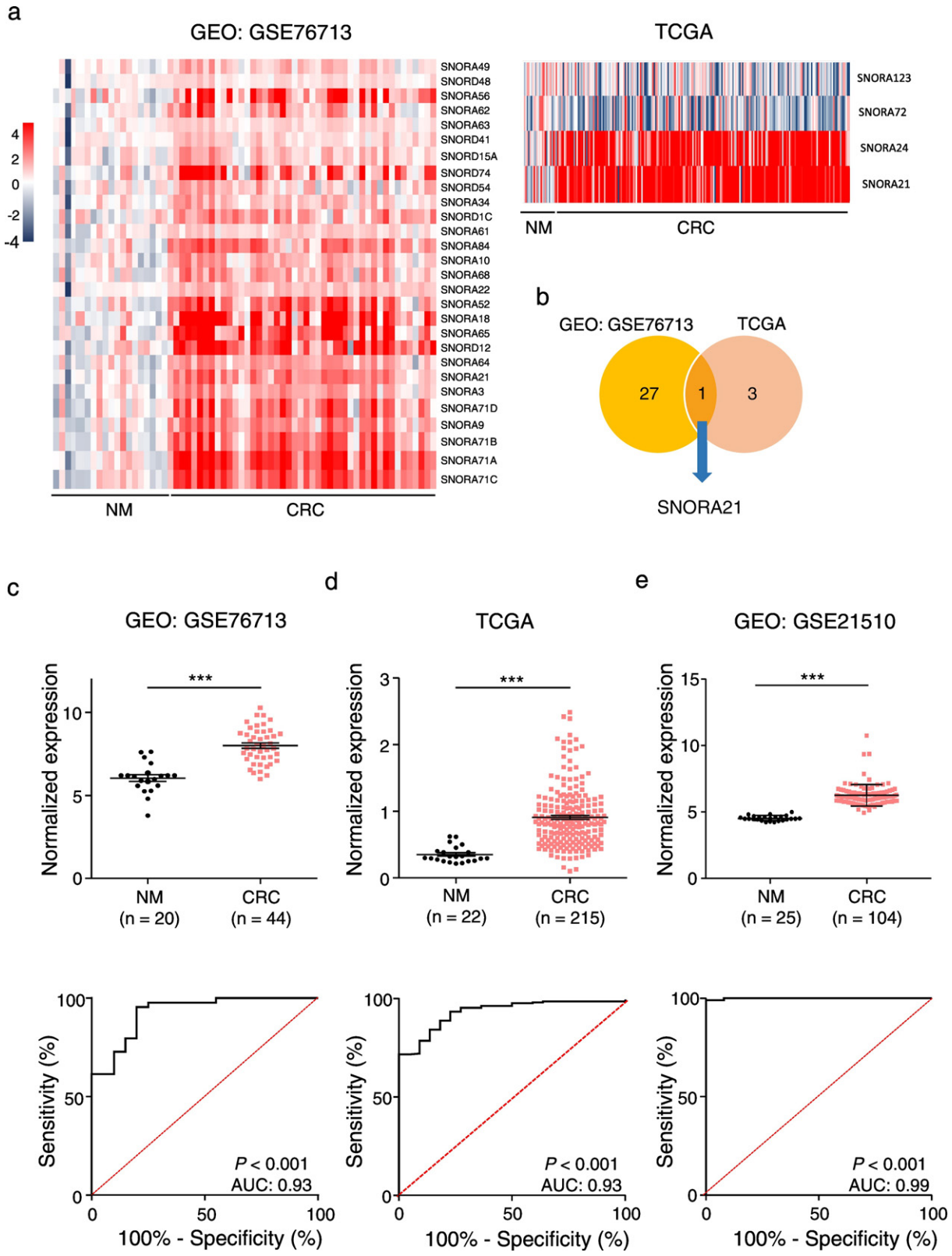

Fig. 1. SNORA21 is overexpressed in colorectal cancer (CRC). (a) Heat maps of differentially expressed snoRNAs (CRCs vs. normal mucosa (NM)) derived from GEO: GSE76713 (left) and TCGA (right) datasets. (b) SNORA21 is dysregulated in CRC compared to NM in both GEO: GSE76713 and TCGA datasets. (c, d, e) SNORA21 is overexpressed in CRC compared to NM (top). Corresponding receiver operating characteristic (ROC) curve of SNORA21 (bottom) (c; GEO: GSE76713, d; TCGA, e; GEO: GSE21510). Abbreviations; CRC, colorectal cancer; NM, normal mucosa. ***$P < 0.001$, by Mann-Whitney U test.

the levels of SNORA21 expression in tissues from patients with pCRC and matched LM sites. SNORA21 expression was significantly upregulated in LM vs. pCRC ($P = 0.009$, Wilcoxon signed-rank test; Fig. 2f), indicating its involvement in metastasis, and the likelihood that patients with high SNORA21 to have higher probability for multiple distant metastases.

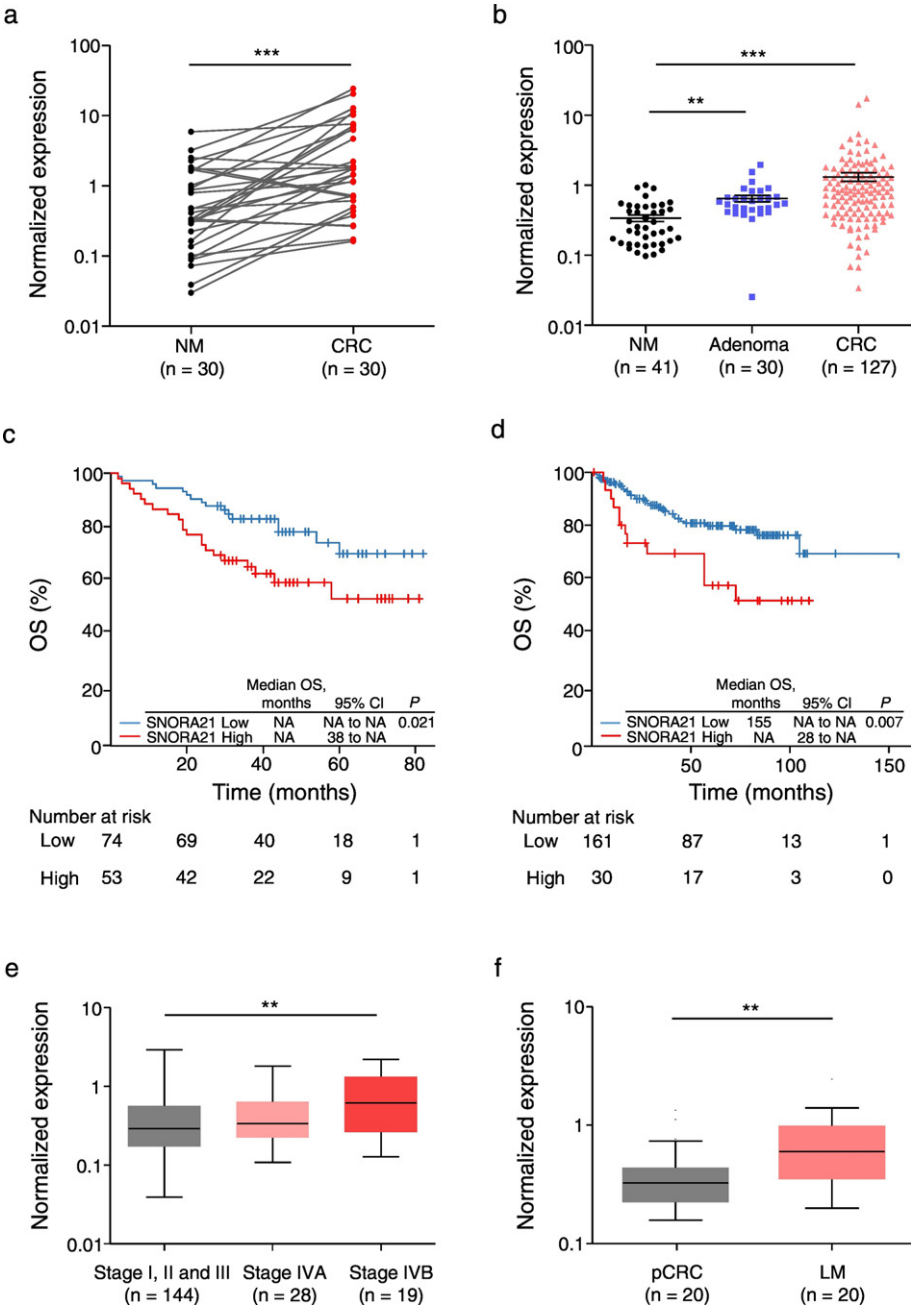

Fig. 2. SNORA21 is a clinically significant snoRNA in CRC. (a) SNORA21 is upregulated in tumor samples compared to adjacent NM ($P < 0.001$, Wilcoxon signed-rank test). (b) SNORA21 is overexpressed in both adenomas and CRCs compared to NM ($P = 0.003$ and <0.001, respectively; Steel-Dwass test). (c, d) CRCs with high SNORA21 expression are associated with poor survival (c: cohort 1, d: cohort 2) ($P = 0.021$ and 0.007, respectively, log-rank test). (e) SNORA21 expression increases with tumor status (Stage I, II and III vs. Stage IVB; $P = 0.004$, Steel-Dwass test). (f) SNORA21 expression is overexpressed in LM compared to pCRC ($P = 0.009$, Wilcoxon signed-rank test). Abbreviations; CRC, colorectal cancer; NM, normal mucosa; OS, overall survival; NA, not available; pCRC, primary CRC; LM, liver metastasis. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5. Inhibition of SNORA21 Suppresses Its Oncogenic Potential in Colorectal Cancer Cells

Considering the clinical significance of SNORA21 in CRC, we next performed functional evaluation of its oncogenic roles in this disease. Since the majority of snoRNAs are primarily located in the nucleolus, it is difficult to knockdown expression of snoRNAs by siRNA or shRNA-based techniques (Ploner et al., 2009). Therefore, we used Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 system (CRISPR/Cas9 system) to suppress the expression levels of SNORA21 in CRC cells. SNORA21 is located in an intron between exons 2 and 3 of *RPL23* gene. A single guide RNA (sgRNA) was designed to target the location shown in the intron (Fig. 3a). To identify most pertinent cell lines for these experiments, we first evaluated steady state expression of SNORA21 in a number of CRC cell lines by qRT-PCR. Although all CRC cell lines showed high SNORA21 expression (the online Supplementary Fig. 2a), SW48 cells had the highest and HCT116 had the lowest SNORA21 expression. Since HCT116 cells were recommended by the manufacturer as optimal for CRISPR experiments, we selected both HCT116 and SW48 cell lines for inhibition of SNORA21 expression. Following the transfection of the CRISPR SNORA21 construct (CRISPR-SNORA21), expression of SNORA21 in HCT116 and SW48 cell lines was confirmed by qRT-PCR. The SNORA21 expression in CRISPR-SNORA21 transfected HCT116 and SW48 cell lines was reduced by

Table 1
Correlation between SNORA21 expression and clinicopathological features of CRC patients in cohort 1.

		No. (%)	Low expression	High expression	*P* value
			$n = 74$, (%)	$n = 53$, (%)	
Age, y	Median (range)	69 (42–90)	67 (42–83)	73 (44–90)	**0.009**[a]
Sex	Female	66 (52.0)	35 (47.3)	31 (58.5)	0.280[b]
	Male	61 (48.0)	39 (52.7)	22 (41.5)	
Tumor location	Colon	80 (63.0)	43 (58.1)	37 (69.9)	0.197[b]
	Rectum	47 (47.0)	31 (41.9)	16 (30.2)	
TNM stage	I	19 (15.0)	15 (20.3)	4 (7.5)	0.280[b]
	II	68 (53.5)	36 (48.6)	32 (60.4)	
	III	35 (27.6)	21 (28.4)	14 (26.4)	
	IV	5 (3.9)	2 (2.7)	3 (5.7)	
Tumor invasion	I	6 (4.7)	6 (8.1)	0 (0.0)	**0.009**[b]
	II	17 (13.4)	14 (18.1)	3 (5.7)	
	III	5 (3.9)	3 (4.1)	2 (3.8)	
	IV	99 (78.0)	51 (68.9)	48 (90.6)	
Lymph node metastasis	Presence	40 (31.5)	23 (31.1)	17 (32.1)	1[b]
	Absence	87 (68.5)	51 (68.9)	36 (67.9)	
Distant metastasis	Presence	5 (3.9)	2 (2.7)	3 (5.7)	0.65[b]
	Absence	122 (96.1)	72 (97.3)	50 (94.3)	
Histology[c]	Well and mod	112 (88.2)	67 (91.8)	45 (88.2)	0.55[b]
	Poor and muc	12 (9.4)	6 (8.2)	6 (1.78)	
Venous invasion	Presence	–	–	–	–
	Absence	–	–	–	
Lymphatic invasion	Presence	–	–	–	–
	Absence	–	–	–	

Bold: differences were statistically significant ($P < 0.05$); Abbreviations; well, well differentiated; mod, moderately differentiated; poor, poor differentiated; muc, mucinous carcinoma.
[a] *P* values were calculated by Mann-Whitney *U* test.
[b] *P* values were calculated by Fisher exact test.
[c] Three patients did not have the information of histology.

>80% and 90%, respectively, compared to those with scrambled control (control) (Fig. 3b), indicating that SNORA21 knockdown cell lines were established successfully.

Subsequently, we conducted various *in vitro* functional assays to determine the effects of SNORA21 suppression on the tumorigenicity of CRC cells. First, we performed MTT (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and colony formation assays to evaluate how suppression of SNORA21 might affect cell proliferation and colony formation. Suppression of SNORA21 expression significantly inhibited cell proliferation in HCT116 and SW48 cells compared with cell lines transfected with the control plasmids ($P = 0.016$ and <0.001, respectively; Fig. 3c). Similarly, SNORA21 downregulation inhibited colony

Table 2
Correlation between SNORA21 expression and clinicopathological features of CRC patients in cohort 2.

		No. (%)	Low expression	High expression	*P* value
			$n = 161$, (%)	$n = 30$, (%)	
Age, y	Median (range)	65 (23–88)	65 (23–88)	65 (45–88)	0.86[a]
Sex	Female	80 (41.9)	68 (42.2)	12 (40.0)	0.84[b]
	Male	111 (58.1)	93 (57.8)	18 (60.0)	
Tumor location	Colon	106 (55.5)	89 (55.3)	17 (56.7)	1[b]
	Rectum	85 (44.5)	72 (44.7)	13 (43.3)	
TNM stage	I	26 (13.6)	21 (13.0)	5 (16.7)	**0.009**[b]
	II	55 (28.8)	53 (32.7)	2 (6.7)	
	III	63 (33.0)	52 (32.1)	11 (36.7)	
	IV	47 (24.6)	35 (21.7)	12 (40.0)	
Tumor invasion	I	10 (5.2)	10 (6.2)	0 (0.0)	0.35[b]
	II	26 (13.6)	20 (12.4)	6 (20.0)	
	III	118 (61.8)	101 (62.7)	17 (56.7)	
	IV	37 (19.4)	30 (18.6)	7 (23.3)	
Lymph node metastasis[c]	Presence	100 (53.5)	81 (50.9)	19 (67.9)	0.11[b]
	Absence	87 (46.5)	78 (49.1)	9 (32.1)	
Distant metastasis	Presence	47 (24.5)	35 (21.7)	12 (40.0)	**0.040**[b]
	Absence	144 (75.5)	126 (78.3)	18 (60.0)	
Histology	Well and mod	177 (92.7)	149 (92.5)	28 (93.3)	1[b]
	Poor and muc	14 (7.3)	12 (7.5)	2 (6.7)	
Venous invasion[d]	Presence	139 (72.9)	114 (72.2)	25 (86.2)	0.16[b]
	Absence	48 (25.0)	44 (27.8)	4 (13.8)	
Lymphatic invasion	Presence	172 (90.1)	143 (88.8)	29 (96.7)	0.32[b]
	Absence	19 (9.9)	18 (11.2)	1 (3.3)	

Bold: differences were statistically significant ($P < 0.05$). Abbreviations; well, well differentiated; mod, moderately differentiated; poor, poor differentiated; muc, mucinous carcinoma.
[a] *P* values were calculated by Mann-Whitney *U* test.
[b] *P* values were calculated by Fisher exact test.
[c] Lymphadenectomy was not performed in four patients.
[d] Four patients did not have the information of venous invasion.

Table 3
Univariate and multivariate analysis in independent two cohorts.

Cohort	Variables	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Cohort 1	Age (<median vs. >median)	2.23	1.10–4.50	**0.026**[a]			
	Sex (Female vs. Male)	0.97	0.51–1.89	0.913[a]			
	T stage (I–II vs. III–IV)	4.94	1.19–20.59	**0.028**[a]			
	Lymph node metastasis (presence vs. absence)	2.28	1.20–4.35	**0.012**[a]	2.17	1.12–4.20	**0.022**[a]
	Distant metastasis (presence vs. absence)	6.59	2.26–19.2	< **0.001**[a]	5.97	1.95–17.9	**0.002**[a]
	Tumor location (colon vs. rectum)	0.40	0.18–0.88	**0.023**[a]			
	Histology (well and mod vs. poor and muc)	2.98	1.30–6.83	**0.010**[a]	3.12	1.34–7.27	**0.008**[a]
	Venus invasion (presence vs. absence)						
	Lymphatic invasion (presence vs. absence)						
	SNORA21 expression (high vs. low)	2.20	1.14–4.21	**0.018** [a]	1.97	1.01–3.85	**0.048**[a]
Cohort 2	Age (<median vs. >median)	0.95	0.52–1.73	0.86[a]			
	Sex (Female vs. Male)	1.08	0.59–2.00	0.80[a]			
	T stage (I–II vs. III–IV)	5.77	1.40–23.87	**0.016**[a]			
	Lymph node metastasis (presence vs. absence)	4.55	2.10–9.84	< **0.001**[a]			
	Distant metastasis (presence vs. absence)	9.408	5.00–17.66	< **0.001**[a]	6.02	3.01–12.06	< **0.001**[a]
	Tumor location (colon vs. rectum)	1.49	0.82–2.72	0.19[a]			
	Histology (well and mod vs. poor and muc)	4.24	1.88–9.60	< **0.001**[a]	4.97	1.92–12.87	< **0.001**[a]
	Venous invasion (presence vs. absence)	4.90	1.51–15.9	**0.008**[a]	4.41	1.20–16.20	**0.026** [a]
	Lymphatic invasion (presence vs. absence)	5.38	0.74–38.13	0.096[a]			
	SNORA21 expression (high vs. low)	2.37	1.24–4.54	**0.01**[a]	2.00	1.00–3.99	**0.049**[a]

[a] *P* values were calculated by Cox proportional hazard models. Bold: differences were statistically significant ($P < 0.05$); Abbreviations; well, well differentiated; mod, moderately differentiated; poor, poor differentiated; muc, mucinous carcinoma; HR, hazard ratio; 95% CI, 95% confidence interval.

formation in both HCT116 and SW48 cell lines ($P = 0.056$ and 0.013, respectively; Fig. 3d). The results suggest that SNORA21 promotes CRC cell proliferation.

To further evaluate whether the cell proliferative function of SNORA21 is associated with apoptosis and/or cell cycle modulation, we performed apoptosis and cell cycle assays on cell lines transfected with CRISPR-SNORA21. While the inhibition of SNORA21 did not affect the numbers of apoptotic cells (the online Supplementary Fig. 2b), cells transfected with CRISPR-SNORA21 showed a significant reduction in S-phase cells in both the HCT116 and SW48 cell lines compared with controls ($P = 0.035$ and 0.005, respectively; the online Supplementary Fig. 2c). Thus, SNORA21 may facilitate tumor progression through modulation of the cell cycle.

Since we identified an association between high levels of SNORA21 expression with tumor invasion and distant metastasis in the CRC clinical cohorts, we performed invasion assays to validate and confirm these findings *in vitro*. Invasion capability was inhibited in both cell lines transfected with CRISPR-SNORA21 compared with that in control cells ($P = 0.013$ and 0.001, respectively; Fig. 3e), although the effect was more pronounced in SW48 cells. These results suggest that SNORA21 not only promotes cell proliferation, but also enhances the invasive ability of CRC, which is consistent with our clinical findings.

3.6. Inhibition of SNORA21 Suppresses Tumor Progression in Xenograft Model

In addition to *in vitro* experiments, we also used a mouse xenograft model to evaluate whether SNORA21 is involved in tumor growth. We subcutaneously injected SW48 cells treated with CRISPR-SNORA21 or the control constructs (1×10^6 cells per mouse) into flanks of nude mice, and evaluated tumor growth. During the initial 12 days, no significant differences were observed in tumor size between treated and control groups; however, at day 12, tumor growth was significantly attenuated in mice injected with cells transfected with CRISPR-SNORA21 vs. those injected with cells transfected with the control construct ($P < 0.001$; Fig. 3f). At 42 days post injection, size and weight of CRISPR-SNORA21 transfected tumors were significantly less than the control tumors ($P < 0.001$; Fig. 3f). These results were collectively indicative that inhibition of SNORA21 expression attenuated tumor growth in an animal model, which is in agreement with our in vitro and clinical findings.

3.7. SNORA21 Modulates the Cancer Related Signaling Pathways

Although this was not the primary aim of this study, we were interested in having preliminary understanding of the potential downstream targets of SNORA21 in CRC cells. We conducted microarray analysis on SW48 cells treated with the CRISPR-SNORA21 vs. control constructs. We identified differentially expressed genes with a false discovery rate (FDR) of <0.05 and an absolute log fold change >2.00. Among the 1027 genes altered, 545 genes were upregulated and 482 genes were downregulated by CRISPR-SNORA21 construct transfection (the online Supplementary Fig. 3a and Supplementary Table 3). Gene ontology (GO) analysis suggested that SNORA21 is involved in biological processes such as epithelial cell differentiation, morphogenesis and cell adhesion (the online Supplementary Fig. 3b), while the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified 10 signaling pathways influenced by SNORA21 inhibition (the online Supplementary Fig. 3c, d and Supplementary Table 4). Intriguingly, these signaling pathways matched biological processes identified by GO analysis. For example, Hippo and Wnt signaling pathways are thought to be involved in regulating pluripotency of stem cells, and hence inhibit cellular differentiation and adhesion (Fevr et al., 2007; Gregorieff et al., 2015). Therefore, SNORA21 could facilitate cell proliferation and CRC progression through modulating multiple cancer related pathways.

4. Discussion

In this study, using a series of logical and comprehensive approaches, we demonstrated functional oncogenic relevance of SNORA21 in CRC. We made several key observations during this study: First, we observed that expression of SNORA21 is upregulated in CRC using multiple publicly available datasets and we demonstrated in clinical specimens that SNORA21 was even overexpressed in colorectal adenomas. Second, while evaluating the clinical significance of SNORA21 in two independent cohorts of tumor specimens from patients with CRC and observed significant associations between high SNORA21 expression with invasion, metastasis, and tumor progression in patients with CRC. Third, we noted that high SNORA21 expression emerged as an independent predictor of poor overall survival in CRC patients. Fourth, in a series of *in vitro* and *in vivo* experiments, we observed that CRISPR-mediated inhibition of SNORA21 resulted in decreased cell

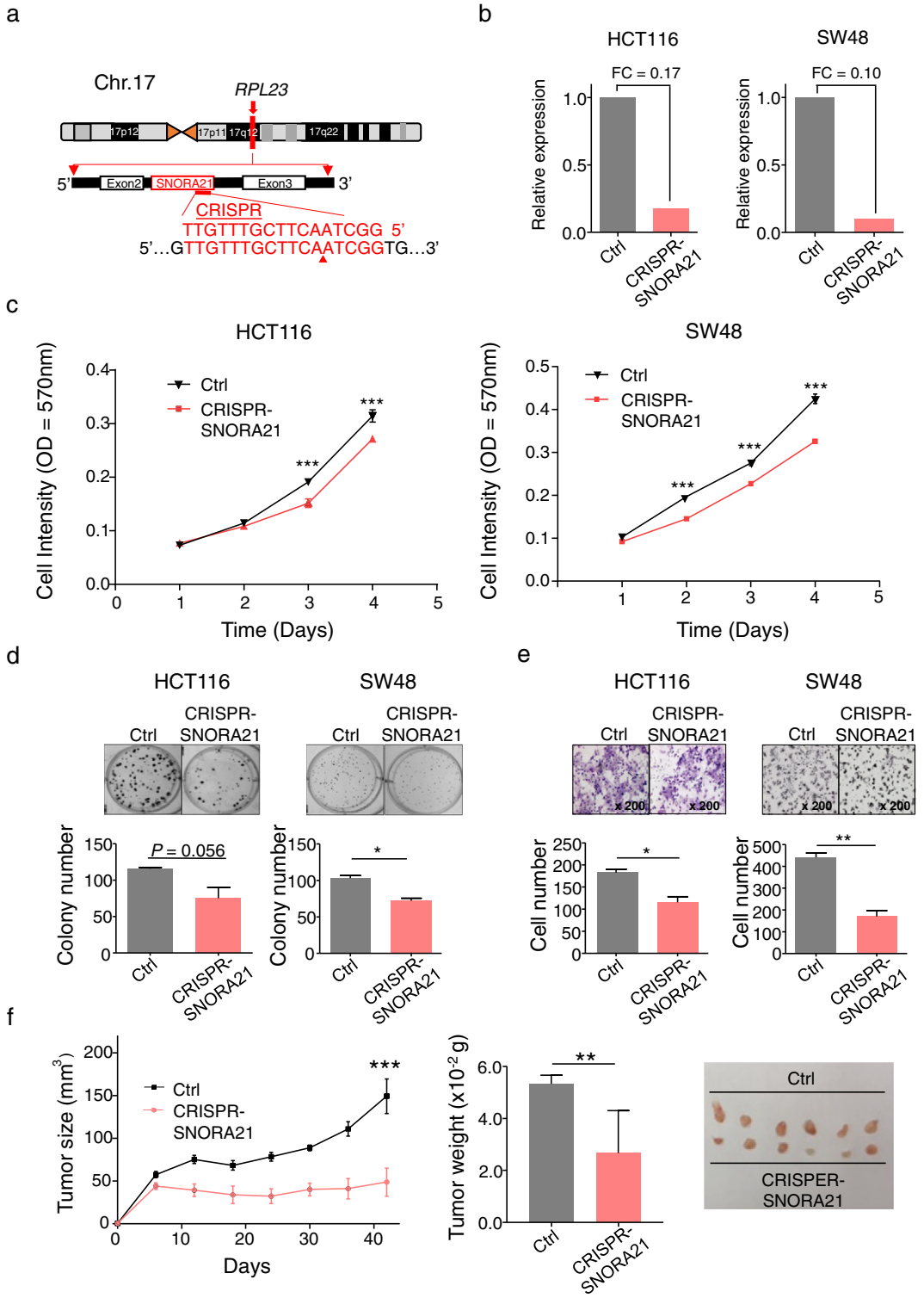

Fig. 3. CRISPR/Cas9-mediated SNORA21 inhibition results in suppression of its oncogenic potential in colorectal cancer cells. (a) The schematic diagram showing the gene location of SNORA21 and single guide (sgRNA) sequences. (b) SNORA21 inhibition efficiency in HCT116 and SW48. (c) Cellular proliferation rate of CRISPR-SNORA21 cells compared to control cells in HCT116 (left) and SW48 (right) cell lines. (d) Colony formation capacity of CRISPR-SNORA21 and control cells in HCT116 (left) and SW48 (right) cell lines. (e) Invasion capacity of CRISPR-SNORA21 and control cells in HCT116 (left) and SW48 (right) cell lines. (f) Xenograft tumor growth of SW48 CRISPR-SNORA21 and control cells. Daily tumor growth (left) Final tumor weight (middle) Representative image of xenograft tumors (right). Abbreviations; FC, fold change; Ctrl, scramble-control construct; CRISPR-SNORA21, CRISPR/Cas9-mediated SNORA21 inhibition; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by Student's *t*-test.

proliferation and the inhibited ability for invasion and metastasis, which corroborated with our findings in patient cohorts.

One of the major findings of the current study is the oncogenic role of SNORA21 in CRC. SNORA21, comprising of 133 nucleotides and located between exons 2 and 3 of the *RPL23* gene, was originally thought to induce rRNA maturation by guiding the psuedouridylation of residues U4401 and U4480 of the 28S rRNA (Ofengand and Bakin, 1997). However, in the present study, through suppression of

SNORA21 expression using the CRISPR/Cas9 system, we provided evidence for its oncogenic role in CRC. Results from our study elucidated that SNORA21 could regulate cell cycle with resultant changes in cellular proliferation and tumor invasion. These results were subsequently validated and were further supported by *in vivo* experiments. To further clarify the oncogenic role of SNORA21, we conducted microarray analysis in CRC cells. Intriguingly, we identified that SNORA21 is involved in several cancer related signaling pathways, including Hippo signaling pathway (Gregorieff et al., 2015), Wnt signal pathway (Fevr et al., 2007) and Axon guidance pathway (Li et al., 2009). Collectively, these data highlights the importance of SNORA21 in regulating key signaling pathways of CRC.

Intriguingly, the oncogenic function of SNORA21 also associated with key clinicopathological features in CRC patients. Previously, we have demonstrated prognostic potential of another snoRNA, SNORA42 (Okugawa et al., 2017). We identified SNORA42 based on published reports from other cancers and our discovery process for CRC-related snoRNA candidates was limited. However, in the present study, we used multiple high-throughput RNA expression profiling datasets to identify potential oncogenic snoRNAs in CRC in an unbiased manner. Based upon such a comprehensive discovery strategy we identified that SNORA21 expression was elevated in CRC and adenomas compared to normal colorectal tissues, and its enhanced expression associated with increased invasion and metastasis. Especially, high levels of SNORA21 in adenoma indicate the possibility that it could facilitate development of CRC, and its expression level could also serve as useful detection biomarkers for CRC. Furthermore, we observed that in primary CRCs with low levels of SNORA21 expression, when these tumors metastasized to the liver, levels of SNORA21 were significantly elevated at the metastatic sites – providing a potential causal link for these observations and the suggestion that it may play an important role in disease progression as well. Moreover, we found that SNORA21 expression was higher in primary cancers from CRC patients with multiple metastatic sites compared to those with no metastasis, suggesting that high SNORA21 expression in the primary cancer may drive cell proliferation and cell invasion ability, and eventual metastasis. Therefore, these findings suggest the clinical utility of SNORA21, and potentially other snoRNA-based prognostic and predictive markers for distant metastasis in CRC. In particular, some of the unique characteristics of snoRNAs make them very attractive for use as biomarkers. Due to their localization within the cell, snoRNAs generally are not affected by hemolysis and are relatively stable in blood by binding to as yet unidentified proteins (Zhang et al., 2012). Collectively, the results of our study highlights the potential of snoRNAs to be used as diagnostic, prognostic markers, and may perform similarly to miRNAs as non-invasive biomarkers (Toiyama et al., 2013, 2014).

One of the limitations of this study is the differences in the patient populations between the two CRC patient cohorts. As shown in Table 1 and 2, there were differences in the number of patients with stage IV CRC between cohort 1 and cohort 2. At first, we validated the clinical significance of SNORA21 expression in cohort 1 to determine the cut-off value of SNORA21 expression based on the comparison of cancerous and matched adjacent normal tissues. Since cohort 1 had a smaller population of patients with stage IV disease, we also validated the SNORA21 expression levels in cohort 2 by including 47 patients with stage IV CRC. In spite of the differences in the populations of two independent cohorts, SNORA21 expression consistently increased according to tumor invasion, distant metastasis and vascular invasion, indicating that SNORA21 may be involved in tumor progression.

In conclusion, we have firstly identified oncogenic roles of SNORA21 through enhanced cellular proliferation and tumor invasion. Furthermore, the expression of SNORA21 in tumors potentially can be used as a diagnostic, a prognostic and a predictive biomarker for distant metastasis in CRC. These data highlight the critical role that snoRNAs may play in cancer biology and suggests that SNORA21 could be a target for therapeutic treatment in CRC.

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Conflicts of Interest

The authors have declared no conflicts of interests.

Author Contributions

Author contributions: Study concept and design (KY, ST, WW, KS, AG); provision of samples (TN, TF, WW, YM, JM, TT); acquisition of data (KY, ST, WW, KS, AG); analysis and interpretation of data (KY, WW, KS, AG, JT); statistical analysis (KY, AG); drafting of the manuscript (KY, ST, AG).

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.07.009.

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