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Clinical significance of SNORA42 as an oncogene and a prognostic biomarker in colorectal cancer

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Abstract

Purpose—Despite recent advances in colorectal cancer (CRC) treatment, the prognosis of CRC patients still remains substandard, and metastatic recurrence following curative surgery is the leading cause of poor prognosis. Therefore, it is imperative to identify prognostic markers to predict the clinical outcome of CRC. Recent evidence revealed the new role of small nucleolar RNAs (snoRNAs) in oncogenesis. Herein, we systematically evaluated dysregulation of snoRNAs in CRC, and clarified the biomarker potential and biological significance of snoRNAs in CRC.

Experimental Design—We analyzed expression levels of four snoRNAs in 274 colorectal tissues from three independent cohorts, and 6 CRC cell lines. The functional characterization for the role of SNORA42 in CRC was investigated through a series of *in vitro* and *in vivo* experiments.

Results—In the screening phase, expression levels of all four snoRNAs were significantly elevated in CRC tissues than in corresponding normal mucosa. In the clinical validation cohort, increased SNORA42 expression was an independent prognostic factor for overall survival and disease free survival, and was an independent risk factor for distant metastasis. SNORA42 expression negatively correlated with overall survival in an additional independent cohort, and

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identified the patients with high risk for recurrence and poor prognosis in stage II CRC. Furthermore, *in vitro* and *in vivo* analysis showed that SNORA42 overexpression resulted in enhanced cell proliferation, migration, invasion, anoikis resistance, and tumorigenicity.

Conclusion—SNORA42 appears to a novel oncogene and could serve as a promising predictive biomarker for recurrence and prognosis in CRC patients.

Keywords

small nucleolar RNA; SNORD76; SNORD78; ACA11; SNORA42; colorectal cancer; biomarker; recurrence

INTRODUCTION

Colorectal cancer (CRC) remains one of the most common and lethal malignancies worldwide, and is the second leading cause of cancer-related deaths in the United States.[1] At the time of initial diagnosis, a large majority of patients have already reached an advanced stage where tumor cell spreading has occurred, and approximately 50% of CRC patients will die from the development of distant metastases.[2, 3] Although progress in treatment options, such as development of novel chemotherapeutic drugs and technical advances in invasive treatment for metastatic lesion, have somewhat improved the prognosis of advanced CRC patients,[4, 5] there still is a clear need for prognostic biomarkers that can help identify high-risk patients who can benefit from intensive post-treatment surveillance protocols for early detection of recurrence.

The central dogma of gene expression is that genetic information flows in a unidirectional fashion of DNA-mRNA-proteins.[6] Therefore, the majority of previous research in molecular and cellular biology has focused on the protein-coding genes and their transcripts, and messenger RNAs (mRNAs). However, in the past decade, noncoding RNAs (ncRNAs) have emerged as frontiers in gene regulation due to their important role in regulating various biological processes in various diseases, particularly cancer development. In this context, with the discovery of microRNAs (miRNAs) and their critical role in biological and physiological process in oncogenesis,[7, 8, 9, 10] other ncRNAs are now beginning to gather increased attention as potential regulators of tumorigenesis.

Small nucleolar RNAs (snoRNAs) are one of the largest groups of single-stranded small ncRNAs with 60 to 300 nucleotides in length,[11] and are categorized into two main groups. Box C/D snoRNAs (SNORDs) serve as guides for the 2′-O-ribose methylation of rRNAs or small nuclear RNAs, whereas box H/ACA snoRNAs (SNORAs) are guides for isomerization of uridine residues into pseudouridine.[12] Thus, in the past, snoRNAs were recognized for housekeeping functions due to their critical roles in rRNA maturation, while causing a relatively low impact on cellular homeostasis. However, recent emerging evidence has revealed a completely new and previously unrecognized role of snoRNAs in the control of cell fate and oncogenesis in various cancers.[13, 14, 15, 16, 17] In addition, recent studies have indicated potential prognostic impact and biomarker potential of snoRNA in solid cancers.[18, 19]

Recently, expression status of SNORD76, SNORD78, ACA11, and SNORA42 was reported to be up-regulated in various cancerous tissues.[20, 21, 22] SNORD76 and SNORD78 are located at 1q25 locus, and are encoded within the intron of a lncRNA, growth arrest-specific transcript 5 gene (GAS5). Although increasing body of literature supports the functional role of GAS5 in cancer development,[13, 14, 23, 24] increased expression of GAS5-related snoRNAs, such as SNORD76 and SNORD78, have also been suggested in NSCLC tissues.[20] In addition, this study also demonstrated that SNORD76 and SNORD78 levels were upregulated in serum of NSCLC patients and suggested that these snoRNAs could be used as noninvasive diagnostic biomarkers in NSCLC patients. Recently, Chu and colleagues revealed oncogenic function of another type of snoRNA, ACA11, which is encoded within intron 18–19 of the WHSC1 gene and demonstrated its unction in binding to heterogeneous nuclear ribonucleoproteins rather than the proteins involved in ribosomal biogenesis in multiple myeloma.[22] Furthermore, another study demonstrated that SNORA42 was frequently overexpressed in NSCLC tissues, and confirmed its oncogenic function through a series of *in vitro* and *in vivo* experiments. In addition, high expression of SNORA42 significantly correlated with poor prognosis in NSCLC patients, suggesting it to be a potentially relevant diagnostic and therapeutic target in NSCLC.[21] In spite of the growing evidence supporting the functional role of snoRNAs in tumorigenesis and their potential as a biomarker, to the best of our knowledge, none of the previous studies have investigated the clinical significance and their functional role in CRC progression.

Previous work from our group has shown that several miRNAs are differentially expressed in CRC, and can be used as biomarkers for diagnosis, prognosis and metastasis prediction in CRC patients.[25, 26, 27, 28, 29] In this study, we systematically investigated the expression of specific snoRNAs using a three-phase study. In the first phase, we focused our attention on four snoRNAs that have previously been reported to be dysregulated in other human cancers.[20, 21, 22] We performed quantitative analyses for determining the expression of these snoRNAs in a subset of clinical specimens from CRC patients to determine their expression pattern in cancers vs. matched normal mucosa. In the second phase, we performed clinical validation of snoRNA expression status using a large cohort of clinical materials. Furthermore, we focused on SNORA42 in an additional, independent cohort of CRC tissues, to confirm its performance as a prognostic biomarker. In the final phase, we performed functional analysis by altering SNORA42 expression in colon cancer cell lines, and undertook extensive *in vitro* and *in vivo* experiments to characterize its biological role in CRC progression.

MATERIAL AND METHODS

Patients and Sample Collection

This study included examination of 274 tissue specimens including 250 formalin-fixed paraffin-embedded (FFPE) primary CRC (pCRC) tissues, and 24 matched corresponding normal mucosa (NM) tissues, from 3 different CRC patient cohorts that were enrolled at Mie University and Okayama University in Japan, as described in Supplementary Table S1. Further information on patient demographics and clinicopathological characteristics is provided in the Supplementary material and methods. Written informed consent was

obtained from all patients, and the study was approved by the institutional review boards of all participating institutions.

SnoRNA expression by qRT-PCR and in-situ hybridization analysis

Total RNA were extracted from FFPE specimens using Total Nucleic Acid Isolation Kits for FFPE tissues (Ambion, Austin, TX, USA). Careful micro-dissection was performed on FFPE tissue slides to enrich for tumor cells. Expression of snoRNAs was analyzed using Custom TaqMan small RNA assays (Applied Biosystems, Foster City, CA, USA), and the average expression levels of snoRNAs were normalized against miR-16. MiR-16 was chosen as the endogenous normalizer for quantitation of snoRNA expression based on the previous findings that miR-16 was one of the most suitable reference genes for relative quantification of small ncRNAs, as well as in line with previous publications demonstrating that miR-16 is a reliable normalizer for non-coding RNAs in tissue specimens.[25, 28, 30, 31, 32]

For in-situ hybridization (ISH) analysis, five-micrometer-thick FFPE tissue sections were hybridized with the SNORA42 probe (LNA-modified and 5′- and 3′-DIG labeled oligonucleotide; Exiqon, Woburn, MA), as described previously.[27, 28] Positive (U6 snRNA, Exiqon) and negative controls (scrambled, Exiqon) were included in each hybridization experiment [27, 28, 32].

Cell Lines

Human colon cancer cell lines Caco2, HCT116, HT29, LoVo, SW480, and SW620 were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). These cell lines were tested and authenticated using a panel of genetic and epigenetic markers on a regular basis. All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% humidified CO_2 atmosphere.

Establishment of SNORA42 overexpressing cell lines

SNORA42 was cloned as described previously.[21] A pCDH vector (System biosciences, Mountain view, CA, USA) was used for ectopic over-expression of SNORA42. The pCDH vector encoding intact sequence of SNORA42 cDNA or empty vector as a control was infected into HEK293T cells together with pPACKH1 Packaging Plasmid mix (System biosciences) for producing viral particles using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Further information is provided in the Supplementary material and methods.

Silencing of SNORA42 expression in CRC cell line

SNORA42 expression was silenced in HCT116 cells using SNORA42 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Human; Applied Biological Materials, Richmond, BC, Canada) which contained SNORA42-specific target sequences and scrambled sequences, according to manufacturer's instruction. Further information is provided in the Supplementary material and methods.

Cell proliferation and colony formation assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma, USA) was used to determine the degree of cell proliferation as described previously.[33] For colony formation assay, the number of colonies with >50 cells were counted after seeding of colon cancer cell lines following manufacture's instruction. Further information is provided in the Supplementary material and methods.

Cell invasion, migration and anoikis assay

Invasion and migration assays were performed using Boyden chambers (Corning, Corning, NY, USA) using 8 um pore size membrane with Matrigel (for invasion assay) or without Matrigel (for migration assay). Anoikis assay was performed as described previously.[34] All experiments were conducted as three independent experiments. Further information is provided in the Supplementary material and methods.

In vivo studies

Male athymic nude mice were obtained from Harlan Laboratories (Houston, TX USA) at 5 weeks of age and kept under controlled conditions (12 h light and dark cycles). The animal protocol was approved by the Institutional Animal Care and Use Committee of the Baylor Research Institute. We generated xenograft tumors using SW480 cell line stably over-expressing SNORA42 or its controls. These cancer cells were suspended in PBS and Matrigel (Corning; 1:1 ratio) and 3×10^6 cells were subcutaneously injected into the abdominal flanks of each mice. Matrigel was used to improve the attachment and differentiation of both SNORA42 stably over-expressed cells and control cells in athymic nude mice. Nine mice were used in each group, and subcutaneous tumors were monitored for 28 days following injection. Further information is provided in Supplementary material and methods.

Statistical Analysis

Results were expressed as means ± S.E, and all statistical analysis was performed using Medcalc version 12.3.0 (Broekstraat 52, 9030; Mariakerke, Belgium). Further information is provided in the Supplementary material and methods.

RESULTS

Overexpression of snoRNAs in colorectal cancer during the screening phase

In the screening step of this study, expression levels of four snoRNAs (SNORD76, SNORD78, ACA11, and SNORA42) were examined in a subset of 16 CRCs and paired adjacent normal mucosa by quantitative real-time PCR. The expression levels of all for snoRNAs were significantly higher in CRC tissues compared to matched normal mucosa tissue specimens (SNORD76, $p<0.01$; SNORD78, $p<0.01$; ACA11, $p<0.01$; SNORA42, $p<0.01$; Figure 1a).

High SNORA42 expression was associated with distant metastasis and poor outcome in colorectal cancer patients during the clinical validation phase

Next, we analyzed the expression patterns for all four snoRNAs with various clinicopathological factors to determine whether their expression status has any prognostic significance in CRC patients by analyzing an independent, large cohort of CRC patients (Supplementary Table 1). In line with our screening phase results, the expression levels of all snoRNAs in CRC tissues were significantly up-regulated in tumor vs. normal mucosa tissues in the clinical evaluation cohort (SNORD76, $p<0.001$; SNORD78, $p<0.001$; ACA11, $p<0.001$; SNORA42, $p<0.01$; Figure 1b). Receiver operating characteristic (ROC) curves were used to evaluate the sensitivity and specificity of each snoRNA expression in distinguishing CRC from normal tissues. Notably, expression of all snoRNAs displayed considerable predictive significance, with an area under curve (AUC) values of 0.79 (95% CI: 0.73–0.82), 0.78 (95% CI: 0.72–0.84), 0.88 (95% CI: 0.83–0.92), 0.75 (95% CI: 0.69–0.81), respectively (Supplementary Figure 1).

Further, to perform the time-to-event analysis in order to evaluate the prognostic impact of these snoRNAs, median value of each snoRNA expression in all patients and curatively resected patients with stage I/II/III disease were used as cut-off thresholds for analyzing prognostic impact of overall survival (OS) and disease free survival (DFS) respectively. CRC patients with expression values higher than the median for each snoRNA were assigned to a high-expression group, and the others to a low-expression group. Although expression status of three snoRNAs (SNORD76, SNORD78, and ACA11) did not significantly correlate with survival or any other clinicopathological factors, high SNORA42 expression associated with venous invasion, lymphatic invasion, distant metastasis, UICC classification (Table 1, Supplementary Table 2) and poor prognosis compared to CRCs in the low-expression group in terms of OS and DFS ($p=0.018$, 0.029, respectively, log rank test, Figure 2a and b). Multivariate Cox's regression analysis showed high SNORA42 expression was an independent prognostic factor for OS (HR:2.11, 95%CI:1.12–3.98, $p=0.021$) and DFS (HR: 3.17, 95%CI:1.32–7.65, $p=0.011$) in CRC patients (Table 2a, 2b). Furthermore, multivariate logistic regression analysis revealed that high expression of SNORA42 was also an independent predictor of distant metastasis in CRC patients (OR:2.66, 95%CI:1.14–6.21, $p=0.023$, Table 2c).

Prognostic impact of SNORA42 expression status in colorectal cancer patients during the performance evaluation phase

To further confirm the prognostic impact of SNORA42 expression in CRC patients, we validated our results in an additional independent CRC cohort. Intriguingly, consistent with the survival outcomes in the clinical validation cohort, high SNORA42 expression was associated with poor prognosis with regards to OS for performance evaluation cohort ($p=0.026$, log rank test, Figure 2c) and total cohort ($p=0.002$, log rank test, Figure 2d). Furthermore, multivariate Cox's regression analysis revealed that high SNORA42 expression was an independent predictor for poor prognosis in the performance evaluation cohort, as well as total cohort (HR:3.00, 95%CI:1.10–8.19, $p=0.033$, HR:2.5, 95%CI:1.51–4.14, $p=0.0004$, respectively, Table 3a and b).

SNORA42 expression status identified high-risk stage II CRC patients

Next, to determine the clinical significance of SNORA42 expression as a predictive biomarker of recurrence and prognosis in stage II CRC patients, we evaluated survival outcomes in stage II patients subdivided on the basis of tissue SNORA42 expression (shown in Supplementary Figure 2a, b, and c). Elevated SNORA42 expression was associated with poor OS and DFS in patients belonging to the clinical validation cohort (p=0.048 and 0.049, respectively, log-rank test). Furthermore, high expression status of SNORA42 was significantly associated with poor prognosis in patients with stage II disease in the total cohort (p=0.039, log rank test). Collectively, our data suggests that SNORA42 expression could identify high-risk patients with stage II CRC.

SNORA42 were highly expressed in cancer cells compared with cancer stroma or corresponding normal mucosa

To further confirm the pathological expression pattern of SNORA42 in clinical specimens, *in situ* hybridization (ISH) staining was performed on 10 primary CRC tissues and corresponding adjacent normal mucosa from the clinical validation cohort. The ISH experiments revealed nuclear staining for SNORA42 in CRC cells, an observation that is consistent with previous reports in non-small cell lung cancer.[21] Furthermore, SNORA42 expression was up-regulated in the primary CRC cells compared with the matched normal mucosa, confirming our qRT-PCR results for its expression in primary CRC and adjacent normal mucosa tissues (Supplementary Figure 3). Based on these results, SNORA42 was overexpressed in CRC cells compared with normal mucosa, and its expression significantly correlated with disease progression in CRC patients. Therefore, we decided to focus the rest of the study on SNORA42 for further assessment of its biological function in colorectal neoplasia.

Overexpression of SNORA42 results in increased cell proliferation, tumorigenicity, migration, invasion and anoikis resistance in colon cancer cells

To investigate whether SNORA42 alters the biological characteristics of colon cancer cells, we first assessed expression levels of SNORA42 in a panel of colon cancer cell lines (Caco2, HCT116, HT29, LoVo, SW480 and SW620) by real-time PCR. Interestingly, the expression of SNORA42 was upregulated in metastatic cell line (SW620) when compared to primary CRC cell line (SW480) derived from the same patient. We selected Caco2 and SW480 cell lines for overexpression studies, since both cell lines showed lowest SNORA42 expression (Figure 3a). The pCDH-SNORA42 and pCDH-controls were transfected into these cell lines, which facilitated significant over-expression of SNORA42 in these cells (Figure 3b).

In order to determine whether ectopic expression of SNORA42 resulted in enhanced cell proliferation in human cancer cell lines, we analyzed rate of cell proliferation by MTT assays using transfected cell lines. Cell proliferation was significantly increased in both SNORA42 overexpressing cell lines, compared with control cell lines (Figure 3c). Next, to examine the colony-forming ability of single cells overexpressing SNORA42, we performed colony formation assays. Both Caco2 and SW480 cells expressing pCDH-SNORA42 demonstrated significantly higher number of colonies compared to pCDH-control cell lines (Figure 3d).

To determine whether ectopic expression of SNORA42 altered cell migration and invasion, we performed *in vitro* migration and invasion assays. As shown in Figures 3e, SNORA42 overexpressing CRC cell lines showed significantly enhanced invasive and migratory potential compared to pCDH-control cells.

Anoikis is a unique form of apoptosis that is induced by detachment of cells from the extracellular matrix.[35, 36] Resistance to anoikis is recognized as one of the oncogenic hallmarks contributing to cancer metastasis.[37, 38, 39] Considering that our clinical data revealed SNORA42 overexpression as an independent risk factor for distant metastasis, we hypothesized that SNORA42 enhances resistance to anoikis in CRC cells. To further confirm whether ectopic expression of SNORA42 increases anoikis resistance, anchorage independent cell viability was assessed after cells were incubated in an ultra-low attachment plate. After induction of anoikis, SNORA42 overexpressing Caco2 and SW480 cells exhibited an increase in the number of viable cells compared with the control cells (Figure 3f).

Inhibition of SNORA42 suppress cell proliferation, tumorigenicity, invasion and anoikis resistance in colon cancer cell

To confirm the oncogenic function of SNORA42, we next performed SNORA42 gene-silencing using CRISPR/cas9 system. We used lentiviral vectors to infect HCT116 cells with two different sequences of SNORA42 sgRNA CRISPR/Cas9 and downregulated SNORA42 expression (Supplementary Figure 4a). Both cell proliferation and colony formation capacity were significantly suppressed in both SNORA42 knock-down cell lines compared with the controls (Supplementary Figure 4b and 4c). Next we assessed whether inhibition of SNORA42 suppresses invasion capacity and anoikis resistance in CRC cells. Invasion assay showed that knock-down of SNORA42 inhibited the invasive ability in CRC cell, while anoikis assay showed suppression of SNORA42 induced anoikis in both SNORA42 knock-down cell lines. Collectively, these results indicate that SNORA42 is involved in CRC pathogenesis by promoting cell growth, colonogenic survival and enhancing invasion, migration, and anoikis resistance.

High SNORA42 expression results in enhanced colorectal cancer growth in an animal model

To assess whether ectopic expression of SNORA42 promotes tumorigenicity *in vivo*, we subcutaneously injected SW480 cells stably over-expressing SNORA42 or control (3×10^6 cells per mouse), into nude mice. During the initial 13 days post-injection, no significant difference in tumor size was observed between two groups. However, mice injected with SNORA42-expressing cells appear to accelerate tumor growth around 16 days post-injection compared to the control group (Figure 3g). Tumor size and weight were significantly higher for SNORA42-expressing cells than those of control animals (Figure 3h, Supplementary Table 3) at 28 days after injection, and over-expression of SNORA42 was maintained in the tumor tissues compared with control cells at the end of experiments (Figure 3i). Collectively, these results clearly demonstrate that high SNORA42 expression enhanced tumor growth *in vivo*, which is consistent with our *in vitro* and clinical findings.

DISCUSSION

Growing evidence supports the role of ncRNAs in regulation of oncogenesis in different cancers.[40, 41, 42] In addition to their significant role in the pathogenesis of CRC, more recent discovery of miRNAs as potential robust biomarkers have paved the way for exploitation of other types of small ncRNAs — a concept that has revolutionized the field of ncRNA biomarkers, for the early detection, predicting recurrence and prognostication in CRC. In contrast to relatively well-characterized role of miRNAs, little is known about the biological significance of snoRNAs. Furthermore, the association between snoRNA expression and their clinical impact as biomarkers for CRC has not been undertaken.

In this study, we systematically investigated potential role of four oncogenic snoRNAs, and provide first evidence of snoRNA dysregulation in CRC. We made several important discoveries during the course of this investigation. First, expression of all four snoRNAs was significantly up-regulated in colorectal cancers compared to normal mucosa. ROC analyses revealed that expression of these snoRNAs considerably discriminated cancer tissues from normal mucosa. Second, we identified that high expression of SNORA42, in particular, correlated significantly with poor OS and DFS in CRC patients. In addition, SNORA42 expression in primary tissues emerged as an independent prognostic risk factor for distant metastasis in CRC patients. Third, we validated prognostic impact of SNORA42 expression in another independent cohort of CRC patients. Fourth, we showed that high expression of SNORA42 could identify patients that are at high-risk for tumor recurrence, particularly in stage II CRC patients. Finally, altered expression of SNORA42 changed not only invasive and migratory capacity of CRC cells but also cell proliferation, tumorigenicity, and anoikis resistance, as evidenced from a series of *in vitro* and *in vivo* experiments.

One of the major findings of our study is that expression status of all four snoRNAs was significantly higher in cancerous tissues compared with normal mucosa from CRC specimens analyzed during the screening and validation phases. Since there were no studies on the role of snoRNAs in colorectal cancer, we purposely focused our study on deciphering the role of these previously reported snoRNAs to determine whether they also play a role in CRC pathogenesis. Nonetheless, our ROC analysis for the expression levels of all 4 snoRNAs demonstrated that these were consistently upregulated in cancer vs. normal tissues, suggesting their potential use as tissue-based diagnostic biomarkers in CRC patients.

Another major finding of our study is the clinical impact of snoRNA expression levels in CRC patients. Our results in the clinical validation cohort showed that elevated expression of SNORA42 was a potential predictor for recurrence and poor prognosis in CRC patients. Interestingly, logistic regression analysis indicated that high SNORA42 expression in cancer tissues was an independent risk factor for predicting distant metastasis in CRC patients. These results were successfully validated in CRC specimens from an independent cohort, suggesting that SNORA42 expression could be used as a prognostic biomarker as it is intimately involved in disease progression in CRC. In line with findings, our results illustrated that high expression of SNORA42 correlated significantly with poor prognosis of DFS and OS in stage II CRC patients. Currently, one of the most clinically relevant need is lack of availability of adequate predictive biomarkers that can identify patients that are at

high risk for developing tumor recurrence, especially in CRC patients with stage II disease. Even though the majority of these patients are cured by surgery alone, a significant proportion of stage II CRC patients develop relapse and subsequently die from disease progression. Furthermore, the effect of treating all patients with stage II CRC with adjuvant chemotherapy remains controversial.[43, 44, 45] Therefore, identification of such high risk CRC patients using molecular biomarkers such as SNORA42 expression will allow use of adjuvant chemotherapy after surgery only in a select subgroup of high risk patients to improve their prognosis.

To further understand the biological function of SNORA42 in CRC progression, we investigated the malignant features of SNORA42 in CRC cell lines, Caco2 and SW480, using pCDH-SNORA42 infection. Recent studies highlighted the oncogenic role of SNORA42 in lung tumorigenesis.[21, 46] Consistent with this previous data,[21] we demonstrated that ectopic expression of SNORA42 enhanced cell proliferation and tumorigenicity in cultured cells and in an animal model of CRC. Our data showed that ectopic expression of SNORA42 exerted distinct oncogenic functions such as increased invasion, migration, and anoikis resistance in colon cancer cell lines, while inhibition of SNORA42 by CRISPR-Cas9 further supported this paradigm. According to the current knowledge, formation of metastasis occurs in multiple steps. In the metastatic process, primary tumor invades into the serosa from the intraluminal epithelial side, followed by cancer cells detachment from the primary tumor and extravasation into the circulation. These cancer cells thereafter adhere to the microvascular endothelium, and proliferate to distant organs.[47, 48]

In summary, this study provides novel evidences for the clinical and biological significance of snoRNAs in CRC. Our results demonstrate the clinical usefulness of SNORA42 as potential of diagnostic and predictive biomarker for risk stratification in CRC patients. Furthermore, our results from a series of in vitro and in vivo experiments supported a mechanistic role of SNORA42 in the CRC tumorigenesis. In this study was successfully revealed the role of SNORA42 as a clinically promising biomarker, and further studies including a broader, unbiased genome wide analysis in future might lead to the identification of other, even more significant snoRNAs in CRC. We conclude that quantification of SNORA42 expression in primary tumors may serve as a clinically useful diagnostic and prognostic biomarker, and potentially as a therapeutic target in CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62:10–29. [PubMed: 22237781]
2. Figueredo, A.; Coombes, ME.; Mukherjee, S. The Cochrane database of systematic reviews. 2008. Adjuvant therapy for completely resected stage II colon cancer; p. CD005390
3. Andre N, Schmiegel W. Chemoradiotherapy for colorectal cancer. Gut. 2005; 54:1194–202. [PubMed: 16009693]
4. Kopetz S, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, et al. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. J Clin Oncol. 2009; 27:3677–83. [PubMed: 19470929]
5. Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. Ann Oncol. 2012; 23:2479–516. [PubMed: 23012255]
6. Mattick JS. The genetic signatures of noncoding RNAs. PLoS Genet. 2009; 5:e1000459. [PubMed: 19390609]
7. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:15524–9. [PubMed: 12434020]
8. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci U S A. 2004; 101:11755–60. [PubMed: 15284443]
9. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005; 102:13944–9. [PubMed: 16166262]
10. Michael MZ, SMOC, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003; 1:882–91. [PubMed: 14573789]
11. Lafontaine DL, Tollervey D. Birth of the snoRNPs: the evolution of the modification-guide snoRNAs. Trends Biochem Sci. 1998; 23:383–8. [PubMed: 9810226]
12. Weinstein LB, Steitz JA. Guided tours: from precursor snoRNA to functional snoRNP. Curr Opin Cell Biol. 1999; 11:378–84. [PubMed: 10395551]
13. Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene. 2009; 28:195–208. [PubMed: 18836484]
14. Mourtada-Maarabouni M, Hedge VL, Kirkham L, Farzaneh F, Williams GT. Growth arrest in human T-cells is controlled by the non-coding RNA growth-arrest-specific transcript 5 (GAS5). J Cell Sci. 2008; 121:939–46. [PubMed: 18354083]
15. Williams GT, Hughes JP, Stoneman V, Anderson CL, McCarthy NJ, Mourtada-Maarabouni M, et al. Isolation of genes controlling apoptosis through their effects on cell survival. Gene Ther Mol Biol. 2006; 10:255–62. [PubMed: 17372619]
16. Dong XY, Guo P, Boyd J, Sun X, Li Q, Zhou W, et al. Implication of snoRNA U50 in human breast cancer. J Genet Genomics. 2009; 36:447–54. [PubMed: 19683667]
17. Dong XY, Rodriguez C, Guo P, Sun X, Talbot JT, Zhou W, et al. SnoRNA U50 is a candidate tumor-suppressor gene at 6q14. 3 with a mutation associated with clinically significant prostate cancer. Hum Mol Genet. 2008; 17:1031–42. [PubMed: 18202102]
18. Gee HE, Buffa FM, Camps C, Ramachandran A, Leek R, Taylor M, et al. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. Br J Cancer. 2011; 104:1168–77. [PubMed: 21407217]
19. Gao L, Ma J, Mannoor K, Guarnera MA, Shetty A, Zhan M, et al. Genome-wide small nucleolar RNA expression analysis of lung cancer by next-generation deep sequencing. Int J Cancer. 2014
20. Liao J, Yu L, Mei Y, Guarnera M, Shen J, Li R, et al. Small nucleolar RNA signatures as biomarkers for non-small-cell lung cancer. Mol Cancer. 2010; 9:198. [PubMed: 20663213]

21. Mei YP, Liao JP, Shen J, Yu L, Liu BL, Liu L, et al. Small nucleolar RNA 42 acts as an oncogene in lung tumorigenesis. Oncogene. 2012; 31:2794–804. [PubMed: 21986946]
22. Chu L, Su MY, Maggi LB Jr, Lu L, Mullins C, Crosby S, et al. Multiple myeloma-associated chromosomal translocation activates orphan snoRNA ACA11 to suppress oxidative stress. J Clin Invest. 2012; 122:2793–806. [PubMed: 22751105]
23. Yin D, He X, Zhang E, Kong R, De W, Zhang Z. Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. Medical oncology. 2014; 31:253. [PubMed: 25326054]
24. Sun M, Jin FY, Xia R, Kong R, Li JH, Xu TP, et al. Decreased expression of long noncoding RNA GAS5 indicates a poor prognosis and promotes cell proliferation in gastric cancer. BMC cancer. 2014; 14:319. [PubMed: 24884417]
25. Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. Journal of the National Cancer Institute. 2013; 105:849–59. [PubMed: 23704278]
26. Toiyama Y, Hur K, Tanaka K, Inoue Y, Kusunoki M, Boland CR, et al. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. Ann Surg. 2014; 259:735–43. [PubMed: 23982750]
27. Han TS, Hur K. MicroRNA-29c mediates initiation of gastric carcinogenesis by directly targeting ITGB1. 2014
28. Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, et al. MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. Gut. 2013; 62:1315–26. [PubMed: 22735571]
29. Takahashi M, Cuatrecasas M, Balaguer F, Hur K, Toiyama Y, Castells A, et al. The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. PloS one. 2012; 7:e46684. [PubMed: 23056401]
30. Link A, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2010; 19:1766–74.
31. Chang KH, Mestdagh P, Vandesompele J, Kerin MJ, Miller N. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC cancer. 2010; 10:173. [PubMed: 20429937]
32. Hur K, Toiyama Y, Schetter AJ, Okugawa Y, Harris CC, Boland CR, et al. Identification of a Metastasis-Specific MicroRNA Signature in Human Colorectal Cancer. Journal of the National Cancer Institute. 2015:107.
33. Okugawa Y, Toiyama Y, Hur K, Toden S, Saigusa S, Tanaka K, et al. Metastasis-associated long non-coding RNA drives gastric cancer development and promotes peritoneal metastasis. Carcinogenesis. 2014
34. Okugawa Y, Tanaka K, Inoue Y, Kawamura M, Kawamoto A, Hiro J, et al. Brain-derived neurotrophic factor/tropomyosin-related kinase B pathway in gastric cancer. Br J Cancer. 2013; 108:121–30. [PubMed: 23175149]
35. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol. 1994; 124:619–26. [PubMed: 8106557]
36. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol. 2001; 13:555–62. [PubMed: 11544023]
37. Guadamillas MC, Cerezo A, Del Pozo MA. Overcoming anoikis--pathways to anchorage-independent growth in cancer. J Cell Sci. 2011; 124:3189–97. [PubMed: 21940791]
38. Frisch SM, Schaller M, Cieply B. Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. J Cell Sci. 2013; 126:21–9. [PubMed: 23516327]
39. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta. 2013; 1833:3481–98. [PubMed: 23830918]
40. Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene. 2012; 31:4577–87. [PubMed: 22266873]

41. Iorio MV, Croce CM. microRNA involvement in human cancer. Carcinogenesis. 2012; 33:1126–33. [PubMed: 22491715]
42. Berindan-Neagoe I, del Monroig PC, Pasculli B, Calin GA. MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin. 2014; 64:311–36. [PubMed: 25104502]
43. Benson AB 3rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2004; 22:3408–19. [PubMed: 15199089]
44. Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet. 2007; 370:2020–9. [PubMed: 18083404]
45. Baddi L, Benson A 3rd. Adjuvant therapy in stage II colon cancer: current approaches. Oncologist. 2005; 10:325–31. [PubMed: 15851790]
46. Mannoor K, Shen J, Liao J, Liu Z, Jiang F. Small nucleolar RNA signatures of lung tumor-initiating cells. Mol Cancer. 2014; 13:104. [PubMed: 24886050]
47. Fidler IJ. Critical determinants of metastasis. Semin Cancer Biol. 2002; 12:89–96. [PubMed: 12027580]
48. Chan DA, Giaccia AJ. Hypoxia, gene expression, and metastasis. Cancer Metastasis Rev. 2007; 26:333–9. [PubMed: 17458506]

SIGNIFICANCE OF THIS STUDY

What is already known about this subject?

- Colorectal cancer (CRC) remains a leading cause of cancer mortality, and is the second leading cause of cancer-related deaths in the United States.
- Small nucleolar RNA (snoRNAs) are one of the largest groups of single-stranded small ncRNAs, and emerging evidence highlights their role in the regulation of cell fate and oncogenesis in various cancers. In addition, recent studies suggest that these RNAs may have prognostic impact and biomarker potential in solid cancers.

What are the new findings?

- This study evaluated snoRNA expression using colorectal tissues from multiple independent tissue cohorts, in a series of experimental assays and approaches.
- This study successfully demonstrated, for the first time, the clinical impact of snoRNA expression as a predictive biomarker of recurrence and poor prognosis in CRC patients.
- We clearly demonstrate that high expression of SNORA42 significantly correlated with distant metastasis in CRC tissues, and SNORA42 expression status is an independent prognostic risk factor for predicting distant metastasis in CRC patients.
- We utilized a series of cell culture experiments to uncover the oncogenic role of SNORA42 in CRC, followed by validation of these findings in an *in vivo* model.

How might it impact on clinical practice in the foreseeable future?

- Our study highlights the clinical significance of snoRNA expression in CRC as a potential diagnostic biomarker, and as a possible predictive biomarker for the identification of high-risk CRC patients, especially stage II patients.
- Moreover, we provide a direct experimental evidence for the functional role of SNORA42 in disease progression of colorectal neoplasia using a series of *in vitro* and *in vivo* experiments. Taken together, these results underscore the potential of SNORA42 expression as a useful molecular biomarker for selecting high-risk patients that may receive more personalized treatments in future.
- The investigation of snoRNAs as potential biomarkers and drivers of disease progression represents an unexplored area of cancer biology and has enormous potential clinical significance.

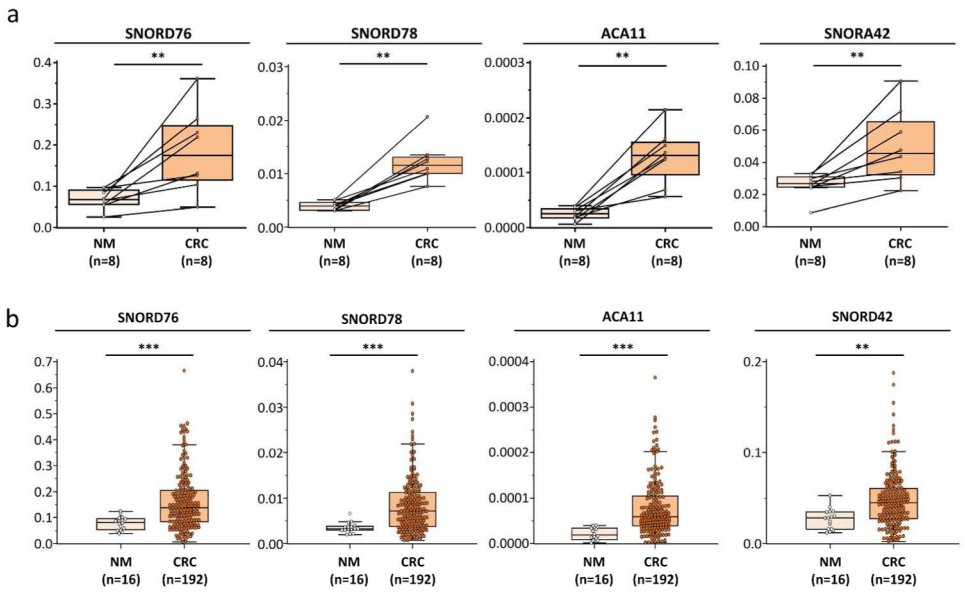


Figure 1. Expression status of oncogenic-candidate snoRNAs in CRC tissues and adjacent normal mucosa in the screening and clinical validation cohorts

(a) Box plots show expression levels of four snoRNAs (SNORD76, SNORD78, ACA11, and SNORA42) in primary tumor tissues (CRC) and corresponding matched normal mucosa (NM) from eight patients enrolled in the screening cohort. The expression of all snoRNAs was significantly higher in CRC tissues than in adjacent normal mucosa. ($p<0.01$, Wilcoxon rank correlation test). **(b)** Box plots show expression levels of four snoRNAs (SNORD76, SNORD78, ACA11, and SNORA42) in CRC (n=192) and NM (n=16) tissues in the clinical validation cohort. Boxes represent interquartile ranges, and the horizontal line across each box indicates median value. The y-axis represents relative expression of four snoRNA, and data were normalized to miR-16 expression. Statistical analysis was performed using Mann-Whitney U tests. All statistical tests were two-sided. **p <0.01; ***p < 0.001

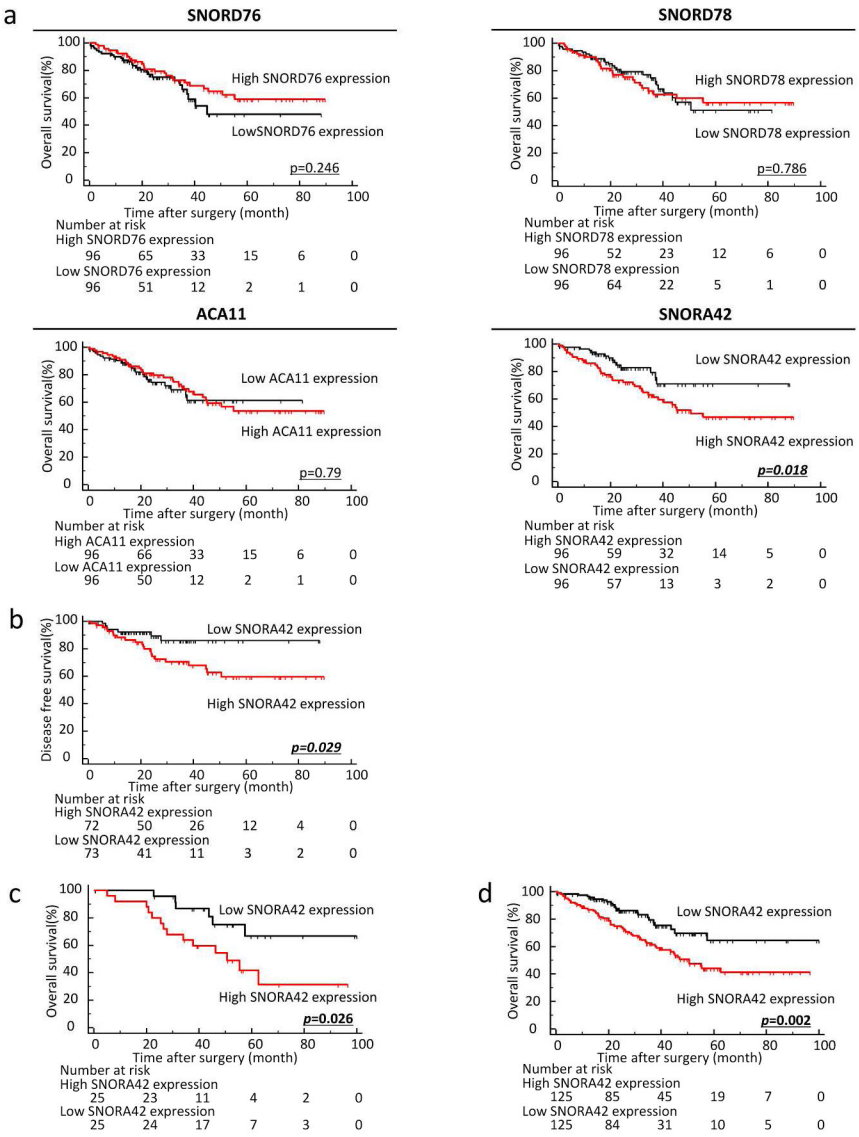
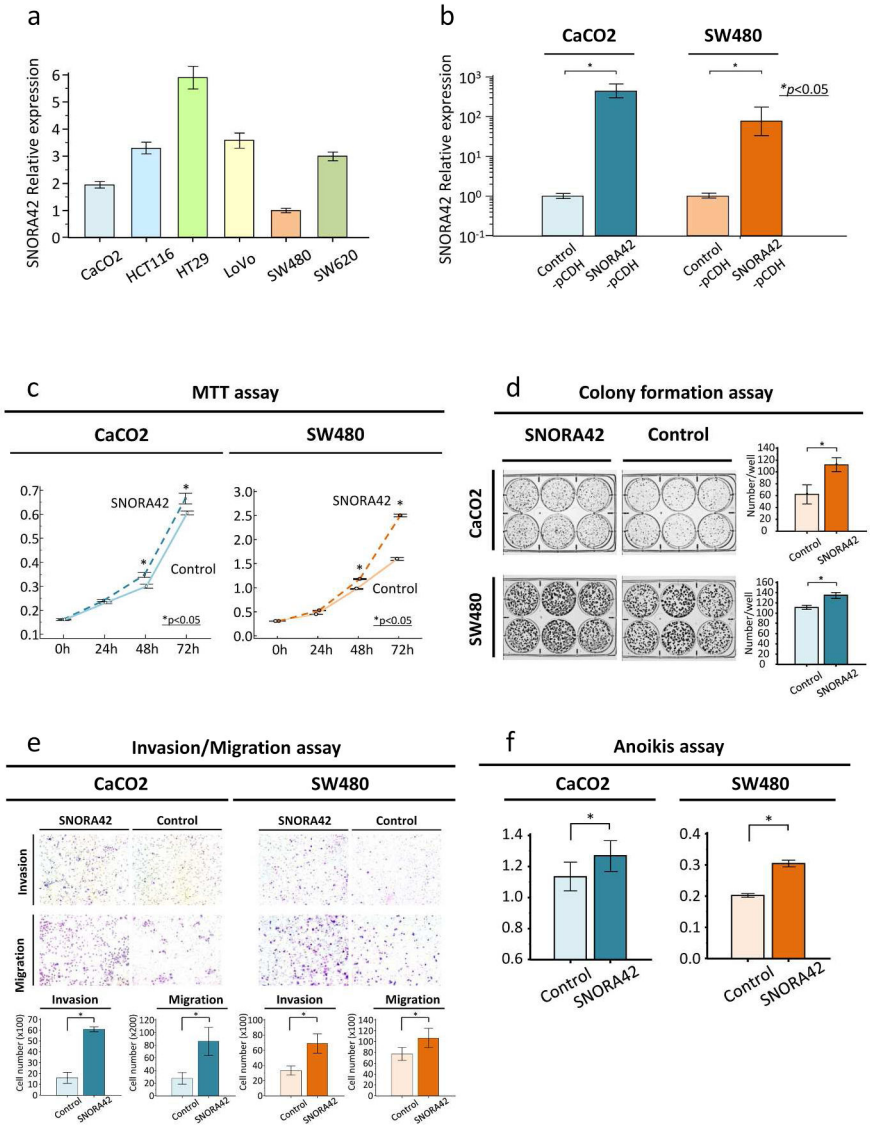


Figure 2. Prognostic impact of snoRNA expression status in CRC patients

(a) Kaplan–Meier survival curves for overall survival (OS) in CRC patients based on the expression of four snoRNAs in the clinical validation cohort (n=192). The OS rate in CRC patients (n=96) with high-SNORA42 expression in tumor tissue was significantly lower than that for those with low-SNORA42 expression (cut-off threshold was median value in this cohort; p=0.018; log-rank test). **(b)** Disease-free survival analyses based on SNORA42 expression status in CRC tissue cohort (cut-off threshold was median value in stage I-III CRC patients; p=0.029; log-rank test). **(c, d)** Overall survival analysis based on SNORA42 expression status in performance evaluation cohort **(c)** (n=50) and total cohort **(d)** (n=250) (cut-off threshold was median value in each cohort; p=0.026, 0.002, respectively; log-rank test). All statistical tests were two-sided.



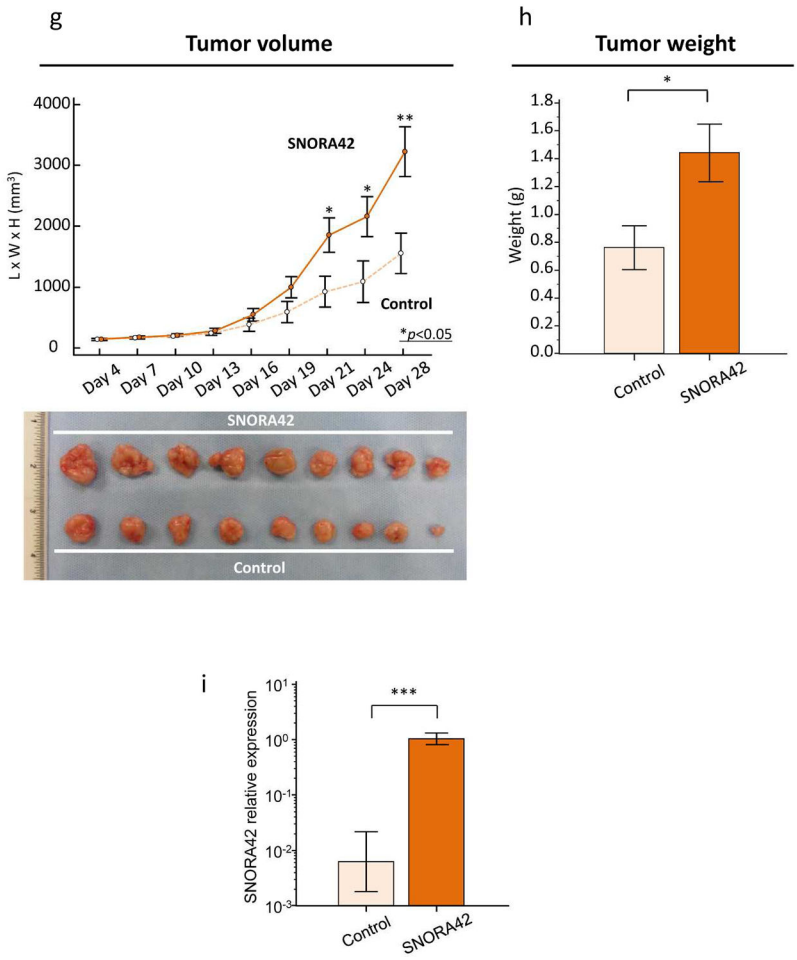


Figure 3. Functional analysis of SNORA42 in cultured cells and an animal model of colorectal cancer

(a) SNORA42 expression status in colon cancer cell lines. **(b)** Ectopic SNORA42 expression in the colon cancer cell lines Caco2 and SW480. **(c)** Effect of SNORA42 over-expression on Caco2 and SW480 cell proliferation as assessed by MTT assay. **(d)** Colony formation assay. The number of colonies with >50 cells were counted after 10 day incubation. **(e)** Cell invasion and migration assay using Matrigel-coated transwell membranes (upper panel illustrates representative image for invasion, bottom panel depicts an image from migrated cells; average counts from five random microscopic fields)). **(f)** Anoikis assay to investigate anoikis resistance in Caco2 and SW480 cells with or without SNORA42 overexpression. After anoikis induction for 48 h, the number of viable floating cancer cells in low attachment plates was calculated by MTT assay. **(g, h)** Effect of SNORA42 ectopic expression in SW480 cells on the xenograft model was assessed by evaluating tumor volume **(g)** and weight **(h)** compared to controls. **(i)** SNORA42 expression levels in xenograft tumors, which were treated with pCDH-SNORA42 or pCDH-control. Statistical analysis was performed using one way ANOVA and Mann-Whitney U tests appropriately. All statistical tests were two-sided. *P < 0.05, **P <0.01, ***P < 0.001.

Table 1

Clinicopathological variables and SNORA42 expression in the clinical validation cohort

Variable		n	SNORA42 expression		*p*
			high (n=96)	low (n=96)	
Gender	Male	109	56	53	0.771#
	Female	83	40	43	
Age (y)	<68 (median)	99	49	50	1.000#
	≧68	93	47	46	
Tumor location	Colon	120	59	61	0.882#
	Rectum	72	37	35	
Histological type	Differentiated	175	89	86	0.611#
	Undifferentiated	17	7	10	
Pathological T category	T1/T2	56	22	34	0.081#
	T3/T4	136	74	62	
Venous invasion	+	83	49	34	**0.041***#
	−	109	47	62	
Lymphatic invasion	+	143	78	65	**0.047***#
	−	49	18	31	
Lymph node metastasis	+	84	45	39	0.467 #
	−	108	51	57	
Distant metastasis	+	45	30	15	**0.017***#
	−	147	66	81	
UICC stage classification	Stage I	44	17	27	**0.028***#
	Stage II	60	31	29	
	Stage III	43	18	25	
	Stage IV	45	30	15	

#Pearson's chi-square test

**p*<0.05

Table 2a

Multivariate analysis for predictors of Overall Survival in the clinical validation cohort

Variables	Univariate			Multivariate		
	HR	**95%CI**	***p value***	**HR**	**95%CI**	***p value***
Gender (Male)	1.04	0.60–1.80	0.89	1.09	0.60–1.98	0.78
Age (≧68 (median))[#]	1.06	0.62–1.82	0.83	1.05	0.60–1.84	0.86
Tumor Location (Rectum)	1.29	0.76–2.22	0.35	1.15	0.66–2.03	0.62
Histological type (Undifferentiated)	3.1	1.56–6.17	**0.0013** *	2.79	1.28–6.10	**0.011** *
T classification (pT3/4)	7.93	2.49–25.3	**0.0005** *	3.1	0.90–10.6	0.073
Node involvement (present)	9.74	4.59–20.7	**<0.0001** *	0.8	0.19–3.34	0.76
TNM stage (Stage 3/4)	12.8	5.46–29.9	**<0.0001** *	10.9	2.25–53.8	**0.003** *
High SNORA42 expression [†]	2.04	1.12–3.73	**0.02** *	2.11	1.12–3.98	**0.021** *

[#]The median age at surgery is 68 years in the clinical evaluation cohort.

[†]Cut-off threshold of SNORA42 expression is median value in all patients in this cohort.

HR: hazard ratio

*$p<0.05$

Table 2b

Multivariate analysis for predictors of Disease Free Survival in the clinical validation cohort

Variables	Univariate			Multivariate		
	HR	**95%CI**	***p value***	**HR**	**95%CI**	***p value***
Gender (Male)	0.75	0.36–1.56	0.45	0.56	0.25–1.28	0.17
Age (≧68 (median))	0.98	0.47–2.03	0.96	0.8	0.38–1.68	0.55
Tumor Location (Rectum)	1.37	0.66–2.83	0.4	1.46	0.65–3.27	0.36
Histological type (Undifferentiated)	1.68	0.51–5.54	0.39	1.1	0.29–4.20	0.89
T classification (pT3/4)	5.15	1.57–16.9	**0.007** *	3.14	0.90–10.9	0.074
Node involvement (present)	7.06	3.20–15.6	**<0.0001** *	6.09	2.64–14.1	**<0.0001** *
High SNORA42 expression ††	2.52	1.07–5.91	**0.035** *	3.17	1.32–7.65	**0.011** *

††Cut-off threshold of SNORA42 expression is median value in curatively resected patients (stage I/II/III) in this cohort.

HR: hazard ratio

*$p<0.05$

Table 2c

Multivariate analysis for distant metastasis in the clinical validation cohort

Vaiables	univariate			Mutivariate		
	Odds ratio	**95%CI**	***p* value**	**OR**	**95%CI**	***p* value**
Gender (Male)	1.06	0.54–2.07	0.88	1.07	0.45–2.52	0.88
Age (≧68 (median))	0.5	0.25–1.00	0.05	0.47	0.20–1.09	0.08
Tumor Location (Rectum)	1.15	0.58–2.28	0.69	0.98	0.41–2.32	0.96
Histological type (Undifferentiated)	3.32	1.20–9.19	**0.021** *	2.82	0.83–9.64	0.09
T classification (pT3/4)	5.61	1.90–16.5	**0.002** *	1.68	0.47–6.02	0.42
Node involvement (present)	18.7	6.93–50.6	**<0.0001** *	16.1	5.57–46.5	**<0.0001** *
High SNORA42 expression †	2.45	1.22–4.94	**0.012** *	2.66	1.14–6.21	**0.023** *

† Cut-off threshold of SNORA42 expression is median value in all patients in this cohort.

OR: odds ratio

* $p<0.05$

Table 3a

Multivariate analysis for predictors of Overall Survival in the performance evaluation cohort

Variables	Univariate			Multivariate		
	HR	**95%CI**	***p value***	**HR**	**95%CI**	***p value***
Gender (Male)	0.92	0.38–2.20	0.84	0.91	0.36–2.29	0.84
Age (≧64 (median) *)	0.56	0.23–1.36	0.2	0.69	0.28–1.73	0.43
Tumor Location (Rectum)	0.76	0.31–1.84	0.54	0.84	0.32–2.22	0.73
Histological type (Undifferentiated)	-	-	0.94	-	-	-
TNM stage (Stage 3/4)	1.29	0.39–4.38	0.69	1.88	0.51–6.89	0.34
High SNORA42 expression	2.84	1.09–7.37	**0.033** *	3.0	1.10–8.19	**0.033** *

* The median age at surgery are 64 years in the performance evaluation cohort.

HR: hazard ratio

* $p<0.05$

Table 3b

Multivariate analysis for predictors of Overall Survival in the total cohort

Variables	Univariate			Multivariate		
	HR	**95%CI**	***p value***	**HR**	**95%CI**	***p value***
Gender (Male)	0.91	0.58–1.43	0.67	1.09	0.67–1.77	0.73
Age (≧67 (median) *)	0.81	0.51–1.27	0.36	0.88	0.55–1.40	0.58
Tumor Location (Rectum)	1.08	0.69–1.69	0.74	1.04	0.65–1.68	0.86
Histological type (Undifferentiated)	3.2	1.73–5.93	**0.0002** *	4.37	2.28–8.35	**<0.0001** *
TNM stage (Stage 3/4)	6.51	3.36–12.6	**<0.0001** *	7.33	3.72–14.4	**<0.0001** *
High SNORA42 expression	2.14	1.31–3.51	**0.0026** *	2.5	1.51–4.14	**0.0004** *

* The median age at surgery are 67 years in the total cohort.

HR: hazard ratio

* $p<0.05$