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Long non-coding RNA HANR as a biomarker for the diagnosis and prognosis of colorectal cancer

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Abstract

Previous work suggests that the long noncoding RNA HCC associated long non-coding RNA (HANR) is associated with hepatocellular carcinoma (HCC) progression, but its significance in the context of colorectal cancer (CRC) remains to be determined. Therefore, in this study we assessed the prognostic and diagnostic value of HANR in patients suffering from CRC.

The HANR expression in 165 pairs of CRC cancer and adjacent non-cancerous prostate tissues was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Student t test was conducted for intergroup comparison. Pearson correlation test was used for correlation analysis. Survival curves were carried out by the Kaplan-Meier method and evaluated using the log-rank test. Multivariable Cox proportional hazard risk regression model was performed to screen the independent factor affected the prognosis of CRC patients.

In this study, levels of HANR were significantly higher in CRC tumor samples relative to adjacent normal tissue samples ($P < .001$). A ROC analysis suggested HANR expression could be reliably used to differentiate between normal and CRC tumor tissue. In addition, elevated HANR expression was positively correlated with more advanced and aggressive CRC features, such as a larger tumor size ($P = .003$), increased invasion depth ($P = .012$), and more advanced TNM stage ($P = .011$). Survival analyses revealed that elevated HANR expression was correlated with worse overall survival ($P = .002$) and disease-free survival ($P = .003$). A multivariate analysis further confirmed the relevance of HANR as an independent predictor of CRC patient outcomes.

In summary, these results indicate that the lncRNA HANR is a promising prognostic indicator in CRC patients.

Abbreviations: CA19-9 = carbohydrate antigen 19-9, CRC = colorectal cancer, DFS = disease-free survival, EMT = epithelial-mesenchymal transition, HCC = hepatocellular carcinoma, lnc RNA = long noncoding RNA, OS = overall survival, TNM = Tumor-Node-Metastasis.

Keywords: bio-marker, colorectal cancer, lnc-HANR, prognosis

1. Introduction

Colorectal cancer (CRC) remains the second deadliest cancer affecting males, resulting in greater than 600,000 deaths each year globally.[1,2] CRC arises over time with a successive series of histological and genetic alterations in the underlying tissue.[3] Those patients with stage I/II CRC have a generally good prognosis owing to recent advances in targeted, surgical, and chemotherapeutic treatments, with a 5-year survival rate of up to 80%.[4,5] Over half of CRC patients, however, exhibit distant metastases at diagnosis, and limited therapeutic options are available for the treatment of such advanced disease, with very complex therapy being essential.[6,7] As such it is important that new biomarkers of diagnostic or prognostic utility be identified in an effort to improve patient survival rates.

Long noncoding RNAs (lncRNAs) are RNA molecules that do not encode protein despite their active transcription.[8] There is clear evidence[9,10] that certain lncRNAs are able to act through a variety of mechanisms in order to influence diverse processes including transcription, mRNA stability, and epigenetic regulatory pathways. In addition, several studies[11,12] have found that certain lncRNAs are dysregulated in tumors, with those being overexpressed often helping to initiate or drive tumor development. As an example, UCC is a lncRNA found to be overexpressed in certain CRC samples, and to be capable of enhancing the metastatic and proliferative activity of CRC cells via sequestering miR-143, with elevated expression of this lncRNA being correlated with more advanced disease.[13] In contrast, Linc00675 has been shown to be downregulated in CRC patients, and in these cells it has been found to act to suppress cellular invasion and proliferation through regulation of Wnt/β-catenin signaling.[14] These results thus demonstrate that in CRC lncRNAs have potential and meaningful prognostic and diagnostic biomarkers.

HCC-associated lncRNA (HCC associated long non-coding RNA [HANR]; RPL13AP20)[15] has been shown to drive the enhanced proliferation, invasion, and epithelial-mesenchymal

transition (EMT) of certain cancer cells.[16,17] Whether HANR exhibits any clinical relevance in patients with CRC, however, is unclear. As such, this study sought to produce novel evidence examining the potential for HANR to be a diagnostic and/or prognostic biomarker in CRC patients.

2. Patients and methods

2.1. Patients and clinical specimens

A total of 165 pairs of CRC tumor tissue and adjacent healthy tissue were collected by Dalian Central Hospital. These patients (101 males, 64 female) were 38 to 76 years old (median: 53.9 years), and had been diagnosed with CRC based upon both clinical and histopathological assessments. Samples used in this study were from patients who had not undergone preoperative chemotherapy or radiotherapy. Samples were snap-frozen using liquid nitrogen prior to −80°C storage. Patient follow-up information and survival was determined based upon medical records and or direct contact with the patients or their families. Patient demographic and clinical characteristics are compiled in Table 1.

The approval of the present study protocol was obtained from the Ethics Committee of the Dalian Central Hospital (Affiliated of Dalian Medical University), and the written informed consent was provided from all patients.

2.2. qRT-PCR

RNA was extracted from samples using the miRNeasy Mini-kit (Qiagen, Xuhui, Shanghai, China), after which Reverse Transcriptase (Transgene, Xuhui, Shanghai, China) was used to prepare cDNA from all samples. A 7300-sequence detection system (Biosystems, CA) was used to conduct qRT-PCR reactions with SYBR Green Master Mix (Biosystems) using 35 cycles of 12 seconds at 95°C and 1 minute at 60°C. For normalization, GAPDH was used as an endogenous control gene. The comparative cycle threshold (CT) approach was used for determining relative HANR expression levels, with primers used shown in Table 2.

2.3. Statistical analysis

SPSS 17.0 (SPSS Inc, IL) was used for statistical testing. Student's t tests were used to compare data between groups, while the association between HANR expression and clinicopathological findings was assessed via chi-squared test. A receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic relevance of HANR expression in CRC patients, while Kaplan-Meier and multivariate analyses were used to gauge the prognostic value of HANR expression. $P < .05$ was the significance threshold.

3. Results

3.1. CRC tumors exhibit elevated HANR expression

To first assess how the lncRNA HANR might be linked to CRC progression, we explored its expression levels in tumor samples from those patients suffering from CRC. This analysis revealed that tumor samples exhibited significantly higher HANR expression relative to normal control samples ($P < .01$) (Fig. 1A). In addition, patients with more advanced CRC exhibited significantly higher HANR expression than those with

Clinical features	Total	Lnc-HANR expression High (N=83)	Low (N=82)	P vaule
Age (years)				0.281
<60	48	21	27	
≥60	117	62	55	
Gender				0.394
Male	92	49	43	
Female	73	34	39	
Tumor location				0.102
Rectum	74	32	42	
Colon	91	51	40	
Tumor size (cm)				0.003
<5	66	24	42	
≥5	99	59	40	
Differentiation grade				0.908
Well	55	27	28	
Moderate	88	44	44	
Poor	22	12	10	
TNM stage				0.011
I	33	18	15	
II	70	26	44	
III	62	39	23	
Depth of invasion				0.012
T1+T2	77	32	45	
T3	65	42	23	
T4	23	9	14	
Lymph node metastasis				0.220
No	103	48	55	
Yes	62	35	27	
Distant metastasis				0.486
No	127	62	65	
Yes	38	21	17	
Adjuvant chemotherapy				0.639
No	78	37	40	
Yes	87	45	42	
CA19-9, kU/L				0.192
<40	124	66	58	
≥40	41	17	24	

HANR = HCC associated long non-coding RNA
CA19-9 carbohydrate antigen 19-9; Pearson chi-square test was used for comparison between subgroups.

less advanced disease ($P < .01$, Fig. 1B). Together these findings suggested the possibility that the lncRNA HANR is expressed at high levels in CRC and may play a role in disease progression.

3.2. The diagnostic value of HANR expression in CRC patients
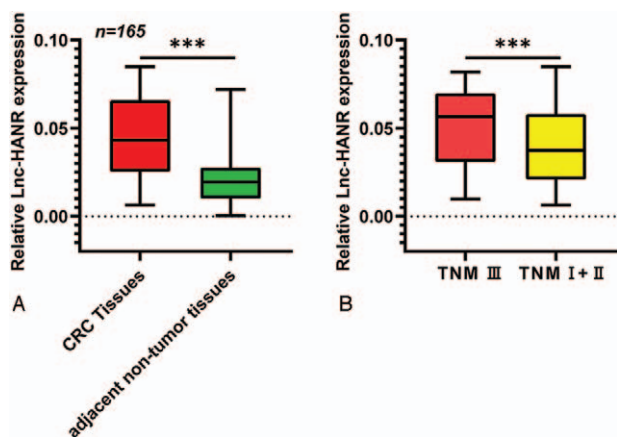
As HANR expression was apparently dysregulated in CRC patient samples, we next explored its potential utility as a

Primer sequence (5'- 3')	
Lnc-HANR (forward)	AAGTACCAGGCAGTGACAGC
Lnc-HANR (reverse)	TTCTCCACGTTCTTCTCGGC
GAPDH (forward)	AGAAGGCTGGGGCTCATTTG
GAPDH (reverse)	AGGGGCCATCCACAGTCTTC

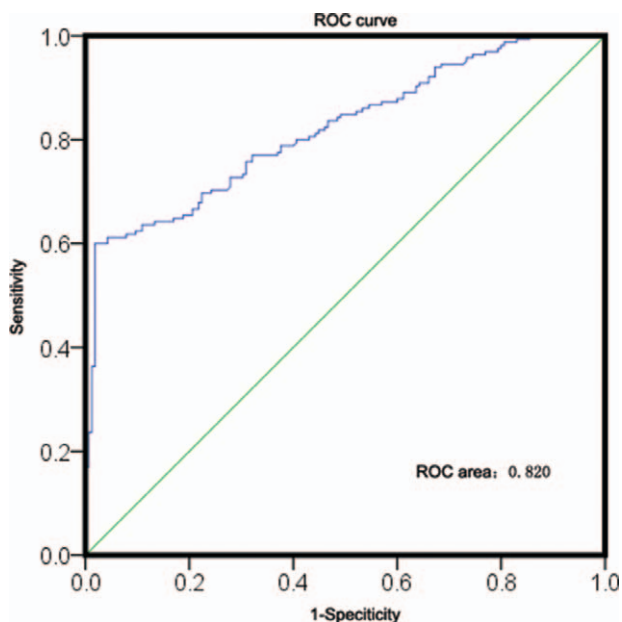HANR = HCC associated long non-coding RNA

Figure 1. CRC tumors exhibit decreased HANR expression. (A) qRT-PCR was used to assess HANR expression in 165 paired normal and CRC tumor tissues. (B) CRC patients with more advanced disease exhibited higher levels of HANR expression. CRC = colorectal cancer, HANR = HCC associated long non-coding RNA.

diagnostic biomarker of CRC. A ROC curve analysis suggests that HANR expression levels allowed for reliable differentiation between normal and CRC tumor tissues (AUC: 0.820; 95% confidence interval: 0.775–0.865) (Fig. 2). The sensitivity and specificity of HANR in this analysis were 0.60 and 0.82, respectively. The lncRNA HANR may thus be a useful diagnostic biomarker for CRC.

3.3. Elevated HANR expression correlated with CRC patient clinical characteristics

To further assess the clinical relevance of HANR expression in patients with CRC, we divided the 165 patient samples according to their levels of HANR expression (HANR-high or HANR-low;

Figure 2. Receiver operating characteristic curve analysis of the diagnostic value of the lncRNA HANR in colorectal cancer. HANR = HCC associated long non-coding RNA

n=83 and 82, respectively) based on the median HANR expression level in CRC tumor tissue samples. Chi-squared tests were then used to compare clinical characteristics between groups, revealing that higher HANR levels were associated with tumor size ($P = .003$), depth of invasion ($P = .012$), and advanced TNM stage ($P = .011$) (Table 1). In contrast, there was no relationship between HANR expression and patient age, gender, histological findings, or tumor site ($P > .05$).

3.4. HANR offers prognostic utility in CRC patients

Finally, we assessed the prognostic relevance of HANR in CRC via a Kaplan-Meier approach, revealing a significant association between elevated HANR expression and reduced overall survival (OS) ($P = .002$, Fig. 3A) as well as disease-free survival (DFS) ($P = .003$, Fig. 3B), meaning that higher levels of this lncRNA are correlated with a worse prognosis. A multivariate analysis was additionally conducted to identify factors predictive of OS and DFS (Table 3), revealing that elevated HANR expression independently predicted reduced OS (HR=2.501, 95% CI: 1.956–4.108, $P = .023$) and DFS (HR=2.314, 95% CI: 1.713–3.956, $P = .012$) in CRC patients.

4. Discussion

CRC is a form of cancer that remains highly aggressive, with many patients succumbing to the disease, making it the 5th leading cause of cancer-associated death in China.[18] Ongoing research has highlighted new avenues for cancer detection and treatment, with specific biomarkers having great promise for the detection and management of CRC.[19,20] Current factors used to guide patient treatment include KRAS sequencing and measures of micro-satellite instability.[21] Several studies[22,23] have highlighted the potential of lncRNAs to serve as tumor diagnostic biomarkers, given that these RNA molecules are often dysregulated in tumors in a manner functionally linked to tumor progression. As high throughput sequencing technologies have become increasingly prevalent, it has become far easier to readily detect expression patterns of many lncRNAs at the same time, making them ideal targets worth of study as putative diagnostic and prognostic biomarkers.[24,25]

Many reports have sought to characterize patterns of lncRNA expression and their functional relevance in CRC.[26] For example, Han et al[27] found lncRNA H19 to be expressed at high levels in CRC in a manner correlated with reduced CRC patient survival and enhanced tumor growth owing to its ability to bind eIF4A3. Iguchi et al[28] found lncRNA-ATB to be expressed at high levels in CRC and to correspond with a worse patient prognosis. Zhang et al[29] found the lncRNA HNF1A-AS1 to similarly be overexpressed in CRC patients in a manner correlating with poorer survival, with in vitro analyses demonstrating the ability of this lncRNA to regulate Wnt/β-catenin signaling so as to control tumor cell invasion. HANR was recently shown to be overexpressed in HCC,[15–17] leading us to investigate its relevance in CRC. Xiao et al[15] firstly observed increased HANR levels in HCC patient tissues and cells, with higher levels of this lncRNA corresponding to poorer survival. When HANR was knocked down, cell proliferation and tumor growth in vivo was impaired, with tumors becoming more sensitive to chemotherapeutic treatment. In contrast, overexpression of this lncRNA had the opposite effect. The authors determined HANR to be capable of binding GSKIP, thereby
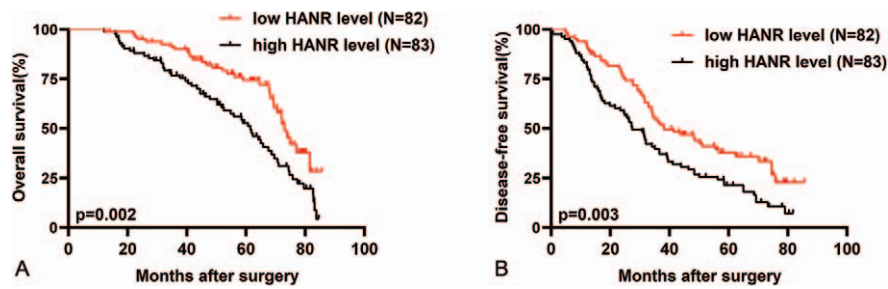
Figure 3. CRC patient survival is associated with HANR expression levels. (A) CRC patients with high HANR expression exhibited significantly reduced overall survival relative to HANR-high patients (P=.002). (B) CRC patients with high HANR expression exhibited significantly reduced disease-free survival relative to HANR-high patients (P=.003). CRC = colorectal cancer, HANR = HCC associated long non-coding RNA.

controlling GSK3β phosphorylation in HCC, potentially thereby promoting tumor growth. Whether HANR is similarly relevant to the prognosis of CRC patients has not been assessed previously.

In line with previous findings, we observed a significant upregulation of HANR in CRC tumors relative to adjacent normal controls, with ROC curve analyses confirming that HANR may be an effective marker well-suited to differentiating between normal and tumor tissue. We further provided novel insight into the clinical relevance of HANR, determining that it was significantly associated with tumor size, depth of invasion, and more advanced TNM stage, indicating that HANR may be positively associated with CRC progression in patients. Importantly, when we assessed patient survival as a function of HANR

expression we found that individuals with higher HANR expression suffered poorer clinical outcomes, with shorter average OS and DFS. We then employed a multivariate analysis to demonstrate that HANR was an independent predictor of OS and DFS in CRC patients, confirming its potential relevance. The limitation of the present study is that:

(1) we have not investigated the targets of onco-HANR such as miR-214, miR-296, or EAG1 in CRC cells. More in-depth study is needed in the future to clarify the role of HANR in CRC.
(2) we employed an arbitrary HANR expression level cut-off value in this study, and future research should seek to identify an optimal clinically relevant cut-off value.

Table 3

Univariate and multivariate Cox regression analyses of overall survival and disease-free survival in CRC patients.

Variables	Univariate analyses			Multivariate analyses		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Overall survival						
Age (years) ≥60/<60	1.632	0.525–4.928	0.188	–	–	–
Gender Male/female	0.947	0.739–1.107	0.629	–	–	–
Tumor location Colon / rectum	1.043	0.605–2.035	0.373	–	–	–
Tumor size (cm) ≥5/<5	2.878	1.602–4.952	0.017	3.162	1.792–7.174	0.011
Differentiation grade Poor+ moderate / well	2.768	1.016–3.275	0.025	2.611	1.145–7.152	0.013
TNM stage III/I + II	3.148	1.905–4.112	0.031	2.826	0.856–3.084	0.085
Depth of invasion pT3–4/pT1–2	2.565	0.541–3.629	0.056	–	–	–
Lymph node metastasis Yes/no	3.353	1.417–8.629	0.022	2.109	1.967–3.264	0.015
Distant metastasis Yes/no	2.633	1.605–4.035	0.007	1.605	0.574–3.856	0.357
Adjuvant chemotherapy Yes/no	0.919	0.662–2.152	0.415	–	–	–
CA19–9, kU/L ≥40/<40	1.172	0.804–2.175	0.083	–	–	–
HANR expression High/Low	2.829	1.824–3.119	0.011	2.501	1.956–4.108	0.023
Disease-free survival						
Age (years) ≥60/<60	2.036	0.525–2.309	0.343	–	–	–
Gender Male/female	0.848	0.739–2.107	0.298	–	–	–
Tumor location Colon / rectum	1.892	0.605–4.035	0.372	–	–	–
Tumor size (cm) ≥5/<5	1.574	1.062–2.952	0.014	1.043	0.992–1.374	0.059
Differentiation grade Poor+ moderate / well	2.906	1.014–4.275	0.012	2.025	1.145–3.152	0.019
TNM stage III/I + II	2.839	1.824–3.112	0.029	1.543	0.559–2.784	0.071
Depth of invasion pT3–4/pT1–2	2.172	1.436–3.609	0.027	1.921	1.767–3.264	0.019
Lymph node metastasis Yes/no	3.759	1.433–4.629	0.022	3.429	1.767–4.265	0.017
Distant metastasis Yes/no	2.087	1.614–4.275	0.033	2.725	1.665–3.656	0.043
Adjuvant chemotherapy Yes/no	0.926	0.804–1.122	0.091	–	–	–
CA19-9, kU/L ≥40/<40	0.908	0.457–1.669	0.145	–	–	–
HANR expression High/Low	2.597	1.486–5.155	0.005	2.314	1.713–3.956	0.012

CA19-9 carbohydrate antigen 19-9.
HANR = HCC associated long non-codingnn RNA, HR = hazard ratio, 95% CI = 95% confidence interval.

(3) In addition, our study population was relatively small, and as such additional research will be needed to confirm that HANR is relevant as a biomarker in CRC patients.

In summary, these results indicate that HANR has potential as a novel biomarker useful for diagnosing CRC and/or for predicting patient prognosis, with higher levels of this lncRNA being correlated with poorer patient prognosis.

Author contributions

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Funding acquisition: Yi-Mo Jia, Xu Sun.
Investigation: Zhi-hang Zhang, Xu Sun.
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Writing – original draft: Meng Xu, Xu Guo, Rong-Di Wang, Yi-Mo Jia, Xu Sun.
Writing – review & editing: Yi-Mo Jia.

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