Original Paper

LncRNA NEAT1 Promotes Deterioration of Hepatocellular Carcinoma Based on *In Vitro* Experiments, Data Mining, and RT-qPCR Analysis

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Key Words

Hepatocellular carcinoma • NEAT1 • Biological functions • Clinical value • Molecular mechanism

Abstract

Background/Aims: Accumulated evidence indicates that lncRNA *NEAT1* has important roles in various malignant tumors. In this study, we conducted a comprehensive analysis to explore the exact role of *NEAT1* in hepatocellular carcinoma (HCC). **Methods:** The effects of *NEAT1* on cell proliferation, apoptosis, migration, and invasion were measured by *in vitro* experiments. The expression level and clinical value of *NEAT1* in HCC was evaluated based on data from The Cancer Genome Atlas (TCGA), Oncomine, and in-house real-time quantitative (RT-qPCR). Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and protein-protein interaction (PPI) network analyses were conducted to investigate the potential molecular mechanisms of *NEAT1*. **Results:** *NEAT1* siRNA not only inhibited proliferation, migration, and invasion of HCC cells but also induced HCC cell apoptosis. A total of four records from TCGA, Oncomine, and RT-qPCR analysis were combined to assess the expression level of *NEAT1* in HCC. The pooled standard mean deviation (SMD) indicated that *NEAT1* was up-regulated in HCC (SMD = 0.54; 95% CI, 0.36–0.73; $P < 0.0001$). The area under the curve value of the summary receiver operating characteristic curve was 0.71. *NEAT1* expression was also related to race ($P = 0.025$) and distant metastasis ($P = 0.002$). Additionally, the results of GO, KEGG pathway, and PPI network analyses suggest that *NEAT1* may promote the progression of HCC by interacting with several tumor-related genes (SP1, MDM4, CREBBP, TRAF5, CASP8, TRAF1, KAT2A, and HIST4H4). **Conclusions:** *NEAT1* contributes to the deterioration of HCC and provides a potential biomarker for the diagnosis and therapy of HCC.

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Introduction

Primary liver cancer is the sixth most common malignancy worldwide and the second leading cause of cancer mortality, accounting for an estimated 40,710 diagnoses and 28,920 deaths in 2017 [1]. In most countries, hepatocellular carcinoma (HCC) represents the majority of liver cancers. Although treatments for HCC have improved and diversified from traditional surgery to more effective therapies, such as liver transplantation, chemotherapy, radiotherapy, and thermotherapy, the survival rates for HCC are unfortunately poor owing to the high invasive ability of HCC cells [2]. Therefore, it is urgent to explore the genetic mechanisms of HCC and to search for personalized treatment options.

Long non-coding RNAs (lncRNAs), which contain more than 200 nucleotides [3], have gradually become a large research focus in recent years. It has been shown that lncRNAs play essential roles in maintaining cellular homeostasis during cell development and are vital elements in the pathophysiology of cancer [3-8]. A growing number of studies have shown that many lncRNAs are associated with HCC. For example, Panzitt et al. [9] found that the upregulation of lncRNA *HULC* could downregulate tumor suppressor gene *P18* and thus promote HCC cell growth. Sui et al. [10] reported that lncRNA *linc-USP16* may negatively regulate migration and invasion of HCC cells by regulating *PTEN* expression. Wang et al. [11] demonstrated that lncRNA *UCA1* inhibited the malignant behavior of HCC cells through downregulation of *miR-216* and activation of the *FGFR1/ERK* pathway. However, there are still numerous novel lncRNAs that remain uncharacterized.

Nuclear paraspeckle assembly transcript 1 (*NEAT1*), a structural component of paraspeckles, consists of two variants: 3.7-kb *NEAT1_v1* (*MENε*) and 23-kb *NEAT1_v2* (*MENβ*) [12, 13]. Some investigators have demonstrated that *NEAT1* plays essential roles in several malignancies, including breast cancer, lung cancer, laryngeal squamous cell cancer, gastric cancer, nasopharyngeal carcinoma, pancreatic carcinoma, and colorectal cancer [14-20]. Our research group previously conducted a comprehensive analysis of the clinical value of *NEAT1* in digestive system malignancies based on 57 microarray and RNA-seq datasets, ultimately finding that *NEAT1* may play different roles in the initiation and progression of digestive system cancers [21]. The biological functions of *NEAT1* in HCC have also been investigated in previous studies. A study conducted by Mang et al. [22] showed that *NEAT1* stimulated the proliferation and invasion of HCC cells *in vitro*. Wang et al. [23] also found that *NEAT1* could boost cell invasion and proliferation in HCC through inhibition of *miR-613* expression.

In this study, we not only studied the biological effects of *NEAT1* on HCC cells *in vitro* but also systematically analyzed the expression level and clinical significance of *NEAT1* in HCC based on RNA-seq and microarray datasets from The Cancer Genome Atlas (TCGA) and Oncomine databases. Additionally, real-time quantitative PCR (RT-qPCR) was conducted to verify the expression of *NEAT1*. Furthermore, we analyzed the genes coexpressed with *NEAT1* according to Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A protein-protein interaction (PPI) network was also constructed. The overall design of the present study is shown in Fig. 1.

Fig. 1. Flow chart of the overall design of the present study.

Materials and Methods

Patients and samples

A total of 101 tumor tissues and adjacent non-tumorous specimens were collected from patients who were diagnosed with HCC pathologically at the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi, People's Republic of China) from March 2010 to December 2011. All cases had not received any adjuvant chemotherapy or radiotherapy before surgery. Adjacent non-tumorous tissues were resected at least 2 cm away from the tumorous node. The Ethical Committee of the First Affiliated Hospital of Guangxi Medical University approved his study.

Cell culture and transfection

Two human HCC cell lines, HepG2 and HepB3, were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously [24]. After being seeded into a 96-well plate (2.5×10^3 cells per well) and incubated at 37°C for 24 h, HCC cells were transfected with a blank control, mock control, scrambled siRNA (5'-GCTTGAATTAACTGCAGGCGTATAT-3'), or *NEAT1* siRNA (5'-GUGAGAAGUUGCUUAGAAACUUUCC-3') at a concentration of 200 nmol/L for up to 96 h with CombiMag Magnetofection (OZ Biosciences, Marseille, France). Then, we collected samples at 0, 24, 48, 72, and 96 h for further analysis. RT-qPCR was utilized to detect *NEAT1* knockdown efficiency.

RT-qPCR

Cellular or tissue RNA isolation was conducted on an ABI PRISM 6100 PrepStation (Thermo Fisher Scientific, Waltham, MA). RT-qPCR was performed with an Applied Biosystems PCR 7900 (Thermo Fisher Scientific) following the manufacturer's instructions. For tissue RT-qPCR, the primer sequences were 5'-TGGCTAGCTCAGGGCTTCAG-3' (*cis*) and 5'-TCTCCTTGCCAAGCTTCCTTC-3' (*trans*). For cellular RT-qPCR, the primer sequences were 5'-CTTCCTCCCTTAACTTATCCATTAC-3' (*cis*), 5'-CTCTTCTCCACCATTACCAACAATAC-3' (*trans*). The *NEAT1* abundance in each sample was normalized to its reference. The expression of *NEAT1* in tissues was calculated using the formula $2^{-\Delta C_q}$, and the change ratio of *NEAT1* in cells was determined using the formula $2^{-\Delta\Delta C_q} \times 100\%$.

Biological function of NEAT1 in HCC by in vitro experiments

Cell proliferation and viability were determined by colorimetric tetrazolium (MTS) assay (CellTiter96 Aqueous One Solution Cell Proliferation Assay G3580, Promega, Madison, WI) and fluorometric detection of resorufin (CellTiter-Blue Cell Viability Assay, G8080, Promega), respectively.

Cell apoptosis was detected using Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) and propidium iodide (PI; Sigma-Aldrich) double-fluorescent chromatin staining. Two reviewers independently counted the viable and apoptotic cells in 10 fields of each well under a 200× magnification microscope after Hoechst 33342/PI staining for 96 h.

Caspase-3/7 activity was detected with a synthetic rhodamine-labeled caspase-3/7 substrate (Apo-ONE Homogeneous Caspase-3/7 Assay, G7790, Promega). Cell migration was monitored using a wound-healing assay. Photographs of the wounds were obtained at 0, 12, 24, and 48 h and analyzed with Adobe Photoshop CS6 software (Adobe, San Jose, CA) to observe cell migration and calculate wound width. The wound widths at 12, 24, and 48 h were compared with the wound width of the blank control at 0 h. A 24-well Transwell assay (Corning Costar, Corning, NY) was conducted to detect cell invasion. Cells were counted in five random fields under a microscope. All experiments were performed following the procedures illustrated in previous studies [24-27].

Statistical analysis

Statistical analysis was conducted using SPSS 22.0 (IBM, Armonk, New York). The results represent three independent experiments. Values are shown as the mean $\pm$ standard deviation (SD). Significance between groups was analyzed via a one-way analysis of variance (ANOVA). For statistically significant ANOVA results, the least significant difference method for multiple comparisons was applied. Statistical significance was determined at the $P < 0.05$ level.

Data mining from TCGA and Oncomine

Based on the following criteria, lncRNA sequencing and microarray datasets available up to August 28, 2017 were downloaded from TCGA [28] and Oncomine [29]: (1) both HCC and normal control tissues were included in the study, and each group included more than two samples; (2) the expression data of *NEAT1* was provided directly or could be calculated; and (3) the species was human.

Two researchers (Z.-A.L. and D.-D.X.) collected the basic information from each dataset: the first author, publication year, region, data source, sample size, and *NEAT1* expression (mean $\pm$ SD) in HCC and normal control groups. All disagreements were resolved by discussion with a third (G.C.) and fourth (R.-L.L.) investigator.

The overall standard mean deviation (SMD) and 95% confidence intervals (CIs) were calculated to assess the pooled expression level of *NEAT1* in HCC. A summary receiver operating characteristic (SROC) curve was generated, and the area under the curve (AUC) value was calculated to further investigate the ability of *NEAT1* in discriminating HCC patients from normal controls. Begg's and Egger's tests were applied to estimate publication bias [30]. All analyses were carried out using STATA 12.0 (Stata Corporation, College Station, TX). The $P < 0.05$ threshold was used to determine statistical significance.

The relationships between *NEAT1* expression and the clinicopathological factors and prognoses were estimated with data from the TCGA. An independent sample *t*-test was employed to assess the relationships between *NEAT1* expression and the clinicopathological parameters. All HCC patients were divided into low-expression and high-expression groups according to the median expression value of *NEAT1*. Then, a Kaplan-Meier survival curve with a log-rank test was applied to evaluate the prognostic value of *NEAT1* in HCC. Univariate and multivariate Cox regression analyses were further conducted to determine whether *NEAT1* was an independent prognostic biomarker.

Bioinformatics analysis of NEAT1 co-expressed genes

The genes co-expressed with *NEAT1* were collected from the cBioPortal website [31]. GO functional annotation and KEGG pathway analyses were performed with the DAVID online tool [32]. We selected coexpressed genes that participated in cancer-related pathways to construct a PPI network using the STRING online database [33]. Then, we selected genes that interacted in the PPI network for further analysis. Independent sample *t*-tests were applied to evaluate the expression levels of these selected genes using expression data from TCGA. A Pearson correlation analysis was employed to assess the associations between the selected genes and *NEAT1*.

Results

Biological effects of NEAT1 on HCC cells in vitro

NEAT1 siRNA inhibited proliferation of HCC cells

In this study, *NEAT1* siRNA effectively decreased *NEAT1* expression in HepG2 and HepB3 cells, with knockdown efficiencies ranging from 75% to 80%. An MTS assay was used, showing a time-independent inhibitory effect of *NEAT1* siRNA on the proliferation of HepG2 and HepB3 cells (Fig. 2A, B). Consistent with the MTS assay result, the fluorometric resorufin viability assay revealed that the viability of cells transfected with *NEAT1* siRNA was markedly decreased compared with those cells transfected with the mock control.

NEAT1 siRNA induced apoptosis of HCC cells

Whether *NEAT1* siRNA influenced apoptosis of HCC cells remained to be clarified. Thus, fluorescent caspase-3/7 assays and Hoechst 33342/PI double-fluorescent chromatin staining analyses were conducted. Caspase-3/7 activity and apoptosis were remarkably increased in HCC cells with *NEAT1* siRNA transfection (Fig. 3A, B, C, D). In addition, viable and apoptotic cells were counted under a microscope (200 $\times$ magnification) following the Hoechst 33342/PI staining for 96 h. The result showed that *NEAT1* siRNA significantly boosted HCC cell apoptosis and decreased cell viability (Fig. 3E).

NEAT1 siRNA suppressed migration and invasion of HCC cells

The effect of *NEAT1* on cell migration and invasion was examined in HepB3 cells using wound-healing and Transwell assays. *NEAT1* siRNA caused significant reductions in the wound-healing rate and invasiveness of the HepB3 cells compared with the blank control (Fig. 4).

*Expression level and clinical significance of NEAT1 in HCC**Expression level of NEAT1 in HCC*

A total of four records containing 574 HCC samples and 174 non-cancerous specimens were included in our study, and the key information on the four records is presented in Table 1.

The expression level of *NEAT1* in each included dataset was visualized using scatter plots and receiver operating characteristic curves (Fig. 5). Owing to the low reliability of individual studies, we combined the four records to obtain a stronger result. The pooled result from a fixed-effects model ($I^2 = 37.7\%$; $P = 0.186$) indicated that *NEAT1* was upregulated in HCC (SMD, 0.54; 95% CI, 0.36–0.73; $P <$

Fig. 2. NEAT1 siRNA inhibited the proliferation and viability of HepG2 and HepB3 cells. (A) Effect of NEAT1 on HepG2 cell proliferation according to the colometric tetrazolium (MTS) assay. (B) Effect of NEAT1 on HepB3 cell proliferation according to the MTS assay. (C) Effect of NEAT1 on HepG2 cell viability according to the CellTiter-Blue Cell Viability Assay. (D) Effect of NEAT1 on HepB3 cell viability according to the CellTiter-Blue Cell Viability Assay. * $P < 0.05$; *** $P < 0.001$.

Fig. 3. NEAT1 siRNA induced apoptosis of HepG2 and HepB3 cells. (A) Effect of NEAT1 on HepG2 cell apoptosis per caspase 3/7 activity as evaluated by the Apo-ONE® Homogeneous Caspase-3/7 Assay. (B) Effect of NEAT1 on HepB3 cell apoptosis per caspase 3/7 activity as evaluated by the Apo-ONE® Homogeneous Caspase-3/7 Assay. (C) Effect of NEAT1 on HepG2 cell apoptosis as evaluated by fluorescence microscopy after Hoechst 33342 and propidium iodide (PI) double-fluorescent chromatin staining. (D) Effect of NEAT1 on HepB3 cell apoptosis as evaluated by fluorescence microscopy after Hoechst 33342/PI staining. (E) NEAT1 siRNA inhibited viability and promoted apoptosis of HepG2 and HepB3 cells. The following cell types were identified: viable cells (Hoechst 33342-positive/PI-negative), early apoptotic cells (Hoechst 33342-positive/PI-negative with blue fragmentation in the cells), late apoptotic cells (Hoechst 33342-positive/PI-positive, with red fragmentation in the cells), and necrotic cells (PI-positive with debris signals). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1. Key information on the four included data sources. HCC, hepatocellular carcinoma; SD, standard deviation; TCGA, The Cancer Genome Atlas; RT-qPCR, real-time quantitative PCR

First author (publication year)	Region	Data source	Sample size		Mean $\pm$ SD	
			HCC	Normal	HCC	Normal
TCGA (2017)	USA	TCGA	374	50	13.32 $\pm$ 1.20	12.36 $\pm$ 1.24
Wurmbach E (2007)	USA	Oncomine	35	10	12.38 $\pm$ 0.77	12.12 $\pm$ 0.82
Mas VR (2007)	USA	Oncomine	64	19	5.81 $\pm$ 0.83	5.59 $\pm$ 0.97
In-house RT-qPCR (none)	China	RT-qPCR	101	101	5.89 $\pm$ 2.55	4.90 $\pm$ 1.89

Fig. 4. NEAT1 siRNA reduced migration and invasion of HepB3 cells. (A) Cell migration was examined in wound-healing assays. (B) Wound width was calculated. (C) Cell invasion was examined with Transwell assays. (D) The number of invasive cells was counted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

0.0001; Fig. 6A). No publication bias was found (Begg's $P = 0.34$; Egger's $P = 0.482$; Fig. 6B).

To further determine the ability of *NEAT1* expression to discriminate HCC from normal controls, we generated an SROC curve. The overall AUC value of *NEAT1* in HCC was 0.70 (95% CI, 0.65–0.73; Fig. 6C), with a sensitivity and specificity of 0.61 (95% CI, 0.42–0.77) and 0.71 (95% CI, 0.42–0.89), respectively.

Clinical significances of *NEAT1* in HCC

NEAT1 expression data and corresponding clinical and survival information were obtained from TCGA. Our results revealed that *NEAT1* was significantly related to race ($P = 0.025$; Fig. 7A) and distant metastasis ($P = 0.002$; Fig. 7B) but not related to gender, age, clinical TNM stage, histological grade, lymph node metastasis, or vascular invasion (Table 2). A Kaplan–Meier survival curve was generated to gain insight into the prognostic role of *NEAT1* in HCC. The results revealed that high *NEAT1* expression indicated higher overall survival (OS; $P = 0.036$; Fig. 7C), while no significant correlation was found between *NEAT1* expression and disease-free survival ($P = 0.257$; Fig. 7D). Then, we further performed univariate and multivariate Cox regression analyses to evaluate the effect of *NEAT1* expression on OS (Table 3). The hazard ratio (HR) from the univariate Cox regression analysis indicated that *NEAT1* expression was significantly related to OS (HR, 0.57; 95% CI, 0.344–0.944; $P = 0.029$). More importantly, the multivariate Cox regression analysis showed that *NEAT1* was still associated with OS (HR, 0.568; 95% CI, 0.329–0.98; $P = 0.042$) after adjusting for age, race, gender, histological grade, stage, lymph node metastasis, distant metastasis, and vascular invasion, which suggests that *NEAT1* is an independent prognostic biomarker for patients with HCC.

Fig. 5. Expression level of NEAT1 in hepatocellular carcinoma (HCC) based on data from (A and B) The Cancer Genome Atlas (TCGA), (C and D) Wurmlich E, (E and F) Mas VR, and (G and H) our in-house RT-qPCR analyses.

Fig. 6. Meta-analysis for NEAT1 expression in hepatocellular carcinoma (HCC). (A) Forest plot of the four records evaluating NEAT1 expression in HCC (fixed-effects model). Standard mean deviation (SMD) > 0 indicates that NEAT1 is upregulated in HCC tissues compared with the noncancerous tissues. (B) Funnel plot assessing publication bias in the four datasets. (C) Summary receiver operating characteristic (SROC) curve for distinguishing HCC patients from the normal controls using NEAT1 expression.

Potential molecular mechanism of NEAT1 in HCC GO and KEGG pathway analyses of co-expressed genes of NEAT1

A total of 1,250 genes coexpressed with NEAT1 were selected for GO and KEGG pathway analyses. The GO terms for biological process, cellular component, and molecular function networks were constructed with the BINGO plugin for Cytoscape 3.3.0 software (Fig. 8). The top five GO

functional annotations and KEGG pathways are presented in Fig. 9. The result of the KEGG pathway analysis revealed that the co-expressed genes are involved in several cancer-related pathways, such as “viral carcinogenesis”, “transcriptional mis-regulation in cancer” and “P53 signaling pathway”.

Construction of the PPI network

In total, 21 genes related to “viral carcinogenesis”, “transcriptional mis-regulation in cancer” and “P53 signaling pathway” were selected to construct a PPI network (Fig. 10). A confidence score greater than 0.4 was set as the threshold for assessing associations between these genes. We found interactions among the following 14 genes: MDM2 (mouse double minute 2), SP1 (transcription factor specificity protein 1), MDM4 (mouse double minute 4), ATM (ATM serine/threonine kinase), CREBBP (cyclic AMP responsive element-binding protein), PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1), TRAF5 (TNF receptor associated factor 5), SERPINB5 (serpin family B member 5), CASP8 (caspase 8), TRAF1 (TNF receptor associated factor 1), KAT2A (lysine acetyltransferase 2A), HIST4H4 (histone cluster 4 H4), HIST1H2BF (histone cluster 1 H2B family member f) and HIST1H4E (histone cluster 1 H4 family member e). Then, we evaluated the expression levels of the 14 genes involved in HCC and further investigated how their expression was correlated with that of *NEAT1*. Because the expression levels of the genes SERPINB5, HIST1H2BF, and

Fig. 7. Clinical value of NEAT1 in hepatocellular carcinoma (HCC). (A) Relationship between NEAT1 expression and race. (B) Relationship between NEAT1 expression and distant metastasis. (C) Relationship between NEAT1 expression and overall survival. (D) Relationship between NEAT1 expression and disease-free survival.

Table 2. Relationships between NEAT1 expression and clinical parameters. SD, standard deviation

Variables	Group	Sample size	NEAT1 expression (mean ± SD)	t-test t	P-value
Race	Non-Asian	201	12.71 ± 1.12	2.254	0.025
	Asian	157	12.45 ± 1.10		
Gender	Male	249	12.54 ± 1.08	1.320	0.188
	Female	121	12.70 ± 1.15		
Age	≥65	149	12.58 ± 1.16	0.122	0.903
	<65	221	12.60 ± 1.07		
TNM stage	I-II	256	12.67 ± 1.10	2.030	0.121
	III-IV	90	12.39 ± 1.14		
Histologic grade	G1-G2	232	12.56 ± 1.09	0.735	0.463
	G3-G4	133	12.66 ± 1.13		
Lymph node metastasis	No	252	12.52 ± 1.11	1.943	0.053
	Yes	117	12.76 ± 1.07		
Distant metastasis	No	266	12.48 ± 1.12	3.069	0.002
	Yes	100	12.87 ± 1.07		
Vascular invasion	No	206	12.58 ± 1.11	0.762	0.447
	Yes	108	12.68 ± 1.11		

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	1.237 (0.875–1.748)	0.228		
Race	1.311 (0.905–1.899)	0.152		
Gender	1.226 (0.86–1.747)	0.260		
TNM stage	2.448 (1.689–3.548)	<0.0001	1.567 (0.206–11.946)	0.665
Histological grade	1.12 (0.781–1.606)	0.539		
Lymph node metastasis	1.512 (1.049–2.179)	0.027	1.177 (0.709–1.955)	0.528
Distant metastasis	1.678 (1.164–2.419)	0.005	1.648 (0.995–2.728)	0.052
Vascular invasion	1.348 (0.89–2.042)	0.159		
NEAT1	0.57 (0.344–0.944)	0.029	0.568 (0.329–0.98)	0.042

Fig. 9. The top five Gene Ontology (GO) items and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the genes coexpressed with NEAT1.

Fig. 10. Protein-protein interaction network of 21 genes that participate in cancer-related pathways.

Fig. 11. Heatmap of the eight genes coexpressed with NEAT1 in hepatocellular carcinoma (HCC) based on data from The Cancer Genome Atlas. The heatmap was generated using the pheatmap package in R.

Fig. 12. Expression of the eight genes coexpressed with NEAT1 in hepatocellular carcinoma (HCC) based on data from The Cancer Genome Atlas: (A) SP1, (B) MDM4, (C) CREBBP, (D) TRAF5, (E) CASP8, (F) TRAF1, (G) KAT2A, (H) HIST4H4.

HIST1H4E were zero in more than 10% of the samples, we removed them from the expression and correlation analyses. Our results indicated that the eight genes SP1, MDM4, CREBBP, TRAF5, CASP8, TRAF1, KAT2A, and HIST4H4 were not only significantly upregulated in HCC (Table 4, Figs. 11 and 12) but also remarkably associated with NEAT1 (Table 4, Fig. 13). Additionally, the expression patterns of the proteins encoded by these eight genes were validated based on the Human Protein Atlas database (<http://www.proteinatlas.org/>), and the results are displayed in Fig. 14. Our results indicate that NEAT1 may exert its unique role in HCC through interactions with these eight genes.

Discussion

With the innovation of genomic and molecular technologies, the functions of lncRNAs in various cancers have received increasing attention in recent years. Accordingly, lncRNAs have been revealed to perform complicated roles in carcinogenesis and cancer progression at the chromatin organizational, translational, and post-translational levels [34].

Table 4. Expression of 11 cancer-related genes in HCC and their correlations with NEAT1. HCC, hepatocellular carcinoma; SD, standard deviation

Gene ID	Sample size		Expression (mean ± SD)		t	t-test P-value	Pearson correlation	
	Normal	HCC	Normal	HCC			r	P-value
MDM2	50	374	11.54 ± 0.61	11.67 ± 0.84	1.379	0.172	0.464	<0.0001
SP1	50	374	10.88 ± 0.52	11.36 ± 0.75	5.745	<0.0001	0.461	<0.0001
MDM4	50	374	8.94 ± 0.61	10.10 ± 0.89	11.78	<0.0001	0.486	<0.0001
ATM	50	374	9.75 ± 0.77	9.91 ± 0.99	1.628	0.11	0.538	<0.0001
CREBBP	50	374	11.06 ± 0.50	11.31 ± 0.84	2.944	0.004	0.45	<0.0001
PMAIP1	50	371	4.62 ± 1.22	4.36 ± 2.01	1.281	0.203	0.291	<0.0001
TRAF5	50	374	6.22 ± 1.02	8.04 ± 1.22	11.5	<0.0001	0.352	<0.0001
CASP8	50	374	8.99 ± 0.65	9.77 ± 0.88	7.55	<0.0001	0.495	<0.0001
TRAF1	50	374	7.47 ± 1.11	8.62 ± 0.96	7.848	<0.0001	0.498	<0.0001
KAT2A	50	374	9.35 ± 0.68	11.22 ± 0.87	17.63	<0.0001	0.433	<0.0001
HIST4H4	50	373	2.59 ± 1.11	3.76 ± 1.30	6.079	<0.0001	0.454	<0.0001

Fig. 13. Correlations in expression between the eight genes and NEAT1 in hepatocellular carcinoma based on data from The Cancer Genome Atlas: (A) SP1, (B) MDM4, (C) CREBBP, (D) TRAF5, (E) CASP8, (F) TRAF1, (G) KAT2A, (H) HIST4H4.

Fig. 14. Immunohistochemistry staining of the proteins encoded by the eight genes coexpressed with NEAT1: (A) low nuclear SP1 expression in normal liver tissue, (B) moderate nuclear SP1 expression in hepatocellular carcinoma (HCC) tissue, (C) low nuclear MDM4 expression in normal liver tissue, (D) high nuclear MDM4 expression in HCC tissue, (E) low nuclear CREBBP expression in normal liver tissue, (F) moderate nuclear CREBBP expression in HCC tissue, (G) moderate cytoplasmic/membranous TRAF5 expression in normal liver tissue, (H) high cytoplasmic/membranous TRAF5 expression in HCC tissue, (I) low cytoplasmic/membranous CASP8 expression in normal liver tissue, (J) high cytoplasmic/membranous CASP8 expression in HCC tissue, (K) low cytoplasmic/membranous TRAF1 expression in normal liver tissue, (L) high cytoplasmic/membranous TRAF1 expression in HCC tissue, (M) low cytoplasmic/membranous KAT2A expression in normal liver tissue, (N) high moderate cytoplasmic/membranous KAT2A expression in HCC tissue, (O) low nuclear HIST4H4 expression in normal liver tissue, (P) high nuclear HIST4H4 protein expression in HCC tissue.

NEAT1, a lncRNA that participates in the formation of the paraspeckle, has also been demonstrated to be involved in the occurrence and development of many malignancies. Peng et al. [35] detected the expression of *NEAT1* in 56 colorectal cancer (CRC) and adjacent non-cancerous tissues and found that *NEAT1* was upregulated in CRC tissues. The authors found that knockdown of *NEAT1* in CRC cells could significantly inhibit cell growth and facilitate cell apoptosis, indicating that *NEAT1* may act as an oncogene in CRC. However, Idogawa et al. [36] silenced *NEAT1* by transferring *NEAT1* siRNA into osteosarcoma U2OS cells and lung cancer A549 cells and discovered that *NEAT1* knockdown could promote cell growth, which suggested that *NEAT1* may be a tumor suppressor. The effect of *NEAT1* on HCC has been explored in previous studies. Fujimoto et al. [37] pursued a characterization of noncoding mutations in liver cancer and found that *NEAT1* contained a significantly high number of mutations in liver cancer, which may cause aberrant expression of *NEAT1* in liver cancer. Then, the authors silenced *NEAT1* expression in HepG2 cells and found that the inhibition of *NEAT1* expression significantly decreased HCC cell invasion but did not affect cell proliferation. A study conducted by Mang et al. [22] showed that knockdown of *NEAT1* inhibited the proliferation and invasion of HCC HepG2 and SMMC-7721 cells. A study performed by Wang et al. [23] also demonstrated that silencing *NEAT1 in vitro* could obviously arrest HCC cell proliferation and invasion. However, the effect of *NEAT1* on HCC cell apoptosis has not been explored to date. In the present study, we detected the effects of *NEAT1* on proliferation, apoptosis, migration, and invasion of HCC cells by transfecting *NEAT1* siRNA into HepB3 and/or HepG2 cells. Our results indicate *NEAT1* siRNA observably inhibits proliferation, migration, and invasion of HCC cells, which was consistent with previous studies [22, 23, 38]. More importantly, we also found that *NEAT1* siRNA could significantly induce HCC cells to undergo apoptosis. These findings indicate that *NEAT1* may promote tumor deterioration in HCC.

To further probe the exact role of *NEAT1* in HCC, we obtained relevant RNA-seq and microarray datasets. Our research group has previously performed a comprehensive analysis of the expression levels of *NEAT1* in digestive system malignancies based on data from TCGA, GEO, ArrayExpress, and Oncomine [21]. The combined result based on 13 RNA-seq and microarray datasets showed that *NEAT1* was downregulated in HCC. However, the result should be interpreted cautiously because a remarkable heterogeneity exists, which may greatly reduce the accuracy and reliability of the result. To avoid the influence of inter-study heterogeneity on our result, we only included RNA-seq and microarray datasets from TCGA and Oncomine. Additionally, we performed RT-qPCR to validate the expression of *NEAT1*. Then, a combined strategy using RNA-seq, gene chip, and RT-qPCR data was employed to systematically investigate the expression levels of *NEAT1* in HCC. We found that *NEAT1* was significantly up-regulated in HCC, which was consistent with previous studies [22, 23, 38]. Compared with those previous studies, our present study included more cases.

A study conducted by Li et al. [39] showed that overexpression of *NEAT1* could drive endometrial adenocarcinoma (EAC) cells in the S phase and thus boost tumor cell proliferation. Additionally, the overexpression of *NEAT1* in EAC cells could upregulate metastasis-related genes, such as matrix metalloproteinase 7 (MMP-7), MMP-2, insulin-like growth factor 1, and c-myc. In the present study, we also found that high *NEAT1* expression was related to metastasis of HCC. We hypothesize that *NEAT1* facilitates HCC metastasis by regulating some metastasis-related genes. Further studies are needed to validate our conjecture.

The prognostic value of *NEAT1* in HCC was also assessed in this study. The results indicated that high *NEAT1* expression was closely associated with better overall survival and could serve as an independent prognostic factor for predicting the prognosis of patients with HCC. A study conducted by Idogawa et al. [36] based on microarray datasets from GEO also showed that the survival rate was higher among cancer patients with high *NEAT1* expression relative to patients with low *NEAT1* expression. The authors analyzed chromatin immunoprecipitation-sequencing data and found that *NEAT1* was a transcriptional target of tumor suppressor P53 and that overexpressed *NEAT1* could enhance the tumor suppressor effect of P53. Therefore, patients with high expression of *NEAT1* have a favorable survival

outcome in several malignancies. In addition, a study performed by Gao et al. [40] showed that enhancing the expression level of *NEAT1* in leukemia cells could significantly increase the sensitivity of tumor cells to chemotherapy. Therefore, we speculate that patients with high expression levels of *NEAT1* in their HCCs will have better chemotherapeutic responses and prognoses. Further large-scale clinical studies are needed to validate our hypothesis.

To preliminarily explore the molecular mechanism of *NEAT1* in HCC, we performed GO and KEGG pathway analyses on 1,250 genes coexpressed with *NEAT1* from cBioPortal, a public database that collects and processes data from TCGA. We found that these co-expressed genes were involved in some cancer-related pathways, such as “regulation of transcription”, “transcriptional mis-regulation in cancer” and “P53 signaling pathway”. Then we selected 21 genes that participated in the above three pathways to construct a PPI network. Among the 21 co-expressed genes, 14 genes were shown to interact with each other. The expression levels of the 14 genes and the correlations between them and *NEAT1* were further evaluated. We found that the genes SP1, MDM4, CREBBP, TRAF5, CASP8, TRAF1, KAT2A, and HIST4H4 were not only upregulated in HCC but also closely linked with *NEAT1*, indicating that *NEAT1* may promote HCC carcinogenesis by interacting with these eight genes.

Accumulated data have demonstrated that the overexpression of CREBBP, SP1, and MDM4 play central roles in various malignant tumors including HCC. Servillo et al. [41] found that CREBBP increased hepatocyte proliferation. Abramovitch et al. [42] demonstrated that CREBBP could promote HCC progression by stimulating tumor cell growth and reducing cell apoptosis *in vitro* and *in vivo*. Huang et al. [43] demonstrated that SP1 significantly upregulated lncRNA *MALAT1* in HCC and further activated tumor progression. Gandhi et al. [44] proposed that SP1 promoted tumor cell proliferation and migration by increasing the expression of lncRNA *HULC* in HCC. MDM4, a P53 binding protein homolog, is a critical negative modulator of tumor suppressor P53 [45]. Pellegrino et al. [46] showed that MDM4 acted as an oncogene in HCC by interacting with the PI3K-AKT/mTOR pathways. The authors also demonstrated that reducing the expression level of MDM4 by inhibiting the PI3K/AKT/mTOR pathways could reactivate tumor inhibition by P53 and subsequently inhibit the progression of HCC.

Both KAT2A and HIST4H4 play central roles in transcriptional regulation and have been demonstrated to exert roles in gastric carcinoma [47, 48]. However, their biological functions in HCC have not yet been studied. Accordingly, further *in vitro* and *in vivo* experiments are necessary to uncover the exact roles of KAT2A and HIST4H4 in HCC.

TRAF1, TRAF5, and CASP8 are apoptosis-related genes [49]. TRAF1 and TRAF5 are members of the TRAF family that have been shown to participate in cell apoptosis by regulating the NF- κ B (nuclear factor kappa B) and ASK1/JNK signaling pathways [50-52]. Previous studies have shown that TRAF1 and TRAF5 are related to various malignancies including HCC [53, 54]. CASP8, an extremely critical molecule in the caspase-dependent apoptotic signaling pathway, is an important member of the cysteinyl aspartate-specific proteinase (caspase) family. The caspase family contains many well-known apoptosis-related proteinases, such as CASP2, CASP3, CASP6, CASP7, CASP8, CASP9, CASP10, and CASP11, which play key roles in cell apoptosis. A large number of studies have provided evidence that these caspases are involved in the occurrence and progression of HCC [55-59]. However, the relationships between these caspases and *NEAT1* have not yet been investigated. In the current study, we verified that *NEAT1* siRNA could induce HCC cells to undergo apoptosis *in vitro*. We hypothesize that *NEAT1* inhibits apoptosis of HCC cells by interacting with these apoptosis-related genes. Further and stricter *in vitro* and *in vivo* investigations are needed to confirm our findings.

Our comprehensive analyses of *in vitro* experiments as well as RNA-seq and microarray datasets and in-house RT-qPCR suggest that *NEAT1* could promote the deterioration of HCC. More importantly, *NEAT1* could serve as a diagnostic and prognostic biomarker that provides a new perspective for molecular-targeted treatment of HCC in clinical settings.

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Disclosure Statement

The authors declare that they have no competing interests.

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