

Original Article

Clinical implication of long non-coding RNA NEAT1 expression in hepatocellular carcinoma patients

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Abstract: Hepatocellular carcinoma (HCC), a primary malignancy of the liver, is associated with high mortality rate and poor prognosis. Emerging evidence showed that novel biomarkers are required toward a better understanding of the biological mechanisms of HCC. NEAT1 (nuclear paraspeckle assembly transcript 1, also known as MEN ϵ / β), a novel long non-coding RNA (lncRNA), serves as a crucial regulator in several cancers. However, the correlation between NEAT1 expression with tumorigenesis and metastasis in HCC tissues remains out of the question so far. In the current study, the aim was to evaluate the potential role of NEAT1 expression in HCC tissues and its relationship with clinicopathological parameters. Method: The expression of NEAT1 was detected by qRT-PCR, in 95 cases of adjacent non-cancerous liver and their paired HCC tissues, respectively. The associations of NEAT1 with clinicopathological features and other biological factors were further analyzed. Result: Our results revealed that NEAT1 appeared to have higher expression in the HCC tissues, compared with the adjacent non-cancerous liver tissues. High levels of NEAT1 promoted the clinical features of HCC, including the number of tumor nodes, metastasis, clinical TNM stage, the status of portal vein tumor embolus, vaso-invasion and the infiltration of tumor cells. Additionally, high NEAT1 expression levels were significantly associated with the expression level of MDTH, NM23 and MALAT1. Conclusion: Our study demonstrates that NEAT1 acts as a pivotal player in tumorigenesis and metastasis of hepatocellular carcinoma.

Keywords: NEAT1, hepatocellular carcinoma, tumorigenesis, metastasis

Introduction

Liver cancer ranks the fifth among all cancers worldwide and it is the third most frequent etiological factor of cancer-caused deaths. Hepatocellular carcinoma (HCC), the predominant form of adult liver malignancies, accounts for between 85% and 90% of liver cancers [1-3]. Several risk factors are related to the tumorigenesis of HCC, including chronic hepatitis virus infection and fatty liver diseases [4-7]. Previous studies have not proven adequate evidences for the prevention, diagnosis and treatment of HCC. Thus, understanding and insight into the mechanisms contributing to tumorigenesis and aggressiveness in HCC is necessary and essential for more precise diagnosis and effective treatments.

Recent evidences have pointed to a relationship between several long non-coding RNAs (lncRNAs) and metastasis, drug resistance and other clinical outcome in several types of cancers [8-13]. Essentially, lncRNAs are involved in the pathogenesis of HCC, such as HOX transcript antisense RNA (HOTAIR), metastasis associated lung adenocarcinoma transcript 1 (MALAT1) and H19 [14-17]. NEAT1, a nuclear-restricted long non-coding RNA, encodes two isoforms: 3700-nucleotide (nt) NEAT1_1 and 23,000-nt NEAT1_2 [18]. This non-coding RNA has been recently revealed to localize specifically to paraspeckles. And NEAT1 is involved in the process of gene expression regulation by retaining mRNAs for editing in the nucleus [19]. Cumulatively, studies suggest that down-regu-

lated NEAT1 expression is involved in lung, liver, esophageal and retinal cancers [20]. Interestingly, evidence also argued that high NEAT1 expression is involved in the metastasis of cancers like lung cancer [21]. To date, the emerging potential role of NEAT1 in HCC is yet unclear.

In light of these findings and previous reports, we hypothesized an appealing association between NEAT1 expression and HCC. To investigate this hypothesis, we evaluated the expression of NEAT1 in adjacent non-cancerous liver and HCC tissues, in a set of 95 primary tumors, and investigated the relationship between NEAT1 expression and clinical, histological, pathological and other biological factors in HCC.

Materials and methods

Patients

Patients were selected from the First Affiliated Hospital of the Guangxi Medical University (Nanning, Guangxi, China) during March, 2010 to December, 2011. Seventy-five males and twenty female individuals were enrolled in the study, with ages between 29 and 82 years old. Additionally, the clinicopathological parameters were collected such as age, gender, differentiation, tumor size, tumor nodes, metastasis, clinical TNM stages, the presence or absence of portal vein tumor embolus, vaso-invasion, capsular infiltration and cirrhosis, and other biomarkers, for instance, serum AFP level detected by ELISA; expression of metadherin (MTDH), NM23, P53, P21, vascular endothelial growth factor (VEGF), as well as microvessel density (MVD) stained by using CD34 with immunohistochemistry; and expression of a lncRNA, MALAT1 by using real time RT-qPCR. **Table 1** showed all these features in detail. The Ethical Committee of First Affiliated Hospital, Guangxi Medical University, China approved the current study. We obtained informed consent from all participating patients. In accordance with the Helsinki Declaration, related research procedure was carried out. All samples were reviewed and diagnosed by two independent pathologists.

RT-qPCR

RNA isolation and RNA normalization were performed by the method reported before [22, 23]. Extracted RNA was analyzed by Real-time qPCR

using Applied Biosystems PCR7900. The expression of NEAT1 was examined by Reverse transcription (RT) and qPCR kits according to the instructions described previously [22, 23]. Primers were as below: NEAT1 Forward-5'-TGGC-TAGCTCAGGGCTTCAG-3', NEAT1 Reverse-5'-TC-TCCTTGCCAAGCTTCCTTC-3'; GAPDH Forward-5'-TGAACGGGAAGCTCACTGG-3', GAPDH Reverse-5'-TCCACCACCCTGTTGCTGTA-3'. The NEAT1 expression was calculated with the formula $2^{-\Delta\Delta Cq}$ [22-24].

Statistics

All statistical analyses were performed using SPSS 20. For the analysis of the significance of difference between two groups, we performed Student's t test. Accordingly, we also applied One-way analysis of variance (ANOVA) test to distinguish its significance of difference, as we divided differentiation into three groups. The strengths of the associations between NEAT1 expression and clinicopathological parameters were tested with the Spearman rank correlation. The effectiveness of NEAT1 for predicting the risk of the clinicopathological parameters was evaluated by ROC curve. We used Kaplan-Meier survival method and log-rank test to estimate the impact of NEAT1 on recurrence of HCC. All tests were 2-sided, and statistical significance was considered as $P < 0.05$.

Results

Overexpression of NEAT1 in HCC

Analysis of 95 matched samples (95 adjacent non-cancerous liver tissues and 95 HCC tissues) by RT-qPCR detected significantly increased levels of NEAT1 in tumor samples compared to the non-tumor group (6.42 ± 3.55 vs. 5.29 ± 3.14 , $P = 0.02$, **Figure 1A**).

Associations of NEAT1 with clinicopathological factors

Subsequently, we explored whether there was an association of NEAT1 expression levels with well-established biological factors in 95 HCC tissues. Correlations of NEAT1 expression of HCC and tumor characteristics were shown in **Table 1**. Most notable findings were the strong associations between high HCC expression levels and some clinicopathological parameters, such as, multi-tumor nodes ($r = 0.223$, $P = 0.030$), metastasis ($r = 0.398$, $P < 0.001$), portal vein

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Table 1. Association of NEAT1 expression with clinicopathological parameters in HCC

Clinicopathological Feature		n	Neat1 relevant expression (2 ^{-ΔCq})		
			Mean ± SD	t	P
Tissue	Adjacent non-cancerous liver	95	5.2859±3.14222	2.34	0.02
	HCC	95	6.4237±3.54655		
Age	≥50	46	6.5646±3.62168	0.373	0.71
	<50	49	6.2914±3.50690		
Gender	male	75	6.5925±3.61357	0.898	0.372
	female	20	5.7905±3.29189		
Differentiation	high	6	4.1500±1.67660	F=1.390 ^a	0.254
	moderate	60	6.6692±3.81677		
	low	29	6.3862±3.12883		
Size	<5 cm	18	6.3317±3.55015	0.122	0.903
	≥5 cm	77	6.4452±3.56866		
Tumor nodes	single	52	5.6040±2.83613	-2.675	0.009
	multi	43	7.4149±4.06767		
Metastasis	Without metastasis	46	4.8935±2.13607	-4.546	<0.001
	With metastasis	49	7.8602±4.00047		
Clinical TNM stage	I~II	22	5.2659±2.47973	-2.192	0.033
	III~IV	73	6.7726±3.75431		
Portal vein tumor embolus	-	63	5.7516±3.17703	-2.465	0.016
	+	32	7.7469±3.90225		
Vaso-invasion	-	59	5.3249±2.47388	-3.709	0.001
	+	36	8.2244±4.27418		
Tumor capsular infiltration	With complete capsule	45	5.0787±2.23320	-3.849	<0.001
	No capsule or infiltration	50	7.6342±4.06132		
HCV	-	63	6.3737±3.59428	-0.192	0.848
	+	32	6.5222±3.50529		
HBV	-	17	6.9465±3.86748	0.669	0.505
	+	78	6.3097±3.48905		
AFP	-	41	6.2549±3.38778	0.47	0.64
	+	38	6.6421±3.92849		
Cirrhosis	-	50	6.1410±3.13986	0.817	0.416
	+	45	6.7378±3.96238		
NM23	-	20	4.5125±2.25753	-3.676	0.001
	+	75	6.9333±3.66313		
MTDH	-	50	5.3994±2.88525	-3.978	<0.001
	+	39	8.2485±3.67527		
P53	-	40	6.2882±3.28013	-0.316	0.753
	+	55	6.5222±3.75514		
P21	-	62	6.4311±3.25098	0.028	0.978
	+	33	6.4097±4.09887		
VEGF	-	25	5.5120±2.99639	-1.507	0.135
	+	70	6.7493±3.68832		
Ki-67 LI	Low	47	5.7415±3.11216	-1.88	0.063
	High	48	7.0917±3.84129		
MVD	Low	47	6.3757±3.75190	-0.13	0.897
	High	48	6.4706±3.37257		
MALAT1	Low	52	5.3667±2.63275	-3.235	0.002
	High	43	7.7019±4.08391		

^aANOVA.

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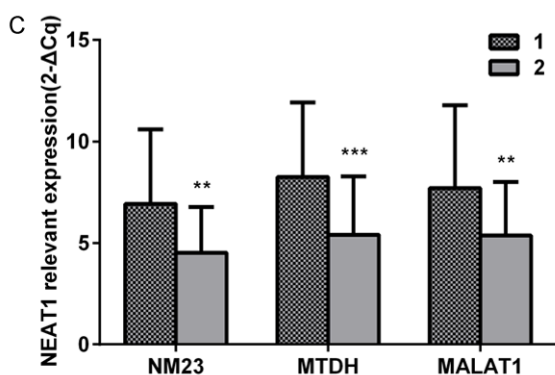
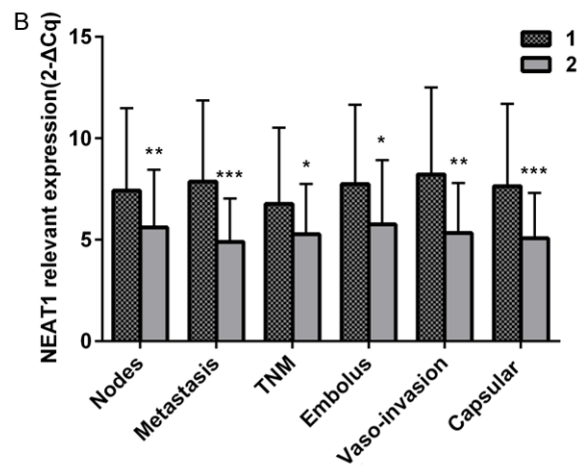
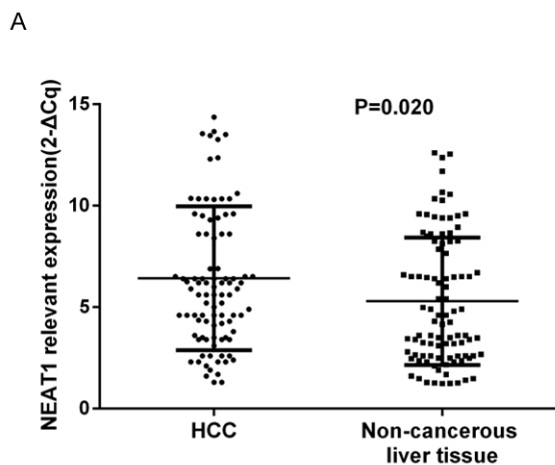


Figure 1. A. Overexpression of NEAT1 in HCC. Higher NEAT1 expression level was observed in HCC compared to that in the adjacent non-cancerous live ($P=0.020$). B. The relationships between NEAT1 clinicopathological features. Nodes: 1. multi-tumor nodes; 2. single tumor node; Metastasis: 1. with metastasis; 2. without metastasis; TNM: 1. TNM III-IV 2. TNM I-II; Embolus: 1. portal vein tumor embolus (+); 2. portal vein tumor embolus (-); Vaso-invasion: 1. (+); 2. (-); Capsular: 1. no capsule or infiltration; 2. With complete capsule; C. Associations of NEAT1 expression with biological factors in HCC. NM23:1. (+); 2. (-); MTDH: 1. (+); 2. low level (-); MALAT1: 1. high expression 2. low expression; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

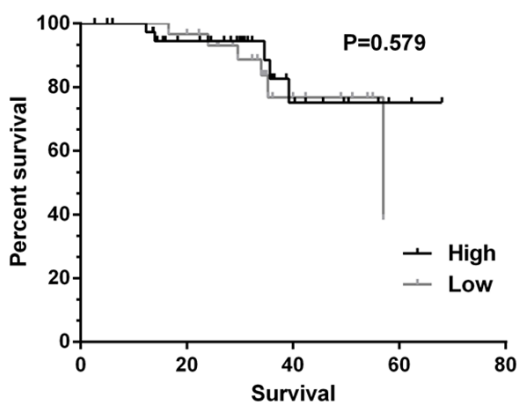


Figure 2. The Kaplan-Meier survival curve for the implication of NEAT1 expression in HCC patients.

tumor embolus ($r=0.255$, $P=0.013$), vaso-invasion ($r=0.341$, $P=0.001$), tumor capsule infiltration ($r=0.324$, $P=0.001$), MTDH level ($r=0.278$, $P<0.001$), NM23 level ($r=0.287$, $P=0.005$) and MALAT1 level ($r=0.180$, $P=0.013$). Meanwhile, NEAT1 level in HCC with multi-tumor nodes was significantly higher (7.41 ± 4.07) than that in the group with single tumor node (5.60 ± 2.84 ,

$P=0.009$). NEAT1 level in HCC tissues with metastasis was remarkably upregulated (7.86 ± 4.00) as compared to that without metastasis (4.89 ± 2.14 , $P<0.001$). Significantly increasing level of NEAT1 was found in clinical TNM stage III-IV (6.77 ± 3.75), as compared to that in the stage I-II (5.27 ± 2.48 , $P=0.033$). NEAT1 appeared to have higher expression in the HCC tissues with the portal vein tumor embolus (7.75 ± 3.90) than that without (5.75 ± 3.18 , $P=0.016$). Moreover, higher NEAT1 expression was detected in vaso-invasion (8.22 ± 4.27) than that without (5.32 ± 2.47 , $P=0.001$). Furthermore, NEAT1 levels were also significantly higher in the group of tumors without capsule or with tumor cell infiltration (7.63 ± 4.06) than that in the corresponding group (5.08 ± 2.23 , $P<0.001$, **Figure 1B**). Additionally, the level of NEAT1 was significantly higher in high MTDH group (8.25 ± 3.68) than in low MTDH group (5.40 ± 2.89 , $P<0.001$). The positive of NM23 also disclosed a higher expression of NEAT1 (6.93 ± 3.66) than the negative one (4.51 ± 2.26 , $P=0.001$). Additionally, the high MALAT1 expression group revealed a

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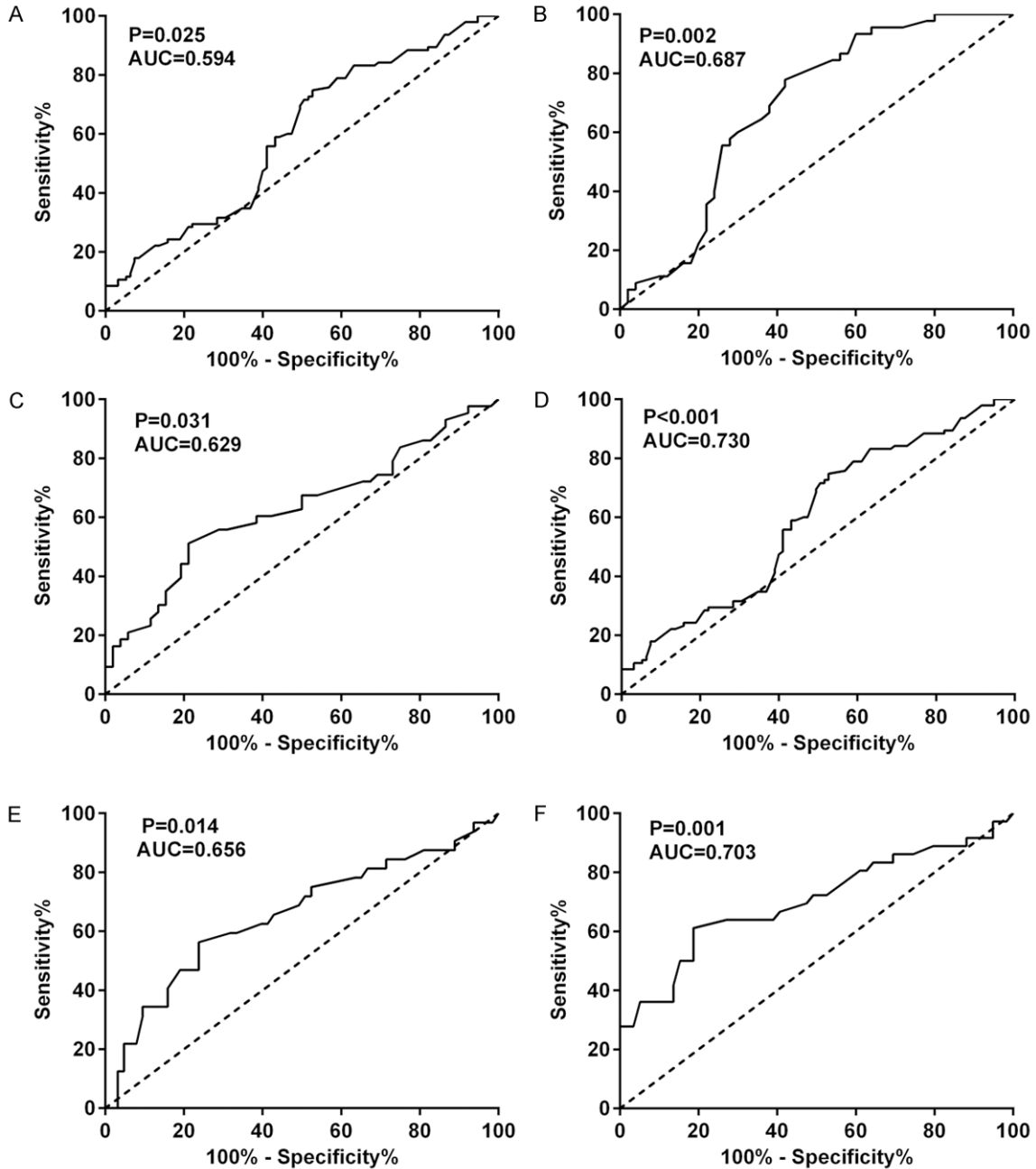


Figure 3. A. The significant diagnostic value of NEAT1 for HCC (AUC=0.594, 95% CI: 51.29%-67.50%, $P=0.025$). B. The predicative value NEAT1 for capsule or infiltration resulted in AUC of 0.687 (95% CI: 57.85%-79.53, $P=0.02$); C. NEAT1 was considered to be a valuable parameter in the prediction of tumor node (AUC=0.629, 95% CI: 51.41%-74.44%, $P=0.031$); D. The AUC was 0.73 for metastasis (95% CI: 51.29%-67.5%, $P<0.001$); E. The AUC was 0.656 for the portal vein tumor embolus (95% CI: 53.33%-77.8%, $P=0.014$); F. The AUC for vaso-invasion was 0.703 (95% CI: 58.65%-81.89%, $P=0.001$).

significantly higher NEAT1 level (7.70 ± 4.08) than the low expression group (5.37 ± 2.63 , $P=0.002$, **Figure 1C**). We next divided the patients into two groups using the median NEAT1 expression as cutoff value (medi-

an=5.90). The mean recurrence time was 58.90 months in patients with higher NEAT1 expression and 50.60 months in patients with lower NEAT1 expression, but there is no statistical significance ($P=0.579$, **Figure 2**).

Diagnostic value of NEAT1 for clinicopathological parameters by ROC curve analysis

As the ROC curve indicated, NEAT1 of 3.7 represented a significant diagnostic cut off value for HCC (AUC=0.594, $P=0.025$, **Figure 3A**). We further explored the predictive value of NEAT1 for other clinicopathological parameters in the 95 HCC samples by ROC curve. NEAT1 of 6.32 in the prediction of capsule or infiltration resulted in AUC of 0.687 ($P=0.02$, **Figure 3B**). As NEAT1 ≥ 6.45 , it was also considered to be a valuable parameter in the prediction of tumor node (AUC=0.629, $P=0.031$), metastasis (AUC=0.73, $P<0.001$), the portal vein tumor embolus (AUC=0.656, $P=0.014$) and vaso-invasion (AUC=0.703, $P=0.001$), respectively (**Figure 3C-F**).

Discussion

Accumulating evidence indicates that the involvement in functional lncRNAs is implicated in HCC pathogenesis [25]. For example, GAS5 expression was decreased in human HCC and was associated with advanced tumor progression [26]. Moreover, MALAT1 expression level was proved to be an independent prognostic factor for HCC. Furthermore, the higher expression of MALAT1 was also found to be related with shortened disease-free survival post liver transplantation [16]. Another lncRNA overexpressed in HCC was HOTAIR. HCC patients with high expression of HOTAIR exhibited significantly poorer prognosis than those with low HOTAIR expression [15].

NEAT1 has been extensively demonstrated to be involved in several cancers. Kim et al. have demonstrated that NEAT1 was up-regulated in stage III serous ovarian carcinoma [27]. A recent study described that upregulation of NEAT1 was observed in acute promyelocytic leukemia samples and cell lines [28]. Dimple et al. conducted the study that NEAT1 could act as one of the differentially regulated lncRNAs in prostate cancer by activating prostate cancer genes [29]. The report by Choudhry et al. also showed that high tumor NEAT1 expression confers a poor outcome in breast cancer patients [30]. The objective of this study was to seek to probe the potentials of NEAT1, a long non-coding RNA recently characterized by our group in HCC. To our knowledge, Gibb et al. was the first

one to indicate a direct association of NEAT1 expression with liver cancers via GEO accessioned human short SAGE libraries. Interestingly, NEAT1 presented a lower expression in liver cancers than in normal tissues in the previous study [20]. Contrary to the result of Gibb et al., our result revealed that high NEAT1 expression was detected in HCC. A possible explanation for this discrepancy is that NEAT1 was detected both in human and non-human origin in their study. In our current study, the significant higher expression of NEAT1 was detected in 95 cases of HCC tissues, compared with adjacent non-cancerous liver tissues. Taken together with the previous studies, we demonstrate that the regulation of NEAT1 contributes to tumorigenesis in HCC. Further large-scale studies might facilitate the discovery of NEAT1 molecular regulation in hepatocarcinogenesis of HCC.

It was interesting to discover the correlation between NEAT1 expression level and clinicopathological parameters in HCC. The expression level of NEAT1 was strongly correlated with the numbers of tumor nodes, metastasis and TNM stage in HCC patients. Moreover, we found significant correlations between high NEAT1 levels and portal vein tumor embolus, vaso-invasion and tumor capsular infiltration. The results also indicate that NEAT1 affects the infiltration of tumor cells, migration and invasion in HCC. More specifically, NEAT1 appears to be associated with MTDH level. The previous studies suggested that MTDH was regarded as a major event in metastasis and poor outcome in HCC patients [31, 32]. Of note, higher NEAT1 expression was detected in the cases of NM23 being positive. RUN et al carried out the study that overexpression of NM23 contributes to the hepatocarcinogenesis in HCC [33]. Given the strong association with NM23 level, the evidence of upregulated NEAT1 expression in HCC suggests a novel pro-oncogenic potential role in hepatocarcinogenesis. All together, our data indicate that the upregulation of NEAT1 increases tumorigenesis and metastasis in HCC.

The biological mechanisms of NEAT1 regulation in HCC remain currently poorly understood. In prostate cancer, NEAT1 is involved in modulation of oncogenic growth by altering the epigenetic landscape [29]. Inducted by hypoxia in HIF transcriptional pathways, NEAT1 also plays an important role in promoting proliferation and

reducing apoptosis, contributing to tumorigenesis [30]. More recently, the cellular levels of NEAT1_1/2 were found to be down-regulated in a particular class of cells in MALAT1-knockout mice, which indicates that MALAT1 transcribed from the adjacent region has an effect on the expression of NEAT1_1/2 in specific cells [34]. In agreement with the previous report, high NEAT1 expression was found to be associated with MALAT1 upregulation in our study. MALAT1 has been associated with metastasis and poor prognosis of HCC according to histology findings. MALAT1 is well-known as an oncogene, because it regulates alternative splicing of endogenous target genes that are involved in cancer [16]. Moreover, we hypothesized that NEAT1 may play an essential role in the progress and metastasis of HCC, by affecting the expression of MALAT1. Taken together, the results suggest NEAT1 may modulate in tumorigenesis and metastasis in HCC. The molecular mechanism driving this regulation is still unclear.

In conclusion, our results suggest that NEAT1 may represent a valuable predictive marker of tumorigenesis and metastasis of human HCC. Additional research of larger series into the molecular mechanism of NEAT1 in HCC is needed.

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Disclosure of conflict of interest

None.

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