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Overexpression of long noncoding RNA HOXC13-AS and its prognostic significance in hepatocellular carcinoma

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Abstract. – OBJECTIVE: The long noncoding RNA HOXC13 antisense RNA (HOXC13-AS) was overexpressed in several tumor specimens, and its overexpression was correlated with cells metastasis of tumors. However, its effects in other tumors remained largely unclear. In this work, we aimed to identify whether HOXC13-AS was abnormally expressed in hepatocellular carcinoma (HCC) and further explore its prognostic value.

PATIENTS AND METHODS: QRT-PCR was applied for the examination of HOXC13-AS levels in 197 paired HCC specimens and matched non-tumor specimens. Chi-square tests were carried out for the verification of the relations between the levels of HOXC13-AS and the clinicopathologic features of HCC patients. The Kaplan-Meier methods were applied for the exploration of the prognostic value of HOXC13-AS. Multivariate analysis was performed using the Cox proportional hazard assays.

RESULTS: Up-regulation of HOXC13-AS was observed in HCC tissues compared to matched normal tissues ($p < 0.01$). Higher levels of HOXC13-AS were associated with TNM stage ($p = 0.024$) and lymph node metastasis ($p = 0.043$). Survival assays showed that HCC patients with high-HOXC13-AS expressions had significantly shorter overall survival ($p < 0.0106$) and disease-free survival ($p < 0.0066$) compared to their counterparts with low-HOXC13-AS expressions. Multivariate analyses suggested HOXC13-AS as an independent prognostic factor for HCC patients.

CONCLUSIONS: We showed that HOXC13-AS might serve as a promising biomarker for prognosis prediction of HCC.

Key Words:
LncRNA HOXC13-AS, Hepatocellular carcinoma, Prognosis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in both men and women worldwide and represents the major cause of cancer-associated death[1]. More than 750,000 men and women succumb to HCC each year[2]. Of note, the global incidence in the last decade maintains a sustained growth. With the extensive applications of surgical resection, liver transplantations, radiotherapy, and adjuvant chemotherapy, those patients diagnosed at an early stage have achieved a favorable clinical outcome[3-5]. However, the five-year overall survivals for patients suffering from HCC diagnosed an advanced stage are less than thirty-five percent due to the potential recurrences and metastasis[6,7]. Besides, the unclear molecular mechanism of HCC progress makes it hard to better prevent metastasis. Thus, identification of the potential biomarkers, which can help the prediction of the prognosis in patients with HCC, is essential for the development of individualized treatment.

Lon noncoding RNA (lncRNAs) are defined as endogenous RNAs with sizes larger than 200 nucleotides. Unlike the protein-coding RNA, they lack open reading frames of protein-coding capabilities[8,9]. With a better understanding of lncRNAs involved in the epigenetic modification of genes, abnormal content or function of lncRNAs have gained extensive attention as novel functional players of biological modulations[10-12]. In disease researches[13,14], several evidence has highlighted the critical roles of lncRNAs in the progression of tumors via various molecular mechanisms by serving as a regulatory element, including HCC. For instance, lncRNA LINC00673 was reported to display a higher level in HCC, and its knockdown led to distinct suppression of metastasis abilities by modulating miR-205 in functional experiments[15]. LncRNA DLX6-AS1, a recently discovered lncRNA whose up-regulation was confirmed in

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HCC tissues, was shown to promote cells proliferation and invasion in tumor cells by the regulation of miR-203a/MMP-2 pathway and further predict a poor prognosis of patients with HCC[16]. The thorough involvement of lncRNAs and their dysregulated expression in tumor development highlighted their imperative potential as novel biomarkers, which may provide necessary information for the management of HCC patients.

Previously, Gao et al[17] identified a new tumor-associated lncRNA, lncRNA HOXC13 antisense RNA (HOXC13-AS) which was found to be overexpressed in nasopharyngeal carcinoma tissues. Functionally, *in vitro* assays confirmed it as a tumor promoter in this disease. Subsequently, the similar expression trend and tumor-promotive roles of HOXC13-AS were also reported in breast cancer[18]. However, the expressions and functions of HOXC13-AS in other tumors remain largely unclear.

Patients and Methods

Patients and Tissue Samples

Fresh HCC specimens and paired adjacent normal specimens were obtained from 197 patients with HCC who underwent primitive surgeries in the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University between May 2011 and September 2014. Collected tumor and normal tissues were immediately snap-frozen and stored at –80°C. All patients were pathologically confirmed as HCC. Before accepting standard resection, all HCC patients did not receive chemotherapy or radiotherapy preoperatively. Based on the severity of the malignancy, postsurgical chemotherapies were further carried out. Our clinical explorations were performed based on the principles which were shown in the Declaration of Helsinki. Informed consents were collected from all 197 patients. In addition, the study protocol was approved by the Ethics committee of the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University.

Isolation of Total RNA and Real-Time PCR Assays

Total RNA was isolated from clinical specimens using TRIzol (Invitrogen, Baoding, Hebei, China), and transcribed by the use of a transcription-PCR System (Takara, Guangzhou, Guangdong, China) based on the constructor's service for the user. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out for the determination of HOXC13-AS levels using SYBR Green real-time PCR master mix (TaKaRa, Guangzhou, Guangdong, China) with a primer application of 250 nM. The able experiments were performed on the ABI 7500 Sequence Detection System (Thermo-Fisher Scientific; Waltham, MA, USA). The PCR conditions were 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15s, 58°C for the 30s, and 72°C for 30s. The relative expressions of HOXC13-AS were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Comparative quantification was established by the use of the $2^{-\Delta\Delta Ct}$ methods. All data were presented using the way of the means ± SD of three independent examinations. Primer sequences used for RT-PCR were the following: HOXC13-AS-F: 5'-TCCGACGACTTTCTTAGGTCA-3', HOXC13-AS-R: 5'-GACTCAATTCCACGGCTCTGC-3'; GAPDH-F: 5'-ATGGGGAAGGTGAAGGTCG-3', GAPDH-R; 5'-GGGGTCATTGATGGCAACAATA-3'.

Statistical Analysis

Statistical assays and charts were generated by performing GraphPad Prism version 7.0 (La Jolla, CA, USA). A Student's *t*-test was used for the examination of the difference of HOXC13-AS levels between HCC tissues and normal non-tumor tissues. Correlations between clinical parameters and HOXC13-AS expressions were determined using the chi-square test. The Kaplan-Meier methods were used to calculate the survival curves, and the difference was further determined using the log-rank tests. Cox proportional hazard regression model was utilized for the exploration of the risk elements for HCC. A value of $p < 0.05$ was considered statistically significant.

Results

HOXC13-AS was Upregulated in HCC Patients

To identify dysregulated lncRNAs in HCC, qRT-PCR was performed to determine the levels of HOXC13-AS in 197 pairs of HCC specimens and their matched normal specimens. As presented in Figure 1, our data revealed that HOXC13-AS l levels were distinctly up-regulated in HCC samples compared with adjacent normal

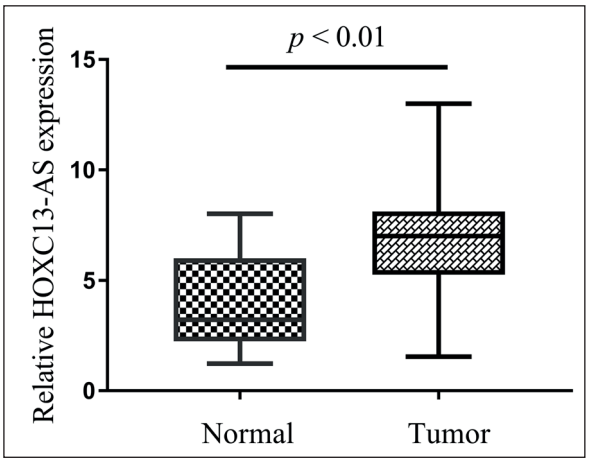

Figure 1. Expressions of HOXC13-AS in 197 pairs of HCC tissues and the corresponding non-tumor specimens by performing RT-PCR.

specimens (p<0.01). Our findings highlighted the importance of HOXC13-AS during the development of HCC.

Correlations Between HOXC13-AS and Clinicopathological Features in HCC Patients

To delve into the possible influence of dysregulated HOXC13-AS in clinical progress of HCC patients, we collected the clinical information of 197 patients and explored the association between HOXC13-AS levels and clinical factors. Using the median value of HOXC13-A expression, which was 6.372 from the results of RT-PCR, all 197 HCC patients were classified into two groups (High: n=100 and Low: n=97). As presented in Table I, statistics results analyzing using chi-square test indicated that high-expression of HOXC13-AS was associated with TNM stage (p=0.024) and lymph node metastasis (p=0.043). On the other hand, the levels of HOXC13-AS were related to age, gender, and other factors (p >0.05). Our results suggested HOXC13-AS as a clinical regulator in HCC patients.

Table I. Association between the clinicopathologic characteristics and expression of HOXC13-AS in HCC.

Characteristics	Number of patients	HOXC13-AS expression		*p*-value
		High	Low	
Age				0.227
< 50	97	45	52	
≥ 50	100	55	45	
Gender				0.307
Male	125	60	65	
Female	72	40	32	
HBsAg				0.358
Positive	102	55	47	
Negative	95	45	50	
Vein invasion				0.235
Negative	128	61	67	
Positive	69	39	30	
Encapsulation				0.367
Complete	116	62	54	
No	81	38	43	
Tumor size (cm)				0.170
≤ 5	133	63	70	
> 5	64	37	27	
AFP (ng/L)				0.695
≥ 400	113	56	57	
< 400	84	44	40	
TNM stage				0.024
I + II	131	59	72	
III-IV	66	41	25	
Lymph node metastasis				0.043
No	148	69	79	
Yes	49	31	18	
Histologic grade				0.174
High	66	38	28	
Low	131	62	69	

The Possible Influence of HOXC13-AS Expressions and the Survival Time of HCC Patients

Overexpression of HOXC13-AS and its roles acting as a positive regulator in clinical progress have been demonstrated in the above experiments. Then, we wondered whether HOXC13-AS was related to the clinical outcome. With investigation by follow up, our group collected five-year clinical survival data of all 197 HCC patients and further performed Kaplan-Meier assays, finding that test, the overall survival (p=0.0106, Figure 2), and disease-free survival (p=0.0066, Figure 3) times of HCC patients with high HOXC13-AS expressions were observed to be distinctly shorter than those of patients with low HOXC13-AS expressions. Furthermore, the clinical associations between survival times and clinical factors, including HOXC13-AS were examined using a multivariate analysis. As shown in Table II, the results after a series of statistical operation revealed that high-expressing HOXC13-AS was an independent prognostic factor for both overall survival (HR=2.894, 95% CI: 1.183-4.223, p=0.015), disease-free survival (HR=3.201, 95% CI: 1.372-4.653, p=0.004), TNM stage, and lymph node metastasis.

Discussion

HCC is a global health problem and hepatitis virus infection whose prevalence in universal in developing countries is a very imperative risk factor for this disease[19]. The long-term survival of HCC patients remains poor due to the limited therapies and tumor develops metastasis, which required sensitive biomarkers of guiding the individualized treatment[20,21]. In recent years, with the tremendous progress of epigenetic inheritance, novel molecular mechanisms involved in genes regulations without changes in DNA sequence have become research highlights for the identification of novel biomarkers[22]. The extensive roles of lncRNAs as novel regulators during the progression of tumors have supported the potential of lncRNAs to be used as promising tumor biomarkers[23]. Notably, the development of sequencing technology promoted the simultaneous achievement of detection of a large number of lncRNAs. Thus, identifying functional lncRNAs, which have a high degree of specificity and sensitivity is of importance for the clinical application of lncRNAs as novel HCC biomarkers. In this report, our group identified a novel HCC-related lncRNA HOXC13-AS.

In recent years, several lncRNAs were identified to display regulator effects in various tumors, and some of them have been well-studied such as lncRNA HOTAIR, lncRNA CCAT2, and lncRNA ANRIL[24-26]. However, due to the huge amounts of lncRNAs in the organism, there are many lncRNAs which remain to be further investigated. HOXC13-AS, a tumor-associated lncRNA, was first identified overexpressed lncRNA in nasopharyngeal carcinoma by Gao et al[17]. In their *in vitro* assays, the promotive roles of HOXC13-AS were demonstrated in the growth and metastasis ability of cells, and its function was mediated by the miR-383-3p/HMGA2 axis.

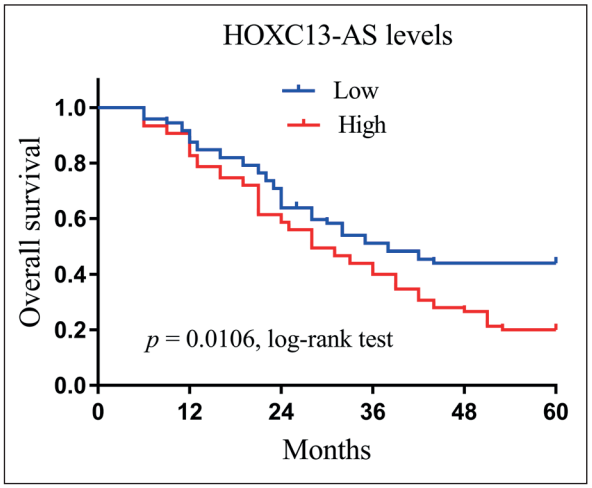

Figure 2. Kaplan-Meier curves of overall survival for HCC patients with high/low HOXC13-AS expressions.

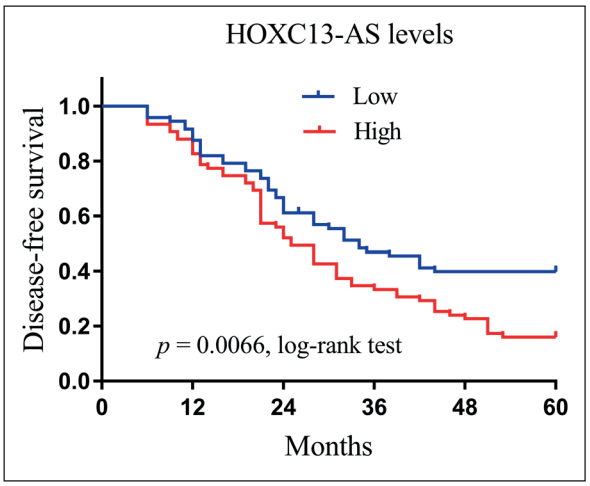

Figure 3. Kaplan-Meier curves of disease-free survival for HCC patients with high/low HOXC13-AS expressions.

Table II. Multivariate analyses for disease-free survival and overall survival by Cox regression model.

Variable	Disease-free survival			Overall survival		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	0.895	0.482-1.572	0.242	0.723	0.672-1.883	0.137
Gender	0.952	0.753-1.983	0.328	0.786	0.892-1.778	0.285
HBsAg	1.216	0.582-2.154	0.185	1.104	0.783-2.331	0.165
Vein invasion	1.387	0.762-1.932	0.138	1.039	0.884-2.218	0.113
Encapsulation	0.852	0.568-2.219	0.114	1.218	0.732-2.019	0.138
Tumor size	1.549	0.872-2.328	0.144	1.892	0.924-2.582	0.113
AFP	0.895	0.657-1.885	0.139	1.139	0.873-1.874	0.125
TNM stage	3.281	1.384-4.882	0.004	3.019	1.219-4.221	0.009
Lymph node metastasis	3.447	1.319-4.774	0.006	2.984	1.128-4.328	0.011
Histologic grade	1.218	0.847-1.994	0.109	1.482	0.924-2.281	0.149
HOXC13-AS expression	3.201	1.372-4.653	0.004	2.894	1.183-4.223	0.015

Next, a similar finding was observed in breast cancer patients. Functional assays performed by Li et al[18] indicated that HOXC13-AS was highly expressed in breast cancer and its knockdown using sh-HOXC13-AS was observed in a series of cellular experiments to suppress breast cancer cells proliferation via modulating the miR-497-5p/PTEN axis. Also, the distinct up-regulation of HOXC13-AS was found in intrahepatic cholangiocarcinoma. Of note, higher levels of HOXC13-AS predicted a poor prognosis in cholangiocarcinoma patients[27]. The previous findings highlighted the fact that HOXC13-AS may serve as a tumor promoter in the above three tumors. However, its expression and potential effects in other tumors remain unknown.

In this work, for the first time, our group provided strong clinical evidence that HOXC13-AS levels were distinctly increased in HCC tissues, which was demonstrated using RT-PCR. Then, by collecting clinical data, the clinical significance of HOXC13-AS was determined. The results revealed a positive influence of dysregulated HOXC13-AS in progress of TNM stage and lymph node metastasis, suggesting that HOXC13-AS may be associated with the outcome of HCC patients. Notably, according to the results of the Kaplan-Meier assays, it was shown that patients with high HOXC13-AS expression had shorter overall survival and disease-free time. In addition, the multivariate analysis suggested that HOXC13-AS served as a novel prognostic indicator for HCC. However, several limitations of current experiments should be noted. First, the relatively small sample capacity may influence the accuracy of our findings. Second, our data revealed HOXC13-AS as a tumor promoter, which was consistent with the roles of HOXC13-AS in breast cancer. However, the functional assays for the determination of roles of HOXC13-AS in HCC cells have not been investigated due to the limitation of experimental condition and funding. In the future, the in-depth investigation involved in the function and molecular mechanisms of HOXC13-AS in HCC needs to be further studied.

Conclusions

LncRNA HOXC13-AS levels were higher and associated with a poorer outcome in HCC patients, suggesting that HOXC13-AS may be a tumor promoter and an ideal prognostic biomarker for HCC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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