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Original Article

Overexpression of long non-coding RNAs DUXAP9 and DUXAP10 is associated with prognosis in patients with hepatocellular carcinoma after hepatectomy

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Abstract: Aim: Hepatocellular carcinoma (HCC) is a common and aggressive malignant tumor with an especially high prevalence in Asian populations. This study aimed to identify differentially expressed lncRNAs using expression microarray and to explore the association between differential expression of lncRNAs and prognosis of HCC. Methods: We retrospectively reviewed 63 patients with primary HCC that underwent a curative liver resection at the Department of General Surgery, Jingmen First People's Hospital. The expression level of lncRNAs DUXAP9 and DUXAP10 were detected by real-time PCR. Prognostic factors were evaluated using Kaplan-Meier curves and Cox proportional hazards models. Results: By microarray profiling of lncRNAs, 256 were found to be differentially expressed, including 162 upregulated and 94 downregulated ($P<0.05$, fold change >2). Two candidate lncRNAs were determined as targets in this study, including DUXAP9 (upregulated by 6.35 fold) and DUXAP10 (upregulated by 4.53 fold). DUXAP9 and DUXAP10 were downregulated in the normal liver cell lines Chang liver, HL7702, THLE-2, THLE-3, FL62891, and AML12, which were significantly lower than HCC cell lines SMMC-7721, Hep3B, HuH7, MHCC-97H, HCC-LM, and SK-Hep-1 ($P<0.05$). Overexpression of lncRNAs DUXAP9 and DUXAP10 were associated with decreasing OS rates, respectively ($P=0.0263$ and $P=0.0285$). Meanwhile, Overexpression of DUXAP9 and DUXAP10 was associated with decreasing PFS rates, respectively ($P=0.0174$ and $P=0.0041$). After adjusting for competing risk factors, we identified microvascular invasion ($P=0.014$), tumor size ($P=0.026$), and lncRNAs DUXAP9 ($P=0.001$) and DUXAP10 ($P=0.036$) expression levels as independent prognostic factors associated with prognosis of patients with HCC. Conclusions: We found that lncRNAs DUXAP9 and DUXAP10 are expressed significantly higher in HCC tissues compared with non-tumorous tissues. Overexpression of DUXAP9 and DUXAP10 were independent risk factors associated with prognosis of patients with HCC.

Keywords: DUXAP9 and DUXAP10, prognosis, HCC

Introduction

Hepatocellular carcinoma (HCC) is prevalent in Asian and developing countries and is becoming more and more common in Europe and North America [1, 2]. Although application of a variety of treatments has been reported to improve the prognosis of hepatocellular carcinoma, hepatectomy is still the main treatment for patients with HCC [3, 4]. The poor prognosis of HCC is largely attributed to a high rate of tumor recurrence after surgery, since intrahepatic metastases develop through invasion of the portal vein and tumor metastases [5]. Moreover, there are other several factors associated with the prognosis of HCC, including completeness of tumor removal, serum alpha-fetoprotein (AFP) levels, tumor size, tumor multifocality, and tumor encapsulation, etc. [6, 7]. Hence, efforts to identify the genes responsible for HCC and to understand the molecular mechanism of gene expression regulation will contribute to the development of new diagnostic tools and therapeutic targets.

Long non-coding RNA (lncRNA) is a type of non-coding RNA with a length varying from 200 nt to 100kb that is widely present in nuclei and cytoplasm. LncRNA is rarely involved in protein coding due to the lack of open reading frame [8-10]. Many studies have demonstrated that lncRNAs have diverse cellular functions including cell

proliferation, differentiation and apoptosis [11-13]. Moreover, lncRNA has been identified as a crucial regulator in a variety of tumor growths or metastases [14]. The database "LncRNA disease" has already included several lncRNAs related to HCC: BLACAT1, H19, MALAT1, AX800134, HULC, HOTAIR and AB074278 [15]. Furthermore, UCA1 is considered as a candidate biomarker of HCC [16].

Along with advances in high-throughput screening and bioinformatics, a growing number of important regulatory lncRNAs in cancer have been discovered. Detection on the biological functions of differentially expressed lncRNAs in HCC could bring new dimensions for diagnosis and prognosis prediction for patients with HCC after hepatectomy. This study identified differentially expressed lncRNAs using lncRNA expression microarray. Several candidate lncRNAs were selected by bioinformatics analysis and we further explored the association between differential expression of lncRNAs and prognosis of HCC. New lncRNAs related to prognosis of HCC were identified from this work.

Materials and methods

Patients and tissue samples

A total of 63 patients with primary HCC underwent a curative liver resection at the Department of General Surgery, Jingmen First People's Hospital, and were included in this retrospective study. All patients were diagnosed as HCC between June 1st, 2012 and December 30th, 2015. The tumor and non-tumor tissues were immediately frozen in liquid nitrogen after surgical removal and stored at -80°C until use. None of the patients recruited in this study had undergone preoperative chemotherapy or radiotherapy. HCC diagnosis was based on WHO criteria. Tumor staging was determined according to the seventh edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The study was approved by the Research Ethics Committee of Jingmen First People's Hospital. Informed consent was obtained from all patients. First, matched cancerous and para-cancerous normal tissues were collected from four males with HCC in September 2012 for microarray analysis. Differential expression of lncRNAs was analyzed by using dual channel lncRNA microarray technology. Four males were aged 49-76 years old with an average age of 54.3 years. All of them had multiple lesions which were not treated by radiotherapy or chemotherapy. According to postoperative pathology, the 4 patients were diagnosed as HCC.

Cell lines and culture conditions

Human HCC cell lines (SMMC-7721, Hep3B, HuH7, MHCC-97H, HCC-LM, and SK-Hep-1 cells) were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC-7721, Hep3B, HuH7, MHCC-97H, HCC-LM, and SK-Hep-1 cell lines were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The normal liver cell lines (Chang liver, HL7702, THLE-2, THLE-3, FL62891, and AML12 cells) were cultured in BEGM (Bronchial Epithelial Medium, Invitrogen, Carlsbad, CA, USA) supplemented with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin dissolved in BEBM medium.

Gene microarray analysis

LncRNA+mRNA Human Gene Expression Microarray V4.0 (Capitalbio, 4×180K, two-channel, containing about 37 thousand lncRNAs and 34 thousand mRNAs) was applied to the profiling of lncRNAs in four cancerous tissues and the paired para-cancerous tissues. Microarray data were normalized and analyzed using Agilent Gene Spring software. Microarray No. was 256740610228_1_1, 256740610227_1_4, 256740610282_1_3 and 256740610282_1_4, respectively.

RNA isolation and real-time quantitative PCR

Total RNA extraction was performed using Trizol reagent (Life, USA) in accordance with the instructions. cDNA was synthesized by reverse transcription using ReverTra Ace® qPCR RT Kit (TOYOBO, Japan). Expression of lncRNAs in each cell lines was determined by RT-PCR using KAPA SYBR Green kit (KAPA, USA). GAPDH was used as the internal reference gene. PCR was conducted under the following conditions: 95°C for 3 min, 95°C for 3 sec, 60°C for 30 sec, 40 cycles. Cycle threshold value (Ct) was obtained and the relative expression of lncRNAs in each cell line was calculated using $2^{-\Delta\Delta CT}$ method. Each sample was examined in triplicate, and the mean values were calculated. mRNA levels

Table 1. Patients' clinicopathologic features (n=63)

Variables		Number
Sex	Female	28
	Male	35
Age	Median	52.4
	Range	20-70
AFP level (μg/L)	≤400	23
	>400	40
HBsAg	Positive	45
	Negative	18
HBeAg	Positive	37
	Negative	26
Liver cirrhosis	Yes	43
	No	20
Edmondson-Steiner grade	I-II	21
	III-IV	42
Diameter (cm)	≤5	32
	>5	31
MVI	Yes	24
	No	39
Tumor number	Single	54
	Multiple	9
TBL (μmol/l)	Median	14.9
	Range	4.1-66.3
Alb (g/dl)	Median	37.2
	Range	21.2-52.5
ALT (U/L)	Median	62.1
	Range	11.2-283.2
DUXAP9	High	30
	Low	33
DUXAP10	High	45
	Low	18
TNM stage	I	17
	II	36
	III	10

Abbreviations: AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis E antigen; MVI, microvascular invasion; TBIL total bilirubin; ALB, albumin; ALT, alanine.

in tumor samples/non-tumorous samples of 0.5-fold was defined as under-expression of the gene, whereas a ratio of 2.0-fold was defined as over-expression.

Follow-up

Postoperative serum AFP and abdominal ultrasound were carried out in all patients monthly. Patients received abdominal contrast-enhanced CT scan or MRI once every 3 months in the first two years after surgery, and once every 6 months thereafter. Further investigations were carried out when clinically indicated or when tumor recurrence was suspected. The follow-up was performed by authors in this study. Outcome definitions: Complete resection was defined as resection of all tumor sites on the basis of surgical findings and postsurgical images. OS (overall survival) was defined as the period from the date of surgery until death or last contact. Patients who did not experience an event were censored on the date of last contact. Progression-free Survival (PFS) was defined as the time from randomization to disease progression or death as assessed by the treating physicians in the study.

Statistical methods

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test; categorical variables were compared using χ^2 or Fisher analysis. Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology [17]. The Greenwood formula was used for the standard deviation. A Cox proportional hazards regression approach [18] was chosen for evaluation of the prognosis. Potential prognostic variables were analyzed by both univariate and multivariate models combining all factors. Results are shown as hazard ratios (HR) and their 95% confidence interval (CI) A HR>1 indicated an elevated risk with respect to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc., Chicago, IL) and GraphPad Prism v6.01 (GraphPad software, Inc.). A value of $P<0.05$ was considered to be statistically significant in all the analyses.

Results

Patients' characteristics

Of all 63 patients recruited into this study, the median follow-up time was 4.5 years (range 3.6 months-8.7 years). The baseline characteristics of patients are summarized in **Table 1**.

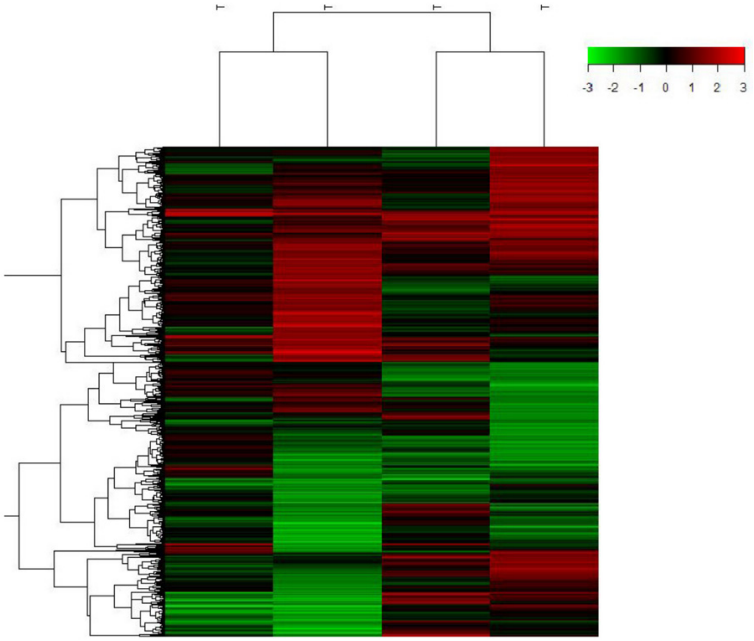

Figure 1. Hierarchical clustering. Hierarchical clustering based on 4 lncRNA microarray images. "Red" indicates upregulation, and "green" downregulation. The clustering tree represents the downregulation and upregulation patterns of lncRNAs in the samples.

Profiling results and candidate lncRNAs screening

By microarray profiling of lncRNAs, 256 lncRNAs were found to be differentially expressed, including 162 upregulated and 94 downregulated (P<0.05, fold change >2) (**Figure 1**). Using the BLAST program, those subjected to alternative splicing that created difficulty for designing high-specificity PCR primers were excluded. LncRNAs with large fold changes that remained were further screened based on the fold change, gene alignment, and analysis of adjacent coding genes. Then after excluding the lncRNAs have been reported previously, 2 candidate lncRNAs were determined as the targets in this study, which were DUXAP9 (upregulated by 6.35 fold), DUXAP10 (upregulated by 4.53 fold).

Gene expression detected by RT-PCR in each cell line

DUXAP9 and DUXAP10 were downregulated in the normal liver cell lines Chang liver, HL7702, THLE-2, THLE-3, FL62891, and AML12, which were significant lower than HCC cell lines SMMC-7721, Hep3B, HuH7, MHCC-97H, HCC-LM, and SK-Hep-1 (P<0.05) (**Figure 2A**, **2B**).

DUXAP9 and DUXAP10 were over-expressed in HCC tissues compared with the non-tumor tissues

DUXAP9 was expressed in 47.6% (30 of 63) of all patients in HCC tissues and DUXAP10 was expressed in 71.4% (45 of 63) of all patients in HCC tissues, which was significantly higher than that in non-tumorous tissues (P<0.001) (**Figure 3A** and **3B**).

Survival descriptions of different subgroups divided by lncRNA DUXAP9 and DUXAP10 expression levels

Descriptive survival statistics and Kaplan-Meier curves suggested that the variable of lncRNA DUXAP9 and DUXAP10 expression level had prognostic significance in this relativly selected cohort. Overexpression of lncRNAs DUXAP9 and DUXAP10 was associated with decreasing OS rates, respectively (P=0.0263 and P=0.0285, **Figure 4A**, **4C**). Meanwhile, Overexpression of DUXAP9 and DUXAP10 was associated with decreasing PFS rates, respectively (P=0.0174 and P=0.0041, **Figure 4B**, **4D**).

Cox proportional hazard analysis

A Cox proportional hazards model was then used to quantify the prognostic significance of risk factors after multivariable adjustment. Univariate Cox proportional hazards analysis demonstrated that TNM stage (P=0.026), tumor number (P=0.031), AFP>400 (ng/ml) (P=0.008), microvascular invasion (P=0.003), tumor size (P=0.012), lncRNA DUXAP9 (P=0.001) and DUXAP10 (P=0.008) expression levels were significantly associated with prognosis of patients with HCC. A multivariate analysis was further performed to assess factors that demonstrated significant effects in univariate analysis. After adjusting for competing risk factors, we identified that microvascular invasion (P=0.014), tumor size (P=0.026), lncRNA DUXAP9 (P=0.001) and DUXAP10 (P=0.036) expression levels as independent prognostic

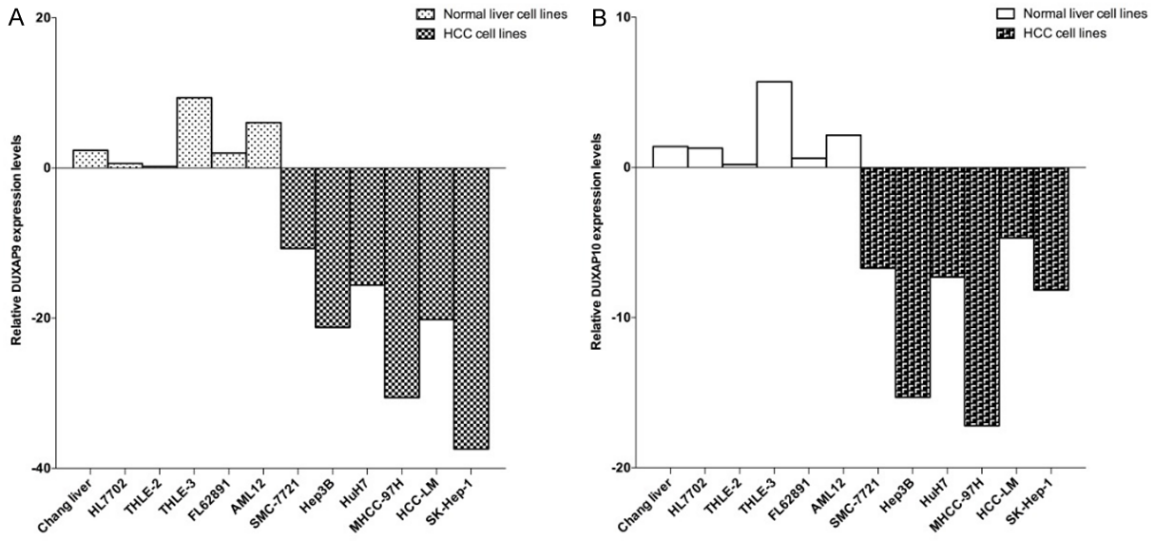

Figure 2. Relative expression of candidate lncRNAs in each cell line. A: The comparison of relative expression of DUXAP9 in HCC and normal cell lines; B: The comparison of relative expression of DUXAP10 in HCC and normal cell lines.

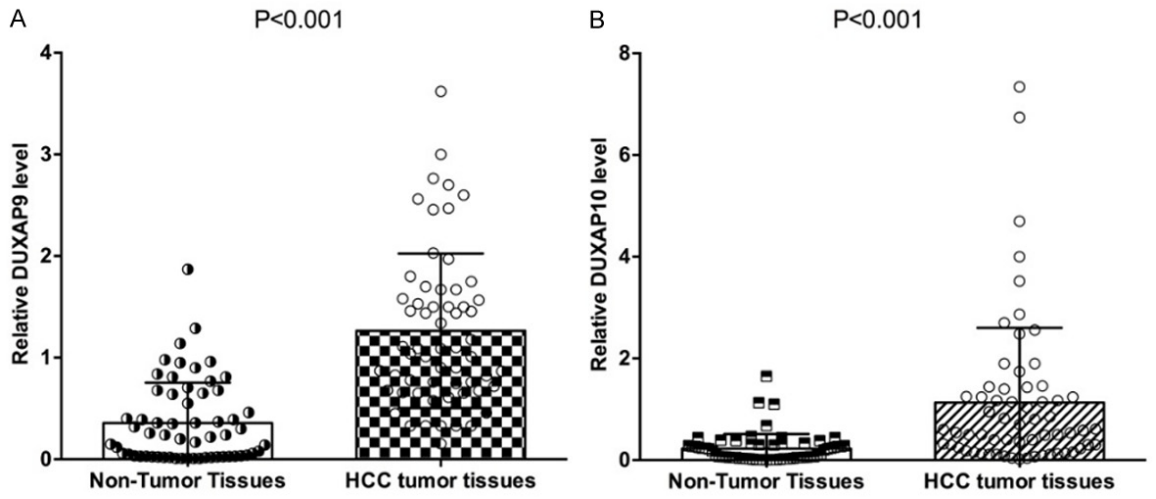

Figure 3. DUXAP9 and DUXAP10 are up-regulated in HCC tissues compared with non-tumor tissues. A: Relative DUXAP9 concentration was detected using Real-Time qPCR; B: Relative DUXAP10 concentration was detected using Real-Time qPCR (P<0.001).

factors associated with prognosis of patients with HCC (**Table 2**).

Discussion

The differences of lncRNA expression profile between four cases of hepatocellular carcinoma tissues and paired adjacent normal tissues were explored by using dual channel lncRNA microarray technology [19]. Candidate lncRNAs DUXAP9 and DUXAP10 were selected through bioinformatics analysis for further analysis to confirm their expression characteristics by RT-PCR. Moreover, survival analysis was performed to validate the prognostic significance of patients with HCC after hepatectomy.

The DUXA (double homeobox A) gene family contains 10 genes which are DUXAP1~10. Homeobox genes encode DNA-binding proteins, many of which are thought to be involved in early embryonic development [20-22]. This pseudogene is a member of the DUXA homeobox gene family. Previous reports suggested

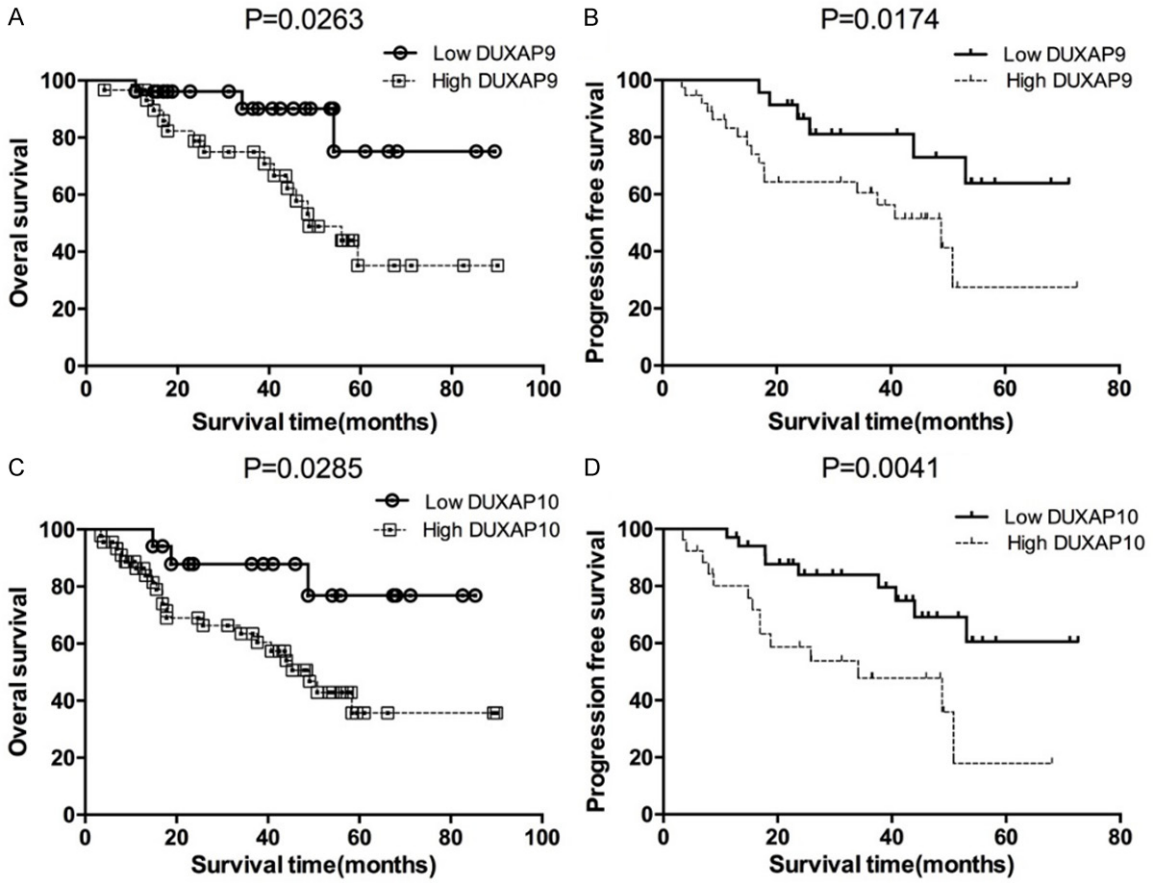

Figure 4. Overall survival and progression free survival estimates. A: OS of patients stratified by DUXAP9 expression levels (*P*=0.0263); B: PFS of patients stratified by DUXAP9 expression levels (*P*=0.0174); C: OS of patients stratified by DUXAP10 expression levels (*P*=0.0285); D: PFS of patients stratified by DUXAP10 expression levels (*P*=0.0041).

Table 2. Cox proportional hazard regression analyses

Variable	Univariate			Multivariate		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age in yr (median range)	1.021	0.991-1.027	0.771			
Gender, male: female	1.008	0.948-1.125	0.901			
HbsAg: positive: negative	1.219	0.941-1.553	0.091			
HBeAg: positive: negative	1.102	0.714-1.242	0.104			
Liver cirrhosis: with: without	1.107	0.865-1.122	0.249			
TBL (μmol/l): >17: ≤17	0.882	0.731-1.231	0.329			
ALB (g/dl): >40: ≤40	1.088	0.827-1.430	0.548			
ALT (U/L): >40: ≤40	1.047	0.902-1.193	0.837			
TNM stage: I: II: III	1.542	1.293-2.735	0.026	1.105	0.756-1.291	0.337
No. tumor: Solitary :Multiple	1.593	1.184-3.024	0.031	1.059	0.921-1.491	0.192
AFP (≤400 μg/L vs >400 μg)	1.629	1.372-3.581	0.008	0.946	0.649-1.302	0.782
Edmondson-Steiner grade: I+II: III	1.199	0.808-1.781	0.397			
Tumor size (≤5 cm vs >5 cm)	1.583	1.216-2.663	0.012	1.377	1.243-2.582	0.026
Micro-vascular invasion (+/-)	1.864	1.392-3.261	0.003	1.402	1.143-3.633	0.014
LncRNA DUXAP9: High: Low	1.905	1.482-2.693	0.001	2.524	1.661-4.015	0.001
LncRNA DUXAP10: High: Low	1.826	1.265-3.254	0.008	1.316	1.251-2.619	0.036

CI: indicates confidence; TBL: total bilirubin; ALB: albumin; ALT: alanine aminotransferase; AFP: alpha-fetoprotein.

that they were restricted to humans and other primates, although they were also found in the mouse genome. DUXAP9 (double homeobox A pseudogene 9) is located in 14q11.2 and DUXAP10 (double homeobox A pseudogene 10) is located in 14q11.1 [23]. Researchers had detected numerous DUXAP9 and DUXAP10 sequences from cancer cell lines. In Ensembl Database, DUXAP9 and DUXAP10 are poorly expressed in normal prostate and testes and in leukemia. These two lncRNAs are not significantly expressed in almost all tissues (http://asia.ensembl.org). In this study, we found that DUXAP9 and DUXAP10 were upregulated significantly in several HCC cancer cell lines, including SMMC-7721, Hep3B, HuH7, MHCC-97H, HCC-LM, and SK-Hep-1 cells. This result implies specific upregulation of DUXAP9 and DUXAP10 in HCC.

LncRNAs have been found dysregulated in many diseases, especially neoplastic disease [24, 25]. Accumulating studies have demonstrated that lncRNAs can be used as tumor biomarkers for prognosis [26, 27]. In present study, novel lncRNAs with specific upregulation in HCC were identified for the first time, namely, DUXAP9 and DUXAP10 by lncRNA expression profiling in HCC. Validation of DUXAP9 and DUXAP10 overexpression in HCC tissues compared with the adjacent nontumor tissues was also performed. After adjusting for competing risk factors, we identified lncRNA DUXAP9 and DUXAP10 are independent risk factors associated with the prognosis of patients with HCC by Cox proportional hazards analysis.

However, there are some limitations of this study: (1) the sample size is too small, and further larger sample size studies are needed to confirm the present experimental results; (2) whether overexpression of lncRNA DUXAP9 and DUXAP10 has the optimal specificity and sensitivity for HCC diagnosis and prognosis also needs future confirmation.

In conclusion, we found that lncRNA DUXAP9 and DUXAP10 are expressed at a significantly higher level in HCC tissues compared with nontumorous tissues. Overexpression of lncRNA lncRNA DUXAP9 and DUXAP10 were independent risk factors associated with prognosis of patients with HCC. They also have potential as therapeutic targets for further research into molecular mechanisms regulating development of HCC.

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Disclosure of conflict of interest

None.

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