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Original Article

HOXD10 acts as a tumor suppressor in hepatocellular carcinoma

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Abstract: Objective: HOXD10, which regulates and maintains cell-differentiated phenotype, has involved in the tumorigenesis of many human cancers. However, the expression patterns and biological functions of HOXD10 in hepatocellular carcinoma (HCC) remain to be unclear. Therefore, we investigated the clinical and biological functions of HOXD10 in HCC in this work. Methods: HOXD10 expression was detected by RT-PCR, western blot and immunohistochemisty in HCC cell line LM3 and 15 cases of HCC. The function of HOXD10 in the LM3 was investigated by using stable transfected-HOXD10 LM3 cells, in which cell cycle, proliferation, apoptosis, migration and invasion ability were analyzed. The tumor formation was performed in BALB/c nude mice. Results: It was found that HOXD10 was significantly down-expressed in HCC tissues compared to that in pericarcinous tissues. Through transfection of HOXD10 to HCC cell line LM3, we observed that over-expression of HOXD10 could significantly increase apoptosis and inhibit proliferation in LM3 in vitro, as causing cell cycle arrest at G1 phase. HOXD10 could also suppress tumor formation in nude mice in vivo. Additionally, the migratory and invasive properties of LM3 cell were inhibited in case of overexpression of HOXD10, partly due to the inhibited expression of ROCK. Conclusion: Taken together, it was suggested that HOXD10 played as a suppressor gene in HCC, and could used as a prognostic marker and a potential therapeutic target for HCC.

Keywords: LM3 cells, cell proliferation, cell apoptosis, tumor metastasis, liver cancer

Introduction

Hepatocellular carcinoma (HCC), making up of 70%-85% of liver cancer in most countries, has been the third leading cause of cancer-related mortality and the fifth most common cancer all around the world. The incidence of HCC is increasing annually, which demands more and more efficient therapeutic strategies in view of a clear explanation of the etiology of HCC [1]. It is reported that HCC usually results from chronic virus infection, such as hepatitis B virus and hepatitis C, and exposure to other risk factors, including aflatoxin B1, obesity, diabetes, tobacco and alcohol usage [2, 3]. Previous studies have verified various molecular pathways involving in the etiology of HCC, such as Jak/Sat pathway, WNT-β-catenin pathway, PI3K/AKT/mTOR pathway [4]. HCC is often accompanied by cirrhosis and hepatic insufficiency, which makes the treatment of HCC more difficult than other cancer [5, 6]. Currently, surgical resection is the most commonly practiced therapy for HCC.

With the advances in cytogenetics and molecular biology, the genetic basis of neoplasia and plenty of critical genes in the pathogenesis of human cancers have been established, which can be used as therapeutic targets in cancer treatment [7, 8]. In HCC, a variety of tumor suppressor genes (TSGs) have shown their potential roles in inhibiting the progression of normal hepatocytes to malignancy in hepatocarcinogenesis, such as TP53, miR122, CTNNB1 and PIK3CA [9, 10].

It was found that abnormal expression of some HOX genes in cells related to human cancers, including colorectal cancer, gastrointestinal cancer, esophageal squamous cell cancer, glioma and lung cancer [11]. Regardless of their broad functions, it is educible that the expression of HOX genes may be either silenced or

overexpressed in different cancers. This indicates that HOX genes can be used as potential therapeutic targets in cancer treatment [12-15]. Hence, some small molecules inhibiting the interaction between HOX genes and their ligand PBX have been developed as anticarcinogen, such as HXR9 [14]. Therefore, characterization of consistent changes in specific HOX genes in particular malignancy would provide novel prognostic markers or therapeutic strategy to improve clinical outcomes.

As a member in HOXD family, HOXD10 can suppress the genes related to extracelluar matrix remodeling and cell migration in endothelial cells in order to maintain their quiescent, differentiated phenotype. Loss or overexpression of HOXD10 in cells commonly is involved in tumorigenesis, in which HOXD10 acts as a tumor suppressor or oncogene for its crucial roles in regulating cancer cell proliferation, survival, invasion and migration in several human malignancies [16, 17]. Recently, Yang et al. reported that HOXD10 was down-regulated and played as a tumor suppressor in cholangiocellular carcinoma through inhibiting cell invasion and cell proliferation [18]. Hakami et al. found that HOXD10 expression varied by stage of head and neck squamous cell carcinoma and produced different effects: high expression of HOXD10 in primary tumor cells providing cancer cells an advantage of proliferation and migration, and low expression in lymph node metastasis cells supporting invasion/metastasis [19]. Hu et al. demonstrated that HOXD10 was significantly down-expressed in glioma cells and tissues to inhibit tumor invasion [20]. However, the role of HOXD10 in malignant progression in HCC remains unclear. Therefore, we investigated the roles of HOXD10 in the development and progression of HCC in this study.

Materials and methods

Collection of tissue specimens

All of the protocols involving tissue specimens in this study were approved by the Ethics Committee of the First Hospital of Shanxi Medical University, Taiyuan, China. Written informed consent was obtained by recruited patients. Fifteen primary glioma tissues and their matched pericarcinous tissues were collected at the Department of Infection Diseases, the First Hospital of Shanxi Medical University, China, from January 1, 2014 to January 30, 2015. The 15 tumor tissues were carefully isolated from the surrounding benign tissues by a certified pathologist, and immunohistochemistry was performed by two independent pathologists to confirm the margin. In addition, none of the patients received blood transfusion, chemotherapy, or radiotherapy before surgery. All samples were immediately frozen and stored in liquid nitrogen after surgical removal until analysis.

Cell culture

Seven human hepatocellular cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

A lentivirus vector encoding complete human HOXD10/GFP open reading frame (HOXD10) and a lentivirus vector encoding a green fluorescent protein open reading frame (GFP) (as negative control, NC) were constructed by using vector purchased from Genechem Biotechnology Inc., Ltd. (Shanghai, China). The following primers were used to amplify the HOXD10 coding sequence by PCR from human genomic DNA: Forward, 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGTCCTTTCCCAACAGCTC-3' and reverse, 5'-TCCTTGTAGTCCATACCAGAAAACGTGAGGTTGGC-3'. The amplified HOXD10 gene was cloned into a mammalian expressing vector GV358, and then transferred to a lentiviral vector Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin to generate the recombinant lentiviral expression vector. A negative control was also packaged in the same manner by cloning GFP gene, defined as LM3-GFP. The transduction efficiency was measured by using a fluorescence microscope through the examination of GFP expression. The LM3 cells were transfected with HOXD10 of high titer (2×10^8 TU/ml) and negative control lentivirus expression vector particles GFP (all with a MOI of 30 transfection concentration) according to the manufacture's instructions. Cells with stable HOXD10 overexpression and negative GFP were obtained by continuous treatment with 2 μg/ml puromycin in LM3 cell lines.

RNA extraction and reverse transcription-PCR

Total RNA was extracted from the frozen tissues and harvested cells using the TRIzol rea-

gent (Invitrogen Life Technologies, Shanghai, China) according to the manufacture's instructions. For mRNA analysis, Oligo (dT) was used as primer to reverse-transcribe 1.0 μg of total RNA into cDNA using a reverse transcription kit (Qiagen, Hilden, Germany). Real-time PCR was subsequently performed in triplicate by using a 1:4 dilution of cDNA as template and the quantification was measured using the Quantitect SyBr green PCR system (Qiagen, Hilden, Germany) on a Rotorgene 6000 series PCR machine (Qiagen, Hilden, Germany). Data were analyzed using the Rotorgene software matched with the PCR machine. The primer for HOXD10 amplification was as following: Forward 5'-CCGAAGTGCAGGAGAAGGAA-3' and reverse 5'-GTGTAAGGGCACCTCTTCTTTCTG-3'. Relative expression levels were calculated using the comparative quantification feature of the Rotorgene software. All mRNA quantification data were normalized to GAPDH expression and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total proteins were extracted and then separated in 10% SDS-polyacrylamide gels, transferred to a cellulose acetate membrane (Millipore, USA). The expression of proteins were detected with primary antibodies, including rabbit anti-human HOXD10 monoclonal antibody, mouse anti-human Cyclin D1 monoclonal antibody, mouse anti-human P27 monoclonal antibody, rabbit anti-human Caspase 3 monoclonal antibody, rabbit anti-human Caspase 8 monoclonal antibody, rabbit anti-human ROCK polyclonal antibody, rabbit anti-human RHOC monoclonal antibody. All of the above antibodies were purchased from Santa Cruz Biotechnology, or a rabbit anti-human-GAPDH antibody (1:2000, Sigma, USA). After incubated with corresponding secondary antibodies, the results were visualized by a commercial ECL kit (Beyotime, China).

Plasmid transfection

When the LM3 cells were grew at 30-40% confluence on an antibiotic-free growth medium approximately 24 h before transfection, constructed plasmids were transfected into cells using using Lipofectamine 2000 (Invitrogen) transfection reagents, according to the manufacturer's instructions. Cells were collected and analyzed 48 h after treatment.

Cell cycle assay

HCCLM3 cells were transfected as previously described. 72 hours later, the cells were collected and washed with cold PBS, resuspended in 200 μl of PBS, and fixed with 100% ethanol of 800 μl at 4°C for 24 h. The fixed cells were washed twice with PBS and stained with solution containing 100 μg/ml PI and 50 ug/ml RNase (Sigma, USA) in PBS at 37°C for 20 min in the dark. Stained cells were filtered with a nylon mesh sieve to remove cell clumps and then were analyzed on a flow cytometry (BD). Data were collected and calculated by the CELL Quest and matched ModFit LT software.

Cell Proliferation and apoptosis assays

To analyze cell proliferation, cells were cultured in a 96-well plate in DMEM with 10% FBS and 100 mg/ml of penicillin/streptomycin for 72 hours. Cell proliferation rates were detected by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay (Roche, USA) according to manufacture's instructions.

To analyze cell apoptosis, cells were pre-labelled with PI and annexin V-FITC, and then measured with an apoptosis detecting kit (Invitrogen, Burlington, Canada) according to manufacture's instructions. Samples were analyzed by a flow cytometry and the results were recorded by matched CellQuest software (Becton Dickinson, San Jose, CA, USA).

Transwell migration and invasion assays

To assess migration, transfected LM3 cells of 5×10^4/well were seeded into 9-well dishes and allowed to attach by culturing. Then, these cells were cultured in serum-free medium for 24 h and harvested, resuspended in DMEM with 0.1% (w/v) BSA. These cells were placed in the top chamber of a 24-well Transwell insert (8 mM: BD Biosciences, Oxford, UK). At last, DMEM was added to the bottom well with 2% (v/v) FBS and incubated overnight at 37°C for 24 h. Migrated cells in the bottom wells were stained with crystal violet (Sigma Aldrich, St. Louis, MO) and recorded.

To assess cell invasion, the wells were prior coated with 100 μl of growth factor-reduced Matrigel (BD Biosciences) diluted in PBS (1:45), and seeded with 2.5×10^4 of cells/well in serum

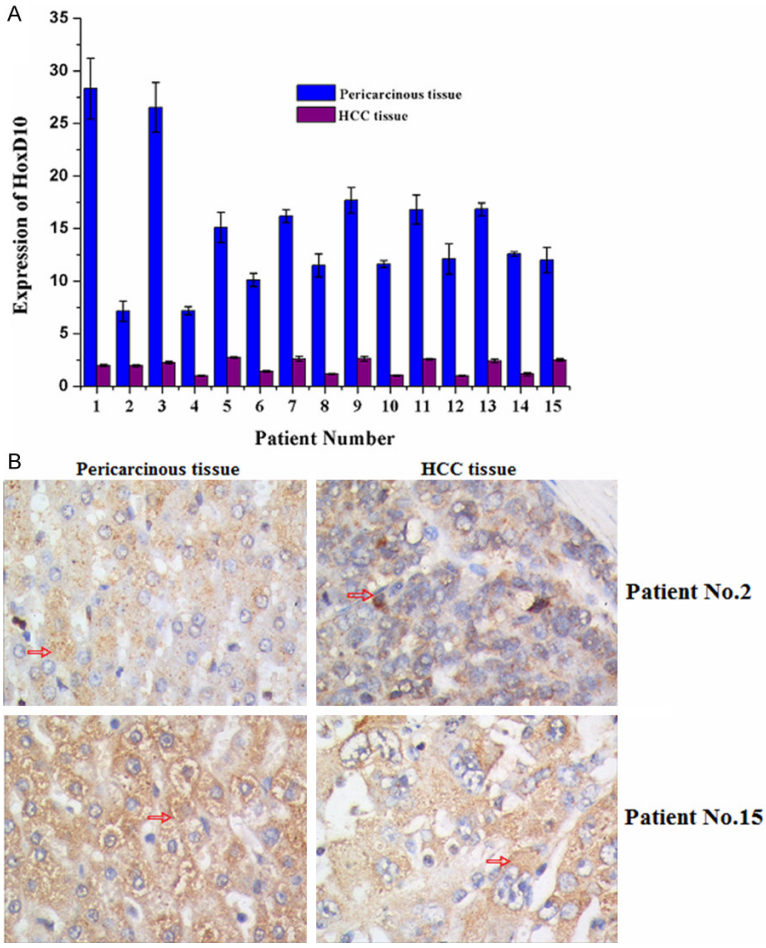


Figure 1. Decreased HOXD10 in the HCC tissues compared to pericarcinous tissues. A. RNA was extracted from 18 tumor tissue samples and patient matched pericarcinous tissues. Expression of the HOXD10 was analyzed by real-time quantitative PCR, and bars represent mean intensity level (± SEM); B. Immunohistochemical stainings for HOXD10 expression in two patient samples. The red arrows indicate the positive expression of HOXD10.

free-medium in the upper chamber for 24 h. The upper wells were placed into the transwell system with the lower wells with complete culture medium with serum and cultured for 24 h. Subsequently, cells in the upper wells were removed using a cotton swab, and the wells were washed twice with PBS before fixed with 4% paraformaldehyde and stained with 0.1% Crystal violet (Sigma Aldrich, St. Louis, MO) and air dried. The filters were removed from the well and mounted on slides. Three separate experiments were performed and the average numbers of the invaded cells were calculated for analysis.

Immunohistochemical analysis

Isolated tumor samples were fixed and processed for paraffin-embedded tumor slides. After antigen retrieval, the slides were subjected to 3% hydrogen peroxide for 10 min for endogenous peroxidase inactivation, The sections were blocked with 1% BSA to reduce non-specific staining. For IHC staining, the sections were incubated with primary antibodies against HOXD10. In negative controls, PBS was replaced the primary antibody. Washing with PBS, the slides were incubated with biotinylated-modified secondary antibody, and then conjugated horseradish peroxidase (HRP)-labeled streptavidin (Dako, Glostrup, Denmark) was added. The slides were incubated with substrate diaminobenzidine (DAB; Sigma, St. Louis, MO, USA). After staining, three high-magnification fields (×400) were randomly selected for each slide, and the images were recorded by using a microscopy (Zeiss, Germany).

Tumor formation in BALB/C nude mice

All experimental procedures involving animals were referred to the Guide for the Care and Use of Laboratory Animals (NIH publication nos. 80-23, revised in 1996) and were performed in compliance with our institutional ethical guidelines for animal experiments. After 24 h of transfection, the cells were suspended in 100 μl of PBS at a concentration of 4×10^6 cells/ml and injected into either flank of the same BALB/C female athymic nude mouse at 5-6 weeks of age.

Statistics analysis

All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) 19.0 software. The data are presented as the mean ± SEM from three separate experiments. Statistical significance was determined by paired or unpaired Student's t-test in cases of standardized expression data. Differences

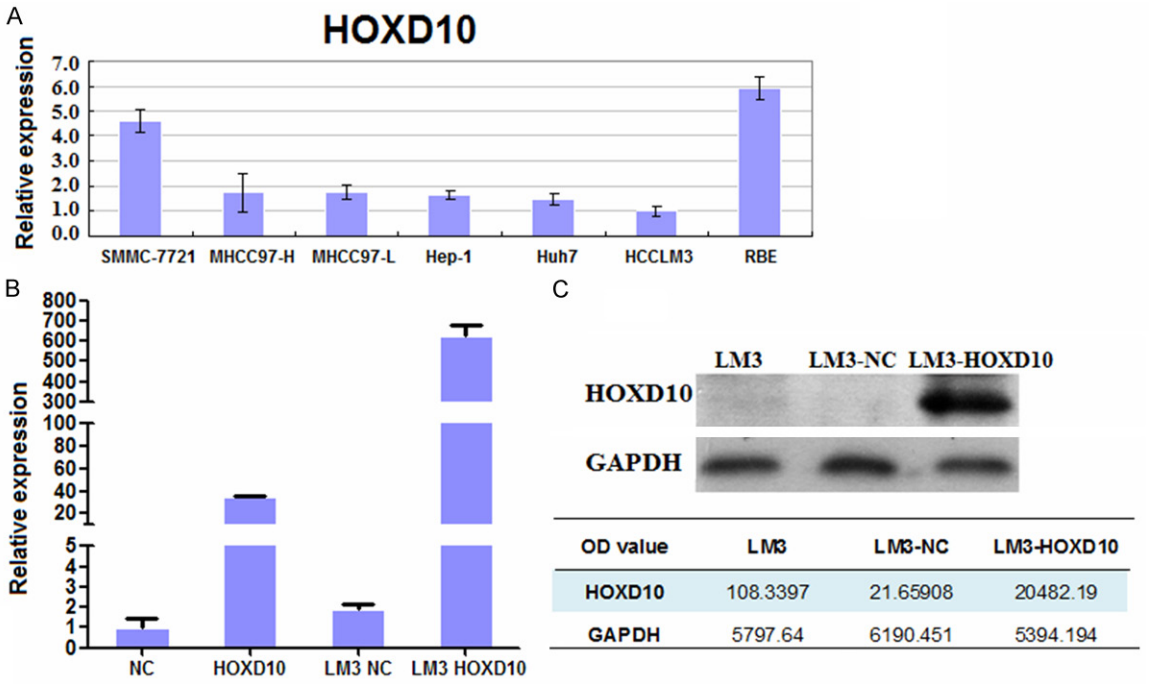


Figure 2. Certification of stable cell line LM3 with HOXD10 overexpression by Lentiviral transduction. A. Expression profiles of HOXD10 in different HCC cell lines; B and C. Relative HOXD10 mRNA and protein expression 5 days after lentivirus transfection. The graph shows the mean ± SEM; NC, negative control; HOXD10, vectors expressed HOXD10, LM3-NC, cells transduced with negative control vector; LM3-HOXD10, cells transduced with positive HOXD10 lentivirus. The data are presented as the average of triplicate values. Error bars, SEM.

were considered as statistically significant at $P<0.05$.

Results

The expression of HOXD10 was reduced in HCC tissues

To determine the role of HOXD10 in HCC, we evaluated HOXD10 expression in tumor or pericarcinous tissues from patients with HCC by using RT-PCR and IHC. In a cohort of 15 matched HCC tissues and pericarcinous tissues (adjacent to tumor with a distance >3 cm), although the HOXD10 expression levels were various among different individuals, HOXD10 was low-expressed in HCC tumor tissues while high-expressed significantly in pericarcinous tissues ($P<0.05$) (**Figure 1A**). In tissues, HOXD10 was expressed in the nucleus and the cytoplasm. This is similar to reports of a number of other HOX genes and may represent shuttling between the nucleus and the cytoplasm [18]. Further IHC analysis also confirmed the pattern with expression lower in the HCC tissues compared with that in pericarcinous tissues (**Figure 1B**). Previous reports demonstrated that HOXD10 acted as a tumor suppressor in human malignancies. Based on these results, we hypothesized that HOXD10 may be an important tumor suppressor in HCC.

Establishment of LM3 cells stably overexpressing HOXD10

We intended to investigate the role of HOXD10 in the HCC by using HCC cell lines. We chose 7 HCC cell lines (SMMC-7721, MHCC97-H, MHCC97-L, Hep-1, Huh7, HCCLM3 and RBE cells) and determined the expression of HOXD10 in these ell lines. The expression in the cultures from cell lines was different, and highest in RBE cells while lowest in HCCLM3 (short for LM3 later) (**Figure 2A**). In order to fully understand the potential inhibition role of HOXD10 in HCC, LM3 cell line with the lowest HOXD10 was used for further experiments.

Firstly, we transfected HOXD10 into LM3 cells through lentivirus. Five days after transfection, the HOXD10 mRNA and protein expressions in LM3 cells were analyzed using RT-PCR and

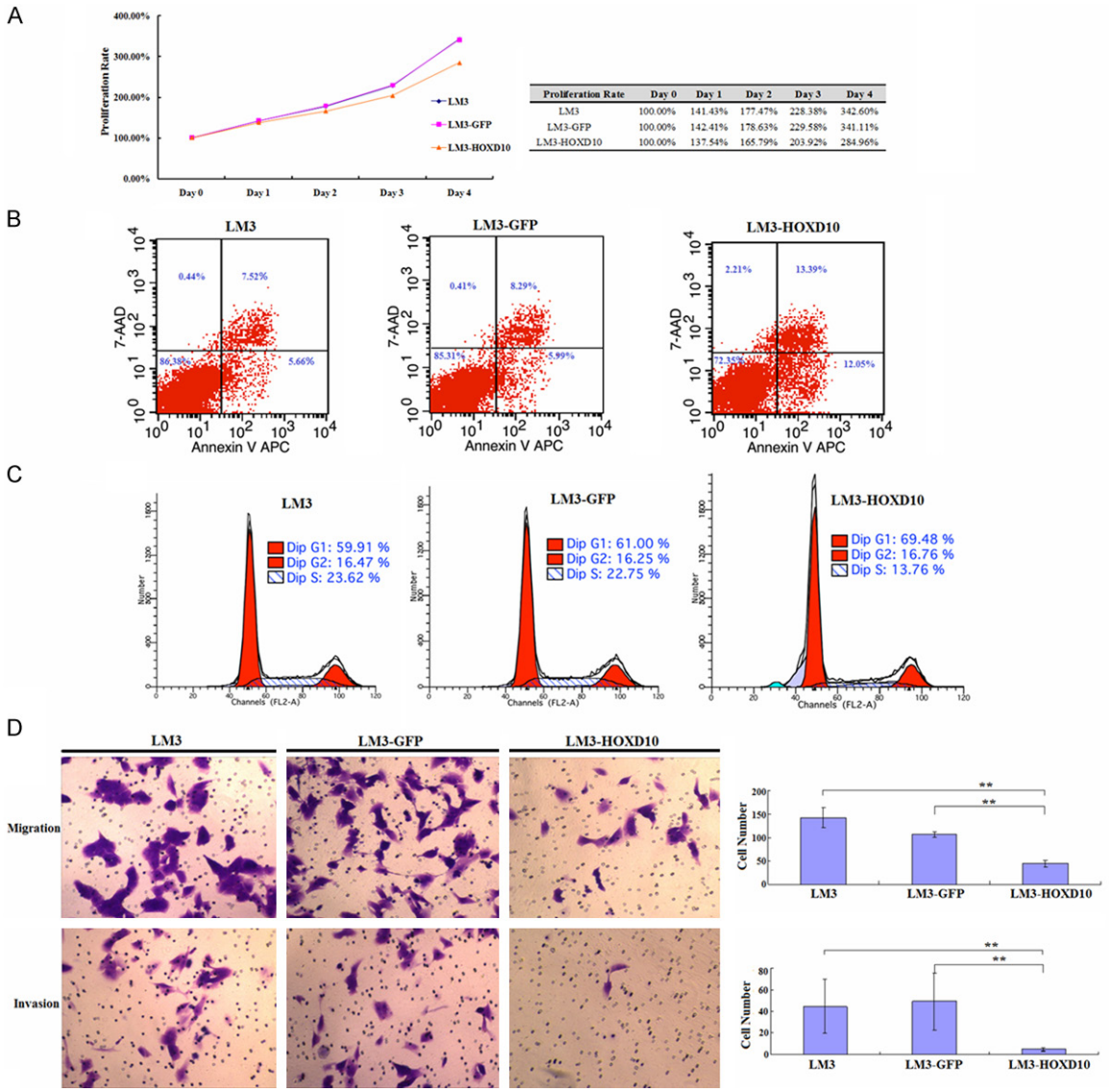


Figure 3. Effects of HOXD10 overexpression on LM3 cells. A. Cell proliferation of untransfected and stably transfected LM3 cells by an MTT assay in the first 4 days; B. Cell apoptosis of untransfected and stably transfected LM3 cells by flow cytometry; C. Cell cycle profile changes were assessed by flow cytometry using PI staining to measure the DNA content. G1 phase cell cycle arrest induced by overexpression of HOXD10 in LM3 cells; D. Cell migration and invasion were assessed by transwell experiments and crystal violet-staining. The data are presented as the average of triplicate values. Error bars, SEM; LM3-GFP, LM3 cells transfected with GFP vector; LM3-HOXD10, LM3 cells transfected with HOXD10 vector.

western blotting, respectively. The levels of HOXD10 mRNA and protein in LM3 cells transfected with recombinant lentivirus HOXD10 were significantly increased, compared with cells transfected with GFP vectors and the blank control cells (**Figure 2B** and **2C**). These results indicated that a lentivirus-mediated overexpression vector effectively and specifically induced HOXD10 overexpression in LM3 cells and stable overexpression of HOXD10 was achieved in the LM3 cell line.

Effects of HOXD10 upregulation on LM3 cell proliferation, cell cycle and apoptosis

Then, we assessed the phenotypic consequences in the manipulated stable cell lines *in vitro*. Increasing the expression of HOXD10 (refer to LM3-HOXD10 cells) resulted in a decrease in cell proliferation, and the proliferation rate in LM3-HOXD10 cells was approximate 17% lower than that in LM3 cells at the 4th day (**Figure 3A**). The percentages of early cell

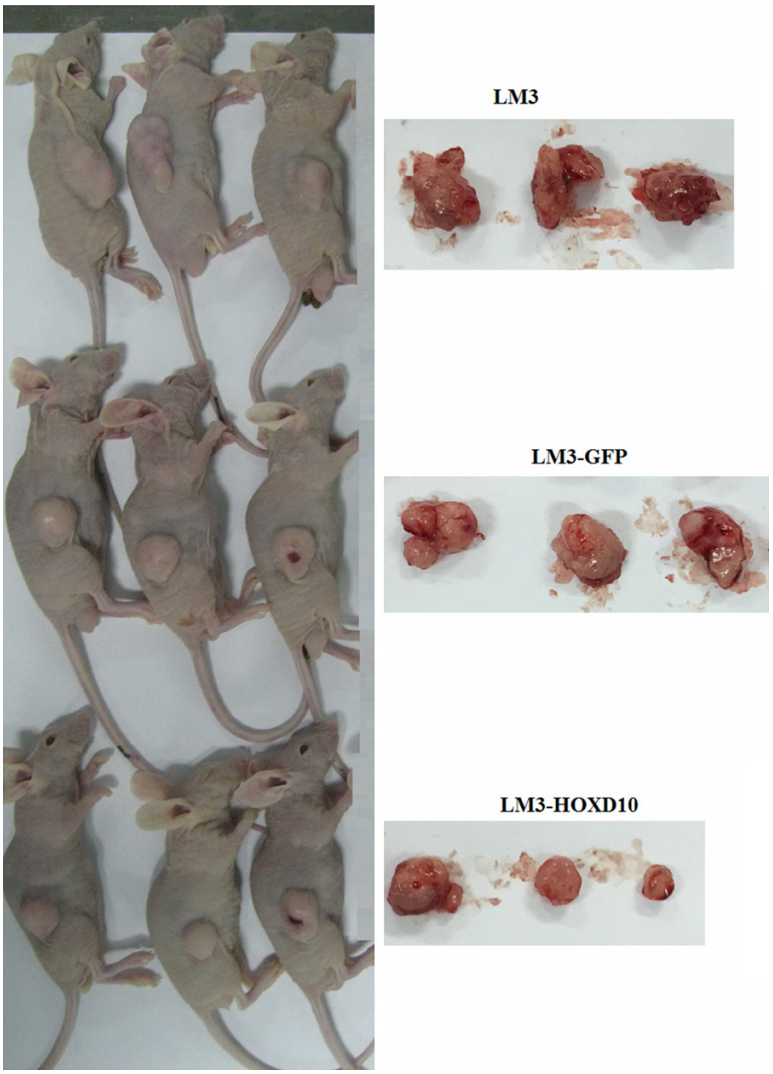


Figure 4. HOXD10 suppresses liver tumor growth in vivo. Tumor formation in nude mice and each group contained ten mice (n=10). Representative photograph of HCC tissues formed in nude mice when NC or HOXD10-treated LM3 cells were injected. LM3-GFP, LM3 cells transfected with GFP vector; LM3-HOXD10, LM3 cells transfected with HOXD10 vector.

apoptosis (right lower quadrant) and late cell apoptosis (right upper quadrant) were markedly increased in the LM3-HOXD10 cell lines. Totally, an obvious increase of proportion of apoptotic cells was seen in LM3-HOXD10 cells, from 13.18% in LM3 cells to 25.44% in LM3-HOXD10 cells (**Figure 3B**). Additionally, the effect of overexpression of HOXD10 on cell cycle progression was examined via PI staining followed by FACS analysis. As shown in **Figure 3C**, LM3-HOXD10 cells accumulated in the G1 phase compared with the control cells and showed a corresponding decrease in cell numbers in the S phase, but unchanged in G2 phase. These results indicated that stable HOXD10 overexpression inhibited LM3 cell proliferation by inducing G1/S phase arrest and apoptosis.

Effects of HOXD10 overexpression on migratory and invasive potential of LM3 cells

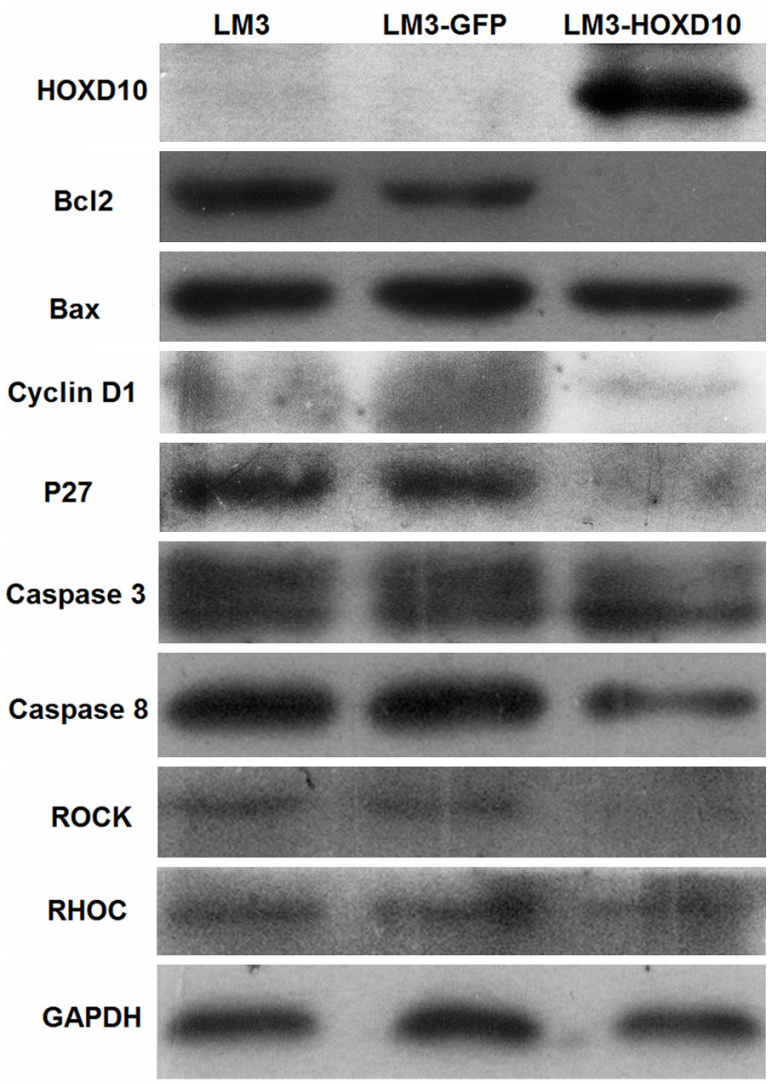
Transwell assays were conducted to confirm the effect of HOXD10 on the migratory and invasive properties of LM3 cell lines. As expected, stable overexpression of HOXD10 significantly decreased both of the migratory and invasive potentials in LM3 cells (left in **Figure 3D**), and the migrated and invaded cell numbers in HOXD10-LM3 cells were significantly lower than those in controls ($P<0.01$). These suggested that HOXD10 was associated with the metastatic capacity of HCC.

Effect of HOXD10 overexpression on tumor growth in vivo

To confirm the tumor suppressor role of HOXD10, we established a BALB/C nude mouse xenograft model using LM3 cells. The LLM3 cells were pretransfected with HOXD10, then injected into female BALB/C nude mice to form tumors. The tumor of the LM3 cells transfected with HOXD10 was significantly small relative to control groups (**Figure 4A**). Representative photographs of isolated HCC tissues formed in nude mice by transplanting HOXD10-LM3 cells showed the increased expression of HOXD10 in tumors would inhibit the tumorigenesis (**Figure 4B**). These results indicated that HOXD10 overexpression significantly inhibited the tumorigenicity of LM3 cells in the nude mouse xenograft model.

Involvement of genes in tumor-suppressive effect of HOXD10

Aforementioned results elucidated that HOXD10 inhibited LM3 cell proliferation by inducing



OD value	LM3	LM3-GFP	LM3-HOXD10
HOXD10	108.3397	21.65908	20482.19
Bcl2	15644.23	10743.57	1.005
Bax	29419.74	27017.52	18637.31
CyclinD1	5159.545	8266.125	781.3752
P27	18424.26	17633.62	2778.472
Caspase3	7957.054	6606.181	4887.668
Caspase8	7485.344	8907.736	2576.594
ROCK	1209.95	1428.727	391.1329
RHOC	1118.347	1375.414	643.3576
GAPDH	5797.64	6190.451	5394.194

Figure 5. Protein profiles of Bcl2, Bax, Cyclin D1, P27, Caspase 3, Caspase 8, ROCK and RHOC in LM3 cells. LM3-GFP, LM3 cells transfected with GFP vector; LM3-HOXD10, LM3 cells transfected with HOXD10 vector.

G1/S phase arrest and apoptosis. Furthermore, the effects of upregulated HOXD10 on the expression of important regulators in these processes were examined using western blot. As shown in **Figure 5**, the protein expression of Bcl2, Caspase 8, Cyclin D1 and P27 was significantly inhibited in the HOXD10-LM3 group compared with the control groups, while no significant change was observed in the expression of Bax and Caspase 3. These results suggested that overexpression of HOXD10 suppressed the growth of LM3 cells, possibly partly by disturbing the expression of Cyclin D1, P27 and Bcl-2. Upregulated expression of HOXD10 resulted in the markedly decreased expression of tumor metastatic-associated gene ROCK, but unchanged expression of RHOC. It was suggested that HOXD10 may inhibit HCC metastasis, including cell migration and invasion by reducing ROCK signaling. As increased expression of ROCK was often observed in human cancers, typically more invasive and metastatic phenotypes, the upregulated HOXD10 could inhibited the expression of ROCK. This suggested that HOXD10 might be used in cancer therapy for blocking of ROCK in HCC.

Discussion

To date, liver resection and LT is the mainstay and golden standard strategy for the treatment of HCC, but the selection criteria for both are controversial according to different clinical guidelines, which results in difficulty in selecting the most suitable therapy in treating HCC [21, 22]. Meanwhile, the golden standards in selecting patients for LT, still possess some limitations that may ignore and exclude some potential patients. Besides of surgical treatment, sorafenib is the first and only available systemic therapy medicine for HCC approved by FDA [23]. Never-

theless, plenty of emerging agents are still under clinical trials and yet show unpromising results. Therefore, it is necessary to investigate the molecular mechanisms involved in the characteristic development and progression of HCC to find out effective therapeutic methods.

Members in HOX family are reported to have a role in the development of various human cancers. For example, HOXA5, HOXA9 and HOXB13 are downregulated in primary breast cancers,, whereas HOXB9 and HOXD10 are upregulated in primary breast cancers [24]. Similar changes in the expression of other HOX genes have been found in thyroid cancers [25]. In HCC, lower expression of HOXA13, HOXA7 and HOXD110 is found in HCC tissues compared with non-tumor tissues [26]. Although low expression of HOXD10 have previously been described in HCC cell line MHCC97-L [27], this is the first study to explore the functional roles of HOXD10 in HCC development. We also demonstrated that HOXD10 expression was involved in pathways of HCC metastases.

In the present study, IHC staining and RT-PCR for HOXD10 was performed in a cohort of HCC patients. HOXD10 was expressed in the nucleus and the cytoplasm in tissues, and the percentage of positive-HOXD10 expressed cells in HCC tissues was markedly lower than that in corresponding pericarcinous tissues. These suggested HOXD10 was involved in the malignant progression of HCC. These results were consistent with those of previous studies in gastric and ovarian carcinoma.

To evaluate the biological functions of HOXD10 in HCC pathogenesis, we used a lentiviral-mediated HOXD10 overexpression vector to effectively and sustainably increase HOXD10 expression in the HCC cell line LM3, which expressed the lowest HOXD10 among the 7 HCC cell lines. We found that ectopic overexpression of HOXD10 significantly inhibited LM3 cell proliferation, which can be partly attributed to the induction of a G1-phase cell cycle arrest and increased apoptosis *in vitro,* suggesting that HOXD10 plays an important suppressive role in HCC cells. The decreased expression of genes related to cell cycle and apoptosis, including Caspase 3, Caspase 8, Cyclin D1, Bcl2 and P27, was accordant with the findings. The HOXD10 also suppressed tumor formation *in vivo* in nude mice. Taken together, HOXD10 played an important role in tumorigenesis of HCC.

Additionally, the overexpression of HOXD10 markedly inhibited the cell migration and invasion potential of LM3 cells, at least partly, by downregulating the expression of invasive factors ROCK, but not that of RHOC. Thus, more studies are needed to determine the precise molecular mechanisms by which HOXD10 overexpression inhibits migration and invasion in LM3 cell lines.

The results of the present study provide evidences for the tumor-suppressive properties of HOXD10 in HCC cell lines. However, the underlying mechanisms of HOXD10 in the malignant progression of HCC, particularly the downstream signaling pathways, remain unclear. ROCK is a major downstream target of the small GTPase RhoA, which also plays important roles in human cancers through regulating cell motility, metastasis, and angiogenesis [28].

Previous studies have reported the biological roles of HOXD10 in certain tumor cell types, which are controversial in different tumors. For example, the HOXD10 were higher expressed in oral squamous cell carcinoma samples relative to those in normal oral mucosa. Down-expression of HOXD10 decreased cell migration and invasion in HCC cell lines MHHC97H and MHCC97L cells through miR-224/HOXD10/p-PAK4/MMP-9 signaling pathway [27]. In addition, HOXD10 could not inhibit the tumor growth of primary breast cancer but markedly suppress the formation of lung metastases in a SCID mouse tumor model. Thus, whether HOXD10 is anti-tumorigenic or pro-tumorigenic gene depending on the cell context and the type of stimulus [29]. In present study, overexpressed HOXD10 may result in the inhibition of HCC malignant progression. However, the precise mechanism of downregulation of HOXD10 in HCC is not clear. In gastric carcinoma, the downregulation or loss of HOXD10 expression occurred due to promoter methylation [29]. By contrast, HOXD10 was negatively regulated by miR-10b via a specific target site within the 3'UTR of HOXD10 at the post-transcriptional level in breast cancer [30]. However, the specific mechanism of HOXD10 downregulation in HCC remains to be investigated.

In conclusion, HOXD10 can suppress HCC development and progression possibly via the

ROCK pathway, suggesting that HOXD10 is a potential target for HCC treatment.

Disclosure of conflict of interest

None.

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References

[1] Shetty S, Sharma N and Ghosh K. Epidemiology of hepatocellular carcinoma (HCC) in hemophilia. Crit Rev Oncol Hematol 2016; 99: 129-133.

[2] Wang Z, Ke ZF, Lu XF, Luo CJ, Liu YD, Lin ZW and Wang LT. The clue of a possible etiology about spontaneous regression of hepatocellular carcinoma: a perspective on pathology. Onco Targets Ther 2015; 8: 395-400.

[3] Knudsen ES, Gopal P and Singal AG. The changing landscape of hepatocellular carcinoma: etiology, genetics, and therapy. Am J Pathol 2014; 184: 574-583.

[4] Wang SD, Chen BC, Kao ST, Liu CJ and Yeh CC. Genistein inhibits tumor invasion by suppressing multiple signal transduction pathways in human hepatocellular carcinoma cells. BMC Complement Altern Med 2014; 14: 26.

[5] Kim SU, Ahn SH, Park JY, Kim do Y, Chon CY, Choi JS, Kim KS and Han KH. Prediction of postoperative hepatic insufficiency by liver stiffness measurement (FibroScan((R))) before curative resection of hepatocellular carcinoma: a pilot study. Hepatol Int 2008; 2: 471-477.

[6] Nishikawa H and Osaki Y. Clinical significance of therapy using branched-chain amino acid granules in patients with liver cirrhosis and hepatocellular carcinoma. Hepatol Res 2014; 44: 149-158.

[7] Talukdar FR, Ghosh SK, Laskar RS, Kannan R, Choudhury B and Bhowmik A. Epigenetic pathogenesis of human papillomavirus in upper aerodigestive tract cancers. Mol Carcinog 2015; 54: 1387-1396.

[8] Wilmott JS, Zhang XD, Hersey P and Scolyer RA. The emerging important role of microRNAs in the pathogenesis, diagnosis and treatment of human cancers. Pathology 2011; 43: 657-671.

[9] Teng YC, Shen ZQ, Kao CH and Tsai TF. Hepatocellular carcinoma mouse models: Hepatitis B virus-associated hepatocarcinogenesis and haploinsufficient tumor suppressor genes. World J Gastroenterol 2016; 22: 300-325.

[10] Loes IM, Immervoll H, Sorbye H, Angelsen JH, Horn A, Knappskog S and Lonning PE. Impact of KRAS, BRAF, PIK3CA, TP53 status and intra-individual mutation heterogeneity on outcome after liver resection for colorectal cancer metastases. Int J Cancer 2016.

[11] Bhatlekar S, Fields JZ and Boman BM. HOX genes and their role in the development of human cancers. J Mol Med (Berl) 2014; 92: 811-823.

[12] Javed S and Langley SE. Importance of HOX genes in normal prostate gland formation, prostate cancer development and its early detection. BJU Int 2014; 113: 535-540.

[13] Gray S, Pandha HS, Michael A, Middleton G and Morgan R. HOX genes in pancreatic development and cancer. JOP 2011; 12: 216-219.

[14] Plowright L, Harrington KJ, Pandha HS and Morgan R. HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer). Br J Cancer 2009; 100: 470-475.

[15] Abe M, Hamada J, Takahashi O, Takahashi Y, Tada M, Miyamoto M, Morikawa T, Kondo S and Moriuchi T. Disordered expression of HOX genes in human non-small cell lung cancer. Oncol Rep 2006; 15: 797-802.

[16] Vardhini NV, Rao PJ, Murthy PB and Sudhakar G. HOXD10 expression in human breast cancer. Tumour Biol 2014; 35: 10855-10860.

[17] Xiao H, Li H, Yu G, Xiao W, Hu J, Tang K, Zeng J, He W, Zeng G, Ye Z and Xu H. MicroRNA-10b promotes migration and invasion through KLF4 and HOXD10 in human bladder cancer. Oncol Rep 2014; 31: 1832-1838.

[18] Yang H, Zhou J, Mi J, Ma K, Fan Y, Ning J, Wang C, Wei X, Zhao H and Li E. HOXD10 acts as a tumor-suppressive factor via inhibition of the RHOC/AKT/MAPK pathway in human cholangiocellular carcinoma. Oncol Rep 2015; 34: 1681-1691.

[19] Hakami F, Darda L, Stafford P, Woll P, Lambert DW and Hunter KD. The roles of HOXD10 in the development and progression of head and neck squamous cell carcinoma (HNSCC). Br J Cancer 2014; 111: 807-816.

[20] Hu X, Chen D, Cui Y, Li Z and Huang J. Targeting microRNA-23a to inhibit glioma cell invasion via HOXD10. Sci Rep 2013; 3: 3423.

[21] Raoul JL. Natural history of hepatocellular carcinoma and current treatment options. Semin Nucl Med 2008; 38: S13-18.

[22] Guthle M and Dollinger MM. [Epidemiology and risk factors of hepatocellular carcinoma]. Radiologe 2014; 54: 654-659.

[23] Kudo M, Matsui O, Izumi N, Iijima H, Kadoya M, Imai Y, Okusaka T, Miyayama S, Tsuchiya K, Ueshima K, Hiraoka A, Ikeda M, Ogasawara S, Yamashita T, Minami T and Yamakado K. JSH Consensus-Based Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma: 2014 Update by the Liver Cancer Study Group of Japan. Liver Cancer 2014; 3: 458-468.

[24] Hur H, Lee JY, Yun HJ, Park BW and Kim MH. Analysis of HOX gene expression patterns in human breast cancer. Mol Biotechnol 2014; 56: 64-71.

[25] Cantile M, Scognamiglio G, La Sala L, La Mantia E, Scaramuzza V, Valentino E, Tatangelo F, Losito S, Pezzullo L, Chiofalo MG, Fulciniti F, Franco R and Botti G. Aberrant expression of posterior HOX genes in well differentiated histotypes of thyroid cancers. Int J Mol Sci 2013; 14: 21727-21740.

[26] Cillo C, Schiavo G, Cantile M, Bihl MP, Sorrentino P, Carafa V, M DA, Roncalli M, Sansano S, Vecchione R, Tornillo L, Mori L, De Libero G, Zucman-Rossi J and Terracciano L. The HOX gene network in hepatocellular carcinoma. Int J Cancer 2011; 129: 2577-2587.

[27] Li Q, Ding C, Chen C, Zhang Z, Xiao H, Xie F, Lei L, Chen Y, Mao B, Jiang M, Li J, Wang D and Wang G. miR-224 promotion of cell migration and invasion by targeting Homeobox D 10 gene in human hepatocellular carcinoma. J Gastroenterol Hepatol 2014; 29: 835-842.

[28] Rath N and Olson MF. Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. EMBO Rep 2012; 13: 900-908.

[29] Wang L, Chen S, Xue M, Zhong J, Wang X, Gan L, Lam EK, Liu X, Zhang J, Zhou T, Yu J, Jin H and Si J. Homeobox D10 gene, a candidate tumor suppressor, is downregulated through promoter hypermethylation and associated with gastric carcinogenesis. Mol Med 2012; 18: 389-400.

[30] Ma L, Teruya-Feldstein J and Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007; 449: 682-688.