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Original Article

Knockdown of kinectin by shRNA causes cell growth and migration inhibition in human hepatoma BEL-7404 cells

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Abstract: Kinectin has initially been proposed to be a membrane anchor in ER for kinesin for intracellular organelles mobility. Previously, we reported that antibody to kinectin may serve as a serum marker for hepatocellular carcinoma. Here the expression of Kinectin in HCC tissues and human HCC cell lines, effects of shRNA targeting kinectin on proliferation, migration and invasion in HCC cell line BEL-7404 were investigated. The expression of kinectin mRNA and protein were detected by QRT-PCR and immunohistochemical staining, respectively. Semi-quantitative RT PCR and western blot were performed to detedct mRNA and protein expression of kinectin in three human HCC cell lines HepG2, BEL-7704, SMMC 7721 and one normal human liver cell line HL-7702. Furthermore, silencing of kinectin by shRNA was performed in the cell line BEL-7404. Cell growth was measured by MTT assay, and cell migration and invasion were measured by transwell assay *in vitro*. The expression of kinectin mRNA present no significant different among the HCC tissues, corresponding adjacent tissues and normal liver tissues, but Kinectin proteins showed higher expression in cancerous regions than paired adjacent non-cancerous regions. Patients with high kinectin expression were statistically susceptible to liver cirrhosis than those with low expression. Kinectin knockdown in BEL-7404 demonstrated that the shRNA inhibition resulted in a statistically significant reduction in cell growth, cell mobility and cell invasion. Our finding present here provides a molecular basis for further investigation into the possible mechanism at proliferation, migration and invasion in HCC.

Keywords: Tumor associated antigens, kinectin, hepatocellular carcinoma, proliferation, migration, invasion

Introduction

Hepatocellular carcinoma (HCC) is the most common malignant tumor of the liver worldwide, and more than fifty percents occurs in China. In spite of advances in surgery, liver transplantation and newer pharmaco/biological therapies, its survival rate remains still low [1]. As we know, HCC is highly associated with multi-gene, multi-factor, multi-step process. Many previous reports have suggested cellular changes and etiological agents can cause HCC, but the molecule pathogenesis of HCC is poorly understood.

Kinectin was initially identified as a kinesin receptor in the endoplasmic reticulum from chick embryonic brain [2]. The gene of kinectin has been separated from fox, mouse, and human genome, and human kinectin protein is about 160 kDa [3-7]. Kinectin can combine to kinesin through its C terminus and stimulate microtuble-dependented organelle mobility [8, 9]. This activity can be facilitated by Rho GTPase [10]. Kinectin-kinesin interaction also facilitates endoplasmic reticulum transport along microtubles to support FA growth [11]. In addition, kinectin anchored translation elongation factor-1 complex in ER membrane to regulate the membrane/secretory protein and cytosolic protein synthesis [12, 13]. Previously we found that Kinectin was a HCC associated antigen by SEREX [14]. We performed *petit* serology to analyze the prevalence of IgG against kinectin

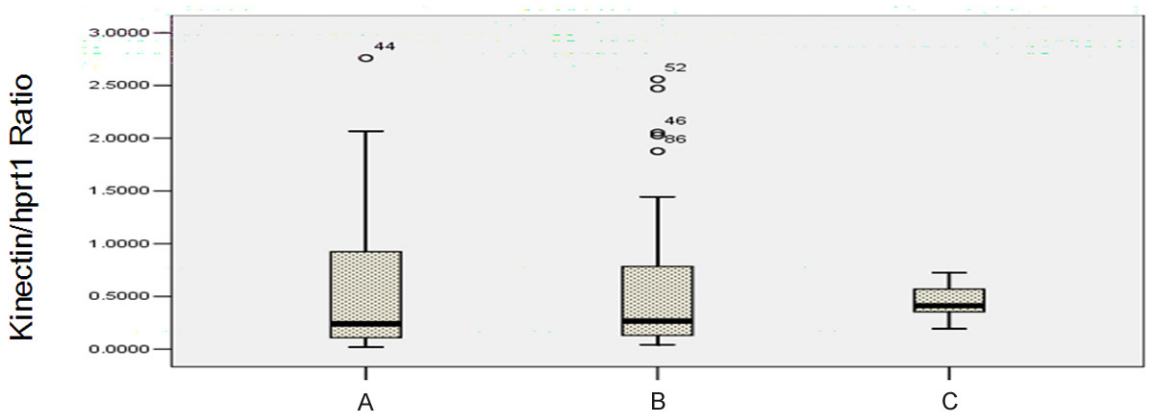

Figure 1. QRT-PCR analysis of kinectin mRNA extracted from HCC tissues, corresponding adjacent non-cancerous tissues and normal hepatic tissues. A pair of primers located in the kinectin conserved region was used to determine the mRNA level of all kinectin variants in 46 HCC tissues, corresponding adjacent tissues and 8 normal 9 cases of nomal hepatic tissues by QRT-PCR. A. HCC tissues, B. Corresponding adjacent non-cancerous tissues, C. Normal hepatic tissues.

in serum from tumor patients, and found that antibody to kinectin presents in a significant number of HCC patients. In contrast, no antibody was detected in glioma, melanoma, lymphoma, renal cell carcinoma, HBV, HCV, cirrhosis and health individuals [15]. In addition, Wang et al. also isolated kinectin cDNA clones with autologous and allogeneic sera from HCC patients by the same approach [16]. Both of these studies suggest that autoantibody to kinectin could be a reliable serum marker for screening HCC patients, and kinectin expression in HCC maybe contribute to the molecule pathogenesis of HCC.

However, the expression of Kinectin in HCC tumor tissues and its function in molecule pathogenesis of HCC is not investigated. So the purpose of this study was to compare the RNA and protein expression of kinectin in HCC tissues and corresponding tissues, and knockdown of kinectin by shRNA-mediated RNA interference in hepatocellular carcinoma cells BEL-7404 in order to identify whether the expression of kinectin is involved in HCC tumorigenesis.

Material and methods

Clinical tissue sample

HCC and corresponding adjacent tissues (distance from the tumor border exceeding 2 cm) were obtained from 46 patients HCC who underwent surgery for HCC and diagnosed as HCC by histological examination at The First Affiliated Clinic Hospital of Guangxi Medical University from 2004 to 2008. And 9 normal hepatic tissues are come from 5 patients with hepatic hemangioma, 2 patients with hepatolithiasis, 1 patient with traumatic liver rupture and 1 patient with acute cholecystitis. All these specimens were collected, immediately frozen in liquid nitrogen and subsequently stored at -70°C for quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) to detect kinectin mRNA expression level. Another 53 surgical specimens for immunochemistry detection were obtained from 53 patients in the same hospital from 2008 to 2009, and they were fixed in formalin, processed through a graded ethanol series, embedded in paraffin immediately. The clinicopathologic data were recorded in detail including gender, age, liver cirrhosis, Edmondson stage, tumor size (cm) and number of tumor, hepatitis B surface antigen, α-fetoprotein (AFP), venous invasion and nodules.

This study was performed in accordance with the guidelines of the Ethical Committee and informed written consent was obtained from all patients before surgery.

Immunohistochemistry

For immunohistochemical examinations, the surgically obtained tissue samples were formalin fixed and paraffine embedded by using the usual procedure. Serial sections 4-μm thick

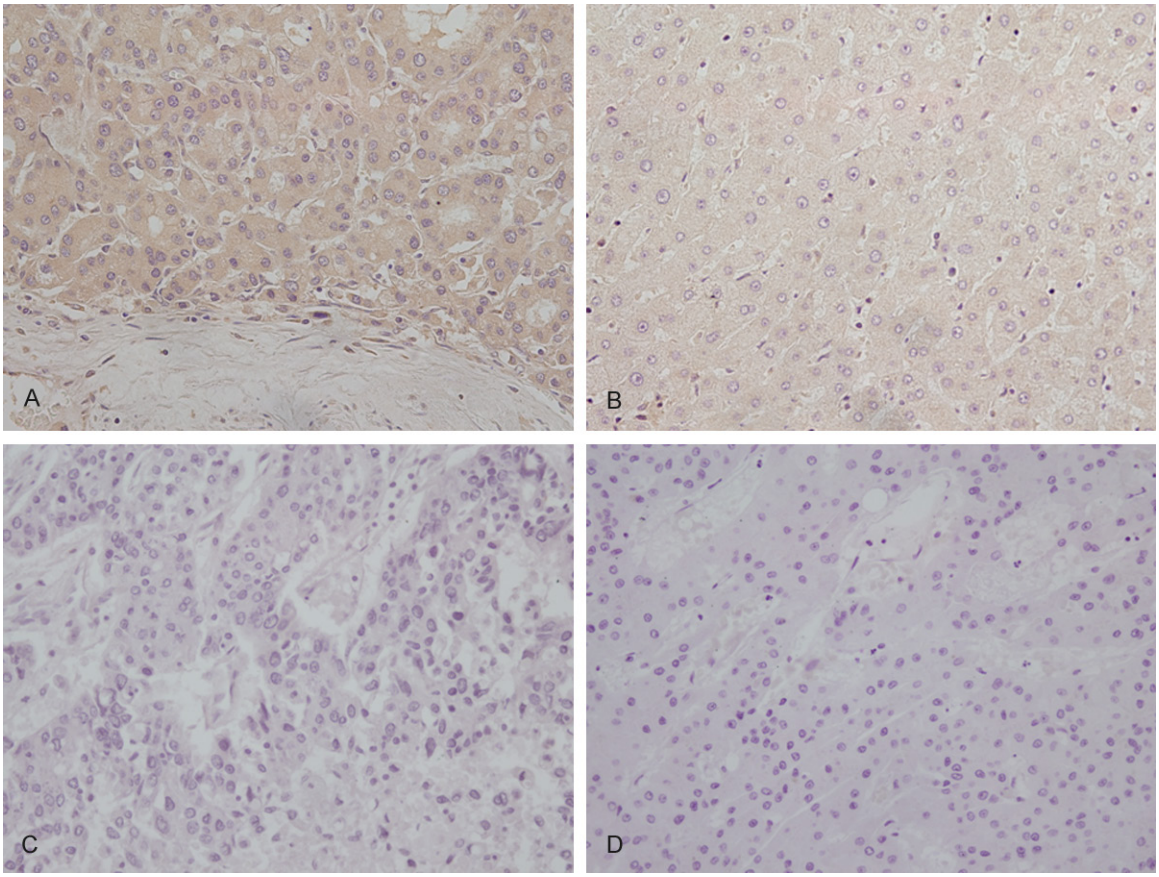

Figure 2. Immunohistochemical staining pattern of Kinectin in hepatocellular carcinoma (HCC) and corresponding adjacent tissues 200x. Kinectin expression is localized to the cytoplasm of tissues in HCC tissues (A) and corresponding adjacent tissues (C). No positive reactivity was observed in these tissues probed with pre-immune serum (B, D).

were mounted on coated slides and the slides were blocked endogenous peroxidase by 0.3% H_2O_2 and unspecific combination with goat serum by dilution of 1:10. Then primary kinectin antibody is applied by a dilution of 1:300 overnight at 4°C, followed by HRP- goat anti-rabbit secondary antibody for 1 h at room temperature. At the end the target protein expression were detected with diaminobenzidine (DAB). Negative controls were performed by replacing the primary antibody with rabbit ascite fluid.

Immunostained sections were measured quantitatively using Image Pro Plus (v. 6.0) software (Media Cybernetics), using the method introduced by Xavier et al. [17]. For each microscopic field, the kinectin-positive area (brown or yellow) was automatically calculated by IPP, At least 5 randomly selected 40 high-power fields were evaluated and scored for the degree of positive expression. The mean integrated optical density (mean IOD) of kinectin positive expression were evaluated and scored by Image-pro plus. The mean density was calculated by this integrated optical density of positive area divided by the corresponding positive area in the microscopic field.

Cell lines

There are four human cell lines used in this study. Three human hepatocellular carcinoma lines BEL-7404, SMMC-7721 and HepG2 and one human immortalized hepatocellular normal cell line HL-7702 were cultured in DMEM (Hyclone) containing 10% new fetal bovine serum in 5% CO_2 incubation at 37°C. The kinectin expression in these cell lines were detected by semi-quantitative RT-PCR and western blot respectively. All of these experiments were preformed in triple.

Table 1. Correlation between Kinectin expression and clinicopathological parameters in HCC

Factors	Cases	Protein expression	*P*-value
Age			
> 50	25	0.052 ± 0.025	0.361
≤ 50	28	0.061 ± 0.023	
Gender			
Male	47	0.060 ± 0.024	0.102
Female	6	0.067 ± 0.013	
HBSAG			
Positive	44	0.061 ± 0.023	0.310
negative	8	0.055 ± 0.30	
AFP			
> 50 μg/L	32	0.061 ± 0.026	0.269
≤ 50 μg/L	21	0.060 ± 0.026	
Tumor size			
> 50 mm	26	0.060 ± 0.022	0.679
≤ 50 mm	27	0.060 ± 0.024	
Lymphatic invasion			
present	2	0.029 ± 0019	0.687
absent	51	0.061 ± 0273	
TNM stage			
I-II	16	0.063 ± 0.024	0.454
III-IV	37	0.059 ± 0.021	
Tumor capsule			
absent	33	0.0549 ± 0.022	0.121
present	23	0.061 ± 0.026	
Live cirrhosis			
absent	19	0.059 ± 0.021	0.034
present	34	0.061 ± 0.028	
Tumor number			
≤ 5	39	0.060 ± 0.024	0.593
≥ 5	14	0.061 ± 0.022	
Tumor Grade			
III	10	0.066	0.570
II	35	0.057	
I	8	0.064	

Transfection of HCC cell line BEL-7404 with shRNA

The interference vector pGUP6/GFP/Neo was used to express double-stranded sequence from the U6 RNA polymerase III promoter. A pair of oligonucleotides which has been exploited and confirmed effective by Niovi Santama *et al.* was cloned into the interference vector. Transfection was performed on BEL-7404 by the lipofectamine 2000 method (Invitrogen, Carlsbad, CA), and the target stable cells (KNT-KD) selected by G418 (500 μg/ml). Both untransfected BEL-7404 and stable cells (KNT-NC) harbouring interference vector containing total random sequence which share the same oligonucleotides with target sequence were used as controls.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out to analyze the mRNA expression of kinectin in cell lines. Total RNA were extracted from normal human hepatic cell HL-7702, human HCC cells HepG2, SMMC-7721, BEL-7404, KNT-NC and KNT-KD using RNA extraction kit. cDNA was synthesized from 3 μg total RNA using revertaid™ first strand cDNA synthesis kits (Fermentas). The following primers were used: kinectin: 5'-CAGCAAGTTCGTGAGCAGATGG-3', 5'-CAACCTAGCATAATCCTGGCGTAG-3' (150 bp); GAPDH: 5'-TGGCGTCGTGATTAGTGATGATG-3', 5'-CAGAGGGCTACAATGTGATGGC-3' (180 bp). The following PCR cycling parameter were employed: at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, 59°C for 1 min, 72°C for 30 s and then 72°C for 8 min. Furthermore, all PCR products were separated by 1.5% agarose gels, and the PCR products were separated by agar gels and analyzed by quantity one software. All of these experiments were preformed in triple.

Western blotting analysis

The kinectin protein expression was assayed by western blot. Total protein extraction from HCC cells were according to the manufacturer' protocol, followed by protein concentration measurement by Bradford method. Fifty micrograms of protein per sample was electrophoresed on 7.5% SDS-PAGE and semi-dry electrophoreticly transferred to PVDF membrane at room temperature for 20 min. Blots were incubated in 5% nonfat milk to block nonspecific binding sites for 1 h, and then incubated with the primary antibody of rabbit polyclonal anti-human kinectin (Santa Cruz Biotechnology) and rabbit polyclonal anti human GAPDH Ab for 1 h. The membrane was washed Three times for 5 min each with PBST secondary antibody were diluted 1:5000 and incubated for 1 h at room temperature. Detection protein expression was performed with diaminobenzidine (DAB).

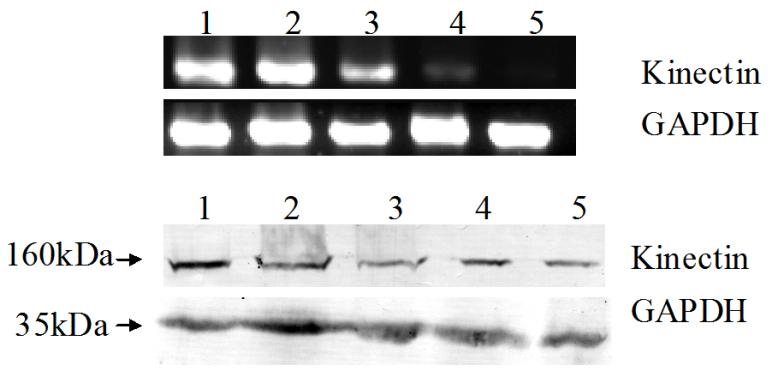

Figure 3. shRNA-mediated down regulation of Kinectin expression in BEL-7404 cells. Total RNA was extracted from 7404 (nontransfection), KNT-NC (cells transfected with vector which don't interfere the studied intrinsic gene) and KNT-KD (cells transfected with pGPU6/KNT shRNA). Semi-quantitative RT-PCR and western blot was performed to analyze kinectin mRNA and protein level respectively. 1. BEL-7404, 2. KNT-NC, 3-5. KNT-KD.

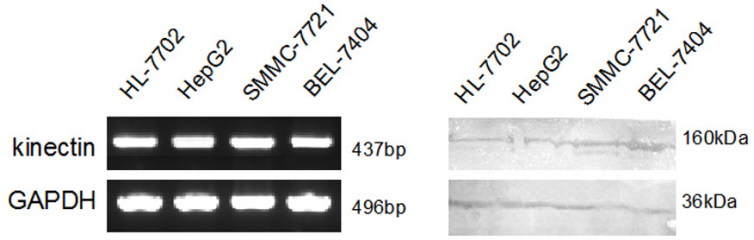

Figure 4. mRNA and protein expression of kinectin in Hepatocellular cell lines. mRNA and protein expression of kinectin in Hepatocellular cell lines Total RNA and protein were extracted from above cell lines, and RT-PCR and Western Blot were performed as described under "Materials and methods". The gel on the left side represents RT-PCR results, and the gel on the right side represents western blots. These figures indicated results from three independent experiments.

In vitro analysis of cell proliferation

Cell proliferation was measured by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenylte-trazoliumbromide (MTT) assay. Cells were seeded in 96-well plates at a density of 1,000 cells/well. The sample were assayed every 24 h for 6 days. MTT were added for at 50 μg/well for 4 h at 37°C and discard the super liquid, followed by adding DMSO 200 μL/well to solubilizing formazan product. After shaking the plates for 10 min, the optical densities were measured at 630 nm immediately. This results were confirmed in three independent experiments.

In vitro cell migration and cell invasion analysis

Cell migration was investigated using 24-well transwell migration plates (Corning Costar, Schiphol-Rijk, Netherland). Prior to plating, cells were serum starved for 12 hrs. The upper chamber was filled with 100 μl of cell suspension (1×10^5 cells) in DMEM/0.1% BSA. The lower chamber contained 600 μl of the DMEM/10% serum. After 24 h, cells remaining on the top side of the filter were removed by soft mechanical scraping. Then the filters were fixed with methanol and stained with hematoxylin and eosin after 48 h's incubation. And the number of cells migrating to the bottom of the filter was counted using a light microscope (in each chamber, six fields were counted at 200x magnification for each condition). Cell invasion assay were performed as the same as cell mobility except for membrane coated by Matrigel® matrix solution (3 mg/ml). All of these experiments were preformed in triple.

Statistical analysis

Results are expressed as mean values ± SD. All statistical analysis was performed with SSPS (v. 18.0) software. Mann-Whitney U test was used for the intergroup comparisons. Correlation analysis between kinectin expression and clinicopathological data was performed using the student's *t*-test (two-tailed) or χ^2. A *P*-value of < 0.05 was considered statistically significant.

Results

Expression of kinectin in HCC tumor tissues with clinicopathological characteristics

Initially, we analyzed kinectin mRNA expression in 46 pairs of specimens obtained from HCC patients and 9 cases of normal hepatic tissues. Kinectin gene has four alternative splicing regions at its C-terminus in HCC tissues which maybe constitute multiple variants [14]. Therefore a pair of primers located in the kinectin conserved region was used to determine the

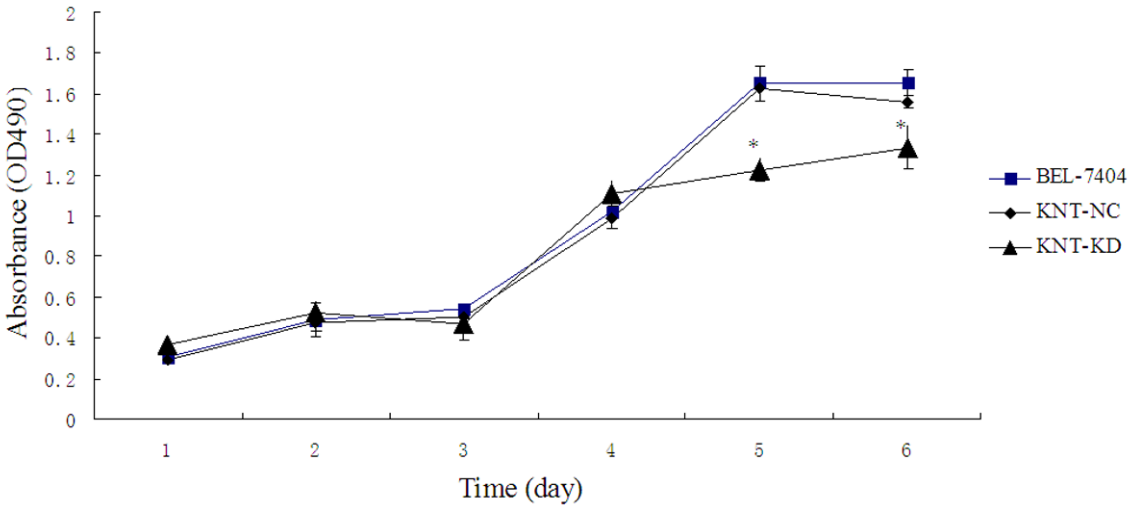

Figure 5. Inhibition of kinectin inhibits cell growth in BEL-7404. Cell growth rate was assayed daily for 6 days in all. Assays were performed in triplicate and are representative for three independent experiments. Values are means ± SD. *Significantly different compared with the noninfection group BEL-7404 and the random sequence group KNT-NC at day 5 and the day 6 of time course, $P < 0.05$.

mRNA level of all kinectin variants in HCC tissues by QRT-PCR. And the results showed that kinectin mRNA expression present no significant different among the HCC tissues, corresponding adjacent tissues and normal hepatic tissues (**Figure 1**).

RNA expression levels do not always reflect levels of translated protein, so we further assessed the kinectin protein expression in HCC tissues and corresponding adjacent tissues by immunochemistry. Immunohistochemical staining for kinectin reveals cells with concentrated staining in the cytoplasm. Kinectin expression in the cytoplasm in HCC tissues with enhanced expression than corresponding adjacent tissues. Representative images of kinectin-positive HCC and the corresponding adjacent tissues are shown in **Figure 2**. The mean density of kinectin expression in HCC and adjacent tissues were 0.067 ± 0.020 and 0.048 ± 0.024 respectively using the IPP software (6.0). Compared with those in corresponding adjacent tissues, kinectin protein expression in cytoplasm were increased significantly ($P < 0.05$).

Then we analyzed the relationship between kinectin protein expression and clinic pathological features by statistical software. The results showed that kinectin protein expression level in HCC tumors with liver cirrhosis was significantly higher than that in HCC tumors without cirrhosis ($P < 0.05$). However its expression level has no relationship between kinectin expression and gender, age, AFP level or tumor capsule (**Table 1**). Although the kinectin protein expression level increased in HCC tumors with less-differentiated (III-IV) tumors compared with HCC tumors in well-differentiated (grades I-II), the statistical analysis results didn't present significant.

In order to determine whether the expression of kinectin was different between HCC cell lines and normal hepatocellular cell line which were cultured under the same conditions, semi-RT-PCR and western blot were carried out to detect the kinectin expression. No significant difference was detected among HCC cell lines and normal hepatocellular cell line at mRNA level. But kinectin protein were increased more in these HCC cell lines compared to normal cell line, especially in BEL-7404 (**Figure 3**).

Decrease in kinectin suppresses human hepatocellular carcinoma cells growth, migration and invasion in vitro

To investigate the role of kinectin in HCC, we transformed the pGPU6/KNT shRNA kinectin to low the expression of kinectin in human hepatocellular carcinoma line BEL-7404. Semi-quantitative RT-PCR and western blots results

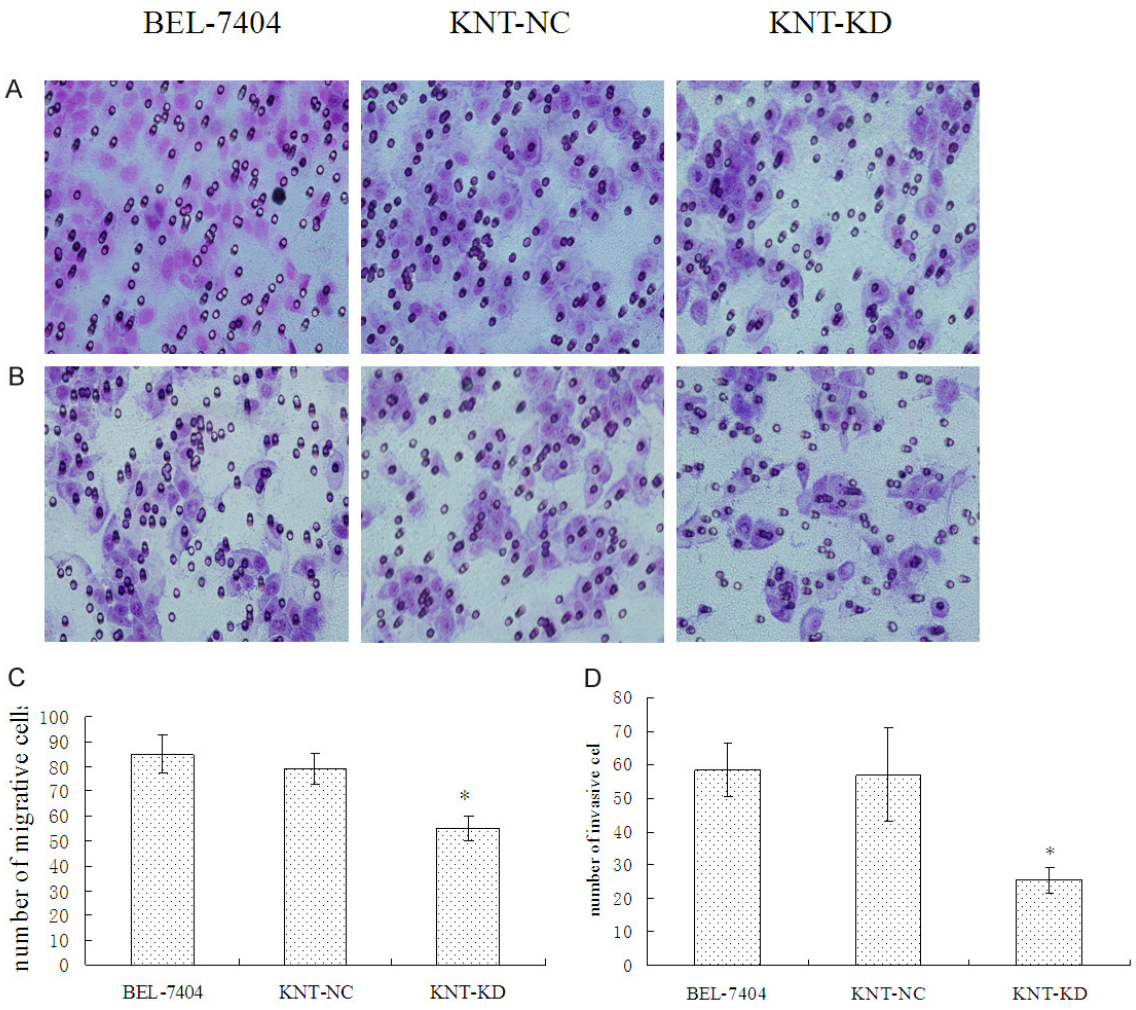

Figure 6. Migration assay and invasion assay in transwell chamber. BEL-7404, KNT-NC and KNT-KD cells imaged with light microscope at 200 magnification. A, C. Migration assay. B, D. invasion assay. *Significantly different compared with the noninfection group and the random sequence group, $P < 0.05$. (Gimsa, x200).

showed that RNAi reduced native kinectin mRNA and protein by more than 80% in stable kinectin knockdown BEL-7404 cells KNT-KD (**Figure 4**). The kinectin knockdown cell exhibited the same morphology with wild BEI-7404 and vector alone cell KNT-NC.

We analyzed cell growth by MTT assay. shRNA has a negative effect on BEL-7404. This growth suppression was noticed from the fifth day after inoculation, and the effect was more prominent in kinectin knockdown cells at the sixth days (**Figure 5**).

To investigate the effect of shRNA of kinectin on the migration and invasion of BEL-7404, cells in serum-free medium migrated past the separating membrane of a Transwell insert towards medium containing 10% serum. The percentage of cell migration refers to the percentage of cells that migrated across the membrane. KNT-KD cells exhibited a 333 ± 11% reduced migration across the membrane in the migration assay compared with wild strains BEL-7404 and KNT-NC cells respectively. The numbers of KNT-KD cells past the Martrigel is also reduced by 27.07 + 1.1% when compared against the numbers of wild strains BEI-7404 and KNT-NC (**Figure 6**). These results indicate that kinectin suppress cell growth, migration and invasion.

Discussion

Specific immunotherapy based on tumor associated antigens (TAAs) emerged as a promising

treatment modality for HCC. And TAAs may also are involved in tumor carcinogenesis. Therefore, more research on tumor associated antigens involved in HCC carcinogenesis is needed. Kinectin is one of HCC associated antigen identified with SEREX, and maybe a promising serum marker for HCC alliance diagnosis especially for patients with AFP negative [15]. Although kinectin has been reported to be associated with HCC, but its expression in tumor tissues is seldom known except the breast cancer [18]. Our present study showed that kinectin is over-expressed in the HCC tissues and cell lines compared with corresponding adjacent tissues and normal hepatocyte cell line, whereas kinectin mRNA didn't show any significance difference between these referred above. This aberration at transcription and translation level also been observed in the expression of other protein in all kinds of normal and tumor cells, such as MMP2, MMP9 and TIMP-1 in cancerous and noncancerous tissues of prostates [19-21]. This poor correlation of mRNA and protein expression to those protein maybe caused by post-transcriptional processing and post-translation modification, as well as the possibility that proteins have very different half-lives. According to that, we hypothesized that this aberration of kinectin is one of the reason that kinectin can elicit a humoral immune response in HCC patients. The underlying mechanism deserves to be illuminated in the next future.

Another group leaded by Wang et al. also found that kinectin can elicit a humoral immune response in HCC patients by SEREX. And they noticed that kinectin presents an array of variants in HCC tissues from the diverse combination with four splicing sites in kinectin mRNA. Furthermore, variant containing D2 splicing region in HCC tissues were over-expressed at mRNA level by normal PCR, which may be tumor associated. But no protein level proof presented in their study [16]. Here, we found protein production of Kinectin increase significantly in tumor issues compared with corresponding adjacent tissues with polyclonal antibody which can react with all of Kinectin variants in theory. And we also use another anti-rabbit polyclonal antibody to kinectin which raised against to amino acid 442-768 mapping near the N terminus of human Kinectin to detect the protein expression in HCC tissues and corresponding adjacent tissues, the same results were achieved (polyclonal antibody is made by our lab, data not shown). Recently, our group has observed kinectin isoforms containing D2 alternative splicing region is highly expressed in HCC tissues compared with corresponding adjacent tissues (data not published). So we proposed that the over-expression of Kinectin protein, especially kinectin isoforms containing D2 alternative splicing region, in HCC tissues probably is one of important reason stimulating body immune response to produce anti-Kinectin antibody.

Due to the aberrant expression of tumor marker in the tumor tissues, we predicted that kinectin may play a role in the tumorigenesis. It is reported that the function of Kinectin involving organelle mobility as a kinesin receptor has been well depicted. However, its function in tumorigenesis has not been identified. Here we found that silencing of kinectin cell restrained the HCC cell proliferation, motility and invasion. Our results are partly coincidence with that of Zhang *et al*.'s group results. But they used siRNA to knockout of kinectin expression in HaLa cell, the kinectin knockout cell exhibited reduced spreading and mobility than those of wild-type [11].

Cell migration contributes to tumor metastasis, which is controlled by many factors [22]. Rho family small GTPases, one number of Ras-super families, are critical regulators of key steps in cell migration. They act as molecular switches that cycle between active GTP-bound states and inactive GDP-bound states. Among the Rho family GTPases, three members Rho, Rac and CDC 42 have been well characterized. Their GTP-bound form can activate downstream effector proteins, which in turn directly or indirectly trigger the initiation of cellular effects, including cell proliferation, survival, adhesion, migration and cell invasion [23]. Kinectin has been identified interact with GTP-bound form of RhoA, Rac1, and Cdc 42, specically interacts with kinectin through coiled-coiled region with amino acid positions from 630-935 of kinectin by two-hybrid method *in vitro* [11, 22]. But the biological significance of the interaction between kinectin and RhoA is worthy of being clarified. Furthermore, kinectin is a key effector of RhoG microtubule-dependent cellular cell activity. RhoG needs binding to kinectin and

requires kinesin activity to establish its morphogenic activity. RhoG also act on kinectin to facilitate the kinesin-kinectin-driven vesicle transport along microtubule [10]. This system is one of reason for the decrease in the cell migration and adhesion in the silencing of kinectin cell. But the further studies are necessary to establish the physiological significance of interaction of the Rho family members with kinectin.

During the process of tumor metastasis and more generally cell migration, cell connects with the surrounding in part through focal adhesions (FAs) [23]. It is also one key regulator controlling cell shape, spreading and migration. It can concentrate integrins and associated cytoskeletal elements as well as a large number of regulatory proteins, including adapters, kinases and small GTPases of the Rho Family [24, 25]. Kinectin also is found to be clustered in Fas [26]. In addition, Kinectin served as a mediator for ER extension along microtubules, has a positive role in FA assembly. Our results are accordance with those referred above. Furthermore silencing of kinectin cell restrained the HCC cell proliferation.

In conclusion, our data demonstrate that kinectin has positive correlation with HCC proliferation, invasion and metastasis. It may be play a role in HCC tumorigenesis. And the mechanism of the effect of kinectin on HCC cell growth is just underway.

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Disclosure of conflict of interest

None.

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