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Effects of Nischarin on the Migration Ability of Hepatocellular Carcinoma Cells

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Abstract: To clarify the effect of Nischarin on the biological behavior of hepatocellular carcinoma cells, two human hepatocellular carcinoma cell lines QGY-7701 and HepG2 were selected. The migration ability of the cells was determined with human normal hepatocyte LO2 as control. Western blotting was used to detect the differential expression of Nischarin and its downstream signals. The expression of Nischarin was knocked down by plasmid transfection to study the changes of cell migration ability, so as to evaluate the effect of Nischarin on hepatocellular carcinoma cells. The results of cell scratch experiment showed that compared with LO2 cells, the migration ability of HepG2 was significantly enhanced ($P < 0.05$), but there was no significant difference in the migration ability between QGY-7701 and LO2 cells. The results of Western blotting showed that the expression of Nischarin in HepG2 was significantly lower than that in LO2 cells ($P < 0.05$). Quantitative RT-PCR was used to detect the mRNA level of Nischarin in cells and found that the Nischarin mRNA level of HepG2 was significantly lower than that of LO2 ($P < 0.01$). When HepG2 cells were transfected with Nis-shRNA to knock down the expression of Nischarin, the cell migration ability was significantly increased ($P < 0.05$). While Nischarin overexpression significantly inhibited the phosphorylation of LIMK1 and cofilin, the important molecules of Rho-GTPase signaling pathway. These results strongly suggest that Nischarin is an endogenous protein with the ability to inhibit the migration of hepatocellular carcinoma cells. In conclusion, we found a protein that can inhibit the migration of hepatocellular carcinoma cells, which may have potential value in the diagnosis and treatment of hepatocellular carcinoma, but its molecular mechanism needs to be further studied.

Keywords: Nischarin, Hepatocellular Carcinoma, Migration

1. Introduction

Hepatocellular carcinoma is currently one of the most common malignant tumors in the world. China is a high-incidence area of primary hepatocellular carcinoma and both the incidence and mortality rate rank first in the world [1, 2]. Because of its fast onset and high tendency to recur and metastasize, hepatocellular carcinoma seriously threatens human health. Studies found that the occurrence and development of hepatocellular carcinoma involves the abnormal expression or activation of many growth factors, oncogenes and tumor suppressor genes. Looking for the target genes closely related to invasion, metastasis and sustained growth of hepatocellular carcinoma and exploring the underlying mechanisms will provide new targets for treatment, which is of great significance for improving the prognosis and prolonging the survival time of patients. We found that the expression level of Nischarin in breast cancer cells was significantly downregulated or even silenced. It has been shown that Nischarin affecting apoptosis and metastasis of breast cancer [3]. Moreover, the absence of Nischarin promotes the metastasis of ovarian cancer cells [4] and the migration of neuronal cells [5]. These findings suggest that

Nischarin may be a potential tumor suppressor, but the expression and function of Nischarin in hepatocellular carcinoma cells are not well understood. In this study, Western blotting, real-time quantitative RT-PCR and cell scratch experiments were performed to study the differentiated expression and function of Nischarin protein in hepatocellular carcinoma cells.

2. Materials and Methods

2.1. Reagents and Instruments

Nischarin antibody was purchased from the BD company (Franklin Lakes, NJ, USA). GAPDH antibody was purchased from Xianzhi Biological Company (Hangzhou, China). Pak1 and LIMK1 antibodies were purchased from the CST company (Boston, USA). BCA protein concentration determination kit, RIPA lysate, protease inhibitor, phosphatase inhibitor PMSF were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Pre-stained protein Marker, electrophoresis device, transfer membrane meter and real-time quantitative THERMAL instrument was purchased from the Bio-Rad company (Hercules, California, USA). PolyJet in vitro DNA transfection reagent was purchased from SignaGen (Rockville, MD, USA). Nis-shRNA and its control plasmid were purchased from Heyuan Biological Co., Ltd (Shanghai, China). Oddesy infrared fluorescence imaging system was purchased from Li-COR Biosciences (Lincoln, NE, USA), and ultra-refrigerated centrifuge was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell Culture and Transfection

Normal hepatocellular cell line LO2, hepatocellular carcinoma cell lines QGY-7701 and HepG2 were purchased from Shanghai Cell Resource Center. LO2 and HepG2 cells were cultured with DMEM containing 10% fetal bovine serum and 1% v/v penicillin/streptomycin. QGY-7701 cells were cultured with RPMI1640 containing 10%fetal bovine serum and 1% v/v penicillin/streptomycin. Cells were transfected with interfering plasmid Nis-shRNA or control plasmid Ctrl-shRNA against the Nischarin protein using PolyJet reagent. After transfection for 48 h, the transfection rate was calculated after taking pictures and observing the transfection effect under an inverted fluorescence microscope. The expression level of Nischarin protein in cells was determined by the Western Blotting method.

2.3. Cell Scratch Experiment

A 100 μl pipette tip was used to scratch the 12 well plate cultured with LO2, QGY-7701 and HepG2 cells. Cells were grouped and photographed at time points of 0, 6, 12 and 24 h, respectively, and the migration distance of the cells was measured with Image J software for quantitative analysis. Migration ability of the cells was calculated as the percentage of the cell migration distance with respect to the initial scratch distance.

2.4. Western Blotting

Lysate buffer was added to a dish cultured with LO2, QGY-7701, or HepG2 cells. After centrifugation, the supernatant was taken to obtain the protein stock. After the protein concentration was determined using a BCA kit, vertical electrophoresis was performed to separate the protein. Subsequently, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane according to the manufacturer's instructions. Blots were placed into blocking solution for 2 h at room temperature (RT), primary antibody was then added and incubated overnight at 4°C. The primary antibodies were mouse monoclonal antibody against Nischarin (1:1000, BD, #558262), rabbit polyclonal antibody against GAPDH (1:1000, Xianzhi, #AB-P-R001), p-LIMK1 (1:1000, CST, #P53667), LIMK1 (1:1000, CST, #P53667), p-cofilin (1:1000, CST, #P23528) and cofilin (1:1000, CST, #P23528). Then DyLight 680- or DyLight 800-conjugated secondary antibody was added and incubated for 2 h at RT with gentle agitation. The LI-COR Odyssey System (LI-COR Biosciences) was applied to visualize the specific protein bands. Quantification of the immunoblot was performed using ImageJ software.

2.5. Quantitative RT-PCR

The total RNA of LO2, QGY-7701 or HepG2 cells was extracted using TRIzol reagent according to the manufacturer's protocol. Then, cDNA was synthesized using a PrimeScript First-Strand cDNA synthesis kit. Primers for Nischarin were as follows: sense 5'-ACCTGCAGTCAG TCAACGTC-3' and antisense 5'-CAGGAAGCAGTGTGT CAGGT-3'. Primers for GAPDH were as follows: sense 5'-TGATTCTACCCACGGCAAGTT-3' and antisense 5'-TGATGGGTTTCCCATTGATGA-3'. Each 20 μl PCR mixture contained 10 μl SYBR Premix, 400 nM primers and 10 ng template. Thermal cycling was performed in a CFX 96 real-time quantitative THERMAL instrument.

2.6. Statistics

All data are represented by mean ± standard error. Image J software was used to process and acquire data from Western blotting and cell scratch experiments. GraphPad Prism software was used for data statistics analysis and histograms. One-way ANOVA was used to determine statistical significance and the statistical differences between groups were analyzed using the Dennett method. The null hypothesis was rejected at the 0.05 level.

3. Results

3.1. Migration Ability of Different Cell Lines

The results of cell scratch experiments showed that the migration ability of hepatocellular carcinoma cells HepG2 was significantly enhanced when compared with normal hepatocellular cell line LO2 ($P<0.05$). However, there was no significant differences between the migration capacity of

hepatocellular carcinoma cells QGY-7701 and LO2 cells (Figure 1A, B).

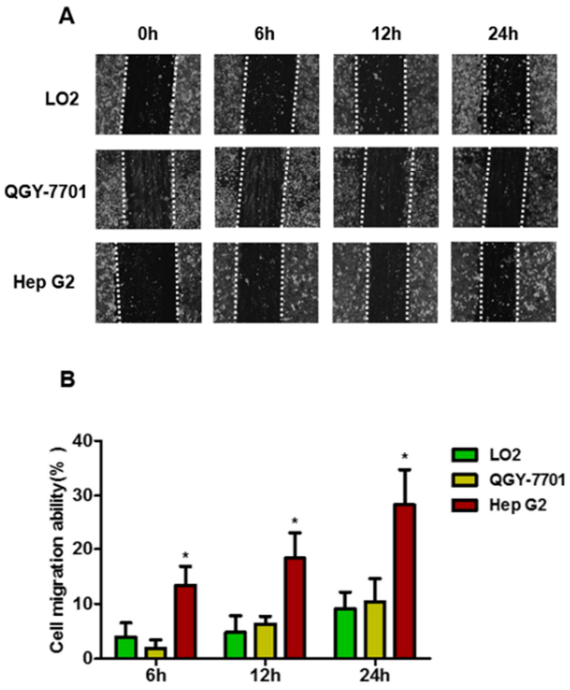

Figure 1. *Comparison of migration ability in different cell lines.*

(A) Typical photos of scratch experiments of different cell lines; (B) Statistical histograms of migration ability of different cell lines. Compared with normal hepatocellular cells LO2, *P<0.05, (n=5).

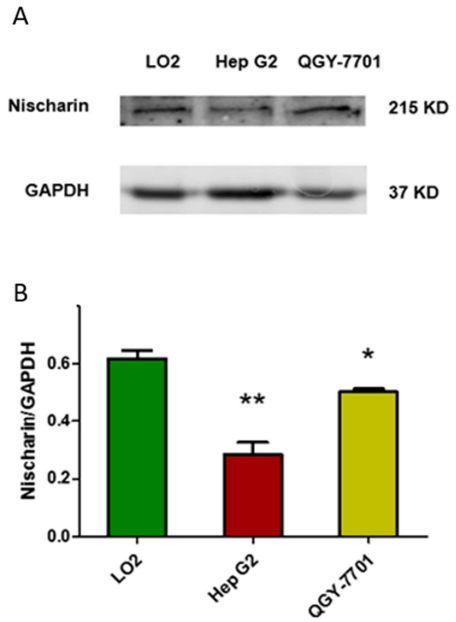

Figure 2. *Comparison of Nischarin expression in different cell lines.*

(A) Western blotting determined Nischarin expression in different cell lines; (B) Statistical chart of Western blotting experimental results. Compared with normal hepatocellular cells LO2, $^{*}P < 0.05$, $^{**}P<0.01$, (n=5).

3.2. Nischarin Expression in Different Cell Lines

The results of the Western blotting experiment showed that among the three different cell lines, expression of Nischarin protein in human normal hepatocellular LO2 cells was the highest, followed by hepatocellular carcinoma QGY-7701 cells. Expression of Nischarin protein in HepG2 cells was the lowest (P<0.05, Figure 2A, 2B).

The results of RT-PCR experiments showed that at the mRNA level, Nischarin expression of LO2 was highest, while that of QGY-7701 and HepG2 was significantly reduced compared with LO2 (P<0.01, Table 1).

Table 1. *Different cellular gene levels Nischarin expression.*

Cell type	Cq1	Cq2	Cq1-Cq2	ΔΔCq	$2^{-\Delta\Delta Cq}$
LO2	24.74	29.90	-5.16	0	1
HepG2	35.63	29.12	6.51	11.67	0.000306**
QGY-7701	34.66	30.78	3.88	9.04	0.001895**

Compared with normal hepatocellular cells LO2, ** P<0.01, (n=5).

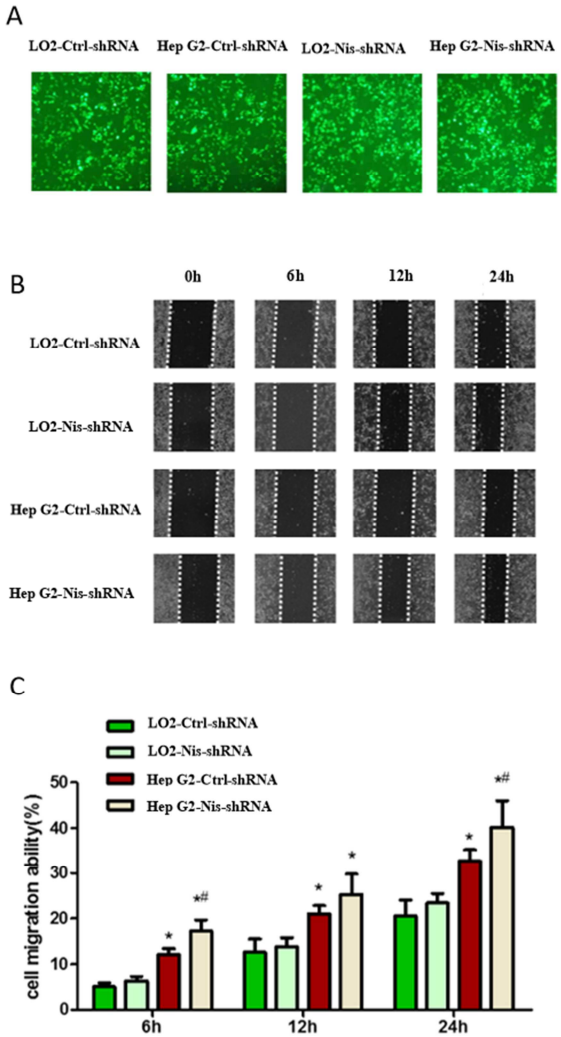

Figure 3. *Changes in the migration ability of cells after knocking down Nischarin expression.*

(A) The immunofluorescence images showed the transfected GFP positive cells under fluorescence microscopy; (B) Typical images of cell scratches after knocking down Nischarin expression; (C) Statistical histograms of cell migration ability after knocking down Nischarin expression. *P<0.05 compared with LO2 cells transfected with the same plasmid, $^{\#}$P<0.05 compared with cells transfected ctrl-RNA, (n=5).

3.3. Effect of Knockdown of Nischarin Expression on Cell Migration Ability

Frstly, transfection efficiency was determined by counting the number of GFP positive cells under a fluorescence microscope. After optimizing the transfection protocol, the transfection efficiency of the HepG2 and LO2 cells reached about 80% after 48 h transfection (Figure 3A), which met experimental requirements. In the subsequent cell scratch experiment, at the three time points of 6, 12, and 24 h, the migration ability of hepatocellular carcinoma cells HepG2 was stronger than that of LO2 cells transfected with the corresponding Nis-shRNA or Ctrl-shRNA (P<0.05, Figure 3B, 3C). Compared with HepG2 cells transfected with Ctrl-shRNA, the migration ability of HepG2 cells transfected with Nis-shRNA was significantly enhanced at both 6 and 24 h (P < 0.05).

3.4. Effect of Nischarin Overexpression on the Rho-GTPase Signaling Pathway

In order to clarify the corresponding molecular mechanism, Western blotting method was used to detect the activity of the Rho-GTPase signaling pathway. The results showed that Nischarin overexpression (Figure 4A, 4B) inhibited the Phosphorylation of LIMK1 and cofilin significantly (P<0.05, Figure 4C, 4D, 4E, 4F).

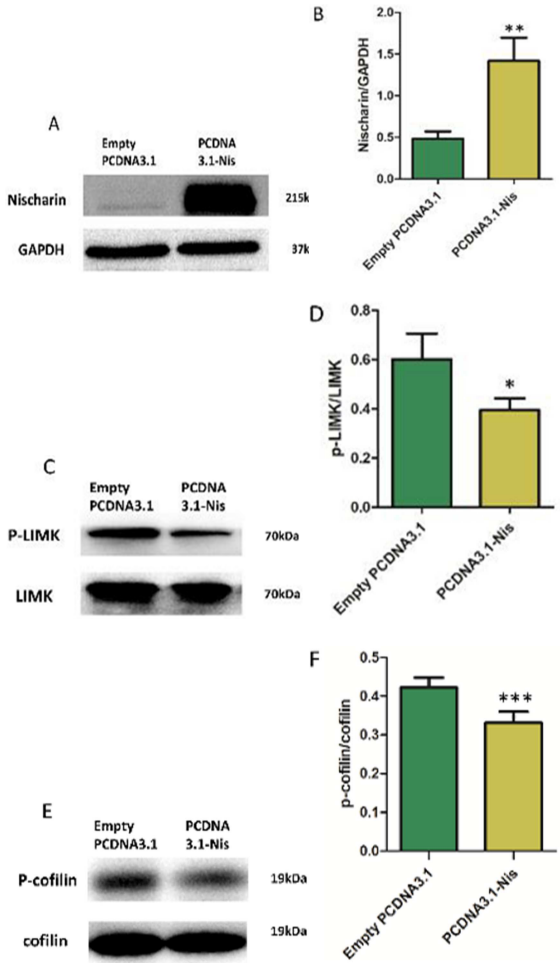

Figure 4. *Effect of Nischarin overexpression on the Rho-GTPase signaling pathway.*

(A) Western blotting determined Nischarin overexpression; (B) Statistical chart of Western blotting experimental results. ** P<0.01 compared with cells transfected with empty PCDNA3.1; (C) Effect of Nischarin overexpression on LIMK1 phosphorylation; (D) Statistical chart of Western blotting experimental results. *P<0.05 compared with cells transfected with empty PCDNA3.1; (E) Effects of Nischarin overexpression on cofilin phosphorylation; (F) Statistical chart of Western blotting experimental results. *** P<0.001 compared with cells transfected with empty PCDNA3.1.

4. Discussion

Hepatocellular carcinoma is the second cause of cancer death, ranks second only behind to lung cancer, with a five-year survival rate of 12% [2]. Nischarin is a cytoplasmic protein screened by yeast double hybridization method from the mouse embryonic gene expression library in 2000 [6], which can bind specifically to the cytoplasmic tail region of the integrin α5 subunit [7, 8]. Studies have shown that Nischarin affects the Rho GTPase to modulate the signaling of the cytoskeleton to inhibit cell migration. Nischarin inhibits cancer cell migration by modulating the Rac1-PAK-LIMK pathway, one of the key downstream pathways of the Rho

GTPase family [9]. The existing research on Nischarin mainly focused on its inhibition of breast cancer cell migration, invasion and the underlying biological mechanisms [10-15], but the expression of Nischarin in cancers of digestion system and its role have not been reported.

In order to clarify the influence of Nischarin on the biological behavior of hepatocellular carcinoma cells and its mechanism, the normal human hepatocyte LO2, human hepatocellular carcinoma cells QGY-7701 and HepG2 were selected for culture, and the migration ability of cells was determined through cell scratching experiments. The migration ability of LO2 cells was the weakest, while the migration ability of hepatocellular carcinoma HepG2 cells was strongest among the three strains. In order to study its molecular mechanism, Western blotting method was applied to detect the expression of Nischarin in different liver cancer cells. The results turned out that compared with the expression of Nischarin in LO2 cells, Nischarin expression in HepG2 cells was significantly reduced, which was corroborated with the results of quantitative RT-PCR experiments. Is the downregulation of Nischarin expression related to the increased migration ability of liver cancer cells? In order to answer the above question, Nis-shRNA plasmid was transfected in HepG2 cells to knockdown the expression of Nischarin, and then cell scratching experiments was performed to investigate the changes in the migration ability of cells. The results showed that the migration ability of hepatocellular carcinoma HepG2 cells was significantly enhanced after knocking down Nischarin expression, suggesting that the downregulation of Nischarin can promote cell migration. To further study the underlying mechanisms, Western blotting experiment was performed after overexpression of Nischarin in HepG2 cells. The results showed that after overexpression of Nischarin, the phosphorylation of Limk1, the key protein of the Rho-GTPase signaling pathway, and its downstream molecule cofilin were significantly reduced. This suggests that upregulation of Nischarin expression can inhibit cell migration by interfering with the activation of LIMK1 and cofilin.

5. Conclusion

In conclusion, this study found for the first time that the expression of Nischarin in HepG2 cells was significantly down-regulated, and the corresponding phosphorylated proteins of LIMK and cofilin downstream of the signaling pathway Rho-GTPase increased with the downregulation of Nischarin expression, which may be a reason for its enhanced migration ability. The findings suggest that Nischarin may be a potential target in the diagnosis and treatment of hepatocellular carcinoma.

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