High NDRG3 expression facilitates HCC metastasis through promoting nuclear translocation of β-catenin

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Running Title: Role of NDRG3 on HCC metastasis

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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Data mining

Five GEO datasets (GSE54238, GSE57958, GSE17548, GSE36376 and GSE14520) and TCGA dataset were used in this study to detect the expression pattern of NDRG3 in HCC. The primary data of GEO datasets can be found at https://www.ncbi.nlm.nih.gov/geo. Besides, the OncoLnc database was used to uncover the prognostic value of NDRG3 in TCGA cohort, (http://www.oncolnc.org/).

Real-time quantitative PCR.

Trizol reagent (Takara, Japan) was used to extract total RNA and PrimeScript RT-PCR kit (Takara, Japan) was used according to the manufacturer's instructions. Quantitative real-time PCR was performed using a 7500 Real-time PCR system (Applied Biosystems, Inc. USA) with SYBR Premix Ex Taq (Takara) in 10μl final reaction volume. The primers as follow: NDRG3-F: 5'-CGCCCATTATAGGAACCCAGGAAG-3', NDRG3-R: 5'-GGCGAATTGTCCCCTACCACCAGT-3'; GAPDH-F: 5'-GTCAACGGATTTGGTCTGTATT-3', GAPDH-R: 5'-AGTCTTCTGGGTGGCAGTGAT-3'. $2^{-\Delta Ct}$ method was used to analyze the data.

Spheroid formation assay

This assay was performed as previously described (1).

Tube formation.

Assay was performed with in vitro angiogenesis assay kit (Millipore, ECM625) according to the manufacturer's protocol. HUNVEs Cells were added CM (conditional medium) from supernatant of NDRG3 knockdown HepG2 cells and control.

Establishment of NDRG3 knockdown cells

Short hairpin RNA (shRNA)-containing plasmids were packaged into lenti-virus and infected into cells in the presence of 6μg/ml polybrene (Sigma,

Shanghai, China). The stable NDRG3 knockdown cells were selected in the presence of 4 μg/ml puromycin and tested by western blotting.

Immunohistochemistry (IHC) and hematoxylin and eosin (HE) staining.

IHC and HE was performed as previously reported. Staining intensities were scored as lower and higher expression in accordance with reported previously (2). All scores were quantified by two independent pathologists in a blinded manner. Antibodies used as followed: NDRG3 (Abcam, ab133715, 1:500), β-catenin (Abcam, ab32572, 1:500), CD31 (Abcam, ab28364, 1:200).

Immunofluorescence（IF）staining.

Assays were performed according to previous description(3).

Luciferase reporter assay.

Sh-NDRG3 and control HCC cells were seeded in 96-well plates and transfected with mixture of 100 ng TOP (TCF reporter plasmid) reporter plasmid (Wnt/β-catenin signaling) and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 24 hours, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI), following the recommended protocol.

Cell viability assay

Cell viability was assessed using a standard Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan). 3000 cells per well were seeded into 96-well plates (100μl per well).

Migration and invasion assay

This assay was performed as previously described (4).

Animal studies

All animals using in this study were approved by the Research Ethics Committee of Changzheng Hospital and adhere to the local or national requirements for the care and use of laboratory animals. Each male BALB/C nude mice (5 in each group, 4-week-old) was orthotopically inoculated in the hepatic lobe with 1×10^6 sh-NDRG3-HepG2 or sh-NC-HepG2 cells. After 6

weeks, mice were sacrificed, and the collected livers were fixed and prepared for histological assessment.

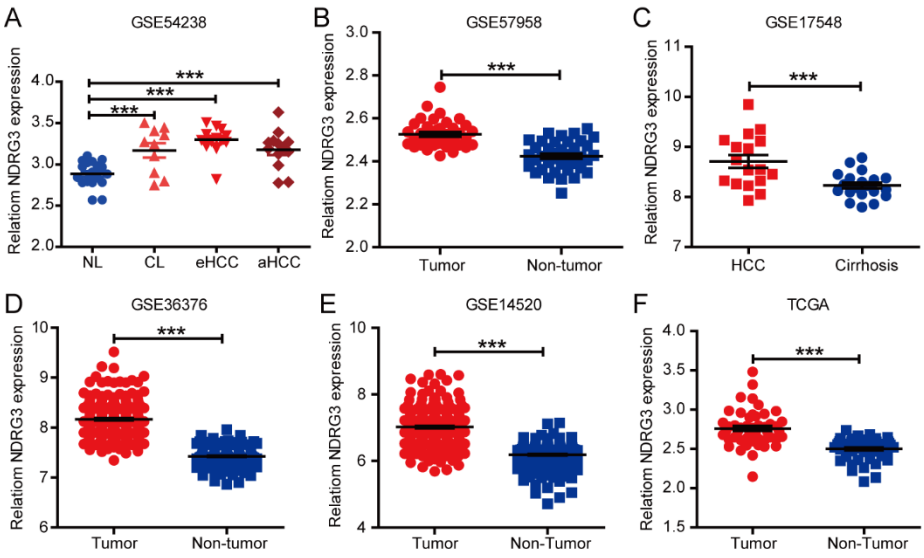
Statistical analyses

SPSS 20.0 (Chicago, IL, USA) and GraphPad Prism 5 software were used to perform statistical analyses. Cumulative survival time was tested by the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate Cox regression analyses were conducted to identify the factors that had a significant influence on survival by Cox proportional hazards model. The chi-square test, or Student's t-test were used for comparison between groups. Statistically significance was accepted at $P < 0.05$.

References

1. Ding, L. J., Li, Y., Wang, S. D., Wang, X. S., Fang, F., Wang, W. Y., Lv, P., Zhao, D. H., Wei, F. and Qi, L. (2016) Long Noncoding RNA lncCAMTA1 Promotes Proliferation and Cancer Stem Cell-Like Properties of Liver Cancer by Inhibiting CAMTA1. *International journal of molecular sciences* **17**.
2. Tian, G. A., Zhu, C. C., Zhang, X. X., Zhu, L., Yang, X. M., Jiang, S. H., Li, R. K., Tu, L., Wang, Y., Zhuang, C., He, P., Li, Q., Cao, X. Y., Cao, H. and Zhang, Z. G. (2016) CCBE1 promotes GIST development through enhancing angiogenesis and mediating resistance to imatinib. *Sci Rep* **6,** 31071.
3. Lu, W. J., Chua, M. S., Wei, W. and So, S. K. (2015) NDRG1 promotes growth of hepatocellular carcinoma cells by directly interacting with GSK-3OI and Nur77 to prevent OI-catenin degradation. *Oncotarget* **6,** 29847-29859.
4. Fu, Y., Feng, M. X., Yu, J., Ma, M. Z., Liu, X. J., Li, J., Yang, X. M., Wang, Y. H., Zhang, Y. L., Ao, J. P., Xue, F., Qin, W., Gu, J., Xia, Q. and Zhang, Z. G. (2014) DNA methylation-mediated silencing of matricellular protein dermatopontin promotes hepatocellular carcinoma metastasis by alpha3beta1 integrin-Rho GTPase signaling. *Oncotarget* **5,** 6701-6715.

SUPPLEMENTARY FIGURES



Supplementary Fig 1. Elevated NDRG3 expression in HCC databases. (A) NDRG3 expression in different pathological stages of liver disease (cirrhotic livers (CL), n=10; early HCC (eHCC),n=13; advanced HCC (aHCC), n=13) compared with normal liver(NL, n=20). (B-F) Expression analysis of NDRG3 in tumors and paired non-tumor tissues in five independent cohorts (B, GSE57958; C, GSE17548; D, GSE36376; E, GSE14520; F, TCGA); (student's t-test, ***P < 0.001).

SUPPLEMENTARY TABLE

Table S1. Clinicopathological correlation of NDRG3 expression in 174 HCC patients

Clinicopathological feature	Total	Expression of NDRG3		P value (χ2 test)
		Low	High	
Age (years)				
≤ 50	90	42	48	0.363
> 50	84	45	39	
Gender				
Male	130	62	68	0.295
Female	44	25	19	
Tumor size				
≤ 5 cm	79	48	31	**0.010**
> 5cm	95	39	56	
Tumor encapsulation				
None	97	35	62	**0.001**
Complete	77	52	25	
Live Cirrhosis				
Yes	136	61	75	**0.010**
No	38	26	12	
Vascular invasion				
Yes	58	22	36	0.024
no	116	65	51	
Thromb				
Yes	34	16	24	0.149
no	134	71	63	
Serum AFP				
≤ 25ng/ml	60	34	26	0.202
> 25ng/ml	114	53	61	
TNM stage				
I	82	49	33	**0.007**
II	28	16	12	
III	64	22	42	

The bold number represents the P-values with significant differences.

Table S2. Univariate analysis of prognostic parameters for survival in patients with HCC

Prognostic parameter	Univariate analysis		
	HR	95% CI	P value
Expression of NDRG3 (low vs. high)	2.351	1.494 - 3.698	**0.000**
Age (≤50vs. >50)	1.039	0.675 - 1.599	0.863
Gender (male vs. female)	0.749	0.444 - 1.263	0,278
Tumor size (≤5 cm vs >5 cm)	3.742	2.290 - 6.116	**0.000**
Tumor encapsulation (none vs complete)	0.626	0.397 - 0.987	**0.044**
Liver cirrhosis (absent vs. present)	1.161	0.681 - 1.980	0.583
Vascular invasion (yes vs. no)	4.516	2.905 - 7.021	**0.000**
Thromb (yes vs. no)	3.780	2.405 - 5.943	**0.000**
Serum AFP (≤25ng/ml vs >25ng/ml)	1.947	1.186 - 3.198	**0.008**
TNM stage (I, II and III)	2,330	1.801 - 3,015	**0.000**

HR: Hazard ratio; CI: Confidence interval.

The bold number represents the *P*-values with significant differences.