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

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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’-CGCCCATTATAGGAA CCCAGGAAG-3’,  NDRG3-R: 5’-GGCGAATTGTCCCCTACCACCAGT-3’;
GAPDH-F: 5’-GTCAACGGATTTGGTCTGTATT-3’,  GAPDH-R: 5’-AGTCTTTCTGGGTGGCAGTGAT-3’. 2^-ΔΔCT 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

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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|
| > 5 cm                      | 95    | 39  | 56   |     |
| Tumor encapsulation         |       |     |      |     |
| None                        | 97    | 35  | 62   | 0.001|
| Complete                    | 77    | 52  | 25   |     |
| Liver 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 (≤50 vs. >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.