NDRG1 as a biomarker for metastasis, recurrence and of poor prognosis in hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 17 May 2011
Accepted 1 June 2011

Keywords:
Hepatocellular carcinoma
NDRG1
Biomarker
Recurrence
Metastasis

ABSTRACT

N-myc downstream-regulated gene 1 (NDRG1) has been reported to be a multifunctional protein associated with carcinogenesis, however, the cellular function of NDRG1 remains elusive in human cancers. Here, our proteomics profile analysis of HCC tissues with different metastatic capabilities revealed that NDRG1 was correlated with metastasis and recurrence in HCC patients after liver transplantation (LT). Immunohistochemical staining of 143 HCC patients after LT showed that NDRG1-positive expression had poor prognosis, either for shorter disease-free survival or overall survival \((P < 0.001)\), compared with NDRG1-negative expression. Multivariate analysis confirmed NDRG1 as an independent prognostic value \((P < 0.001)\). In addition, in vitro experiments HCC cells with small interfering RNA against NDRG1 significantly suppressed its proliferation, colony formation, invasion and migration ability. Microarray analysis revealed that NDRG1 modulated the expression of genes associated with transmembrane transporter activity, chemoattractant activity, immune response, cell adhesion and cell proliferation process. Taken together, these results suggested that NDRG1 was an important molecule in controlling HCC metastasis and thus suggested as a novel biomarker for predicting HCC recurrence after LT.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for the third cause of cancer-related death due to poor prognosis. There were 626,000 new cases estimated around the world annually, with about half in China alone [1]. The overwhelming majority of HCC is caused by exposure to aflatoxins, infection with hepatitis B and C viruses. It has been shown that small size of HCC can be cured by surgical resection or liver transplantation. Unfortunately, the disease is often diagnosed at an advanced stage when conventional and effective treatment options become unavailable [2]. Localized disease can be treated by means of surgical resection, liver transplantation, radiofrequency ablation and chemoembolization [2–5]. However, HCC represents a tumor type of high invasion and aggression. Recurrence of HCC following treatments remains to be one of the most prevalent causes leading to poor long-term survival. Therefore, studying the molecular pathogenesis of cancer recurrence can guide our evaluation of the treatment and prognosis of patients.

Hepatocarcinogenesis is a complex process with mutations in numerous protooncogenes and tumor suppressor genes, such as p53, Rb, PTEN, GSTP1, Smad2/4, IGF-2, b-catenin, c-myc, and cyclin D1 [6]. In addition, our center and some other groups have found that aberrant expression of microRNAs were also involved in the process of
HCC development [7]. The major signaling pathways, such as Wnt/\(\beta\)-catenin-signaling pathway, MAPK pathway, PI3K/Akt/mTOR pathway, activated in HCC also play pivotal roles in the carcinogenesis. In spite that many molecules in the pathogenesis of liver cancer have been in-depth studied, they are still insufficient for recognizing those patients who are at high risk of recurrence [8]. Currently, we still use vascular invasion, the number and size of nodules, and high serum alpha-fetoprotein levels as indicators of recurrence, which are not fully reliable yet [9]. Therefore, molecular “signatures” of recurrence will be essentially important for properly control of HCC.

Identification of new biomarkers of recurrence for early detection, prediction of prognosis, and the response to treatment will be greatly useful to guide the treatment procedure of HCC. Proteomics analysis is currently considered to be a powerful tool for the discovery of new cancer biomarkers and prognostic targets. We undertook this systemic biological approach to identify protein “signatures” that significantly discriminate HCC with recurrence from that without recurrence. A total of 89 differently expressed proteins were identified. N-myc downstream-regulated gene 1 protein (NDRG1), a hypoxia-associated protein, also altered by DNA damage, oncogenes, and tumor-suppressor genes, was chosen to verify its biological and clinicopathologic significance [10–12]. We have performed immunohistochemistry analysis for clinical HCC materials. Furthermore, the function of NDRG1 in the HCC cells was studied by RNA interference assay. The results revealed that NDRG1 had over-expression in the most of HCC tissues, and NDRG1 protein level in HCC was significantly associated with HCC recurrence. NDRG1 could play a significant role in cancer cell growth and invasion, and be a potential target for the development of therapeutic agents, as well as a novel biomarker for recurrence.

2. Materials and methods

2.1. Patients and samples

HCC tissues and their corresponding normal liver tissues had been obtained earlier from patients in our hospital (The First Affiliated Hospital, Zhejiang University, China) with informed consent. The selection criteria were as follows: (a) confirmed by pathologic diagnosis, (b) without pre-operative anti-cancer treatment and distant metastases before liver transplantation, (c) curative liver transplantation, except perioperative death, (d) with a complete clinicopathological and follow-up data. A total of 143 patients were selected to elucidate the relationship between NDRG1 expression and the recurrence/invasive phenotype of HCC. All tumors were staged on the basis of the pTNM pathologic classification of the American Joint Committee on Cancer/International Union Against Cancer. In order to discover recurrence of HCC-related biomarkers by proteomics analysis, tissues were immediately snap-frozen in liquid nitrogen after surgical resection and stored at \(-80^\circ\text{C}\). Pathologic features of those samples were examined by hematoxylin and eosin stain, of which only \(>90\%\) homogenous properties were included in the study. For the validation studies, 143 paraffin-embedded HCC tissues, paired fresh HCC and tumor-adjacent normal liver tissues from 18 patients were obtained to compare the NDRG1 expression levels. Follow-up procedures and diagnostic criteria of recurrence according to a uniform guideline described previously [13]. This study was approved by the Ethic Committee of Zhejiang University.

2.2. Experimental materials

Hepatoma cell lines of HepG2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were maintained in DMEM with high glucose supplemented with 10% heat-inactivated fetal bovine serum. Urea, thiourea, Tris, dithiothreitol, CHAPS and immobile dry strips were provided by GE Healthcare (Piscataway, USA). Sequencing grade modified trypsin was a product of Promega (Madison, USA). The other reagents were purchased from Sangon Biotech Co. Ltd. (Shanghai, China).

2.3. Protein extractions and proteomics

HCC tissues (50 mg) were lysed in 1 ml lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 2% IPG buffer (pH 4–7), 1 mM Na2VO4, 1 mM PMSF on ice and disrupted by a four cycle sonication protocol. Protein concentration was quantified using the Bradford’s method. Isoelectric focusing (IEF) and SDS/PAGE were carried out to separate proteins according to their isoelectric points and molecular weights as described [7]. Taking into account the complexity of tissue samples, we adopted Cup-based sample application to improve protein-focusing patterns, using immobile dry strips (pI 4–7, 24 cm, GE Healthcare), and each cup hold 200 \(\mu\)g protein sample. Proteins were gradient focused till the maximum voltage 8000 V for 8 h and 15 min to generate a total energy of 74732 kVh. After gel fixation, the protein spots were visualized with silver staining as described in protocol [7]. Digitalized images of gels were captured using a high-resolution scanner (Amersham Biosciences) and analyzed with ImageMaster 2D software (version 6.0). After background subtraction, normalization, matching, relative protein expression levels of matched protein spots were measured by percentage volume. Statistically significant spots were those mean fold change \(\geq 2\) or \(\leq 0.5\), and paired t-test less than 0.05. Spots of interest were excised from wet gels and digested in gel trypsin digestion, and subsequently sequenced by using mass spectroscopy (4800 Proteomics Analyzer, Applied Biosystems).

2.4. Immunoblot analysis

Paired fresh HCC and tumor-adjacent normal liver tissues from 18 patients were obtained from the specimen library of Division of Hepatobiliary and Pancreatic Surgery in our Hospital. Tissue samples were lysed in RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). The extracted proteins were separated by SDS–PAGE and transferred to PVDF membranes. After being blocked by TBS-T...
buffer containing 5% non-fat powder milk for 2 h, the membranes were immunoblotted using primary antibody against NDRG1 (1:1000, sc-100786, mAb, Santa Cruz) overnight. HRP-conjugated goat antimouse IgG antibody was used as secondary antibody. β-actin (1:5000, A5441, mAb, SIGMA–ALDRICH) was used as an internal control. Immunoreactive bands on the blots were visualized with enhanced chemiluminescence reagent ECL kit (Beit Haemek, Israel) and intensities were quantified using Image-Pro plus 6.0 software.

2.5. Immunohistochemistry

Immunostaining was done on formalin-fixed, paraffin-embedded clinical tissue specimens. Briefly, sections were dewaxed in xylene, rehydrated through graded alcohols to water, quenched endogenous peroxidase activities with hydrogen peroxide and renovated antigen by microwave. Subsequently, blocking antibody (5% normal goat serum) was applied to the slides for 60 min at room temperature. Next, slides were incubated overnight with anti-NDRG1 antibody (sc-100786, mAb, Santa Cruz) at a 1:300 dilution, then incubated with the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin for 30 min at room temperature. The results were visualized by reaction with diaminobenzidine (DAB, 3, 3-diaminobenzidine tetrahydrochloride) and counterstaining with hematoxylin. Because the intensity of staining within each liver tissue was mostly homogenous, the intensity of NDRG1 staining was semiquantitatively evaluated over five visual representative fields by two independent investigators without prior knowledge of clinical pathologic data using criteria as previously described: NDRG1 positive, strong intensity was mostly homogenous, the intensity of NDRG1 staining was less than 50% of tumor cells staining. Normal liver tissues adjacent to resection margins were used as negative expression for control [14].

2.6. Small interfering RNA

Target sites for NDRG1 siRNA were searched based on GenBankTM cDNA sequence (NM_006096), and its target sequence was chosen 5′-GGA GTC CTT CAA CAG TTT G-3′, as described [15,16]. The sense (5′-CAC CGG AGT CCT TCA ACA GTT TGT TCA AGA GAC AAA CTG TTG AAG GAC TCC TTT TTT G-3′) and antisense (5′-GAT CCA AAA AAG GAG TCC TTC TCT CTT GAA CAA ACT GTT GGA GGA CTC C-3′) oligonucleotides were synthesized by genePharma Inc (shanghai, China), and annealed to generate siRNA duplexes. The NDRG1 siRNA was cloned into pGPU6-GFP-Neo –siRNA vectors at the Bbs I/BamHI site and sequenced. The specific shRNA or negative control shRNA was transfected into HepG2 cells by using Lipofectamine2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. Twenty-four hours after transfection, 1000 μg/mL G418 was added to the DMEM medium supplemented with 10% FBS to select for the stably transfected cells for a month. The stably transfected HepG2 cells maintained in Dulbecco’s modified Eagle’s medium (Hyclone) containing 10% fetal bovine serum and 200 μg/mL G418 for further study.

2.7. Cell proliferation assay and colony-forming assay

To observe cell proliferation, the stably transfected HepG2 cells with NDRG1 shRNA or control vectors were seeded in 96-well plates at a cell density of 1000 cells per well, and cultured in DMEM complete medium containing 200 μg/mL G418 for 24, 48 and 72 h. Cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories, Japan) according to the instructions of the manufacturer. The number of viable cells was assessed by measurement of absorbance optical density at 450 nm using a microplate reader (ELx800, BioTek). All experiments were independently repeated at least seven times. To assay the effect of NDRG1 on colony formation, cells were trypsinized, resuspended in DMEM with 200 μg/mL G418 to prepare single-cell suspensions. They were then inoculated into 35-mm dishes at a density of 2000 cells/dish. After 12 days, colonies were washed twice with PBS, dyed with crystal violet and photographed. All experiments were independently repeated at least three times under identical conditions.

2.8. Cell cycle and apoptosis analysis

The stably transfected HepG2 cells with NDRG1 shRNA or control vectors were trypsinized and washed three times with pre-chilled PBS buffer and resuspended in 100 μL PBS at a concentration of about 1 × 10⁶ cells/mL. For cell cycle analysis, each sample cell was mixed with 50 μL DNA Prep LPR (Coulter DNA-Prep Reagents kit, Beckman Coulter, USA) and incubated in the dark at room temperature for 20 min, subsequently, DNA was stained and RNA was removed by mixing 0.5 mL DNA Prep Stain in the dark at room temperature for another 20 min. The stained cells were then analyzed of DNA content by the BD LSR II (BD Biosciences, USA) and each histogram was constructed with the data from at least 10,000 events. Data were analyzed to calculate the percentage of cell population in each phase using ModFit LT software. The apoptotic HepG2 cells were evaluated by using FITC-conjugated Annexin-V and PI kit according to the manufacturer’s recommendation and analyzed using the same flow cytometer and FlowJo software.

To further clarify the NDRG1 in the pathogenesis of liver cancer, we used 5-ethyl-2'-deoxyuridine (EdU) labeling methods to studying cell cycle kinetics [17]. Like [3H] thymidine and BrdU labeling methods, EdU is a standard method for direct measurement of cells in the S-phase. HepG2 cells mentioned above were grown on 96-well plates in DMEM supplemented with 10% bovine calf serum and 200 μg/mL G418 for 24 h, then labeled by incubation with 50 μM EdU for 2 h (Cell-Light EdU DNA Proliferation Kit, RiboBio Co. Ltd, China). After fixation, cells were stained by incubating with Alexa594-azide for 30 min. After staining, the cells were washed several times with PBS containing 0.5% Triton X-100, then counterstained with Hoechst33342. Labeling of proliferating cells was imaged by fluorescence microscopy, and overlay of the
2.9. Cell migration and invasion assay

The capability of cell migration was assessed using a wound-healing assay. The stably transfected HepG2 cells were cultured as confluent monolayers and manually wounded by scraping the cells with a 1000 μl pipette tip. The cell culture medium was replaced and migration was assessed at 24 h. Basement Membrane Invasion Assay was performed using a Transwell (Corning, NY). In brief, 1 × 10⁵ cells were cultured in a serum-free DMEM on an insert coated with matrigel (BD Bioscience, Bedford, MA) for 24 h. Then the lower compartment was changed with DMEM complete medium containing 200 μg/ml G418. Cells were incubated for 48 h. After fixation and staining, the number of cells on the bottom surface that invaded across the membranes was counted.

2.10. Microarrays

Total RNA from HepG2 cells stably transfected with NDRG1 shRNA or control vectors was isolated using TRIzol (Invitrogen) according to standard protocol, and was further purified with RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was used to perform Agilent Oligonucleotide Microarray (Agilent Whole Human Genome Oligo Microarray, 44 K) between NDRG1 shRNA and control clones according to the manufacturer’s instructions. After washing in Agilent washing buffer, the slide was scanned with Agilent Microarray Scanner G2565BA. Agilent Feature Extraction software (Version 10.7) was used to convert the image (Scan resolution 5 μm, PMT 100%, 10%) into gene expression data. Data were normalized by the Quantile method. The 3-fold change between two groups was the threshold for significant regulation.

2.11. Quantitative real-time PCR (Q-RT-PCR)

To validate the results in microarray analysis, a series of randomly selected different genes were measured by quantitative real-time RT-PCR, using a SYBR Green PCR master mix (Applied Biosystems). Quantifying the transcripts of the genes in each sample normalized to a housekeeping gene, GAPDH; 2^(-ΔΔCt) normalization was used to calculate the results. Primer sequences are available in Table S1.

2.12. Gene ontology and network analysis

To elucidate the precise mechanisms of NDRG1 in HCC pathogenesis, the list of differentially expressed genes obtained from the primary analysis (Microarrays section) was further analyzed by SBC analysis system (http:// www.ebioservice.com/), a web-based statistical software. GO enrichment analysis was applied to obtain NDRG1 transcriptional targets genes. To further study the function of NDRG1, GeneSpring was applied to assess the altered transcripts in the enriched GO categories for canonical pathways and interaction networks, which generated from information in the Human Interaction DataBase.

2.13. Statistical analysis

The Student t-test was used to compare two groups data represented as the mean ± SEM. All data of clinicopathologic variables of HCC patients in the study were evaluated with SPSS version 16.0 software (SPSS Inc.). Kaplan–Meier curves were calculated for cumulative recurrence and survival probability, and differences were assessed using the log-rank test. Differences were considered significant at values of P < 0.05.

3. Results

3.1. Differentially expressed proteins in HCC tissue among recurrence and without recurrence

In order to find a biomarker of HCC recurrence, HCC tissues which are from recurrence or without recurrence after liver transplantation were analyzed in triplicate by 2-DE. After gel-to-gel matching and normalization, 141 protein spots were found to display differential expression in HCC tissues among recurrence and without recurrence by one-way ANOVA. Fig. S1 shows a representative 2-DE gel image of HCC tissues with and without recurrence. Those deregulated spots were excised from the gels and digested with trypsin enzyme for MS analysis, 89 proteins were successfully identified by MALDI-TOF/TOF (Table S2). They are involved in metabolism, cell cycle, cell proliferation, apoptosis, molecular chaperoning, protein folding, DNA and RNA processing, protein transport, anti-apoptosis and cytoskeleton. One protein spot, which was significantly increased in recurrence HCC tissue compared with that without recurrence, was identified as NDRG1 (Fig. 1A and B), with high Mascot score and sequence coverage.

3.2. Selection and validation of the candidate biomarkers for HCC recurrence

To select the candidate biomarkers of HCC recurrence from differentially displayed proteins identified, we first applied bioinformatics tool for interpretation of proteomics data, and found hypoxia-inducible factor 1 (HIF-1) could regulate NDRG1 expression [18]. To investigate whether NDRG1 expression was abnormal, NDRG1 expression in HCC, we examined the expression levels of NDRG1 between HCC and matched non-cancerous liver tissues from 18 HCC patients. We found that most HCC had higher NDRG1 expression, which was in accord with our proteomics result (Fig. 1C).

3.3. Suppression of liver cancer cell proliferation and colony formation by silencing NDRG1 expression in HepG2 cells

To evaluate NDRG1 functions in HCC cells, we observed the effect of NDRG1 suppression on cell proliferation and colony formation. HepG2 cells were transfected with shRNA-NDRG1 or shRNA-NC, and cell growth was assessed daily over 3 days. The results showed that the silencing of NDRG1 significantly reduced the growth of HepG2 cells, as compared with the control groups transfected by a negative control shRNA (shRNA-NC) (P < 0.05; Fig. 2A). In colony formation assay, upon 12 day continuous culture, decreased exogenous NDRG1 expression suppressed colony formation of HepG2 cells relative to cells transfected with negative control shRNA (Fig. 2B). These findings indicate that NDRG1 has potential oncogenic properties in hepatocarcinogenesis.

3.4. GI arrest and apoptosis induced by silencing NDRG1 expression in HepG2 cells

HIF1α, an important transcription factor, is generally considered to be a promoter of tumorigenesis. Up-regulation NDRG1 expression induced by HIF1α, suggests that NDRG1 can affect cell cycle kinetics and the survival ability. To evaluate NDRG1 functions in HCC cells, we used siRNAs that targeted NDRG1 to knock down the endogenous NDRG1 expression in HepG2 cells. The results demonstrated that the silencing of NDRG1 induced an accumulation of HepG2 cells in G1 phase and reduction of cells in S phase (Fig. 3A and B).
To investigate whether NDRG1 affects survival of liver cancer cells, we examined apoptosis cells in HepG2 cells. These results suggested or showed that knockdown of NDRG1 by the specific siRNA enhanced apoptosis and death of HepG2 cells (Fig. 3C). In this way, up-regulation of NDRG1 in HCC may contribute to both tumor oncogenesis and progression.

3.5. Inhibition of invasion and migration of HCC cells by silencing NDRG1 expression

In order to elucidate the function of NDRG1, herein, we employed wound-healing and Matrigel assays to assess the effects of NDRG1 expression on cell migration and invasion. In vitro invasion assays were performed with HepG2 cells in the absence and presence of NDRG1 expression by Small RNA interference. Knockdown of NDRG1 decreased invasion by 35.7% ($P < 0.001$ versus shRNA-NC), indicating an increased invasive behavior of NDRG1. Next, the wound-healing assay revealed that cell migration was inhibited by NDRG1 silence in HepG2 cells, and representative photography indicated accelerated closure in shRNA-NC transfected cells (Fig. 2C and D). Thus, these findings indicate that up-regulation of NDRG1 contributes to HCC progression, such as vascular invasion and intrahepatic metastasis, by promoting cell migration and invasion.

3.6. Identification of NDRG1 downstream genes in HCC

To identify the downstream genes mediating the effects of NDRG1 in HCC cells, an Agilent Whole Human Genome Oligo Microarray (4 x 44 K) was performed between shRNA-NC and shRNA-NDRG1 clones. Data were analyzed using SBC Analysis System (ShanghaiBio Corporation, Shanghai, China). With a 3.0-fold cut cutoff, expressions of 1117 different oligonucleotides (734 genes) were modulated in shRNA-NDRG1 clones compared with shRNA-NC clones. Furthermore, 26 of these deregulated
genes were chosen for validation microarray data. The results demonstrated the significant correlation of Q-RT-PCR mRNA levels with the microarray transcript levels (Fig. 4, Supplementary microarrays data). Enrichment analysis for GO categories was showed in Table S3, and the most highly enriched clusters of the gene ontology categories included cell proliferation, cell adhesion, chemorepellent activity, chemotactant activity, transmembrane transporter activity, enzyme inhibitor activity, response to external stimulus, regulation of multicellular organismal process, immune response and regulation of immune system process. The pathway enrichment analysis identified a set of statistically significant enriched pathways. The most highly enriched pathways included AKT, EGF, ErbB, Jak-STAT, MAPK, FAS, Calcium signaling pathway, and Cyclins and Cell Cycle Regulation, which play a key role in the tumor development and metastasis. Importantly, our result showed that NDRG1 was able to modulate immune response through several signaling pathway, including complement pathway, antigen dependent B cell activation, cytokines and inflammatory response, stress induction of HSP regulation, which is not report by other researchers.

The genes identified in our research provided a new insight into how overexpression of NDRG1 resulted in the observed tumorigenic pheno-

Fig. 2. Effect of NDRG1 knockdown on cell growth, colony formation, invasion and migration in HCC Cells. (A) HepG2 cells transfection with shRNA-NDRG1 or shRNA-NC vector, the viable cells were detected by CCK-8 assay after 24, 48, and 72 h. Cell numbers were significant different between these two groups ($P < 0.05$). (B) HepG2 cells stably transfected with NDRG1 shRNA or control vectors were scraped and plated on dishes and cultured in G418 for 12 day. The representative dishes showed the inhibition of silencing NDRG1 expression on colony formation. (C) Invasion assay. $1 \times 10^5$ HepG2 cells were placed in each chamber at the beginning of the assay. The number of invaded cells was significantly decreased in shRNA-NDRG1 cells as compared with vector control (shRNA-NC) ($P < 0.001$). (D) HepG2 cells with silencing NDRG1 by siRNA migrated slowly compared with control cells (24 h after scratch).
types such as enhanced cell proliferation, resistance to cell death, increased cell migration, and avoided immune response. Representative examples of networks with enrichment genes in cell adhesion and immune response indicated that NDRG1 transforms multiple signaling pathway and the expression of important molecules, to achieve its biological behavior in HCC (Fig. S2).

3.7. NDRG1 overexpression in HCC patients predicts a poor prognosis

To further verify the biological and clinicopathologic significance of NDRG1, we examined the expression of NDRG1 protein by immunohistochemistry from 143 HCC patients who underwent liver transplantation. Correlation analysis found that NDRG1 positive expression levels were significantly higher in HCC patients with increased tumor size ($P = 0.003$), vascular invasion ($P = 0.002$), pTNM stage ($P = 0.002$), AFP 400U/L ($P < 0.001$) and recurrence ($P < 0.001$; Table 1). Based on immunohistochemical results, all 143 patients with HCC were divided into two groups: positive-expression ($n = 74$) and negative-expression group ($n = 69$). Patients with NDRG1-positive expression had significantly worse prognosis than those with NDRG1-negative expression, either shorter disease-free survival or overall survival ($P < 0.001$; Fig. 5). The recurrence rates of NDRG1-positive HCC patients were much higher than those of NDRG1-negative. Our results indicated that up-regulation of NDRG1 could potentially be an important predictive biomarker of recurrence and poor outcome for patients with HCC.

**Fig. 3.** Cell cycle and apoptosis analysis. (A) Cell cycle distribution of HepG2 cells stably transfected with NDRG1 shRNA (left) or control vectors (shRNA-NC, right). HepG2 cells with silencing NDRG1 expression were arrested in G1 phase. The data were from one of three typical experiments. (B) Fluorescent microscopy images of HepG2 cells nuclei labeled with EdU and Hoechst33342. EdU stain showed red light while Hoechst33342 stain showed blue light, then overlay of the EdU and Hoechst 33342 stain images by Image-pro plus software. As EdU staining cell in S-phase, more S-phase cells in shRNA-NC group (right) than NDRG1 shRNA group (left), and this result was in accord with cell cycle distribution that HepG2 cells proliferation was inhibited and G1 was arrested by silencing of NDRG1 expression. (C) Survival and apoptosis of HepG2 cells with stably transfected NDRG1 shRNA (right) or control vectors (left). Knockdown of NDRG1 induced more cells death and apoptosis.
4. Discussion

Despite advances in surgical and nonsurgical therapies for hepatocellular cancer, metastasis and recurrence still remain the major challenges in clinical practice, and represent the most cause of death in patients with HCC [5,10,19]. The present practical evaluation of the clinical prognosis of liver cancer, including tumor size, number, pTNM stage, venous invasion, may be useful for predicting patient outcome roughly, but those indicators do not have a good linear relationship with HCC metastasis, recurrence and survival [9,20,21]. Therefore, prognosis and treatment of HCC with different biological behavior will benefit from the identification of novel tumor markers with higher specificity and sensitivity. It also will be helpful to improve the clinical strategy and outcome of HCC [22].

In this study, we compared the global protein profiles of HCC tissues among recurrence cancer tissues and non-recurrence cancer tissues, using a 2-DE and MS/MS-based approach. We found that many proteins possibly participated in the processes associated with metastasis and recurrence of HCC after surgical therapy [23,24]. Most of these proteins were involved in the biological processes, such as metabolism, cell motility and invasion, and signal transduction, which usually play key roles in the malignant and metastasis progress (Table. 1). Considering the process of invasion and metastasis, cancer cells are loss of the supply of oxygen and nutrients until plant to the new environment. Thus, genes induced by hypoxia may contribute to cancer cell growth, invasion and metastasis. NDRG1 is up-regulated in hypoxia, therefore, NDRG1 became the subsequent focus of the study [16]. To confirm the consistency of protein abundance changes analyzed with 2-DE between different cancer samples, the expression alteration of NDRG1 was performed by Western Blot analysis. NDRG1 expression occurred at very low level in paired tumor-adjacent normal liver tissues, and it remarkably increased in the HCC samples analyzed. In these contexts, NDRG1 might be assessed as a new diagnostic tissue marker for HCC metastasis and recurrence.

Analyzing the association of NDRG1 expression with pathological characteristics in 143 HCC patients with liver transplantation by immunostaining revealed a significant correlation of NDRG1 expression level with tumor size, vascular invasion, TNM stage, AFP 400 U/L and recurrence. Kaplan–Meier analysis showed that patients with HCC who had NDRG1 positive expression in general had worse prognosis than those with negative expression, both disease-free survival and overall survival. Univariate and multivariate analyses revealed that NDRG1 was an independent prognostic factor for both recurrence and survival in HCC patients. Our clinical data, together with other
research reports, showed that NDRG1 expression was associated with HCC progression, such as PVTT, suggesting that overexpression of NDRG1 contributed to tumor invasion and metastasis [14,25]. Therefore, NDRG1, a sensitive and specific biomarker, its protein expression level may be a powerful prognostic indicator for HCC recurrence and survival.

Hypoxia is a hallmark of cancer, especially in HCC tissue. NDRG1, also known as Drg1 or Cap43, is a member of the N-myc downstream-regulated gene (NDRG) family and is involved in cellular differentiation, activation of p53, spindle checkpoint, apoptosis resistance, metastasis, and hypoxia [11,26]. NDRG1/Case43 expression is regulated with nickel, hypoxia, oxidative stress, vitamins A and D, steroids, histone deacetylase-targeting drugs, oncogene (N-Myc/C-Myc) and tumor suppressor gene (p53 and VHL) [27,28]. Hypoxia induces expression of NDRG1 through Egr-1 binding motif present in the promoter of NDRG1 [29]. Thus NDRG1, a hypoxia-associated gene, may play an essential role in an environment poor in oxygen and nutrients which appears in HCC tissue. But NDRG1 gene in carcinogenesis still remains elusive. High expression of NDRG1 showed lower invasive and metastatic potential in colorectal, prostate and pancreatic cancer [30–33]. On the other hand, overexpression of NDRG1 has been observed in human HCC with aggressive invasion, metastasis and poor patient survival [14]. Therefore, further study on NDRG1 function in HCC is necessary.

The effect of NDRG1 in regulating liver cancer cell motility and invasion was directly showed in a series of in vitro assays. Our experimental data showed that silencing NDRG1 expression could inhibit proliferation, induce malignant cell apoptosis, suppress cancer cell invasion and metastasis. The results of the present study showed that NDRG1 was involved in the invasive/metastatic HCC phenotype. In an effort to illuminate the biological impact of NDRG1 in HCC, we have been successfully used microarray approach to identify NDRG1 targets. A total of 734 genes with at least 3-fold change were identified as potential NDRG1 targets. A number of those genes were in direct or indirect correlation with tumor growth and metastasis. The genes identified as being NDRG1-regulated fell into several enriched GO categories, including extracellular region, immune response, response to biotic stimulus, cell adhesion, cell proliferation, transmembrane transporter activity, enzyme inhibitor activity, extracellular matrix, which have been implicated in the growth and invasion of tumors.

As a multifunctional protein, NDRG1 must possess multiple substrates, thus, affect through multiple signaling pathways. Several pathways modified by NDRG1 were identified in our study, especially including the AKT, EGF, ErbB, p53, Wnt/β-catenin, MAPK, Jak-STAT, apoptotic, stress induction of HSP regulation, cyclins and cell cycle regulation signaling pathway [23,34,35]. Wnt/β-catenin signaling pathway is a master regulator of hepatocarcinogenesis, and NDRG1 may have been implicated in this essential pathway. Our current study identified three genes, and they were CCND2, FOSL1 and PRICKLE1, which act as positive regulators of the Wnt/β-catenin pathway [36]. NDRG1 also functioned downstream of Wnt/β-catenin by interacting with β-catenin and HSP90, which have been reported to link with HCC malignancy [37,38]. Moreover, caspase9, caspase10, STAT1 and Fasl, induced expression by NDRG1, were implicated in apoptotic signaling pathway. Zheng et al. also reported that expression of NDRG1 delayed activation of protein kinase C delta (PKCδ), and made acute myeloid leukemic (AML) cells resistant to apoptosis [15].

Interestingly, we found that hepatoma cells expression of NDRG1 could regulate immune and immune evasion, which never reported by others before. Hepatocellular carcinoma cells with strong expression of NDRG1, seemed to inhibit the production of proinflammatory danger signals and avoid active immune cells. Microarrays results showed that chemokine, such as CCL2, CCL23, CCL26, CCL5, CXCL10, CXCL11, CXCL16, SHC3, STAT1 and VAV3, were increased in NDRG1-knocked down HepG2 cells. Toll-like receptor signaling pathway was blocked and lymphocytes activation was negatively regulated. Thus, our data indicated that overexpression of NDRG1 in hepatoma cells

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<th>Variable</th>
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<td>≥55</td>
<td>25</td>
</tr>
<tr>
<td>HBsAg</td>
<td>Negative</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>42</td>
</tr>
<tr>
<td>MELD score</td>
<td>&lt;20</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>≥20</td>
<td>3</td>
</tr>
<tr>
<td>Tumor number</td>
<td>&lt;3</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>≥3</td>
<td>15</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>≤5 cm</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>&gt;5 cm</td>
<td>16</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>Yes</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>55</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>I–II</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>III–IV</td>
<td>50</td>
</tr>
<tr>
<td>TNM stage</td>
<td>I–II</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>III–IV</td>
<td>21</td>
</tr>
<tr>
<td>AFP</td>
<td>&lt;20</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>≥20</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>&gt;400</td>
<td>56</td>
</tr>
<tr>
<td>Recurrence</td>
<td>Present</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>60</td>
</tr>
</tbody>
</table>

AFP, serum alpha-fetoprotein; HBsAg, hepatitis B surface antigen; MELD, model for end-stage liver disease. TNM stage for HCC was based on The American Joint Committee on Cancer/International Union Against Cancer staging system.

* Chi-Square test, P values less than 0.05 were considered statistically significant; NS, not significant between any groups.
represented a potentially important mechanism for tumor immune evasion and promoted tumor invasion without activating inflammatory responses.

In summary, our current findings indicate that NDRG1 is generally up-regulated in HCC tissues, particularly in the recurrence and metastatic HCC. Clinically, overexpression of NDRG1 in HCC is associated with metastasis, recurrence and poor prognosis, thus, NDRG1 is suggested as a tumor biomarker. NDRG1 is found to be associated with metastasis and recurrence of HCC by immune escape for the first time, although the precise mechanism remains to be elucidated. These interesting data suggest that NDRG1 serves as one of many contributors to tumor growth and progression in HCC, in which immune escape, cooperation with signaling pathways responsible for cell proliferation, invasion and other malignant features could have a synergic role in HCC metastasis and recurrence. These findings might give a more profound insight into the mechanism of HCC recurrence, metastasis and novel strategies for HCC management.

Grant support

This study was financially supported by National Basic Research Program of China (2009CB522407) and Major National S&T Program (2008ZX10002-026).

Conflict of interest

The authors declare no conflict of interest related to this work.

Acknowledgements

We gratefully acknowledge the clinical data support by Jiejie Fu, Chenxi Zhang, Shen Yan, Jun Yu; technical assistance by Prof. Bo-Xiong Zhong (Zhejiang University). The authors are grateful to Dr. Grace Marquez (Samford University, Birmingham, AL, USA) and Sunyi Ye for revision of this manuscript.
Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.06.001.

References