Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma

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We investigated the association of p42.3 expression with clinicopathological characteristics and the biological function of p42.3 in human hepatocellular carcinoma (HCC).

METHODS: We used reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR and western blotting to detect p42.3 mRNA and protein expression in hepatic cell lines. We examined primary HCC samples and matched adjacent normal tissue by immunohistochemistry to investigate the correlation between p42.3 expression and clinicopathological features. HepG2 cells were transfected with a PIRE5-EGFP-p42.3 expression vector to examine the function of the p42.3 gene. Transfected cells were analyzed for their viability and malignant transformation abilities by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation assay, and tumorigenicity assay in nude mice.

RESULTS: p42.3 is differentially expressed in primary HCC tumors and cell lines. Approximately 69.6% (96/138) of cells were p42.3-positive in hepatic tumor tissues, while 30.7% (35/114) were p42.3-positive in tumor-adjacent normal tissues. Clinicopathological characteristics of the HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation (P = 0.031). However, p42.3 positivity was not related to tumor-node-metastasis classification, hepatitis B virus status, or hepatoma type. Regarding p42.3 overexpression in stably transfected HepG2 cells, we discovered significant enhancement of cancer cell growth and colony formation in vitro, and significantly enhanced tumorigenicity in nude mice. Western blot analysis of cell cycle proteins revealed that enhanced p42.3 levels promote upregulation of proliferating cell nuclear antigen, cyclin B1 and mitotic arrest deficient 2.

CONCLUSION: p42.3 promotes tumorigenicity and tumor growth in HCC and may be a potential target for future clinical cancer therapeutics.

Key words: p42.3; Hepatocellular carcinoma; HepG2; Overexpression; Tumorigenicity

Core tip: p42.3 is a novel tumor-specific and mitosis phase-dependent expression gene. It is believed to be involved in tumorigenesis in gastric and colorectal cancer.
cer. To the best of our knowledge, this is the first study to investigate the expression and function of p42.3 in hepatocellular carcinoma (HCC). We found that p42.3 promotes tumorigenicity and tumor growth in HepG2 cells and is overexpressed in HCC. These results suggest that p42.3 may act as a novel tumor biomarker and aid in the development of improved therapeutic strategies.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. The annual number of new HCC cases worldwide is over one million, making it the 5th most common cancer and the 3rd leading cause of cancer-related deaths,[1] accounting for more than 1 million deaths annually.[2] Despite improvements in monitoring and clinical treatment strategies, HCC prognosis remains poor.[3,4] Discovering novel biomarkers that correlate with HCC development or progression may present opportunities to reduce the severity of this disease through early and novel therapeutic interventions.

In our previous research, we cloned the full-length cDNA of the p42.3 gene by using mRNA differential display in a synchronized gastric cancer (GC) cell lines. We found that p42.3 expression is frequently upregulated in primary tumors and embryonic tissues but not in normal tissues from adult organs. Moreover, stable silencing of p42.3 in BGC823 cells suppresses tumorigenicity and cell proliferation with accumulation of cells at G2/M stage of the cell cycle.[5] In addition, Jung et al.[6] reported that the expression of p42.3 mRNA was significantly elevated in colorectal cancer (CRC) tissues compared to normal tissues. All these data indicate that p42.3 plays an important role in tumorigenesis, suggesting that it may be a potential tumor biomarker. In order to elucidate the role of p42.3 in tumorigenesis, we characterized p42.3 expression and validated its biologic significance in HCC.

### MATERIALS AND METHODS

#### Patients and tissues

HCC specimens (n = 138) were collected from 98 men and 40 women (age, 31-74 years; mean ± SD, 52.6 ± 8.7 years) who were inpatients at Beijing Cancer Hospital, Beijing, China, from January 2006 to September 2009. Patient data are shown in Table 1. All patients underwent a radical resection with curative intent and had sufficient clinical information available. No patients had received chemotherapy or radiation therapy. Moreover, 114 adjacent normal hepatic tissues (at least 5 cm distant from the tumor edge) were also collected from HCC patients. Tumor stage was classified according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification. The investigation project and its informed consent have been examined and certified by the Ethics Committee of Beijing Cancer Hospital.

#### Tissue microarray immunohistochemistry

The hepatic tissue microarray was constructed using a tissue array instrument as previously described[7]. For immunohistochemistry studies, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation in 3% H2O2 solution for 10 min at room temperature. After blocking with 5% skim milk, sections were incubated with specific murine p42.3 mAb (1:1000, our lab) at 4 °C overnight, followed by the incubation with the peroxidase-based EnVision TM kit (Dako Cytomation, Cambridgeshire, United Kingdom) for 30 min at room temperature. The reaction product was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark) for 5 min at room temperature. Sections were counterstained with hematoxylin.

Purified IgG from normal mouse sera was used as a negative control. The number of tumor cells or normal hepatic cells was evaluated by two independent pathologists. A specimen with more than 20% immunostained cells was classified as a positive case.

#### Cell lines and cell culture

The 6 human HCC cell lines MHCC97L, MHCC97T3,

| Tissues parameters      | No. of cases | Positive | Negative | P value |
|-------------------------|--------------|----------|----------|---------|
| Gender                  |              |          |          |         |
| Male                    | 98 (71.0)    | 42 (42.9)| 56 (57.1)| NS      |
| Female                  | 40 (29.0)    | 23 (58.0)| 17 (42.0)|         |
| Age at diagnosis (yr)   |              |          |          |         |
| < 60                    | 117 (84.8)   | 52 (44.4)| 63 (55.6)| NS      |
| ≥ 60                    | 21 (15.2)    | 12 (57.1)| 9 (42.9) |         |
| Carcinoma and adjacent tissue |     |          |          | 0.0008  |
| Carcinoma tissue        | 138 (54.8)   | 96 (69.6)| 42 (30.4)|         |
| Adherent tissue         | 114 (45.2)   | 35 (30.7)| 79 (69.3)|         |
| Degree of differentiation|              |          |          | 0.031   |
| Well                    | 42 (30.4)    | 11 (26.2)| 31 (73.8)|         |
| Moderate                | 87 (63.0)    | 39 (44.8)| 48 (55.2)|         |
| Poor                    | 9 (6.5)      | 6 (66.7)| 3 (33.3) |         |
| TNM classification      |              |          |          | NS      |
| Stage I / II            | 101 (73.2)   | 43 (42.6)| 58 (57.4)|         |
| Stage III / IV          | 37 (26.8)    | 19 (51.4)| 18 (48.6)|         |
| HBV                     |              |          |          | NS      |
| Negative                | 41 (29.7)    | 15 (36.6)| 26 (63.4)|         |
| Positive                | 97 (70.3)    | 47 (48.5)| 50 (51.5)|         |
| Type of hepatoma        |              |          |          | NS      |
| Nodular                 | 94 (68.1)    | 44 (46.8)| 50 (53.2)|         |
| Massive                 | 35 (25.4)    | 13 (37.1)| 22 (62.9)|         |
| Diffuse                 | 9 (6.5)      | 5 (55.6)| 4 (44.4) |         |

TNM: Tumor-node-metastasis; NS: Not significant; HBV: Hepatitis B virus.
BEL7402, Huh7, HepG2, and SMMC7721 and the immortal human hepatocyte line HL7702 were routinely maintained as previously described. HL7702 was cultured in Roswell Park Memorial Institute medium (RPMI 1640; Gibco, Grand Island, NY, United States), supplemented with 20% fetal bovine serum (FBS; Gibco). BEL7402 and SMMC7721 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. The remaining cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS. All media contained 100 units/mL penicillin and 100 µg/mL streptomycin. All cell lines were maintained at 37 °C in 5% CO₂.

Reverse transcription-polymerase chain reaction and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using TRIzol (Qiagen, United States). The prepared RNA (5 µg) was mixed with oligo-dT primers and reverse-transcribed with moloney murine leukemia virus reverse transcriptase (Promega, United States) for 60 min at 37 °C, followed by polymerase chain reaction (PCR) amplification with specific primers for p42.3 (forward: 5′-TGAGCTGG-GGCGCTGAAG-3′; reverse: 5′-ACTCCATCGCTGT-GTTTCAAT-3′). PCR amplification was performed in 20 µL using a thermocycler (Biometra, Germany) with the following PCR program: pre-denaturation for 5 min at 94 °C, denaturation for 45 s at 94 °C, annealing for 45 s at 61 °C, extension for 45 s at 72 °C, and a final elongation at 72 °C for 10 min. β-Actin served as an internal positive control (forward: 5′-TCACCCACACTGTGGCCTAC-TCAAG-3′; reverse: 5′-CAGCGGAACCGCTCATTGC-CAATGG-3′). PCR was performed for 24 or 32 cycles (β-actin 24 cycles; p42.3 32 cycles). PCR products were separated by electrophoresis on a 1.5% agarose gel. Quantitative real-time reverse transcription-PCR (Q-RT-PCR) using SYBR-Green Master PCR mix (Applied Biosystems, Carlsbad, CA, United States) was performed and yielded similar results.

Western blotting

Cell lysates were prepared by incubating cells at 4 °C for 1 h in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 2 mmol/L dithiothreitol, 5 mmol/L ethylene diamine tetraacetic acid, 100 mmol/L NaCl, and 2 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of protein were electrophoresed on a 12% sodium dodecylsulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using standard techniques. We used four specific antibodies obtained from Santa Cruz Biotechnology: proliferating cell nuclear antigen (PCNA) (diluted 1:300; F-2), cyclin B1 (diluted 1:500; H-433), cell division cycle 25 A (Cdc25A) (diluted 1:500; DCS-122), and cell division cycle 25 homolog C (Cdc25C) (diluted 1:500; C-20). The following specific antibodies were also used: mitotic arrest deficient 2 (MAD2) (diluted 1:1000; Ab70383; Abcam, United Kingdom), actin (diluted 1:10000, AC-15; Sigma, United States), and p42.3 (diluted 1:1000; our lab). Nonspecific binding was blocked using a 5% fat-free milk solution. Signals were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Plasmid construction and cell transfection

The whole coding region of p42.3 was cloned into the pIRESE2-EF1-GFP vector at the BamHI and HindIII sites. Nucleotide sequences of the subcloned DNAs were verified by sequencing. HepG2 were selected and cultured at 60%-70% confluence in 35 mm plates. Cells were transfected with recombinant p42.3 plasmids or an empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, United States). At 48 h post-transfection, cells were seeded for 21 d in selection medium containing 400 µg/mL G418 to screen for stable clones. To confirm the transfection efficiency, RT-PCR and Western blot analysis were performed.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and soft agar colony formation assay

Stably transfected cells in were seeded (2 × 10⁵) in duplicates into each well of a 96-well culture plate and grown in 200 µL DMEM with 5% FBS; 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Genview, Florida, United States) (5 mg/mL) was added at 0, 24, 48, 72, 96 and 120 h. The MTT was then removed after 4 h incubation; 100 µL of dimethylsulfoxide (Amresco, Cochrane, United States) was added to each well, then incubated for 30 min. Absorbency was measured at 570 nm using an iMark Microplate Reader (Bio-Rad, CA, United States).

For the soft agar assay, cells (2 × 10⁴) were trypsinized and resuspended in 4 mL of 0.3% agarose gel. Quantitative real-time reverse transcription-PCR (Q-RT-PCR) using SYBR-Green Master PCR mix (Applied Biosystems) was performed. Cells were plated in triplicate (p42.3 32 cycles). p42.3 was then injected subcutaneously into the left dorsal flank of 10, 4-wk-old female nude mice. As a control, the right side was inoculated with HepG2-vector. Tumor diameters were checked every 3 d, and tumor volume was calculated according to a²/2 (a ≥ b). Tumor specimens were collected at 15 d after injections and split. Immunohistochemistry (IHC) analysis was used to detect p42.3 protein expression. Three independent experiments were performed and yielded similar results.

Statistical analysis

To evaluate the possible differences of p42.3 expression in different hepatic specimens, we performed Pearson's
χ² test. The Student’s two-sided t-test was used to compare test and control sample values in MTT assay, soft agar colony formation assay and tumorigenicity assay. All statistical analyses were carried out using the SPSS statistical software package 16.0 (SPSS Inc., United States). P values < 0.05 were considered statistically significant.

RESULTS

p42.3 protein expression in human tumor cell lines
p42.3 mRNA and protein expression were examined in 6 human HCC cell lines and the immortal human hepatocyte HL7702. RT-PCR and Q-RT-PCR showed that p42.3 mRNA was expressed in all 7 cell lines (7/7, 100%), and the lowest expression was found in HepG2 cells (Figure 1A and B). Consistent with mRNA expression levels, p42.3 protein was expressed at high levels in all cell lines except HepG2 cells (6/7, 85.7%) (Figure 1C and D). Thus, we confirmed that the HepG2 cell line is a p42.3-deficient line and could therefore be used as a model to investigate p42.3 protein function.

p42.3 protein levels in human primary tumors
To characterize p42.3 expression in HCC specimens, IHC was performed on tumor tissues and tumor-adjacent normal tissues. We found p42.3 protein was detected in 69.6% (96/138) of hepatic tumor tissues. However, p42.3 expression was less apparent, with significantly less positive cells (30.7%, 35/114) in tumor-adjacent normal tissues (P < 0.001, Table 1 and Figure 1E). The results indicate that p42.3 protein is highly expressed in primary HCC tissues rather than tumor-adjacent normal tissues. Analysis of the clinicopathological characteristics of the 138 HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation (P = 0.031, Table 1). However, we found no relationship between p42.3 positivity and tumor TNM classification, hepatitis B virus status, or type of hepatoma.

Overexpression of p42.3 induces PCNA, cyclin B1 and MAD2 expression in HepG2 p42.3-deficient cells
To examine the gene function of p42.3 overexpression on HCC cells, we stably transfected the pIRES2-EGFP-
p42.3 expression vector into HepG2 cells. A cell line that stably expresses p42.3 (HepG2-p42.3) was generated and analyzed by western blotting. As shown in Figure 2, p42.3 protein was not detected in cells stably transfected with the empty vector. However, p42.3 protein was significantly increased in the p42.3 overexpressing cells, HepG2-p42.3-1 and HepG2-p42.3-2. These results indicated that the eukaryotic vector for p42.3 used in this study sufficiently upregulates p42.3 expression in HepG2 cells.

Since p42.3 is a novel cell cycle-dependent protein, we investigated cyclin B1 and other M phase-related proteins in p42.3-expressing HepG2 cells and control cells (HepG2-vector). We found that p42.3 expression resulted in a significant upregulation in PCNA, cyclin B1 and MAD2 protein levels. However, Cdc25A and Cdc25C protein levels only slightly changed with p42.3 expression (Figure 2).

**Overexpression of p42.3 promotes growth and colony formation in HepG2 cells**

The effects of p42.3 overexpression on the viability of HepG2 cells were measured using an MTT colorimetric assay. We found that transfection with pIRES2-EGFP-p42.3 promotes HepG2 cell growth. A stable single clone of HepG2-p42.3-1 and HepG2-p42.3-2 cells grew much faster over a 6-day period when compared to parental HepG2-vector cells, indicating that p42.3 may confer a strong growth capability in HepG2 cells ($P < 0.01$, Figure 3A).

The colony formation assay was used to evaluate the ability for anchorage-independent growth of cells in soft agar medium. Our data showed a significant increase in HepG2-p42.3-1 and HepG2-p42.3-2 colony formation in both number and size; however, the HepG2-vector cells only formed a few small colonies ($P < 0.01$, Figure 3B and C). This suggests that p42.3 confers anchorage-independent growth to cells.

**Overexpression of p42.3 promotes HepG2 cell tumorigenicity**

We tested p42.3 tumorigenicity in nude mice. HepG2-p42.3 cells were injected subcutaneously into the left dorsal flank of female nude mice (BALB/c), the right side was injected with HepG2-vector cells as a control. Of the 5 animals injected with HepG2-p42.3-1 or HepG2-p42.3-2 cells, tumors appeared faster and were larger than in the controls (HepG2-vector) ($P < 0.01$, Figure 4A and B). After the animals were sacrificed, the xenografts were removed and collected for immunohistochemistry analysis. The results showed that p42.3 protein was found in all HepG2-p42.3 xenografts, but that p42.3 protein was not found in HepG2-vector xenografts (Figure 4C). These results further confirmed that the p42.3 overexpression promotes tumorigenicity of HepG2 cells.

**DISCUSSION**

p42.3 is a highly conserved mammalian gene and strong G2 induction[5,9-11]. Moreover, p42.3 is involved in Chromosome segregation[12], it may play a key role in tumorigenicity. Our previous studies have shown that p42.3 was overexpressed in GC tissue and its expression is cell cycle dependent in the BGC823 cell line, expression peaked at early G1 phase[5,13]. Additionally, reduced proliferation and tumorigenic properties were detected in the BGC823 cell line that lacked p42.3[9], suggesting that p42.3 is involved in gastric carcinogenesis. While most studies have focused on the roles of p42.3 in GC development[5,13,14],
the roles of p42.3 in other cancer remain to be elucidated. Therefore, we characterized p42.3 expression in HCC tissues from patients. Moreover, we investigated p42.3 functions and potential mechanisms of action in HepG2 cells.

In 7 human HCC cell lines, consistent with the mRNA expression, p42.3 protein was highly expressed with the exception of the HepG2 line. In concert with our previous study in GC cells, we found that the p42.3 gene was highly expressed in the majority of the tested tumor cell lines. This suggests that the p42.3 gene is overexpressed in tumor cells. In previous research, data showed that the p42.3 gene is closely related to GC and CRC [5,6]. Similarly, our current data revealed that the p42.3 protein was expressed in 64.7% of hepatic tumor tissues compared to only 35.3% in tumor-adjacent normal tissues. The clinical p42.3 data in GC, CRC and HCC tissues suggest that upregulation of p42.3 may be a common feature in a variety of tumors.

Our results further support the hypothesis that p42.3 might stimulate cellular viability and malignant transformation since overexpression of p42.3, by stable transfection of the pIRES2-EGFP-p42.3 into HepG2 cells, significantly promotes cancer cell growth by MTT colo-
Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. Discovering novel biomarkers that correlate with disease and tumor stage is important for improving treatment outcomes. In this study, we investigated the expression and function of p42.3 in HCC. The authors found that p42.3 promotes tumorigenesis and tumor growth in HepG2 cells and is overexpressed in HCC.

**Innovations and breakthroughs**

Recent reports have highlighted that p42.3 is involved in GC and CRC. This is the first study to investigate the expression and function of p42.3 in HCC. The authors found that p42.3 promotes tumorigenesis and tumor growth in HepG2 cells and is overexpressed in HCC.

**Applications**

In understanding the expression and function of p42.3 in HCC, this study may represent a future strategy as a therapeutic target and/or improve clinical cancer HCC treatment.

**Peer review**

The authors examined the expression of p42.3 and its function in HCC. These data revealed that p42.3 was increased in HCC and in all HCC cells with the exception of HepG2 cells. Moreover, p42.3 expression was correlated with tumor differentiation. p42.3 promotes tumorigenesis and tumor growth in HCC; therefore, it may be used as a potential target to improve the clinical treatment of HCC.

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