Liver Cancer

Overexpression of p28/ gankyrin in human hepatocellular carcinoma and its clinical significance

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Overexpression of p28/gankyrin plays an important role and contributes to the metastasis potential in the process of carcinogenesis. p28/gankyrin may become a specific biological tissue marker for the pathological diagnosis of HCC.

INTRODUCTION

Human primary hepatocellular carcinoma (HCC) is one of the most common types of malignant cancer in Asia and Africa where hepatitis virus infection and exposure to specific liver carcinogens are prevalent[1-4]. HCC has ranked second in cancer mortality in China since the 1990s and is increasing in frequency among males in many countries[5,6]. Although the major viral and environmental risk factors for HCC development have been unraveled[7,8], the oncogenic pathways leading to malignant transformation of liver cells have long remained obscure[9]. It has been widely reported that some tumor suppressor genes such as p53 and p16[9-11] play a vital role in the development of HCC[10,11], while few oncogenes that control growth behavior or metastatic potential of HCC have been underscored.

p28 gene was initially cloned in human cDNA library by comparing the amino-acid sequence of a subunit isolated from the purified bovine erythrocyte PA700 complex with protein structures in databases of Homo-Protein cDNA Bank[12-14]. p28 protein is one of the non-ATPase subunits of the 26 S proteasome with other proteins. Recently, another gene named ‘gankyrin’ was cloned by cDNA subtractive hybridization in HCC may be associated with the ubiquitin-proteasome pathway[22-24]. So far, the mechanism of up-regulation of p28/gankyrin in HCC is still unknown.

In order to elucidate the role of p28/gankyrin in carcinogenesis of HCC and its correlation with clinical parameters, the following study was carried out.

MATERIALS AND METHODS

Sample collection and processing

All 64 HCC specimens and their para-carcinoma tissues (more than 2 cm away from the focus), were sampled from 64 patients who had undergone curative hepatectomy (58 men and 6 women; mean age 46.4±10.5 years). Patients who had received radiotherapy or chemotherapy before hepatectomy were excluded. Non-tumor liver tissues were obtained from...
22 patients who had received hepatic hemangiectomy. Ten different types of human normal tissues were from 2 men of accidental deaths. They were all cases from 1999 to 2000 in Eastern Hepatobiliary Surgery Hospital in Shanghai, China. Informed consent was obtained from all patients for subsequent use of their resected tissues. These specimens were immediately dissected into small pieces under aseptic condition within half an hour, quickly-frozen and preserved in liquid nitrogen before subsequent procedures. The specimens used for immunohistochemistry (IHC) were routinely processed, formalin-fixed and paraffin-embedded, at least 2 serial paraffin sections of 4 mm-6 mm thick were made, one for hematoxylin and eosin (HE) staining and the other for p28\textit{/gankyrin} protein detection.

**Cells lines** A series of cell lines (ATCC, Rockville, MD) were investigated in this study, including HepG2 (ATCC HB-8065), HuH-7, SK-Hep-1 (ATCC HTB-52), Chang liver (ATCC CCL-13), and a human fetal hepatocyte cell line WRL-68 (ATCC CL-48). They were maintained, as specified by the suppliers, in Dulbecco’s modified Eagle medium or other recommended mediums supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO\textit{2} in air.

**Northern blot analysis of p28\textit{/gankyrin} transcript** Preparation and labeling of the probe Polymerase chain reaction (PCR) of a human fetal liver cDNA library (provided by Max-Planck Institute) was performed in a final volume of 50 ml containing all four dNTPs (each at 200 mmol/L), 1.25 mmol/L MgCl\textit{2}, 2.5 units of Taq (TaKaRa Biotech, Dalian, China) and each primer at 0.5 mmol/L. The following temperature program was used: 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 40 s, 52°C for 30 s and 72°C for 55 s, followed by a final extension at 72°C for 5 min. Primers used for amplification were human p28\textit{/gankyrin} sense primer corresponding to nucleotides 2-19 (5’- GCCGATCCAGTGTGCTGGGACAGC-3’), and antisense primer complementary to nucleotides 830-847 (5’- GCCAATTCCGGACAAGAGTCACATG-3’) with the BamHI and EcoRI restriction sites at their 5’ strand ends respectively. The PCR product was cloned into pcDNA3.1 vector (Invitrogen, Groningen, Netherlands) to generate the clone full.hup28_pcDNA3.1, which was confirmed by sequencing with an automatic DNA sequencing (ABI model 3700). The purified PCR product was labeled with α-32P-dATP by a random primer labeling method as described\textsuperscript{[25]} with Prime-a-Gene Labeling System (Promega, Madison, WI).

**RNA extraction and preparation of the hybridization membrane** Samples subjected to RNA analysis were isolated from surgical specimens of HCC and the para-carcinoma liver tissues of 64 patients and from a series of non-tumor liver samples, normal human tissues and hepatoma cell lines by the acid guanidinium thiocyanate-phenol-chloroform extraction method as previously described\textsuperscript{[26)}. Total RNA (40mg) was denatured and separated by electrophoresis in a 1.0% agarose gel containing 2.2 mol/L formaldehyde and then transferred to nitrocellulose membranes (BA85, Schleicher Schuell, Germany). The membranes were dried in a vacuum drying oven at 80°C for 2 h and sealed in a plastic bag for use.

**Northern blot analysis** Hybridization of the membranes was performed by using the labeled p28\textit{/gankyrin} cDNA as the probe at 42°C for 2 h in a solution containing 50% formamide, 5×SSC, 0.1% SDS and 5× Denhardt’s after the membranes had been pre-hybridized in the same solution with 0.1 mg/ml salmon sperm DNA at 42°C for 4 h. After this hybridization, the membranes were rinsed in stringent conditions (65°C for 30 min in a washing buffer of 0.1×SSC and 0.1% SDS) and then exposed to Kodak X-ray film at -80°C for 14 days. The expression level of mRNA was normalized with ethidium bromide-stained 18 s rRNA as internal standard and analyzed by Phosphor Imager (FLA 2000, Fujifilm, Japan).

**Immunohistochemical analysis of p28\textit{/gankyrin} protein** Preparation of the polyclonal antibody A polyclonal antibody against p28\textit{/gankyrin} protein (226 amino acid) was prepared. Briefly, a 683-bp fragment from nucleotide 2 to 684 (amino acid 1-221) was generated by PCR and subcloned into the fusion expression vector pPROEX-HTb (Gibco/BRL Life Technologies, NY) using BamHI and XhoI. The expression vector was then transformed into competent Escherichia coli DH5α for induction of 6×histidine-fused protein with 0.6 mmol/L isopropyl-1-thio-b-D-galactopyranoside (IPTG) at 37°C for 3 h. The cells were harvested and lysed with ice-cold TS buffer containing 50 mmol/L Tri- HCl (pH 8.0), 150 mmol/L NaCl, 1 mg/ml lysozyme, 5 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.2 mg/L aprotinin and 1% Triton X-100. The lysates were purified by affinity chromatography with Ni-NTa agarose column (Qiagen, Germany) and identified by electrophoresis on a 10% SDS-PAGE gel. The purified fusion protein emulsified with Freund’s adjuvant (Gibco/BRL Life Technologies, NY) was used to immunize two New Zealand rabbits (male, 2.5-3.0 kg) for preparation of the polyclonal antibody. Antibody valence was determined by double immunodiffusion test on 1% agar gel. For the specificity, affinity chromatography was applied to purify the antibody from the immune sera by an agarulum column.

**Immunohistochemical staining** p28\textit{/gankyrin} protein was detected in HCCs, para-carcinoma liver tissues and SK-Hep1 cell line by using peroxidase-labeled secondary antibody two-step IHC technique with the DAKO EnVision system (DAKO Corporation, carpinteria, USA). All paraffin embedded samples were deparaffined and rehydrated, and the SK-Hep1 cells cultured on coverslips were fixed in 4% formalin for 30 min, then pretreated with citrate buffer (0.01 mol/L citric acid: pH 6.0) for 15 min at 100°C in a microwave oven. After being treated with 0.3% H\textsubscript{2}O\textsubscript{2} for 10 min to block the endogenous peroxidase, the sections were incubated with 10% fetal calf serum for 30 min to reduce nonspecific binding, and then the primary p28\textit{/gankyrin} antibody was applied to the sections at 4°C overnight. The sections were subsequently incubated with horseradish peroxidase (HR) labeled goat anti-rabbit immunoglobulin for 30 min, followed by incubation with prepared liquid 3,3’-diaminobenzidine (DA) + substrate-chromogen solution for 10 min at room temperature. Finally, the sections were counterstained with hematoxylin. The negative controls were conducted by substituting rabbit normal serum for the primary antibody to verify the possibility of false-positive responses from the secondary antibody.

**Clinical data and histopathological parameters** The following variables were evaluated: age, sex, HBsAg, accompanying cirrhosis, serum α-fetoprotein (AFP) level, tumor size, satellite nodules, formation of tumor capsule, fibrous capsular infiltration, portal vein tumor thrombus, microscopic invasion of the tumor into hepatic vein, and histological grade of HCC according to Edmonson and Steiner’s classification\textsuperscript{[27]}.

**Statistical analysis** Data of p28\textit{/gankyrin} mRNA levels were presented as means...
RESULTS

Clinical profiles and histopathologic examination

64 patients with primary HCC were recruited in this study, including 58 males and 6 females with ages ranging from 24 to 74 years (mean, 46.4 ± 10.5 years). Serum anti-hepatitis B virus was detected in 45 (70.3%) of 64 patients and positive anti-hepatitis C virus in serum was found in 6 (18.8%) of 32 patients examined. 50 (78.1%) patients showed increased serum AFP level and levels above 1000 mg/L were observed in 29 of them. Histopathologically, 47 of 64 HCCs and 15 of 22 non-tumor liver tissues displayed cirrhosis. Among the 46 patients with fibrocapsule formation around HCC, 21 had broken through and 19 of them had microscopic hepatic vein involvement. Portal vein tumor thrombus was found in 18 (28.1%) patients and 32 (50.0%) patients had satellite nodules in primary HCC. According to Edmonson and Steiner's classification, 20 (31.3%) and 44 (68.7%) patients were classified to grade I-II group and grade III-IV group, respectively.

Tissue distribution of p28/gankyrin mRNA

Northern blot analysis showed that p28/gankyrin was expressed as one single transcript of 1.5 kb, corresponding well with the size of the cloned cDNA (Figure 1 A). p28/gankyrin was strongly expressed in brain and heart, whereas weaker expression was found in lung and spleen. Faint signals were discerned in muscle, but no signals were observed in digestive system including liver, pancreas, stomach, small and large intestine.

Figure 1 Northern blot of p28/ gankyrin in a series of human normal tissues, hepatoma cell lines, HCC and non-tumor liver tissues. (A) p28/gankyrin expression in ten different normal adult human tissues. (B) p28/gankyrin expression in five hepatoma cell lines and NIH/3T3 cell. (C) p28/gankyrin expression in HCC and para-carcinoma liver tissues. L indicates the para-carcinoma liver tissues, and K indicates the HCC tissues. (D) Comparison of p28/gankyrin expression in normal human liver tissues, liver cirrhosis and HCC tissues. Each bottom panel showed equal amount of total RNA loading as indicated in 28S and 18S rRNA.

p28/gankyrin expression in hepatoma cell lines

In the 5 human hepatoma cell lines, p28/gankyrin mRNA was detected as the expected size with various expression levels (Figure 1 B). It was expressed strongly in SK-Hep1, weakly in HepG2 and no expression has been found in NIH/3T3 cell line.

Figure 2 Densitometric analysis. The ratio of the optical density of p28/gankyrin mRNA to the corresponding 18S rRNA signal was calculated and expressed as means ± SD. (A) Comparison of p28/gankyrin expression in human liver cirrhosis, para-carcinoma liver tissues and HCCs. *The expression in HCCs was significantly higher than that in liver cirrhosis (P < 0.05). **The expression in HCCs was significantly higher than that in para-carcinoma liver tissues (P < 0.01). (B) Clinicopathological significance of p28/gankyrin expression in HCCs. p28/gankyrin mRNA levels were significantly higher (P < 0.021) in portal vein thrombus group than in non-thrombus group, higher (P = 0.047) in microscopic hepatic vein involvement group than in non-hepatic vein involvement group.

Correlation of p28/gankyrin mRNA expression with clinicopathological parameters

Comparison of clinical profiles of HCC patients with p28/ gankyrin mRNA expression was summarized in Table 1. From these results, age, sex, HBsAg, and accompanying cirrhosis were not significantly correlated with the expression levels of p28/gankyrin (P > 0.05). Relationships of p28/gankyrin mRNA expression with histopathological parameters were listed in Table 2. These results showed that there were no significant correlation between p28/gankyrin mRNA expression and various parameters, including serum AFP level, tumor size, satellite nodules, formation of tumor capsule, fibrous capsular

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infiltration, and histological grade of HCC. However, it was noted that p28/gankyrin mRNA levels (Figure 2B) were significantly higher ($P=0.021$) in portal vein thrombus group ($n=18$) than in non-thrombus group ($n=46$), higher ($P=0.047$) in microscopic hepatic vein involvement group ($n=19$) than in non-hepatic vein involvement group ($n=45$). This fact implies that tumors with high levels of p28/gankyrin mRNA expression tend to be more metastatic than those with low levels.

**Immunohistochemical examination**

Immunohistochemistry was performed in HCCs and corresponding para-carcinoma liver tissues to determine the exact cellular site of p28/gankyrin protein. p28/gankyrin was moderately to intensely present in the cytoplasm of most hepatocytes in HCC tissues, while nuclear immunostaining was also occasionally observed (Figure 3A). In para-carcinoma liver tissues, p28/gankyrin was weakly present in the cytoplasm of hepatocytes (Figure 3B). The histospecific expression of p28/gankyrin was absent in the bile duct cells, blood vein endometrial cells and other interstitial cells in liver tissues (Figure 3C). In SK-Hep1 cell line immunostaining, p28/gankyrin was mainly present in the cytoplasm, while occasionally in the nucleus (Figure 3D).

**Table 2** Clinicopathological features of patients with HCC: comparison with p28 mRNA expression

| Variables                          | n  | mRNA level± | Df | p-valueb |
|------------------------------------|----|-------------|----|----------|
| Serum AFP (ng/ml)                  |    |             |    |          |
| <25                                | 14 | 2.439±0.362 |    | 0.362    |
| 25–1000                           | 21 | 2.469±0.273 |    | 0.273    |
| >1000                             | 29 | 2.821±0.389 |    | 0.389    |
| Tumor size (cm)                    |    |             |    |          |
| <5                                | 21 | 2.393±0.206 |    | 0.124    |
| 5–10                              | 27 | 2.852±0.468 |    | 0.468    |
| >10                               | 16 | 3.001±0.652 |    | 0.652    |
| Capsule formation                  |    |             |    |          |
| Positive                           | 46 | 2.661±0.421 |    | 0.242    |
| Negative                           | 18 | 3.016±0.460 |    |          |
| Fibrous capsular infiltration      |    |             |    |          |
| Positive                           | 21 | 2.833±0.636 |    | 0.240    |
| Negative                           | 25 | 2.513±0.226 |    |          |
| Hepatic vein involvement           |    |             |    |          |
| Positive                           | 19 | 3.726±0.306 |    | 0.047    |
| Negative                           | 45 | 2.442±0.532 |    |          |
| Satellite nodules                  |    |             |    |          |
| Absent                             | 32 | 2.467±0.209 |    | 0.243    |
| Present                            | 32 | 2.931±0.554 |    |          |
| Portal vein thrombus               |    |             |    |          |
| Absent                             | 46 | 2.390±0.230 |    | 0.021    |
| Present                            | 18 | 3.971±0.384 |    |          |
| Histological grade                 |    |             |    |          |
| I–II                               | 20 | 2.899±0.538 |    | 0.466    |
| III–IV                             | 44 | 2.859±0.655 |    |          |

Abbreviations: serum AFP, serum alpha-fetoprotein.

*Results were expressed as the mean±standard deviation.

*P*-value was based on the analysis of variance (ANOVA) or Student’s *t*-test, variances were adjusted by Bartlett’s method.

*According to Edmonson and Steiner’s classification.

Figure 3 Immunohistochemical staining of P28/Gankyrin in HCC, para-carcinoma liver tissue and SK-Hep1 cell line (A) p28/gankyrin was moderately to intensely present in the cytoplasm of most hepatocytes in HCC tissue, and occasionally present in the nucleus of hepatocytes. (B) In contrast, p28/gankyrin was weakly present in the cytoplasm of hepatocytes in para-carcinoma liver tissue. (C) p28/gankyrin protein signals were specifically present in the cytoplasm of hepatocytes, while absent in bile duct cells, blood vein endometrial cells and other interstitial cells. (D) In SK-Hep1 cells, p28/gankyrin was mostly located in cytoplasm and occasionally in the nucleus.
DISCUSSION

*p28/gankyrin* gene has been identified recently as an oncogene expressed in HCC and its up-regulation is not related to the grade or stage of the tumors[21]. However, the above mentioned study did not address the potential importance of normal or cirrhosis liver, in the context of high *p28/gankyrin* expression observed in HCC, nor did it investigate *p28/gankyrin* mRNA expression with respect to histopathological parameters of HCC. In the present study, *p28/gankyrin* mRNA expression was examined in the normal liver, liver cirrhosis, and HCC. *p28/gankyrin* mRNA was absent in normal liver samples, weakly expressed in liver cirrhosis samples and intensively expressed in 62 of 64 (96.8%) HCC samples compared with the para-carcinoma liver tissues by northern blot analysis. These findings indicate that *p28/gankyrin* is a useful biological marker of HCC and plays an important role in the process of liver carcinogenesis.

Interestingly, *p28/gankyrin* mRNA levels were significantly higher in HCCs with portal vein tumor thrombosis than in those without. Microscopic hepatic vein involvement is also an important correlated parameter with regard to *p28/gankyrin* mRNA expression. It is well known that hepatocellular carcinoma has a strong propensity to invade vessel and duct systems[26-30]. Rapid invasion and metastasis of HCC seriously hold back the clinical therapy and also significantly affect the occurrence of HCC, which is like the alteration of serum AFP levels in patients with HCC[34,35]. This incident implies that to understand the mechanism of liver carcinogenesis promoted by *p28/gankyrin*, the involvement of *p28/gankyrin* in liver development process should be considered.

*p28/gankyrin* was strongly expressed in SK-Hep1 cells, while weakly in HepG2 cells. It should be noted that SK-Hep1, not HepG2, has the tumorigenic potential when inoculated in nude mice[36]. Thus, the higher expression of *p28/gankyrin* in SK-Hep1, to some extent, contributes to the cell tumorigenesis. This conclusion coincides well with our experiment that *p28/gankyrin* caused cell transformation after being transfected into NIH/3T3 cells (data not shown).

By using our specific polyclonal antibody against *p28/gankyrin* protein, the cellular localization of *p28/gankyrin* expression in HCC was determined. *p28/gankyrin* protein was mainly localized in the cytoplasm of hepatocytes, from weakly in hepatocytes of para-carcinoma cirrhosis liver to intensely in hepatocytes of HCC. Occasionally, the nuclear localization of *p28/gankyrin* in malignant hepatocytes of HCC was also observed. It is interesting that *p28/gankyrin* protein can not be detected in bile duct cells, blood vein endometrial cells and other interstitial cells in liver tissues. The prospective application of *p28/gankyrin* in clinical pathological diagnosis as a specific tissue marker should be investigated further by collecting more examination data and statistical analysis.

In conclusion, *p28/gankyrin* mRNA is markedly overexpressed in HCC compared with para-carcinoma liver, normal liver or liver cirrhosis. In addition, in HCC, *p28/gankyrin* mRNA expression correlates significantly with metastasis potential of this cancer. The *p28/gankyrin* protein is particularly highly expressed in the hepatocytes of HCC. These findings suggest that *p28/gankyrin* plays an important role in the carcinogenesis of HCC by influencing tumor metastasis behavior and that it may serve as a new specific tissue marker for the pathological diagnosis of HCC.

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