Gankyrin-Mediated Dedifferentiation Facilitates the Tumorigenicity of Rat Hepatocytes and Hepatoma Cells

Wen Sun,1* Jin Ding,1* Kun Wu,1 Bei-Fang Ning,2 Wen Wen,1 Han-Yong Sun,1 Tao Han,1 Lei Huang,1 Li-Wei Dong,1 Wen Yang,1 Xing Deng,2 Zhong Li,1 Meng-Chao Wu,1 Gen-Sheng Feng,1,3 Wei-Fen Xie,2 and Hong-Yang Wang1

Gankyrin is a critical oncoprotein overexpressed in human hepatocellular carcinoma (HCC). However, the mechanism underlying gankyrin-mediated hepatocarcinogenesis remains elusive. Herein, we provide evidence that gankyrin expression was progressively elevated in liver fibrosis, cirrhosis, and HCC. Levels of gankyrin expression were closely associated with the dedifferentiation status of hepatoma in patients. Decrease of hepatocyte characteristic markers and increase of cholangiocyte-specific markers were observed in rat primary hepatocytes with enforced gankyrin expression and diethylnitrosamine (DEN)-triggered rat hepatocarcinogenesis. Overexpression of gankyrin also attenuated the hepatic function of primary hepatocytes, which further suggests that gankyrin promotes the dedifferentiation of hepatocytes. Moreover, elevated expression of gankyrin closely correlated with the expression of HCC stem/progenitor cell markers in DEN-triggered hepatocarcinogenesis and human HCCs. Hepatoma cells derived from suspension-cultured spheroids exhibited a higher gankyrin level, and enforced gankyrin expression in hepatoma cells remarkably enhanced the expression of CD133, CD90, and epithelial cellular adhesion molecule (EpCAM), indicating a role of gankyrin in hepatoma cell dedifferentiation and stem/progenitor cell generation. In contrast, down-regulation of gankyrin in hepatoma cells by lentivirus-mediated microRNA delivery significantly improved their differentiation status and attenuated malignancy. Interference of gankyrin expression in hepatoma cells also diminished the proportion of cancer stem/progenitor cells and their self-renewal capacity. Furthermore, gankyrin was found to bind hepatocyte nuclear factor 4α (HNF4α), which determines hepatocyte differentiation status and enhances proteasome-dependent HNF4α degradation in hepatoma cells. The inverse correlation of gankyrin and HNF4α was further confirmed in primary hepatocytes, DEN-induced hepatocarcinogenesis, and human HCCs. Conclusion: Gankyrin-mediated dedifferentiation of hepatocytes and hepatoma cells via, at least partially, down-regulation of HNF4α facilitates HCC development, and interference of gankyrin expression could be a novel strategy for HCC prevention and differentiation therapy. (HEPATOLOGY 2011;54:1259-1272)

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third most frequent cause of cancer death in the world.1 The high heterogeneity of HCC makes it difficult to eliminate the cancer cells with chemotherapy alone. Meanwhile, surgical resection and liver transplantation
are limited to the early stage of HCC. Late diagnosis, recurrence, and metastasis result in the poor prognosis of HCC worldwide. The 5-year survival rate of patients having undergone surgical resection is disappointingly low. It is necessary to elucidate the underlying mechanisms of hepatocarcinogenesis and identify novel targets for HCC prevention and therapy.

Hepatocyte dedifferentiation is a key cellular event during hepatocarcinogenesis, which is featured by an alteration of morphology and loss of hepatic function. Hepatoma has been characterized as a heterogeneous complex that includes undifferentiated cells with infinite proliferative capacity. Induction of hepatoma cell differentiation has been considered as a promising strategy for HCC treatment. Clinicopathological studies have demonstrated that poorly differentiated HCCs are associated with poorer convalescence, and that a higher differentiation grade of HCCs is related to a lower recurrence rate. Assessment of differentiation grades of HCCs is not only important for the evaluation of pathological diagnosis and prognosis, but is also critical for the optimization of therapeutic strategies. The maintenance of liver architecture and hepatic function is cross-regulated by a set of liver-enriched nuclear factors, termed hepatocyte nuclear factors (HNFs), which includes HNF1α, HNF3β, and HNF4α, and so on. Among the members of the HNF family, HNF4α is most closely associated with the differentiation status of HCC. Our previous study has indicated that forced reexpression of HNF4α was, to some extent, sufficient to induce the differentiation of hepatoma cells into hepatocytes.

Gankyrin, also named 26S proteasome non-ATPase regulatory subunit 10, was reported to be an oncoprotein principally overexpressed in human HCC by our and other studies. It has been documented that the interaction of gankyrin and CDK4 facilitates the degradation of Rb. Gankyrin also binds MDM2 directly and accelerates MDM2-dependent ubiquitination and degradation of p53. Fujita at el. showed that IGFBP-5 levels were up-regulated by gankyrin in hepatoma cells, and their data suggested that gankyrin may play an oncogenic role at the early stages of human hepatocarcinogenesis. We also reported that gankyrin binds RelA in the nucleus of HCC cells and transports nuclear factor kappa light-chain enhancer of activated B cells (NF-kB) back to the cytoplasm, thus inhibiting the activation of NF-kB. Our most recent data also clarified that gankyrin overexpression accelerates HCC invasiveness and metastasis via phosphoinositid 3-kinase/AKT/hypoxia-inducible factor alpha pathways, and HCC patients with high gankyrin expression in general had worse prognosis than those with low gankyrin expression, which indicates that gankyrin could be a candidate gene for the risk prognostication of HCC. However, the role of gankyrin in the differentiation of hepatocytes or hepatoma cells has not been reported, so far. In this study, we clarified, for the first time, that gankyrin expression was inversely correlated with the differentiation of HCC, and overexpression of gankyrin significantly enhanced the degradation of HNF4α in hepatoma cells, which indicates that gankyrin-mediated dedifferentiation of hepatocytes and hepatoma cells was, at least partially, via an HNF4α-dependent mechanism. Moreover, we showed that differentiation induced by gankyrin interference dramatically reduced the proportion of cancer stem cells in hepatoma cells and induced the differentiation of hepatoma cells, implying a novel strategy for HCC prevention and differentiation therapy.

Materials and Methods

A detailed description of the materials and methods used in this study can be found in the Supporting Information.

Results

Expression of Gankyrin Is Enhanced During Hepatocarcinogenesis. To study the expression of gankyrin during hepatocarcinogenesis, liver samples from diethylnitrosamine (DEN)-adminis-tered Wistar rats were subjected to immunohistochemical staining. As shown in Fig. 1A, livers of rats exhibited distinct fibrosis and cirrhosis upon DEN treatment, and all the animals developed HCC at week 22 after DEN administration. Intriguingly, gankyrin expression was progressively increased during the process of hepatocarcinogenesis in rats (Fig. 1B; Supporting Fig. 1A). In addition, the expression of gankyrin was assessed in carbon tetrachloride (CCL4)- (Fig. 1C,D) and bile duct ligation (BDL) (Supporting Fig. 1B,C)-triggered mouse cirrhosis model, and the results showed that gankyrin
Fig. 1. Expression of gankyrin is enhanced during hepatocarcinogenesis. (A) Livers of Wistar rats sacrificed at week 18 (cirrhosis) or week 22 (HCC) after DEN administration were collected and subjected to hematoxylin and eosin staining and immunohistochemistry. Arrows indicate HCC nodules. (B) Livers of DEN-administrated rats were collected and subjected to western blotting assay. (C) C57 mice were sacrificed at indicated time intervals after CCl4 administration, and liver sections were subjected to Sirius red staining and immunohistochemistry. (D) Livers of CCl4-administrated mice were collected and subjected to western blotting assay. Scale bar = 100 µm.
expression was notably increased in fibrotic or cirrhotic liver relative to healthy tissue.

**Gankyrin Expression Inversely Correlates With Hepatocyte Dedifferentiation.** To explore the relevance of gankyrin expression with hepatocyte differentiation status, we performed real-time polymerase chain reaction (PCR) using 59 pairs of human HCC and paracancerous tissues. Enhanced gankyrin expression was in concomitance with the down-regulation of hepatic function-related genes and the up-regulation of the dedifferentiation-associated genes in human HCC tissues (Fig. 2A,B). Tissue microarray revealed the overexpression of gankyrin in 76 of 91 human HCCs (Fig. 2C). More important, the expression of gankyrin significantly correlated with the differentiation status of HCC (Supporting Table 4). Poorly differentiated HCCs exhibited a higher expression level of gankyrin, whereas in those well-differentiated HCCs, gankyrin expression was comparatively low (Fig. 2D,E). As expected, the expression of gankyrin was further increased in portal vein tumor thrombus, indicating its role in the progression of HCC (Fig. 2F).

**Gankyrin-Mediated Hepatocyte Dedifferentiation Facilitates Hepatocarcinogenesis.** It has been reported that cultured hepatocytes undergo a time-dependent process of dedifferentiation in vitro. We, therefore, isolated the primary hepatocytes of Wistar rats and tested the expression of gankyrin in this process. As expected, the level of gankyrin in primary hepatocytes was progressively increased in concomitant with the decrease of ammonia detoxification which was considered as a functional index of hepatocyte differentiation status (Fig. 3A,B). To further assess the effect of gankyrin on hepatocyte differentiation, primary hepatocytes of Wistar rats were infected by Ad-GANK or adenovirus expressing green fluorescent protein (Supporting Fig. 2A). Intriguingly, the expression of hepatocyte and epithelial cell markers was dramatically down-regulated, whereas cholangiocyte and mesenchimal cell markers were significantly up-regulated, suggesting gankyrin overexpression promotes the dedifferentiation of hepatocytes (Supporting Fig. 2B). Moreover, ammonia detoxification of primary hepatocytes was impaired by Ad-GANK infection and recovered by adenovirus expressing microRNA against gankyrin (Ad-MirGANK) (Supporting Figs. 2C and 3C). Expression of hepatic differentiation-related genes was examined during DEN-induced rat hepatocarcinogenesis, and consistent results were achieved (Fig. 3D,E). Moreover, gankyrin expression was much higher in HCC cell lines, in comparison with that in normal cells, and the expression level was correlated with the malignancy of HCC cells, implying that gankyrin might mediate the dedifferentiation of hepatoma cells, as well (Supporting Fig. 2D).

**Gankyrin Enhances the Generation of Hepatoma Stem/Progenitor Cells.** Considering the important role of cancer stem cells in tumorigenesis, we investigated the effect of gankyrin overexpression on hepatoma stem/progenitor cells. Expression of cluster of differentiation (CD)133, CD90, and epithelial cellular adhesion molecule (EpCAM), which are considered as potential markers of hepatoma stem cells and are closely associated with the dedifferentiation of hepatoma cells, was markedly up-regulated in parallel with the increase of gankyrin during DEN-induced hepatocarcinogenesis (Fig. 4A; Supporting Fig. 3). Enforced gankyrin expression in hepatoma cells enhanced the expression of CD133, CD90, and EpCAM (Fig. 4B; Supporting Fig. 4). SMMC-7721 and LM3 cells, derived from suspension-cultured spheroids, exhibited a much higher level of gankyrin, compared with the cells from monolayer culture (Fig. 4C). More important, enhanced gankyrin expression was concomitant with the up-regulation of CD133, CD90, and EpCAM in human HCC tissues, and the statistical correlation was observed between gankyrin expression and the level of CD133, EpCAM, alpha fetal protein, and tumor size (Fig. 4D; Supporting Table 3). A small fraction of gankyrin-expressing hepatoma cells were also positive for CD133 expression, suggesting that gankyrin-mediated dedifferentiation of hepatoma cells might give rise to cancer stem/progenitor cells (Fig. 4E).

**Down-regulation of Gankyrin Promotes the Differentiation of Hepatoma Cells.** To evaluate the effect of gankyrin interference on hepatoma cell differentiation, we established LM3 and Huh7 cells stably expressing Mir-GANK or vector control using lentivirus (Supporting Fig. 5A). Morphological change and differentiation-related gene expression were observed (Supporting Fig. 5B; Fig. 5A). Colony formation and invasion potential of MirGANK stably transfected hepatoma cells were also positive for CD133 expression, suggesting that gankyrin-mediated dedifferentiation of hepatoma cells might give rise to cancer stem/progenitor cells.

**Gankyrin-Mediated Hepatocarcinogenesis**

1. SUN ET AL. HEPATOLOGY, October 2011

2. 1262
Fig. 2. Gankyrin expression inversely correlates with hepatoma differentiation. (A) Total RNA of 59 pairs of human HCC and paracancerous tissues were isolated and subjected to real-time PCR. Data were analyzed using the paired t-test. (B) Representative images of immunohistochemical assay for gankyrin and differentiation-related markers using consecutive sections of human HCC with surrounding noncancerous tissues. Scale bar = 100 μm. (C) Expression of gankyrin was analyzed in HCC and paracancerous tissues of 91 HCC patients using tissue microarray. (D) Comparison of gankyrin expression in 91 differentially differentiated human HCC tissues using tissue microarray. Data were analyzed using the least significant difference Test (*P < 0.05; **P < 0.01). (E) Representative view of immunohistochemical staining of gankyrin in differentially differentiated human HCC tissues from tissue microarray. Scale bar = 100 μm. (F) Comparison of gankyrin expression in human HCC and the corresponding portal vein tumor thrombus by real-time PCR. Data analysis was performed by the paired t-test.
Fig. 3. Gankyrin-mediated hepatocyte dedifferentiation facilitates hepatocarcinogenesis. (A) Western blotting assay of gankyrin expression in cultured primary hepatocytes isolated from Wistar rats. (B) Ammonia concentration in the supernatant of cultured hepatocytes was significantly decreased on a time-dependent manner. (C) Ammonia concentration in the supernatant of primary cultured hepatocytes infected by adenovirus, as indicated, was measured 36 hours postinfection. (D) Total RNA of the livers from rats exposed to DEN was isolated and subjected to real-time PCR. Results showed the gene-expression folds of DEN-treated rat liver versus healthy liver, respectively. (E) Representative images of immunohistochemical assay for E-cadherin, vimentin, and gankyrin in livers of DEN-administrated rats. Scale bar = 100 µm.
Fig. 4. Gankyrin enhances the generation of hepatoma stem/progenitor cells. (A) The mRNA level of CD90, CD133, and EpCAM in the livers of rats administrated by DEN for 17 weeks was measured by real-time PCR. (B) HepG2 and SMMC-7721 cells were infected with Ad-GANK or Ad-GFP, followed by real-time PCR assay 48 hours post infection. (C) Spheroid assay of SMMC-7721 and LM3 cells was performed, and the mRNA of spheroids was extracted and subjected to real-time PCR. (D) The mRNA level of gankyrin, CD90, CD133, and EpCAM in 59 pairs of human HCC and paracancerous tissues was examined by real-time PCR. Scatter plot showing the correlation of gankyrin with CD133, CD90, and EpCAM in 59 human HCC tissues. Data were analyzed using the paired t-test, and correlation analysis was performed using Pearson's correlation test. (E) Representative images of human HCC frozen section stained with anti-gankyrin (red) and anti-CD133 (green) antibodies. Arrows indicate dual-stained cells. Blue shows the nuclei counterstained by 4′,6-diamidino-2-phenylindole. Scale bar = 100 μm.
suggesting that the interference of gankyrin promotes the differentiation of hepatoma cells (Fig. 5D).

**Suppression of Gankyrin Reduces Hepatoma Stem/Progenitor Cells.** EpCAM- or CD133-positive Huh7 and LM3 cells, which have been considered as potential hepatoma stem cells, exhibited enhanced gankyrin expression, as compared with the majority of CD133 or EpCAM-negative cells (Fig. 6A; Supporting Fig. 7). The expression of CD133, CD90, and EpCAM was apparently decreased in Huh7 MirGANK and LM3 MirGANK cells, in comparison with control cells, and the portion of CD133- or EpCAM-positive

![Image of bar graphs and microscopy images](image-url)
cells was significantly reduced as well, suggesting the close association between gankyrin and hepatoma stem/progenitor cells (Fig. 6B,C). LM3 cells infected by Ad-MirGANK or LM3 MirGANK stable transfectant formed much less spheroids in suspension culture than control cells, which further indicates that the inhibition of gankyrin reduces the proportion of hepatoma stem/progenitor cells (Fig. 6D,E).
Gankyrin promotes the degradation of HNF4α. It has been well accepted that HNFs play a critical role in the maintenance of hepatic differentiation status. We, therefore, explored the correlation between gankyrin and HNFs. The expression of HNF4α, but not HNF1α, was much higher in Huh7 MirGANK cells.
than that in control cells (Supporting Fig. 8A). Consistent data were achieved in Ad-GANK-infected Huh7 and HepG2 cells (Supporting Fig. 8B). HNF4α expression was notably down-regulated by Ad-GANK infection and up-regulated by Ad-MirGANK infection in hepatoma cells (Fig. 7A; Supporting Fig. 8C). Interestingly, HNF4α messenger (m)RNA was not affected by gankyrin overexpression in primary hepatocytes (Supporting Fig. 9A) or gankyrin interference in hepatoma cells (Supporting Fig. 9B). Treatment of HepG2 cells with cycloheximide (CHX) did not influence Ad-GANK-mediated down-regulation of HNF4α, whereas decrease of HNF4α by gankyrin introduction or increase of HNF4α by gankyrin interference were dramatically blocked at the presence of MG132, which indicates that gankyrin promotes the degradation of HNF4α through a proteasome-dependent mechanism (Fig. 7B). In further study, an immunoprecipitation assay was performed, and the results suggested the direct interaction between gankyrin and HNF4α in hepatoma cells (Fig. 7C,D; Supporting Fig. 10). To further confirm the correlation between gankyrin and HNF4α in HCC development, an in vivo study was performed. HNF4α expression was notably decreased in parallel with the increase of gankyrin during DEN-induced rat hepatocarcinogenesis (Fig. 8A). The negative regulation of HNF4α by gankyrin was also confirmed in primary hepatocytes (Supporting Fig. 11). Significant up-regulation of gankyrin and down-regulation of HNF4α were detected in human HCCs, and a significant inverse correlation between gankyrin and HNF4α was found (Fig. 8B,C). Additionally, immunohistochemistry of human HCC and surrounding noncancerous tissue clearly demonstrated the inverse correlation between gankyrin and HNF4α in vivo (Fig. 8D).

Discussion

Hepatocyte dedifferentiation has been viewed as a key cellular event during hepatocarcinogenesis, although the underlying molecular mechanism remains elusive.17 In the present study, we observed elevated gankyrin expression in hepatocarcinogenesis and the close association of gankyrin expression with hepatocyte dedifferentiation. Consistent, enforced gankyrin expression induced dedifferentiation of primary liver cells and increased the “stemness” gene expression in hepatoma cells. Moreover, down-regulation of gankyrin, using lentivirus-mediated RNA interference in hepatoma cells, significantly improved their differentiation status and reduced the proportion of cancer stem cell/progenitor cells, indicating that differentiation induction of hepatoma cells via gankyrin interference could be a promising strategy for HCC therapy.

Oncogene overexpression is implicated in the initiation, promotion, and progression of a variety of human cancers and, therefore, provides a potential target for gene therapy.18 Gankyrin has been identified as an oncoprotein, playing a potent role in the regulation of cell cycle and apoptosis by inhibiting p53 and Rb.11,12 Our previous study showed that knockdown of gankyrin elicited apoptosis in p53-mutant or -deficient hepatoma cells, indicating that the role of gankyrin in HCC development is not restricted to its interaction with p53.10 Herein, we report that gankyrin expression was elevated during DEN-induced rat HCC and BDL or CCl4-triggered cirrhosis, implying the involvement of gankyrin in both liver cirrhosis and hepatocarcinogenesis. We have previously reported that gankyrin expression is elevated in human cirrhotic livers.19 The vast majority of HCC occurs in cirrhotic livers with hepatitis B virus/hepatitis C virus–induced hepatitis, and the pathological process is recapitulated in DEN-elicited rodent HCC models.20,21 Therefore, we believe that aberrant expression of gankyrin could be a shared mechanism in an animal model and human patients for cirrhosis and HCC. Further analysis of DEN-induced hepatocarcinogenesis and human HCCs suggested the correlation between gankyrin and dedifferentiation of hepatocytes or hepatoma. More important, overexpression of gankyrin in rat primary hepatocytes triggered the typical gene-expression profile of hepatocyte dedifferentiation. These findings were consistent with the clinical observation that elevated expression of gankyrin was associated with the malignancy of human HCCs. To our knowledge, this is the first report concerning the role of gankyrin in hepatocyte dedifferentiation.

In the last few years, growing evidence indicates that tumors consist of heterogeneous cell populations with different biological properties, and the capability of tumor formation and growth exclusively resides in a minor subset of cells termed cancer stem cells.22 The existence of cancer stem cells was first reported in the context of acute myelogenous leukemia23,24 and was subsequently verified in breast25 and brain tumors.26,27 More recently, identification of cancer stem cells has also been documented in human prostate and ovarian cancers.28 Emerging evidence also suggests that HCC development is attributed to the propagation of hepatic cancer stem/progenitor cells, which display distinct surface markers.29-34 However, the precise cell(s) of origin, molecular genetics, and specific markers of
Fig. 8. Negative correlation between gankyrin and HNF4α in HCC. (A) Immunohistochemistry of gankyrin and HNF4α in DEN-induced rat hepatocarcinogenesis. (B) Expression analysis of gankyrin and HNF4α in 39 pairs of human HCC and surrounding noncancerous tissues by immunohistochemistry. Data were analyzed using the paired t-test. (C) Correlation analysis between gankyrin and HNF4α in the 39 human HCCs, using Pearson’s correlation test. (D) Representative images of immunohistochemical assay for HNF4α and gankyrin using consecutive sections of human HCC and surrounding noncancerous tissues. Scale bar = 100 μm.
HCC stem cells remain poorly understood. It was reported that CD90-positive cells in human liver cancer exhibit cancer stem cell properties in certain conditions.\textsuperscript{32} CD133-positive HCC cells isolated from cultured SMMC-7721 cells or primary hepatoma tissue possess a high capacity of tumorigenesis.\textsuperscript{30} Moreover, EpCAM may serve as a cancer stem/progenitor cell marker of HCC because of its significant up-regulation in premalignant hepatic tissues.\textsuperscript{33} In the present study, we observed the correlation between elevated gankyrin levels and the expression of CD133, CD90, and EpCAM during DEN-triggered rat hepatocarcinogenesis and human HCC. We also showed that gankyrin overexpression significantly induced the expression of CD133, CD90, and EpCAM in hepatoma cells and enhanced the self-renewal capacity of them. These findings suggest a mechanism that gankyrin promotes HCC development by driving dedifferentiation of hepatocytes and hepatoma cells and facilitating HCC stem/progenitor cell generation.

Numerous studies have documented that the growth and progression of tumor is controlled by cancer stem cells, which possess robust proliferative potential and resistance of chemotherapies.\textsuperscript{35} Failure to eliminate the cancer stem cells usually renders the recurrence and metastasis of tumor after conventional therapy. Identification of key molecules and pathways regulating hepatoma stem/progenitor cell differentiation will give rise to the targeted differentiation therapy of HCC.\textsuperscript{36} In this regard, our results demonstrated that interference of gankyrin, using lentivirus-mediated miRNA delivery, remarkably improved the differentiation status of hepatoma cells and reduced the proportion of cancer stem/progenitor cells. The malignancy of MirGANK-transfected hepatoma cells was dramatically attenuated, and the self-renewal capacity was significantly impaired. Therefore, gankyrin might be one of the optimal targets for differentiation therapy of HCC.

Most chronic hepatitis and cirrhosis patients are at high risk of liver cancer, which renders the prevention of HCC rather important. Our data show that gankyrin expression was up-regulated in DEN-induced rat hepatocarcinogenesis and clarified the critical role of gankyrin in hepatocyte dedifferentiation via down-regulating HNF4α. Considering that the pathological process and gene-expression profile of DEN-elicited rodent HCC are similar to those of human HCC triggered by hepatitis/cirrhosis,\textsuperscript{20,21} we predict that blocking gankyrin in the human cirrhotic liver might attenuate hepatocarcinogenesis in humans, a point that will be investigated in our future studies.

In conclusion, our results suggest that the pharmacological inhibition of gankyrin might be a promising approach for HCC prevention and therapy. Despite the current improvement in diagnosis and treatment, the majority of HCC patients are not eligible for surgical resection because of the late diagnosis.\textsuperscript{37} Meanwhile, the high heterogeneity of HCC makes it almost impossible to eradicate the cancer cells with chemotherapy alone. The maximal therapeutic effects are likely to be achieved by providing patients with comprehensive treatments. Therefore, the combination of conventional treatment with differentiation therapy targeting gankyrin is highly worthy of anticipation.

Acknowledgments: The authors thank Dong-Ping Hu, Shan-Na Huang, Dan-Dan Huang, Shan-Hua Tang, Lin-Na Guo, and Dan Cao for their technical assistance.

References

1. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907-1917.
2. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557-2576.
3. Portolani N, Coniglio A, Ghidoni S, Giovanelli M, Benetti A, Tiberio GA, et al. Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. Ann Surg 2006;243:229-235.
4. Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004;5:215-219.
5. Ishiyama T, Kano J, Minami Y, Iijima T, Morishita Y, Noguchi M. Expression of HNFs and C/EBP alpha is correlated with immunocytological differentiation of cell lines derived from human hepatocellular carcinomas, hepatoblastomas, and immortalized hepatocytes. Cancer Sci 2003;94:757-763.
6. Lerose R, Molinarí R, Rocchi E, Manenti F, Villa E. Prognostic features and survival of hepatocellular carcinoma in Italy: impact of stage of disease. Eur J Cancer 2001;37:239-245.
7. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. Hepatology 2003;38:1331-1347.
8. Tanaka T, Jiang S, Hotta H, Takano K, Iwanari H, Sumi K, et al. Dysregulated expression of P1 and P2 promotor-driven hepatocyte nuclear factor-4α in the pathogenesis of human cancer. J Pathol 2006;208:662-672.
9. Yin C, Lin Y, Zhang X, Chen YX, Zeng X, Yue HY, et al. Differentiation therapy of hepatocellular carcinoma in mice with recombinant adenovirus carrying hepatocyte nuclear factor-4α gene. Hepatology 2008;48:1528-1539.
10. Li H, Fu X, Chen Y, Hong Y, Tan Y, Cao H, et al. Use of adenovirus-delivered siRNA to target oncoprotein p28GANK in hepatocellular carcinoma. Gastroenterology 2005;128:2029-2041.
11. Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kidó T, et al. Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. Nat Med 2000;6:96-99.
12. Higashitsuji H, Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, et al. The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquititation and degradation of p53. Cancer Cell 2005;8:75-87.
13. Umemura A, Itoh Y, Itoh K, Yamaguchi K, Nakajima T, Higashitsuji H, et al. Association of gankyrin protein expression with early clinical stages and insulin-like growth factor-binding protein 5 expression in human hepatocellular carcinoma. Hepatology 2008;47:493-502.
14. Chen Y, Li HH, Fu J, Wang XF, Ren YB, Dong LW, et al. Oncoprotein p28 GANK binds to RelA and retains NF-kappaB in the cytoplasm through nuclear export. Cell Res 2007;17:1020-1029.

15. Fu J, Chen Y, Cao J, Luo T, Qian YW, Yang W, et al. p28(GANK) overexpression accelerates hepatocellular carcinoma invasiveness and metastasis via phosphoinositol 3-kinase/AKT/hypoxia-inducible factor-1alpha pathways. HEPATOLOGY 2010;53:181-192.

16. Berasain C, Herrero JL, Garcia-Trevijano ER, Avila MA, Esteban JI, Mato JM, et al. Expression of Wilms' tumor suppressor in the liver with cirrhosis: relation to hepatocyte nuclear factor 4 and hepatocellular function. HEPATOLOGY 2003;38:148-157.

17. Lazarevich NL, Cheremnova OA, Varga EV, Ovchinnikov DA, Kudratjavtseva EI, Morozova OV, et al. Progression of HCC in mice is associated with a down-regulation in the expression of hepatocyte nuclear factors. HEPATOLOGY 2004;39:1038-1047.

18. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001;1:222-231.

19. Fu XY, Wang HY, Tan L, Liu SQ, Cao HF, Wu MC. Overexpression of p28/gankyrin in human hepatocellular carcinoma and its clinical significance. World J Gastroenterol 2002;8:638-643.

20. Ning BF, Ding J, Yin C, Zhong W, Wu K, Zeng X, et al. Hepatocyte nuclear factor 4 alpha suppresses the development of hepatocellular carcinoma. Cancer Res 2010;70:7640-7651.

21. Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, et al. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat Genet 2004;36:1306-1311.

22. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. Oncogene 2004;23:7274-7282.

23. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730-737.

24. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 1994;367:645-648.

25. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983-3988.

26. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396-401.

27. Galli R, Bindu E, Orfanelli U, Cipelletti B, Grittì A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004;64:7011-7021.

28. Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene 2006;25:1696-1708.

29. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542-2556.

30. Yin S, Li J, Hu C, Chen X, Yao M, Yan M, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 2007;120:1444-1450.

31. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun 2006;351:820-824.

32. Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008;13:153-166.

33. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008;68:1451-1461.

34. Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, et al. Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. Cancer Res 2008;68:4287-4295.

35. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-111.

36. Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, et al. Liver stem cells and hepatocellular carcinoma. HEPATOLOGY 2009;49:318-329.

37. Bruix J, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. HEPATOLOGY 2002;35:519-524.