Review

DOI: 10.5582/bst.2016.01176

Advances in the study of oncofetal antigen glypican-3 expression in HBV-related hepatocellular carcinoma

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Summary **Early specific diagnosis and effective treatment of hepatocellular carcinoma (HCC) are crucial. Expression of membrane-associated heparan sulfate proteoglycan glypican-3 (GPC-3) was recently found to increase as part of the malignant transformation of hepatocytes, and this increase is especially marked in patients with hepatitis B virus (HBV) infection, periportal cancerous embolus, or extra-hepatic metastasis. According to data from basic and clinical studies, the oncofetal antigen GPC-3 is a highly specific diagnostic biomarker of HCC and an indicator of its prognosis, and GPC-3 is also a promising target molecule for HCC gene therapy since it may play a crucial role in cell proliferation, metastasis, and invasion and it may mediate oncogenesis and oncogenic signaling pathways. This review summarizes recent advances in the use of oncofetal antigen GPC-3 to diagnose HBV-related HCC, estimate its prognosis, and its targeted therapy.**

Keywords: Glypican-3, hepatocellular carcinoma, HBV infection, diagnosis, prognosis, targeted therapy

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Its incidence is still increasing with a multi-factorial, multi-step, complex process, and very poor prognosis (*1,2*). The development and progression of HCC occurs within the context of a chronic, persistent infection with the hepatitis B virus (HBV) or hepatitis C virus (HCV) along with alcohol and aflatoxin B1 intake (*3,4*). Most patients with HCC soon die because of its rapid progression. Hepatic resection, radio-frequency ablation, and transplantation are potential treatments for HCC, but the options are rather limited. HCC can become resistant to radiotherapy or chemotherapy, it has a higher rate of recurrence and a considerably shorter 5-year survival after surgery, and it readily metastasizes (*5,6*). Therefore, HCC needs to be diagnosed early and effective treatments need to be identified (*7,8*).

All glypicans share a structure characterized by a conserved pattern of 14 cysteine residues that may form intra-molecular disulphide linkages (*9*), and glypicans play important roles in cellular growth, differentiation, and migration. Glypican-3 (GPC-3) belongs to a family of heparan sulfate proteoglycans with 6 sub-types (GPC_{1-6}). In this family, the proteoglycans are linked to the exocytoplasmic surface of the plasma membrane by a glycosyl-phosphatidylinositol anchor (*10*). The GPC-3 gene is located on the X human chromosome (Xq26) and it encodes a 70-kDa core protein that can be cleaved by furin to generate a 40 kDa N-terminal- and a 30 kDa C-terminal-protein containing two heparan sulfate glycan chains. $Serine^{560}$ is predicted to be a cleavage site in GPC-3, allowing GPC-3 to bind Wnt, Hedgehog (Hh), and fibroblast growth factor-2 through its core protein and/or the HS chains (*11,12*). GPC-3 contributes to cell migration, invasion, angiogenesis, and apoptosis, possibly through its interactions with the Wnt, Hh, bone morphogenetic protein-7, and insulin-

Released online in J-STAGE as advance publication October 29, 2016.

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like growth factor (IGF) signaling pathways (*13-15*). Abnormal expression of hepatic GPC-3 is associated with HCC progression (*16*). The current review focuses on recent advances in the use of oncofetal antigen GPC-3 to diagnose HCC, estimate its prognosis, and treat the disease.

2. GPC-3 as a promising marker of HCC

2.1. *Dynamic expression of GPC-3*

Hepatocyte oncogenesis may be induced by GPC-3 *via* activation of the IGF-II pathway, *via* regulation of zinc fingers and homeoboxes 2, or *via* expression of AFP regulator 2 during liver regeneration. Dynamic changes in hepatic GPC-3, GPC-3 mRNA, and serum GPC-3 expression were investigated in a rat model of hepatocarcinogenesis induced by 2-fluorenylacetamide (2-FAA), and results indicated that GPC-3 has value in diagnosing the early stages of HCC. Positive GPC-3 staining in the liver cytoplasm revealed the morphological stages of granule-like degeneration, atypical hyperplasia (a precancerous stage), and malignant transformation of hepatocytes. A previous study by the current authors found that the level of GPC-3 mRNA expression in the liver was 100%, expression of GPC-3 in the liver was 100%, and expression of GPC-3 in serum was 77.8% in patients with HCC; the level of GPC-3 mRNA expression in the liver was 100%, expression of GPC-3 in the liver was 100%, and expression of GPC-3 in serum was 66.7% in patients with precancerous lesions; the level of GPC-3 mRNA expression in the liver was 83.3%, expression of GPC-3 in the liver was 83.3%, and expression of GPC-3 in serum was 38.9% in patients with degenerated hepatocytes; and GPC-3 was not detected in the control group (*17*). Expression of GPC-3 mRNA in the liver was closely correlated with expression of total RNA in the liver, the level of GPC-3 protein in the liver, and the level of GPC-3 in serum, indicating that abnormal expression of GPC-3 mRNA or protein (which are associated with the malignant transformation of hepatocytes) should be a promising molecular marker for early diagnosis of HCC (*18*).

2.2. *Diagnosis and differential diagnosis*

Although many markers for diagnosis of HCC have been used in clinical practice (*19,20*), only a few markers such as HS-GGT (*21*), AFP-L3 (*22*), and Wnt3a (*23*) have sufficient sensitivity and specificity. The level of circulating AFP is a routine marker for diagnosis of HCC. However, an elevated AFP level results in a higher false positive rate in patients with benign liver diseases, greatly hampering the differential diagnosis (Figure 1) (*24,25*). Circulating GPC-3 increases in patients with HCC, which implies that

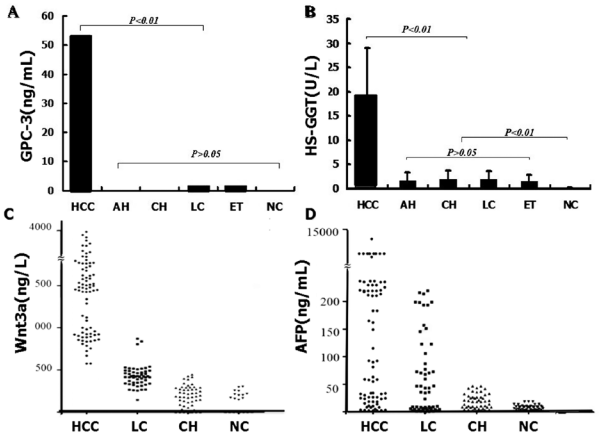

Figure 1 Comparative analysis of 4 markers for HCC diagnosis. (A), the levels of GPC-3 expression in patients with different liver diseases; **(B)**, the levels of HS-GGT expression in patients with different liver diseases; **(C)**, the levels of Wnt3a expression in patients with different liver diseases; and **(D)**, the levels of total AFP expression in patients with different liver diseases. HCC, hepatocellular carcinoma; GPC-3: glypican-3; AFP, total α-fetoprotein; HS-GGT, hepatoma-specific γ-glutamyl transferase.

GPC-3 may be very valuable in diagnosing HCC and monitoring its progression. Serum GPC-3 is superior to AFP in terms of specificity, positive or negative predictive value, and the accuracy with which it diagnoses HCC. Moreover, GPC-3 is also superior to AFP in formulating a treatment strategy and predicting the survival of patients with HCC. Yao M *et al*. found that serum GPC-3 was detectable in 52.8% of patients with HCC with a specificity of 97.1%, and only 1.4% to 2.0% of patients had other liver diseases (*26*). AFP is not accurate at diagnosing HCC because of its higher false positive rate (14.3% to 35.0%) in benign liver diseases. AFP-L3 has a sensitivity of 53.3% and a specificity of 88.9%. Serum GPC-3 levels could be used to differentiate HCC from non-malignant chronic liver disease and other liver cancers. Although serum GPC-3 has a higher level of specificity at diagnosing HCC, the combination of circulating levels of GPC-3, positive GPC-3 mRNA, and AFP significantly improves the accuracy of HCC diagnosis (*27-29*).

3. GPC-3 in relation to disease stage and prognosis

Clinical studies have reported that GPC-3 is specifically over-expressed in HCC and that it is a valuable marker for diagnosis of HCC and estimation of its prognosis (*17,24*). GPC-3 promotes the growth of HCC by stimulating canonical Wnt signaling. There is increasing evidence indicating that the structural requirements for GPC3 activity are cell type-specific, and its core protein is processed by a furin-like convertase. GPC-3 is expressed like a carcinoembryonic antigen, it stains dark brown deep within HCC tissues, and it is found in the cytoplasm and cell membrane (*30*). Immunohistochemistry has revealed oncofetal GPC-

3 expression in the cytoplasm and cell membrane at levels of 70-100% in HCC or hepatoblastoma, 6%-75% in fibrolamellar carcinoma or high-grade dysplasia, and 0-10% in cholangiocarcinoma (*31,32*).

3.1.*Clinical staging*

Wang *et al*. found that 80.6% of patients with HCC tested positive for GPC-3, 41.7% of their para-cancerous tissues tested positive, and none of the tissues distant from the cancer tested positive. The intensity of GPC-3 in HCC is significantly higher than that in surrounding tissues (*33*). Hepatic GPC-3 expression gradually increases in different stages, with dark staining in the advanced stage. A previous study by the current authors staged 69 specimens of cancerous tissue in accordance with the clinical staging criteria of the IUAC for HCC, and 11 specimens had stage I carcinoma (15.9%, 11 of 69), 19 had stage II (27.6%, 19 of 69), and 39 had stage III or IV (56.5%, 39 of 69). High levels of GPC-3 expression were noted in 45.5% of stage I HCC tissue specimens while low levels were noted in 54.5%, high levels were noted in 52.6% of stage II specimens while low levels were noted in 47.4%, and high levels were noted in 100% of stage III or IV specimens while low levels were noted in 0% (33).

GPC-3 is a developmentally-regulated oncofetal protein that is a clinically relevant biomarker for diagnosis of HCC and is one of the first transcripts to appear during the hepatocyte malignant transformation; about 50% of high-grade dysplastic macro-nodules in the cirrhotic liver express GPC-3 (*34,35*). GPC-3 can provide a molecular signature of early HCC since it is expressed in all HCC tissues and not expressed in any of the dysplastic nodules. Thus, GPC-3 should be a specific biomarker for diagnosis of HCC. An examination of hepatic fine needle aspirates (FNA) found that GPC-3 immunoreactivity was from 83% to 90% in cases of HCC, whereas there was no reaction in any benign lesions or metastatic carcinomas (*36,37*). The usefulness of GPC-3 when examining specimens is as an aid to distinguish HCC from metastatic tumors and benign liver lesions.

3.2. *Monitoring metastasis*

Metastasis occurs as cancerous cells enter the circulation and eventually grow into a lethal tumor in distant organs (*38*). GPC-3 mRNA from peripheral blood mononuclear cells (PBMC) is of value in monitoring HCC with extra-hepatic metastasis. Hepatic and blood GPC-3 mRNA are associated with extra-hepatic metastasis of HCC (*39*). Metastasis is the final stage in tumor progression and is thought to be responsible for up to 90% of deaths from HCC as cancerous cells enter the circulation and eventually grow into a lethal tumor in distant organs, reflecting inherent differences within the disseminating cells of distinct tumors (*2*). Amplification of fragments of the GPC-3 gene and verification of its identity by sequencing has revealed GPC-3 mRNA in most cancerous tissues or circulating PBMCs from patients with HCC but not in tissues distant from the cancer or cells from benign liver diseases (*26*).

Transcription of the GPC-3 gene in circulating PBMCs was associated with extra-hepatic metastasis of HCC. GPC-3 mRNA was expressed in 74.8% of primary and recurrent HCC but only in 3.2% of normal livers according to Northern blotting (*31*). The expression of GPC-3 mRNA was low or absent in the normal liver, focal nodular hyperplasia, and liver cirrhosis. Fragments of the GPC-3 gene were amplified and the identity of the gene was verified with DNA sequencing, but the gene was not found in tissue distant from the cancer or cells from benign liver diseases. Circulating GPC-3 mRNA is related to the TNM stage, periportal cancerous embolus, and extra-hepatic metastasis ($p < 0.001$) (*18*). Interestingly, a higher level of GPC-3 mRNA expression was found in patients with stage I-II HCC, HBV infection, and a small tumor, and GPC-3 is highly expressed in early and small HCC, and especially in patients with periportal cancer embolus (100%) or extra-hepatic metastasis (100%) (*32*), suggesting that up-regulation of circulating GPC-3 mRNA could be a more sensitive and specific biomarker with which to monitor the metastasis of HCC (*18*).

3.3. *Prognostic value*

The prognosis for HCC remains poor because of its late diagnosis and high rate of recurrence after surgery. New findings regarding the use of circulating GPC-3 as a marker have recently been reported, with GPC-3 displaying prognostic value in patients with HCC and HBV-associated cirrhosis after liver transplantation (*40-42*). GPC-3-positive patients had lower 5-year survival and disease-free survival rates than GPC-3-negative patients (38.2% vs. 75.4%; 30.8% vs. 69.7%) (*33*). Multivariate Cox regression analysis revealed that GPC-3 is an independent risk factor for the 5-year survival ($p = 0.031$) and disease-free survival rates ($p = 0.047$). Together with tumor differentiation, the Milan criteria, and preoperative AFP, GPC-3 is a potential indicator of a poor prognosis after liver transplantation in patients with HCC and HBV-associated cirrhosis (*43,44*).

Early detection of HCC and monitoring its recurrence after surgery would improve prognosis and justify screening programs for at-risk populations, such as chronic carriers of HBV and individuals with cirrhotic HCV (*3,6*). Basic and clinical studies have indicated that GPC-3 is a specific indicator of the prognosis for HCC (*45*). As expected, the over-expression of hepatic GPC-3 was significantly related to the 5-year survival of 69 patients with HCC ($p < 0.001$). Cox regression univariate analysis indicated that

certain HCC clinical prognostic factors, such as liver cirrhosis (p = 0.007) and HBV infection (p = 0.014), were significantly correlated with the 5-year survival rate. All of these factors were entered in multivariable analysis. A high level of GPC-3 expression ($p < 0.001$), liver cirrhosis (p = 0.008), and HBV infection (p = 0.006) were all identified as independent predictive factors for worse outcomes from HCC (*46*). Kaplan-Meier survival curves indicated that patients with HCC and high levels of GPC-3 expression had a significantly shorter survival time than those with low levels or no GPC-3 expression (*33*).

4. GPC-3 as a novel target for HCC therapy

Molecularly targeted therapy offers an effective option for non-surgical management of HCC that is highly chemo-resistant or that fails to respond to medication (*5,7*). However, molecular therapy remains a challenge mainly due to lack of specific targets. Use of the GPC-3 antigen as a target for HCC has been investigated with siRNA (*47*), vaccines (*48,49*), T lymphocytes (*50-52*), and anti-GPC-3 antibodies *in vitro* and *in vivo* (*53,54*). Some advances on the use of GPC-3 as the novel target for HCC therapy have been made (*55*). Of these, antibody-based therapy is the most clinically advanced. Given that GPC-3 increases in early, high-grade dysplastic macronodules and since a significant proportion of overt HCC is immunoreactive to anti-GPC-3, a therapeutic mAb (GC33, aa524-563) in the C-terminal portion of GPC-3 has been generated in MRL/lpr mice against a GST-fusion protein fragment, indicating that the antibodies are cytotoxic and significantly inhibit HCC growth both *in vitro* and *in vivo* (*56*).

4.1. *Studies in vitro*

The over-expression of hepatic GPC-3 plays an important role in HCC transformation, proliferation, and metastasis (*18*). Therefore, GPC-3 should be a specific molecular target for HCC therapy. Intervening in gene transcription with specific short hairpin RNA (shRNA) or microRNA (miRNA) inhibits cell proliferation *via* apoptosis *in vitro* (*47*,*57,58*). Silencing the GPC-3 gene with specific shRNA inhibits HCC cell proliferation, with inhibition of 71.1% in cells transfected with shRNA and 80.1% in cells transfected with shRNA and treated with sorafenib (100 μmol/L). A total of 65.6% of the cells were arrested in the G1 phase. Cell apoptosis increased significantly to 66.8% in comparison to cells that were not transfected with shRNA (6.9%) (*59*). By up-regulating key molecules (cyclin D1, β-catenin, and GSK3β) in the Wnt/β-catenin signaling pathway, oncofetal GPC-3 stimulates cell proliferation, suggesting that the GPC-3 gene should be a novel therapeutic target for HCC, but further studies should focus on the combination of miRNA and multi-targeting strategies for HCC therapy (*60*).

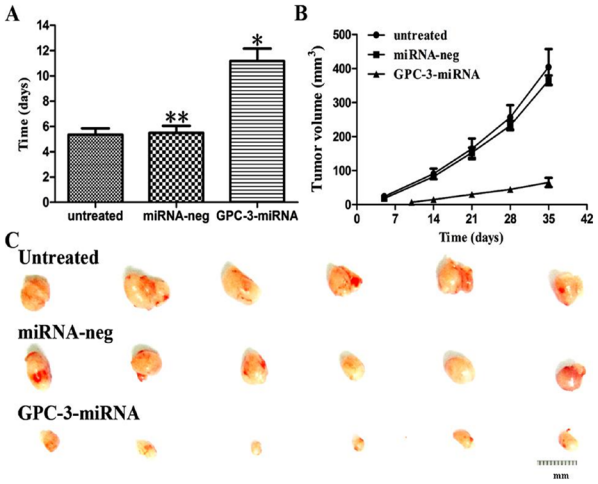

Figure 2 Silencing *GPC-3* inhibited tumor development when a liver cancer cell line was xenografted to nude mice. **(A)**, the time for a tumor to develop after nude mice were injected with stable HepG2 cells; **(B)**, the tumor volume in different groups (n = 6); **(C)**, the dissected tumor and its actual size. Scale bar, 100μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

4.2. *Studies in vivo*

Xenograft models are commonly used in tumor studies because of their convenience, high success rate, short latent period, and ease of monitoring. Besides anti-GPC-3 antibodies, siRNA targeting GPC-3 has also displayed therapeutic efficacy in a model of HCC (*54,61*). Tumor formation and growth are significantly inhibited by miRNA in a model of HepG2-cell-induced HCC in nude mouse in comparison to the same mice not treated with miRNA (Figure 2, unpublished data). Immunohistochemical analysis indicated that down-regulation of GPC-3 with miRNA significantly ($p < 0.01$) decreases expression of β-catenin, p-GSK3β, and cyclin D1. β-catenin and GSK3β are known to play an important role in regulating metabolism, transcription, embryonic development, and other processes and to also play a key role in the Wnt/β-catenin-induced phosphorylation of GSK3β, which results in the dissolution of the complex responsible for β-catenin degradation (*62,63*). A therapeutic intervention targeting GPC-3 is a promising approach for the clinical management of HCC.

5. Conclusion

In conclusion, HCC is one of the most common malignancies worldwide, and treatment outcomes generally remain poor (*64,65*). The GPC-3 gene is located upstream of the Wnt signaling pathway, which is involved in tumor development and progression, and GPC-3 is up-regulated in HBV-related liver malignancies. Activation of several key signaling

molecules regulates the expression of inflammation response- or cancer related-genes. An interesting finding is that GPC-3 levels and HBV infection have been identified as independent predictive factors for worse outcomes of HCC, that is, patients with HCC and higher levels of GPC-3 expression have a shorter survival time than those with lower levels or no expression. GPC-3 is specifically expressed in HCC but not in benign liver diseases. This means that GPC-3 is both a promising diagnostic or independent prognostic factor and also a therapeutic target for HCC. Further work should explore the combination of specific siRNA plus multi-targeting strategies in HCC therapy.

Acknowledgements

This project was supported by Grants-in-Aid from the Jiangsu Fund for Projects in Medicine (BE2016698, JS-2014-208, and JS-2014-209), the National Natural Science Foundation (NO:81673241), and the International S. & T. Cooperation Program (2013DFA 32150) of China.

References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin. 2016; 66:115-132.
2. Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: Clinical frontiers and perspectives. Gut. 2014; 63:844-885.
3. Riviere L, Ducroux A, Buendia MA. The oncogenic role of hepatitis B virus. Recent Results Cancer Res. 2014; 193:29-74.
4. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterol. 2012; 142:1264-1273.
5. Bruix J, Reig M, Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterol. 2016; 150:835-853.
6. El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011; 365:1118-1127.
7. Jain S, Singhal S, Lee P, Xu R. Molecular genetics of hepatocellular neoplasia. Am J Transl Res. 2010; 2:105-118.
8. Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. CA Cancer J Clin. 2012; 62:394-399.
9. Filmus J, Capurro M. The role of glypican-3 in the regulation of body size and cancers. Cell Cycle. 2008; 7:2787-2790.
10. Theocharis AD, Skandalis SS, Tzanakakis GN, Karamanos NK. Proteoglycans in health and disease: Novel roles for proteoglycans in malignancy and their pharm- acological targeting. FEBS J. 2010; 277:3904-3923.
11. Gao W, Kim H, Feng M, Phung Y, Xavier CP, Rubin JS, Ho M. Inactivation of Wnt signaling by a human antibody that recognizes the heparan sulfate chains of glypican-3 for liver cancer therapy. Hepatol. 2014; 60:576-587.
12. Dong Z, Yao M, Wang L, Yang JL, Yao DF. Down-regulating glypican-3 expression: Molecular-targeted therapy for hepatocellular carcinoma. Mini Rev Med Chem. 2014; 14:1183-1193.
13. Magistri P, Leonard SY, Tang CM, Chan JC, Lee TE, Sicklick JK. The glypican 3 hepatocellular carcinoma marker regulates human hepatic stellate cells *via* Hedgehog signaling. J Surg Res. 2014; 187:377-385
14. Lai JP, Sandhu DS, Yu C, *et al*. Sulfatase 2 up-regulates glypican 3, promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma. Hepatol. 2008; 47:1211-1222.
15. Cheng W, Tseng CJ, Lin TT, Cheng I, Pan HW, Hsu HC, Lee YM. Glypican-3 -mediated oncogenesis involves the insulin-like growth factor-signaling pathway. Carcinogen. 2008; 29:1319-1326.
16. Baghy K, Tátrai P, Regős E, Kovalszky I. Proteoglycans in liver cancer. World J Gastroenterol. 2016; 22:379-393.
17. Yao M, Yao DF, Bian YZ, Zhang CG, Qiu LW, Wu W, Sai WL, Yang JL, Zhang HJ. Oncofetal antigen glypican-3 as a promising early diagnostic marker for hepatocellular carcinoma. HBPD Int. 2011; 10:289-294.
18. Wang L, Yao M, Dong Z, Zhang Y, Yao DF. Circulating specific biomarkers in diagnosis of hepatocellular carcinoma and its metastasis monitoring. Tumour Biol. 2014; 35:9-20.
19. Abd El Gawad IA, Mossallam GI, Radwan NH, Elzawahry HM, Elhifnawy NM. Comparing prothrombin induced by vitamin K absence-II (PIVKA-II) with the oncofetal proteins glypican-3, alpha fetoprotein and carcinoembryonic antigen in diagnosing hepatocellular carcinoma among Egyptian patients. J Egypt Natl Canc Inst. 2014; 26:79-85.
20. Huang TS, Shyu YC, Turner R, Chen HY, Chen PJ. Diagnostic performance of alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein, des-gamma carboxyprothrombin, and glypican-3 for the detection of hepatocellular carcinoma: A systematic review and meta-analysis protocol. Syst Rev. 2013; 2:37.
21. Yao DF, Jiang D, Huang Z, Lu JX, Tao QY, Yu ZJ, Meng XY. Abnormal expression of hepatoma specific gamma-glutamyl transferase and alteration of gamma-glutamyl transferase gene methylation status in patients with hepato- cellular carcinoma. Cancer. 2000; 88:761-769.
22. Wu CS, Lee TY, Chou RH, Yen CJ, Huang WC, Wu CY, Yu YL. Development of a highly sensitive glycan microarray for quantifying AFP-L3 for early prediction of hepatitis B virus-related hepatocellular carcinoma. PLoS One. 2014; 9:e99959.
23. Pan LH, Yao M, Zheng WJ, Gu JJ, Yang XL, Qiu LW, Cai Y, Wu W, Yao DF. Abnormality of Wnt3a expression as novel specific biomarker for diagnosis and differentiation of hepatocellular carcinoma. Tumour Biol, 2016; 37:5561-5568.
24. Liu XF, Hu ZD, Liu XC, Cao Y, Ding CM, Hu CJ. Diagnostic accuracy of serum glypican-3 for hepatocellular carcinoma: A systematic review and meta-analysis. Clin Biochem. 2014;47:196-200.
25. Enan ET, El-Hawary AK, El-Tantawy DA, Abu-Hashim MM, Helal NM. Diagnostic role of glypican 3 and CD34 for differentiating hepatocellular carcinoma from nonmalignant hepatocellular lesions. Ann Diagn Pathol. 2013; 17:490-493.
26. Yao M, Yao DF, Bian YZ, Wu W, Yan XD, Yu DD, Qiu LW, Yang JL, Zhang HJ, Sai WL, Chen J. Values of circulating GPC-3 mRNA and alpha-fetoprotein in detecting patients with hepatocellular carcinoma. HBPD

Int. 2013; 12:171-179.
27. Wang SK, Zynger DL, Hes O, Yang XJ. Discovery and diagnostic value of a novel oncofetal protein: Glypican 3. Adv Anat Pathol. 2014; 21:450-460.
28. Li B, Liu H, Shang HW, Li P, Li N, Ding HG.Li P, Li N, Ding HG. Diagnostic value of glypican-3 in alpha fetoprotein negative hepatocellular carcinoma patients. Afr Health Sci. 2013; 13:703-709.
29. Lagana SM, Salomao M, Bao F, Moreira RK, Lefkowitch JH, Remotti HE. Utility of an immunohistochemical panel consisting of glypican-3, heat-shock protein-70, and glutamine synthetase in the distinction of low-grade hepatocellular carcinoma from hepatocellular adenoma. Appl Immunohistochem Mol Morphol. 2013; 21:170-176.
30. Li L, Jin R, Zhang X, Lv F, Liu L, Liu D, Liu K, Li N, Chen D. Oncogenic activation of glypican-3 by c-Myc in human hepatocellular carcinoma. Hepatol. 2012; 56:1380-1390.
31. Zaakook M, Ayoub M, Sinna EA, El-Sheikh S. Role of glypican-3 immunocyto- chemistry in differentiating hepatocellular carcinoma from metastatic carcinoma of the liver utilizing fine needle aspiration cytology. J Egypt Natl Canc Inst. 2013; 25:173-180.
32. Nassar A, Cohen C, Siddiqui MT. Utility of glypican-3 and survivin in differentiating hepatocellular carcinoma from benign and preneoplastic hepatic lesions and metastatic carcinomas in liver fine-needle aspiration biopsies. Diagn Cytopathol. 2009; 37:629-635.
33. Wang L, Pan L, Yao M, Cai Y, Dong ZZ, Yao DF. Expression of oncofetal antigenglypican-3associates significantly with poor prognosis in HBV-related hepatocellular carcinoma. Oncotarget. 2016; 7:42150-42158.
34. Li J, Gao JZ, Du JL, Wei LX. Prognostic and clinicopathological significance of glypican-3 overexpression in hepatocellular carcinoma: A meta-analysis. World J Gastroenterol. 2014; 20:6336-6344.
35. Xiao WK, Qi CY, Chen D, Li SQ, Fu SJ, Peng BG, Liang LJ. Prognostic significance of glypican-3 in hepatocellular carcinoma: A meta-analysis. BMC Cancer. 2014; 14:104.
36. Kandil D, Leiman G, Allegretta M, Trotman W, Pantanowitz L, Goulart R, Evans M. Glypican-3 immunocytochemistry in liver fine-needle aspirates: A novel stain to assist in the differentiation of benign and malignant liver lesions. Cancer. 2007; 111:316-322.
37. Ibrahim TR, Abdel-Raouf SM. Immunohistochemical study of glypican-3 and HepPar-1 in differentiating hepatocellular carcinoma from metastatic carcinomas in FNA of the liver. Pathol Oncol Res. 2015; 21:379-387.
38. Geramizadeh B, Seirfar N. Diagnostic value of arginase-1 and glypican-3 in differential diagnosis of hepatocellular carcinoma, cholangiocarcinoma and metastatic carcinoma of liver. Hepat Mon. 2015; 15:e30336.
39. Wang Y, Shen Z, Zhu Z, Han R, Huai M. Clinical values of AFP, GPC3 mRNA in peripheral blood for prediction of hepatocellular carcinoma recurrence following OLT: AFP, GPC3 mRNA for prediction of HCC. Hepat Mon. 2011; 11:195-199.
40. Cui X, Li Z, Gao PJ, Gao J, Zhu JY. Prognostic value of glypican-3 in patients with HBV-associated hepatocellular carcinoma after liver transplantation. HBPD Int. 2015; 14:157-163.
41. Wang YL, Zhu ZJ, Teng DH, Yao Z, Gao W, Shen ZY. Glypican-3 expression and its relationship with recurrence of HCC after liver transplantation. World J Gastroenterol. 2012; 18:2408-2414.
42. Chen IP, Ariizumi S, Nakano M, Yamamoto M. Positive glypican-3 expression in early hepatocellular carcinoma predicts recurrence after hepatectomy. J Gastro-enterol 2014; 49:117-125.
43. Jeon Y, Kim H, Jang ES, Hong S, Kim JW, Yoon YS, Cho JY, Han HS, Jeong SH. Expression profile and prognostic value of glypican-3 in post-operative South Korean hepatocellular carcinoma patients. APMIS. 2016; 124:208-215.
44. Feng J, Zhu R, Chang C, Yu L, Cao F, Zhu G, Chen F, Xia H, Lv F, Zhang S, Sun L. CK19 and glypican 3 expression profiling in the prognostic indication for patients with HCC after surgical resection. PLoS One. 2016; 11:e0151501.
45. Liu X, Wang SK, Zhang K, Zhang H, Pan Q, Liu Z, Pan H, Xue L, Yen Y, Chu PG. Expression of glypican 3 enriches hepatocellular carcinoma development- related genes and associates with carcinogenesis in cirrhotic livers. Carcinogen. 2015; 36:232-242.
46. Haruyama Y, Yorita K, Yamaguchi T, Kitajima S, Amno J, Ohtomo T, Ohno A, Kondo K, Kataoka H. High preoperative levels of serumglypican-3 containing N-terminal subunit are associated with poor prognosis in patients with hepato- cellular carcinoma after partial hepatectomy. Int J Cancer. 2015; 137:1643-1651.
47. Maurel M, Jalvy S, Ladeiro Y, Combe C, Vachet L, Sagliocco F, Bioulac-Sage P, Pitard V, Jacquemin-Sablon H,Zucman-Rossi J, Laloo B, Grosset CF. A functional screening identifies five microRNAs controlling glypican-3: Role of miR-1271 down-regulation in hepatocellular carcinoma. Hepatol. 2013; 57:195- 204.
48. Kawashima I, Kawashima Y, Matsuoka Y, Fujise K, Sakai H, Takahashi M, Yoshikawa T, Nakatsura T, Ishihara T, Ohno T. Suppression of postsurgical recurrence of hepatocellular carcinoma treated with autologous formalin-fixed tumor vaccine, with special reference to glypican-3. Clin Case Rep. 2015; 3:444- 447.
49. Sawada Y, Yoshikawa T, Ofuji K, *et al*. Phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for hepatocellular carcinoma patients. Oncoimmunol. 2016; 5:e1129483.
50. Iwama T, Uchida T, Sawada Y, Tsuchiya N, Sugai S, Fujinami N, Shimomura M, Yoshikawa T, Zhang R, Uemura Y, Nakatsura T. Vaccination with liposome-coupled glypican-3 derived epitope peptide stimulates cytotoxic T lymphocytes and inhibits GPC3-expressing tumor growth in mice. Biochem Biophys Res Commun. 2016; 469:138-143.
51. Sayem MA, Tomita Y, Yuno A, Hirayama M, Irie A, Tsukamoto H, Senju S, Yuba E, Yoshikawa T, Kono K, Nakatsura T, Nishimura Y. Identification of glypican-3 -derived long peptides activating both CD8+ and CD4+ T cells; prolonged overall survival in cancer patients with Th cell response. Oncoimmunol. 2015; 5:e1062209.
52. Dargel C, Bassani-Sternberg M, Hasreiter J, *et al*. T cells engineered to express a T-cell receptor specific for glypican-3 to recognize and kill hepatoma cells *in vitro* and in mice. Gastroenterol. 2015; 149:1042-1052.
53. Gao W, Kim H, Ho M. Human monoclonal antibody targeting the heparan sulfate chains of glypican-3 inhibits HGF-mediated migration and motility of hepato- cellular carcinoma cells. PLoS One. 2015; 10:e0137664.
54. Hanaoka H, Nagaya T, Sato K, Nakamura Y, Watanabe

R, Harada T, Gao W, Feng M, Phung Y, Kim I, Paik CH, Choyke PL, Ho M, Kobayashi H. Glypican-3 targeted human heavy chain antibody as a drug carrier for hepatocellular carcinoma therapy. Mol Pharm. 2015; 12:2151-2157.
55. Wang L, Yao M, Pan LH, Qian Q, Yao DF. Glypican-3 as special biomarker or promising target molecule for primary liver cancer. HBPD Int. 2015; 14:361-366.
56. Feng M, Ho M. Glypican-3 antibodies: A new therapeutic target for liver cancer. FEBS Lett. 2014; 588:377-382.
57. Huang N, Lin J, Ruan J, Su N, Qing R, Liu F, He B, Lv C, Zheng D, Luo R. MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3. FEBS Lett. 2012; 586:884-891.
58. Miao HL, Lei CJ, Qiu ZD, Liu ZK, Li R, Bao ST, Li MY. MicroR-NA-520c-3p inhibits hepatocellular carcinoma cell proliferation and invasion through induction of cell apoptosis by targeting glypican-3. Hepatol Res. 2014; 44:338-348.
59. Yu D, Dong Z, Yao M, Wu W, Yan MJ, Yan XD, Qiu LW, Chen J, Sai WL, Yao DF. Targeted glypican-3 gene transcription inhibited the proliferation of human hepatoma cells by specific short hairpin RNA. Tumour Biol. 2013; 34:661-668.
60. Yao M, Wang L, Qiu L, Qian Q, Yao D. Encouraging microRNA-based thera- peutic strategies for hepatocellular carcinoma. Anticancer Agents Med Chem. 2015; 15:453-460.
61. Yao M, Wang L, Dong Z, Qian Q, Shi Y, Yu DD, Wang SY, Zheng WJ, Yao DF. Glypican-3 as an emerging molecular target for hepatocellular carcinoma gene therapy. Tumour Biol. 2014; 35:5857-5868.
62. Gao W, Tang Z, Zhang YF, Feng M, Qian M, Dimitroy DS, Ho M. Immunotoxin targeting glypican-3 regresses liver cancer *via* dual inhibition of Wnt signalling and protein synthesis. Nat Commun. 2015; 6:6536.
63. Capurro M, Martin T, Shi W, Filmus J. Glypican-3 binds to Frizzled and plays a direct role in the stimulation of canonical Wnt signaling. J Cell Sci 2014; 127:1565-1575.
64. Tagliamonte M, Petrizzo A, Tornesello ML, Ciliberto G, Buonaguro FM, Buonaguro L. Combinatorial immunotherapy strategies for hepatocellular carcinoma. Curr Opin Immunol. 2016; 39:103-113.
65. Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

(Received September 18, 2016; Revised October 10, 201; Accepted October 19, 201)