Clinical values of AFP, GPC3 mRNA in peripheral blood for prediction of hepatocellular carcinoma recurrence following OLT

Yuliang Wang 1, Zhongyang Shen 1*, Zhijun Zhu 1, Ruifa Han 2, Mingsheng Huai 1

1 Orient Organ Transplant Center, First Central Clinic Institute of Tianjin Medical University, Key Lab for Critical Care Medicine of the Ministry of Health, Tianjin, China
2 Tianjin Institute of Urology, Tianjin Medical University, Tianjin, China

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Annually, about 200,000 patients died of HCC in China. Liver transplantation (LT) holds great theoretical appeal in treating HCC. However, the high recurrence rate after transplantation is the most important limiting factor for long-term survival.

Objectives: To assess the value of alpha-fetoprotein (AFP) messenger RNA (mRNA), Glypican-3 (GPC3) mRNA-expressing cells in the peripheral blood (PB) for prediction of HCC recurrence following orthotopic liver transplantation (OLT).

Patients and Methods: 29 patients with HCC who underwent OLT with a minimum clinical follow-up of 12 months were included in this retrospective study. We detected AFP mRNA, GPC3 mRNA-expressing cells in the PB by TaqMan real-time reverse transcriptase-polymerase chain reaction (RT-PCR), pre-, intra- and post-operatively. The early recurrence of patients was evaluated.

Results: 8 (28%), 15 (52%), and 9 (31%) patients had AFP mRNA detected pre-, intra-, and post-operatively, respectively. With 12 months of follow-up, HCC recurred in 7 (24%) patients. Univariate analysis revealed that positive pre- and post-operative AFP mRNA, TNM stage as well as vascular invasion were significant predictors for the HCC recurrence. Multivariate analysis revealed that being positive for AFP mRNA pre-operatively remained a significant risk factor for HCC recurrence after OLT. GPC3 mRNA was expressed in all PB samples. There was no significant difference in the expression levels of GPC3 mRNA between the HCC and control groups. There were no significant differences in GPC3 mRNA expression values between those patients with and without tumor recurrence.

Conclusions: The pre-operative detection of circulating AFP mRNA-expressing cells could be a useful predictor for HCC recurrence following OLT. GPC3 mRNA-expressing cells in PB seem to have no diagnostic value.

ARTICLE INFO

Article type: Original Article

Article history:
Received: 19 Jul 2010
Revised: 26 Nov 2010
Accepted: 18 Dec 2010

Keywords:
Liver transplantation
Hepatocellular carcinoma
Alpha-fetoprotein
Glypican-3
Recurrence

Implication for health policy/practice/research/medical education:
We suggest reading this interesting article to internists, gastroenterologists, hepatologists and infectious diseases specialists with regard to early diagnosis of HCC post liver transplantation.

Please cite this paper as:
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. Hepat Mon. 2011;11(3):195-199.

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a global incidence of 500,000 new cases per year; 82% of cases (and deaths) occur in developing countries (with 55% in China) (1, 2). HCC is up to four times more common in men than in women; 60%-90% of these tumors develop in a cirrhotic liver (3). Each year, about 200,000 patients died of HCC in China. Liver transplantation (LT), which offers the potential to both resect the entire potentially tumor-bearing liver and eliminate the cirrhotic tissues, holds great theoretical appeal in treating HCC. However, recurrence of HCC is a significant cause of mortality after LT (4, 5). Even with the implementation of Milan criteria, recurrence rates have been shown to be 8%-15% in most stud-
ies (6-8). The high recurrence rate after transplantation is the most important limiting factor for long-term survival. Establishment of a direct and accurate method to predict tumor recurrence and/or micrometastasis after LT is needed. The human α-fetoprotein (AFP) messenger RNA (mRNA) is generally accepted as a tumor-specific marker. There are some reports to identify AFP mRNA for detecting isolated tumor cells (ITC) in peripheral blood (PB) (9, 10). It sounds theoretically reasonable to consider the detection of circulating HCC cells as being predictive for HCC recurrence after LT. We have established an accurate and sensitive method for the detection of circulating HCC cells by RT-PCR method to quantify AFP mRNA (11). Glypican-3 (GPC3) is a member of the glypican family of cell-surface heparin-sulfate proteoglycans, which is linked to the cell surface through a glycosylphosphatidylinositol (GPI) anchor (12). There has been considerable interest in GPC3, because it is markedly overexpressed in a high proportion of HCCs and promotes the growth of HCCs (13-15). Some clinical research studies reported GPC3 overexpression in HCC both at mRNA and protein levels (16, 17). Based on these results, it has been proposed that GPC3 could be used as a serum and histochemical marker for HCC. Despite this clinical interest in GPC3, it is still unclear whether GPC3 mRNA detectable in PB is related to HCC diagnosis and predicts its recurrence.

Objectives

In the present study, we applied TaqMan real-time RT-PCR to assess the usefulness of detecting AFP mRNA and GPC3 mRNA-expressing cells in the PB for predicting the risk of HCC recurrence after orthotopic liver transplantation (OLT).

Patients and Methods

Patients and Sample Collection

Twenty-nine liver transplant recipients with HCC (27 men, 2 women; mean age: 48 years, age range: 37–62 years) who underwent OLT in Orient Organ Transplant Center during 2008 were included in this retrospective study. The diagnosis of HCC was confirmed by pathologic study by experienced pathologists. The criteria for OLT in patients with HCC are absence of extrahepatic malignancies, macroscopic tumor thrombosis, or extrahepatic metastasis of HCC. Of the 29 studied patients, 27 had hepatitis B virus (HBV)-induced cirrhosis, and two patients had hepatitis C virus (HCV)-induced cirrhosis. Twelve patients had received treatment for HCC; treatments included resection, radiotherapy or transcatheter hepatic arterial chemoembolization (TACE) prior to transplantation. The remaining 17 patients had not received any treatments. Clinical characteristics of HCC patients are summarized in Table 1. All recipients had undergone successful OLT (livers were from cadaveric donors who voluntarily donated). No organs were obtained from executed prisoners. The present study was approved by the Institutional Ethics Committee of our institute. The informed consent was obtained from each patient. The procedure met all applicable institutional guidelines of our institute, and governmental regulations concerning the ethical use of donated organs.

RNA extraction and real-time RT-PCR

Total RNA was extracted with PB mononuclear cells (PBMCs) with TRIzol reagent (GIBCO-BRL, USA). The real-time RT-PCR used for the detection of AFP mRNA was performed as described previously (11). The sequences of primers and TaqMan probe of GPC3 were: forward primer (5’-AGAGGCTTTGAAATGCTAGC-3’), reverse primer (5’-AAATCTTTAGGTCAGTC-3’), and probe (5’-FAM-ATGCCAAGAACTACA CCAATGC-TAMRA-3’). The conditions for every PCR reaction were 15 min at 95 °C, followed by 40 cycles of denaturation for 20 seconds at 95 °C and annealing/extension for 60 seconds at 60 °C. Data were analyzed with Sequence Detection Software. The level of expression was calculated using the formula (18):

Relative expression = \[
\frac{\text{Copy number of target molecule}}{\text{Copy number of β-actin}}
\]

Statistical analysis

Kaplan-Meier survival analysis and log-rank test were used to derive the survival curve. To assess the risk factors, univariate and multivariate analyses were performed using the Kaplan-Meier method (log-rank test) and Cox’s proportional hazards model. Comparison between two groups was made by independent Student’s t-test for numerical variables, and x² test for categorical variables. One-way ANOVA with Bonferroni post hoc test was used for comparisons between more than two groups. A p value <0.05 was considered statistically significant. Analyses were made using SPSS® software ver 12 for Windows® (SPSS, Chicago, IL, USA).

Results

All patients had at least one year of follow-up. HCC reoccurred...
Figure 1. Recurrence-free survival of those with negative pre-operative AFP mRNA was higher than those with positive pre-operative AFP mRNA (Kaplan-Meier method).

Discussion

It is a well-developed and mature technique in recent years that marked RNA of tumor cells in PB was amplified using RT-PCR to confirm the presence of tumor cells in blood (9, 10). Previous studies showed that AFP mRNA may be investigated as a surrogate marker for isolated tumor cells (ITC) of HCC, and may predict HCC recurrence after curative hepatectomy (19, 20). However, the specificity and prognostic value of AFP mRNA for circulating HCC tumor cells remains questionable (21). Furthermore, the clinical significance of this molecular technique remains controversial in patients for prediction of HCC recurrence after LT. A recent study by Marubashi, et al, showed that the detection of AFP mRNA in PB pre-operatively could be associated with the HCC recurrence and might serve as a useful predictor for the HCC recurrence in liver transplant patients (22). Whereas Cheung, et al, showed that plasma AFP mRNA level revealed insignificant association with the HCC recurrence after LT (23). A higher risk of HCC recurrence in patients with pre-operative AFP mRNA or in patients with AFP mRNA persistently positive pre-operatively has been reported in the literature (23, 24). This study showed that the positive expression rate of AFP mRNA was 28% (8/29) in PB samples pre-operatively. Several literature reports showed that the positive rate of pre-operative AFP mRNA was 20%-50% in hepatic resection and transplantation (24-27). This variability can be attributed to in vitro instability of mRNA, differences concerning laboratory techniques, primer selection, time between sample collection and processing, and different patient populations. Pre-operative presence of AFP mRNA in PB may represent shedding of cells from primary HCC; another possibility is the presence of unfound occult micrometastasis that was undetectable pre-operatively.

Operative manipulation would enhance the dissemination of HCC cells into PB; this cell spread could be partly responsible for tumor recurrence after LT, although this is an intermittent and transient phenomenon. However, it has been shown that surgical intervention induces a release of non-neoplastic liver cells into the circulation and increases false-positive signals of AFP mRNA. To reduce the likelihood of false-positive results, the post-operative blood samples were taken one week after transplantation, as liberated non-neoplastic cells are presumably filtered from the PB within one week, as previously proposed by Ijichi, et al (24). Our study showed that consistent positive results of AFP mRNA (three times test after operation) should be considered a real post-operative positive result. One possible explanation for post-operative positive result is that a proportion of cancerous...
cells released from the surgical procedure would still stay in the PB for more than one week in an immunosuppressed patient. Another possibility would be the presence of unresected occult metastases that left undetectable at the time of surgery. The overall recurrence rate in our study was 24% during the first year post-OLT, which is in keeping with rates reported in other studies (28, 29). According to the results of both univariate and multivariate analyses, only pre-operative presence of AFP mRNA was an independent risk factor for HCC recurrence. This test would help to identify patients who may benefit from more intensive post-LT surveillance, altered immunosuppressive regimen or adjuvant chemotherapy. GPC3 expression is frequently increased in HCC (30). In fact, GPC3 may be reactivated in HCC as frequently as AFP, which has been used extensively as a marker of this cancer. In fact, GPC3 may be reactivated in HCC as frequently as AFP, which has been used extensively as a marker of this cancer. GPC3 expression is frequently increased in HCC (30). In fact, GPC3 may be reactivated in HCC as frequently as AFP, which has been used extensively as a marker of this cancer. GPC3 expression is frequently increased in HCC (30). In fact, GPC3 may be reactivated in HCC as frequently as AFP, which has been used extensively as a marker of this cancer. GPC3 expression is frequently increased in HCC (30). In fact, GPC3 may be reactivated in HCC as frequently as AFP, which has been used extensively as a marker of this cancer.

In conclusion, AFP mRNA test should be considered in the pre-operative workup to adequately select suitable patients and the most relevant therapeutic option; evaluation of AFP mRNA may provide important clues for selecting those patients who need adjuvant chemotherapy and intensive follow-up after OLT. But, we should admit that AFP mRNA is only recommended as complementary tests to the conventionally diagnostic methods used. Future studies with higher numbers of patients and longer follow-up need to confirm the clinical usefulness of this assay.

### Financial support
None declared.

### Conflicts of interest
None declared.

### References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55(2):74-108.
2. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132(7):2557-76.
3. Thimme R, Neagu M, Boettler T, Neumann-Haefelin C, Kersting N, Geissler M, et al. Comprehensive analysis of the alpha-fetoprotein-specific CDH1-T cell responses in patients with hepatocellular carcinoma. Hepatology. 2008;48(5):1822-33.
4. Tanaka K, Yamada T. Current status and issues of living donor liver transplantation for hepatocellular carcinoma. Clin Ital. 2005;57(2):347-53.
5. Zavaglia C, De Carli I, Alberti AB, Minola E, Benni LS, Slim AO, et al. Predictors of long-term survival after liver transplantation for hepatocellular carcinoma. Am J Gastroenterol. 2005;100(1):2708-16.
6. Hanje AF, Yao FY. Current approach to down-staging of hepatocellular carcinoma prior to liver transplantation. Curr Opin Organ Transplant. 2008;13(3):234-40.
7. Song TJ, Ip EW, Fong Y. Hepatocellular carcinoma: current surgical management. Gastroenterology. 2004;127(5 Suppl 1):S248-60.
8. Befeler AS, Hayashi PH, Di Bisceglie AM. Liver transplantation for hepatocellular carcinoma. Gastroenterology. 2005;132(6):1752-64.
9. Morimoto O, Nagano H, Miyamoto A, Fujiwara Y, Kondo M, Yamamoto T, et al. Association between recurrence of hepatocellular carcinoma and alpha-fetoprotein messenger RNA levels in peripheral blood. Surg Today. 2005;35(2):1039-44.
10. Jeng KS, Sheen IS, Tsai YC. Does the presence of circulating hepatocellular carcinoma cells indicate a risk of recurrence after resection? Am J Gastroenterol. 2004;99(5):1059-9.
11. Wang YL, Li G, Wu D, Liu YW, Yao Z. Analysis of alpha-fetoprotein mRNA level on the tumor cell hematogenous spread of patients with hepatocellular carcinoma undergoing orthotopic liver transplantation. Transplant Proc. 2007;39(1):166-8.
12. Song IH, Filmus J. The role of glypican-3 in mammalian development. Biochim Biophys Acta. 2002;1573(3):241-6.
13. Capurro MI, Shi W, Sandal S, Filmus J. Processing by convertases is not required for glypican-3-induced stimulation of hepatocellular carcinoma growth. J Biol Chem. 2005;280(50):41904-9.
14. Capurro MI, Xiao Y, Lobe C, Filmus J. Glypican-3 promotes the
growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res. 2005;65(14):6245-54.

15. Huang JS, Chao CC, Su TL, Wey SH, Chen DS, Chen CT, et al. Diverse cellular transformation capability of overexpressed genes in human hepatocellular carcinoma. Biochem Biophys Res Commun. 2004;325(4):595-9.

16. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology. 2003;125(1):89-97.

17. Yasuda E, Kumada T, Toyoda H, Kaneoka Y, Maeda A, Okuda S, et al. Evaluation for clinical utility of GPC3, measured by a commercially available ELISA kit with Glypican 3 (GPC3) antibody, as a serological and histological marker for hepatocellular carcinoma. Hepatol Res. 2010;40(5):477-85.

18. Zhang Y, Wang YL, Liu YW, Li Q, Yuan YH, Niu WY, et al. Change of peripheral blood mononuclear cells IFN-gamma, IL-10, and TGF-beta mRNA expression levels with active human cytomegalo-virus infection in orthotopic liver transplantation. Transplant Proc. 2009;41(2):1767-9.

19. Chen XP, Zhao H, Zhao XP. Alternation of AFP-mRNA level detected in blood circulation during liver resection for HCC and its significance. World J Gastroenterol. 2002;8(5):818-21.

20. Kamiyama T, Takashashi M, Nakagawa T, Nakanishi K, Kamachi H, Suzuki T, et al. AFP mRNA detected in bone marrow by real-time quantitative RT-PCR analysis predicts survival and recurrence after curative hepatectomy for hepatocellular carcinoma. Ann Surg. 2006;244(3):450-63.

21. Witzigmann H, Geissler F, Benedix F, Thiery J, Uhlmann D, Tannapfel A, et al. Prospective evaluation of circulating hepatocytes by alpha-fetoprotein messenger RNA in patients with hepatocellular carcinoma. Surgery. 2003;133(1):34-43.

22. Maruhashi S, Dono K, Nagano H, Sugita Y, Asaoka T, Hama N, et al. Detection of AFP mRNA expressing cells in the peripheral blood for prediction of HCC recurrence after living donor liver transplantation. Transpl Int. 2007;20(7):756-82.

23. Cheung SJ, Fan ST, Lee YJ, Chow JP, Ng SO, Fong DY, et al. Albumin mRNA in plasma predicts post-transplant recurrence of patients with hepatocellular carcinoma. Transplantation. 2008;85(1):487.

24. Ijichi M, Takayama T, Matsumura M, Shiratori Y, Omata M, Makuuchi M. alpha-Fetoprotein mRNA in the circulation as a predictor of postsurgical recurrence of hepatocellular carcinoma: a prospective study. Hepatology. 2002;35(4):853-60.

25. Jeng KS, Sheen IS, Tsai YC. Circulating messenger RNA of alpha-feto-protein: a possible risk factor of recurrence after resection of hepatocellular carcinoma. J Surg Oncol. 2004;90(10):1055-60.

26. Liu Y, Wu MC, Qian GX, Zhang BH. Detection of circulating hepatocellular carcinoma cells in peripheral venous blood by reverse transcription-polymerase chain reaction. Hepatobiliary Pancreat Dis Int. 2002;1(2):12-6.

27. Schmolzovitz-Weiss H, Stemmer SM, Liberzon E, Avigad S, Sulkjes J, Belinki A, et al. Quantitation of alpha-fetoprotein messenger RNA for early detection of recurrent hepatocellular carcinoma: a prospective pilot study. Cancer Detect Prev. 2006;30(2):204-9.

28. Parfitt R, Marotta P, Alghamdi M, Wall W, Khakhkar A, Suskin NG, et al. Recurrent hepatocellular carcinoma after transplantation: use of a pathological score on explanted livers to predict recurrence. Liver Transpl. 2007;13(4):543-51.

29. Shimoda M, Gohbrial RM, Carmody IC, Aneulmo DM, Farmer DG, Yeruz I, et al. Predictors of survival after liver transplantation for hepatocellular carcinoma associated with Hepatitis C. Liver Transpl. 2004;10(12):1478-86.

30. Huo HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporal-spatial distribution. Cancer Res. 1997;57(2):379-84.

31. Zygier DR, McCallum JC, Luan C, Chou PM, Yang XJ. Glypican 3 has a higher sensitivity than alpha-fetoprotein for testicular and ovarian yolk sac tumour: immunohistochemical investigation with analysis of histological growth patterns. Histopathology. 2010;56(6):750-7.

32. Baumhofer D, Tornillo L, Stadlmann S, Roncalli M, Diamantis EK, Terracciano LM. Glypican 3 expression in human nonneoplastic, pre-neoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples. Am J Clin Pathol. 2008;129(6):899-906.

33. Di Tommaso L, Destro A, Seik YJ, Ballardore E, Terracciano L, Sangiovanni A, et al. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. J Hepatol. 2009;50(4):746-54.

34. Jia HI, Ye QH, Qin LX, Budhu A, Forgues M, Chen Y, et al. Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Arch Surg. 2007;142(4):413-9.

35. Kandil DH, Cooper K. Glypican-3: a novel diagnostic marker for hepatocellular carcinoma and more. Adv Anat Pathol. 2009;16(2):125-9.

36. Shirakawa H, Kurokuma T, Nishimura Y, Hasebe T, Nakano M, Gotoda N, et al. Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int J Oncol. 2009;34(3):649-56.