DKK1 in relation to HCV induced liver cirrhosis and HCV induced HCC curative resection

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Abstract:

Background: **hepatitis C virus (HCV) is an RNA virus that induces hepatocarcinogenesis by mechanisms other than integration in the host cell genome. Dickkopf-1 (DKK1) expression has been studied mostly in hepatitis B virus induced HCC but not properly investigated in HCV induced HCC.**

Aim: **we aimed to assess serum DKK1 in HCV induced HCC, HCV induced liver cirrhosis and viral infection-free controls. Moreover, we assessed serum DKK1 level after curative resection of HCC.**

Methods: **Serum DKK1 was measured by ELISA in 20 HCV induced HCC patients; both pre-resection and post-resection, 20 HCV induced liver cirrhosis patients and 20 viral infection-free controls.**

Results: **DKK1 levels were significantly higher in HCC than cirrhosis patients (P = 0.000). DKK1 did not differ significantly between cirrhotic patients and controls (P = 0.11). DKK1 levels significantly reduced 5 days post-resection compared to their pre-resection levels (P = 0.000).**

Conclusion: **We documented serum DKK1 as a marker for detection of early HCC in HCV infected patients. Significant reduction of DKK1 5 days after curative resection might indicated it as a follow up marker for recurrence in surgically resected HCV induced HCC patients. Larger scale studies to follow up its level at various intervals postoperatively and evaluate its pre-resection level as a prognostic marker in HCV induced HCC patients will be needed.** (Acta gastroenterol. belg., **2016**, 79, **309-313**).

Key words : HCV, Cirrhosis, HCC, DKK1

Introduction

Dickkopf-1 (DKK1) is a secreted protein implicated in embryogenesis. Wnt-1 protein specifically binds to the frizzled receptor (Fz) and the low-density lipoproteinreceptor-related protein-5/6 (LRP5/6), triggering signals essential for cell proliferation via β-catenin. Dickkopf-1 binds to LRP5/6 (1) and blocks interaction with Wnt-1 leading to β-catenin degradation and consequences on cell proliferation (2).

Hepatocellular carcinoma (HCC) is one of the long-term complications of hepatitis C virus (HCV) infection. In most cases, HCV induced HCC is the last step of a progressive course from hepatitis to HCC (5). Although aspects of this pathogenesis have been investigated, its underlying mechanisms are still evasive. Unlike hepatitis B virus (HBV) DNA genome that incorporates in the infected cell genome, HCV harbors an RNA genome that does not integrate in the host cell genome (3). As a result, indirect mechanisms of HCV induced hepatocarcinogenesis have been suggested.

Like alpha fetoprotein (AFP), which is a fetal serum protein (4), that is also expressed in HCC, DKK1 (protein implicated in embryonic development) expression has been studied mostly in HBV induced HCC (5,6,7,8). Umer et al, (9) studied methylation pattern of Wnt pathway inhibitors including DKK1 in various HCV disease stages. However, serum DKK1 level was poorly investigated in different disease stages of HCV infected patients. In this study, we aimed to assess serum DKK1 in HCV induced HCC, HCV induced liver cirrhosis and viral infection-free apparently healthy control subjects. Moreover, we assessed serum DKK1 level after curative resection of HCC and followed up HCC surgically resected patients for recurrence.

Subjects and methods

Study Population

The protocol and the procedures followed, were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2013. Informed consent was obtained from all participants in the study prior to blood and data collection.

This prospective study was done in the period between December 2014 and January 2016. The study included 20 patients with HCV induced HCC underwent liver resection for primary HCC. Their ages were 58.20 ± 7.27 years. They were 14 males and 6 females. The surgeries were done at the hepatobiliary surgery department, National Liver Institute, Menoufia university – Egypt. Twenty HCV induced liver cirrhosis patients were also recruited. Their ages were 54.20 ± 6.10 years. They were 13 males and 7 females. HCV infection was documented both serologically and by PCR for HCV-RNA. Moreover, 20 viral infection-free controls were enrolled in the study. Their ages were 55.35 ± 6.27

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years. They were 10 males and 10 females. HCC and cirrhosis were age and gender matched (P = 0.0671 and P = 0.7389 respectively). Cirrhosis and controls were age and gender matched (P = 0.5601 and P = 0.3434 respectively).

Cirrhosis diagnosis was documented by radiographic findings using ultrasonography and computed tomography (CT). HCC patients were diagnosed according to radiographic findings and/or pre-operative serum alpha-fetoprotein (AFP). The diagnosis was documented after resection by histopathology with proved negative resection margins.

Pre-operative workup including complete medical history, clinical examination, radiological investigations [ultrasonography, CT and magnetic resonance imaging (MRI)], HCV and HBV serology, and other routine laboratory investigations including complete blood picture, kidney function tests, liver function tests, and serum AFP was done for each patient. HCC patients were all Barcelona Clinic Liver Cancer (BCLC) stage A. Neoadjuvant therapy for HCC was not received by any patient and curative resection was the initial HCC treatment.

Post-operative 3-month-interval follow up included liver function tests, serum AFP level, ultrasonography and contrast-enhanced triphasic CT scans. Chest CT and bone scan were also performed to diagnose extra-hepatic recurrence. We reported 9 months follow up. Seven patients were excluded from follow up as they died. Three of them died early post operative, 1 week in 2 patients (one due to massive pulmonary embolism and the other due to portal vein thrombosis) and 2 weeks in one patient, due to sepsis occurring after management of iatrogenic intestinal leak. The other 4 patients died 2-5 months post operative (mean = 3.75 months) due to hepatic decompansation.

Serum DKK1 level:

Preoperative and 5 days postoperative serum DKK1 level was measured using Human DKK1 ELISA Kit (BOSTER BIOLOGICAL TECHNOLOGY, Valley Ave, Pleasanton, CA, USA). It is a standard sandwich enzyme-linked immune-sorbent assay technology. The 96-well plates are pre-coated using monoclonal antibody from mouse specific for DKK1. Standards and properly diluted test samples were added into the wells, a biotinylated detection polyclonal antibody from goat specific for DKK1 is added subsequently and then followed by washing. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away. HRP enzymatic reaction was visualized using HRP substrate TMB. A blue color product was formed and changed into yellow after adding acidic stop solution. The yellow color density is proportional to the human DKK1 sample concentration.

Statistical Analysis:

Quantitative data were expressed as mean ± standard deviation (mean ± SD) and analyzed by applying student t-test for comparing two groups of normally distributed variables and Mann Whiteny test for comparing two groups of abnormally distributed variables. Qualitative data were analyzed by applying chi-square test. $P < 0.05$ was considered significant.

Results

Assessment of 20 viral infection-free controls, 20 patients with HCV induced liver cirrhosis and 20 patients with chronic HCV infection underwent liver resection for primary HCC both pre-resection and post-resection revealed data presented in table 1.

Comparing viral infection-free controls and HCV induced liver cirrhosis revealed that total bilirubin, alanine transaminase, aspartate transaminase and AFP were significantly lower in infection free normal controls than cirrhotic patients (P = 0.000 for all). Serum albumin was significantly higher in controls than cirrhotic patients (P = 0.000). DKK1 did not differ significantly between controls and cirrhosis patients (P = 0.11)

Comparing HCV induced liver cirrhosis and HCV induced HCC revealed that total bilirubin, alanine transaminase, aspartate transaminase were significantly lower in HCC than cirrhosis patients (P = 0.000 for all). Albumin, AFP and DKK1 levels were significantly higher in HCC than cirrhosis patients (P = 0.000 for all). (Table 1)

AFP and DKK1 levels significantly reduced 5 days post-resection compared to their pre-resection levels (P = 0.000 for both). Serum aspartate transaminase significantly increased post-resection compared to its pre-resection level (P = 0.003). Pre-resection and 5 days post-resection levels of bilirubin, albumin and alanine transaminase did not differ significantly (P = 0.10, P = 0.32 and P = 0.10 respectively).

Box plots representing DKK1 levels in viral infection-free controls, patients with HCV induced liver cirrhosis, patients with HCV induced HCC pre- resection and post-resection are shown in figure 1.

Nine months follow up, revealed that number of patients with AFP level more than 100 ng/ml in both pre-resection and post-resection assessment did not differ significantly between non-recurrent and recurrent HCC (P = 0.22 and P = 0.11 respectively). Both pre-resection and post-resection DKK1 did not differ significantly between non-recurrent HCC and recurrent HCC patients (P = 0.23 and P = 0.68 respectively). (Table 2)

Table 1. — **Comparison of studied parameters among viral infection-free controls, HCV induced cirrhosis, HCV induced HCC pre-resection and HCV induced HCC post-resection.**

	Controls (n=20) mean ± SD	HCV-cirrhosis (n=20) mean ± SD	HCV-HCC pre-resection (n=20) mean ± SD	HCV-HCC post-resection (n=20) mean ± SD	P
Serum total bilirubin (mg/dl)	0.67 ± 0.14	2.36 ± 0.56	1.48 ± 0.27	1.53 ± 0.24	0.000[1]
					0.000[2]
					0.10[3]
Serum albumin (gm/dl)	4.37 ± 0.30	2.00 ± 0.43	3.16 ± 0.38	3.08 ± 0.35	0.000[1]
					0.000[2]
					0.32[3]
Serum alanine transaminase (U/L)	15.15 ± 4.90	130.85 ± 34.32	49.49 ± 12.17	56.05 ± 18.04	0.000[1]
					0.000[2]
					0.10[3]
Serum aspartate transaminase (U/L)	22.00 ± 5.59	129.15 ± 38.39	53.15 ± 12.35	61.15 ± 14.15	0.000[1]
					0.000[2]
					0.003[3]
Alpha fetoprotein (ng/ml)	2.75 ± 1.20	119.00 ± 44.24	1586.50 ± 968.01	406.00 ± 261.32	0.000[1]
					0.000[2]
					0.000[3]
DKK1 (pg/ml)	201.82 ± 71.70	248.00 ± 64.55	8100.00 ± 2301.71	1569.00 ± 646.85	0.11[1]
					0.000[2]
					0.000[3]

[1]Viral infection-free controls versus HCV induced cirrhosis.
[2]HCV induced cirrhosis versus HCV induced HCC pre-resection.
[3]HCV induced HCC pre-resection versus HCV induced HCC post-resection.

Table 2. — **Comparison of alpha fetoprotein and DKK1 between non-recurrent HCC and recurrent HCC both pre-resection and post-resection.**

	Non-recurrent HCC (n=5) mean ± SD	Recurrent HCC (n=8) mean ± SD	P
Pre-resection			
Alpha fetoprotein >100 ng/ml	5/5 (100%)	6/8 (75%)	0.22
DKK1(pg/ml)	9420.00 ± 2140.56	8075.00 ± 2524.59	0.23
Post-resection			
Alpha fetoprotein >100 ng/ml	5/5 (100%)	5/8 (62.5%)	0.11
DKK1 (pg/ml)	1480.00 ± 676.01	1655.00 ± 816.38	0.68

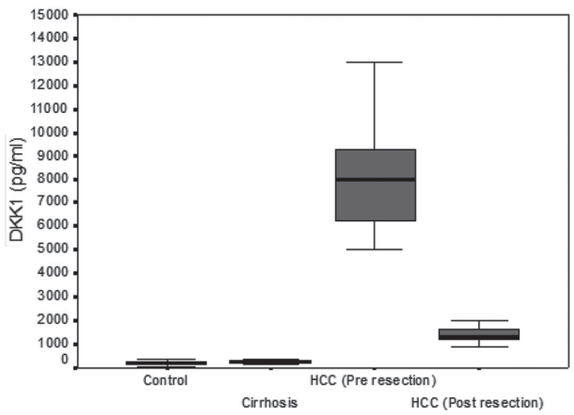

Fig. 1. — Box blots representing DKK1 levels in viral infection-free controls, HCV induced cirrhosis, HCV induced HCC pre-resection and HCV induced HCC post-resection.

Discussion

Serum DKK1 level was poorly investigated in different disease stages of HCV infected patients. In this study we investigated serum DKK1 in patients with HCV induced liver cirrhosis and HCV induced HCC. An observation that emerged from our results was that DKK1 did not differ significantly between HCV induced liver cirrhotic patients and viral infection-free controls. However, serum DKK1 level was significantly higher in HCV induced HCC than HCV induced cirrhosis.

It was found that HCV core protein activates Wnt/β-catenin signaling through multiple regulation of upstream molecules. It upregulates gene expression of canonical Wnt ligands, Fz receptors and LRP5/6 co-receptors. However, Wnt antagonists; Secreted Frizzled-Related Protein (SFRP) 3, 5 and Dkk1 were moderately repressed (10). Umer et al, (9) reported DKK1 promoter region was hypermethylated in liver biopsies from HCV infected patients at different stages of disease progression when compared to normal controls. Comparing DKK1 promoter methylation level among HCV diseased groups (chronic hepatitis, liver cirrhosis, HCC) revealed no significant differences (9, 11). Actually, it has been shown that DKK1 is a downstream target of Wnt/β-catenin signalling, and is regulated under a negative feedback loop in the Wnt/β-catenin pathway (12, 13). Liang et al, (14) reported higher mRNA levels of β-catenin in the DKK1 silencing cells compared with controls. After the interference of 5-aza-2'-deoxycytidine, the mRNA levels of Dkk-1 significantly increased and the mRNA levels of β-catenin decreased.

One can assume that throughout HCV disease stages, HCV core protein activates Wnt/β-catenin signaling together with DKK1 (Wnt inhibitor) promoter hypermethylation and DKK1 repression. Wnt signaling dysregulation promotes hepatocyte tumorigenesis, that is not accompanied by a corresponding change in DKK1 promotor methylation levels observed as the disease progresses from chronic hepatitis to HCC, could explain the significant elevation of serum DKK1 in HCV induced HCC than HCV induced liver cirrhosis and the non significant difference of serum DKK1 between HCV induced cirrhosis and viral infection-free controls.

However, Tung et al, (5) reported some HCC patients with high expression of DKK1 transcript, but having no or insignificant β-catenin staining. This indicates that, in addition to Wnt/β-catenin signalling, other signaling pathway(s) may regulate DKK1 expression. Tung et al, (5) revealed that serum DKK1 levels in the HBV carriers was not significantly different from that of the cirrhosis patients. In contrast, there was a stepwise increase in the serum DKK1 level from cirrhosis through the early HCC to advanced HCC. The serum DKK1 levels were significantly higher in the two HCC groups as compared to the HBV carrier and cirrhosis groups. The serum DKK1 level was also significantly higher in the advanced than the early HCC. Shen et al, (6) study showed that levels of DKK1 in serum were significantly higher in patients with HCC than all controls (chronic HBV infection, cirrhosis and healthy controls). Recently, Ge et al, (8) reported insignificant difference in DKK1 levels among three control groups (chronic HBV infection, cirrhosis and healthy controls).

Interestingly, our results revealed significant reduction in serum DKK1 5 days following HCC curative resection. This may be explained by excision of the tumor mass.

Tung et al, (5) reported that serum DKK1 level was significantly lower in the group of HCC patients after treatment when compared with advanced HCC group.

Nine months follow up of our living patients did not show significant difference between recurrent and non-recurrent patients in both pre-resection and post-resection serum DKK1 levels. The mean post- resection level was higher in recurrent than non recurrent patients (1655.00 vs. 1480.00 pg/ml) but this did not reach the statistical significance. Lack of significant difference in pre-resection serum DKK1 level between recurrent and non-recurrent patients indicated an absent prognostic role for pre-resection serum DKK1 in HCC curative resection in HCV infected patient.

Intra-hepatic metastasis and tumor recurrence, both of which are related to venous invasion (15). Tung et al, (5) did not find significant association between serum DKK1 and venous invasion or tumor stage. They performed multivariate analysis on patients' survival and they found that DKK1 (over-expression vs. no over-expression) did not significantly affect overall and disease-free survival of HCC

patients. However, They reported high tumor tissue DKK1 transcript association with venous invasion and advanced tumor stage. Yu et al, (20) reported that elevated expression of tumor tissue DKK1 was associated with cytoplasm/nuclear β-catenin accumulation and was a prognostic predictor for HCC after curative resection. Huang et al, (7) revealed that overexpression of tumor tissue DKK1 was involved in HCC metastasis and recurrence after orthotopic liver transplantation. In these studies (5, 7, 16) HCV was not referred as the inducing agent for HCC in their subjects.

Even though DKK1 levels; in our study do not seem to predict recurrence, these data should be interpreted with caution since numbers of included subjects are small. Studies that follow up both serum and tumor tissue DKK1 level in larger number of HCV induced HCC surgically resected patients for recurrence will be needed in order to clarify its potential role in intra-hepatic metastasis and tumor recurrence and hence to verify anti-DKK1 monoclonal antibody as potential therapeutic tool against tumor metastasis and recurrence.

Conclusion

We documented serum DKK1 as a marker for detection of early HCC in HCV infected patients. Serum DKK1

is not significantly different between HCV induced cirrhosis and viral infection-free controls; supporting previous report on DKK1 methylation levels in HCV infected patients. Significant reduction of DKK1 5 days after curative resection might indicated it as a follow up marker for recurrence in surgically resected HCV induced HCC patients. Larger scale studies to follow up its level at various intervals postoperatively and evaluate its pre-resection level as a prognostic marker in HCV induced HCC patients will be needed.

Compliance with Ethical Requirements

Conflict of Interest: The authors declare that they have no conflict of interest.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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