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RESEARCH ARTICLE

Risk of Early Hepatocellular Carcinoma Recurrence after Curative Ablation: Association with Cytokeratine 18

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Abstract

Introduction and aim: Evidence has now accrued that hepatocellular apoptosis plays a central role in chronic liver diseases and hepatocellular carcinoma (HCC). Cytokeratin 18 (CK18) fragments have been examined as potential tumor markers in many different types of epithelial cell carcinomas, including HCC. Therefore we evaluated the serum level of CK18 to predict early recurrence of HCC after curative local ablation and macroscopic vascular invasion. **Methods:** A case control study was done on 108 patients with HCC of various Barcelona Clinic Liver Cancer (BCLC) Staging underwent percutaneous local ablation and were followed up for local recurrence, compared to 110 Control subjects classified into 80 cirrhotic patients proved by liver biopsy and 30 healthy control subjects. Serum level of CK18-M30 Apoptosense was analyzed in all subjects. **Results:** CK18 level was significantly elevated in HCC patients when compared to control groups ($p < 0.001$). There was no significant difference in the level of CK18 pre and post successful local ablation (percutaneous ethanol injection or radiofrequency ablation) ($p = 0.15$), whereas there was significant increase in basal CK18 level in patients with recurrent local lesion versus those with no recurrence ($p < 0.001$). **Conclusion:** CK18 M30 could be used as a prognostic marker for prediction of early local recurrence of HCC, but unfortunately, there was no value in detection of successful ablation. In addition to older age- more than 60 years- and larger tumor diameter-3 to 5 cm- were two independent risk variables.

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INTRODUCTION

Cytokeratin is a highly complex subclass of the intermediate filaments which represents a stable composition in each type of epithelial cell and has been used in identification of different epithelial tissues and their neoplasms.^[1, 2, 3] In the normal human liver, hepatocytes have a very simple keratin composition: CK 8 and 18 which are required for the maintenance of hepatocyte integrity,^[4, 5] and altered expression of keratin gene is known to be related to liver diseases, including chronic hepatitis, increased hepatocyte fragility and decreased bile secretion.^[6] CK 7 and 19 are useful markers of bile ducts.^[7, 8]

Keratin molecules of cancer cells have been investigated for many years which identified modulation of cytokeratin 18 (CK18) during tumor transformation in human hepatocellular carcinoma (HCC).^[6] Most of the hepatocellular adenomas and carcinomas are observed in HE-stained sections were positive for CK8/18 that

overexpression may drive neoplastic transformation.^[9, 10] Evidence has now accrued that hepatocellular apoptosis plays a central role in chronic liver disease including hepatocellular carcinoma, viral hepatitis, alcoholic hepatitis, nonalcoholic steatohepatitis, cholestatic liver disease and hepatic fibrosis and cirrhosis. CK18 fragments have been examined as potential tumor markers in many different types of epithelial cell carcinomas, including hepatocellular carcinoma.^[10, 11, 12]

Hepatocellular carcinoma (HCC) is a primary liver cancer and is the fifth most common cancer worldwide.^[13] HCC is difficult to diagnose at early stage, and has a very poor survival rate when diagnosed at a late stage.^[14]

When surgery is not possible, there are several minimally invasive options for tumor ablation either chemical or thermal.^[16, 17, 18] The most frequent event observed during the follow up of curatively treated HCC patients is intrahepatic recurrence.^[19, 20, 21] Intrahepatic recurrence found in patients with HCC may be either local tumor progression or intrahepatic distant recurrence. Local tumor progression (LTP) occurs along the peripheral margin of the ablative lesion and intrahepatic distant recurrence (IDR) is a new HCC tumor remote from the margin of the ablative lesion.^[15]

In cases of liver cirrhosis, many HCC recurrences develop in a multi-centric fashion, it is estimated that approximately 50% of HCC is already multi-centric in the early stage; but frequently HCC recurrence also shows intrahepatic metastasis, even at a relatively early stage.^[22] The present study aims to evaluate the value of serum CK-18 caspase cleaved M30 in different Barcelona Clinic Liver Cancer (BCLC) stages of HCC and to find its relation with recurrence..

Material and methods:

The present case-control study included 108 HCC patients recruited prospectively from out and inpatient clinic of Mansoura University hospital during the period from June 2009 till September 2012. In addition to 110 matched age and sex subjects who were included as a control group; they were classified into 30 healthy volunteers and 80 post HCV cirrhotic patients (by biopsy they were 6/6 evaluated in the context of interferon therapy). Informed consent was obtained from all subjects.

All studied HCC patients were preceded by HCV cirrhotic liver. They were classified according to Barcelona Clinic Liver Cancer (BCLC)^[23] staging into 91 patients BCLC-A (applicable for percutaneous ablation) and 17 patients BCLC-C to screen the serum level of M-30 in vascular invaded patients (the most common cause of recurrence was vascular invasion). Moreover, they were classified according to Child Pugh staging; they were 72 Child A and 36 Child B. All studied patients had portal hypertension and most of treated patients had non-significant elevated alpha-feto protein level (less than 200 ng/ml).

Exclusion criteria included patients with multifocal >3 lesions, large size >5 cm, Child-C, haemostatic disorders, patients failed to achieve successful percutaneous ablation as well as other etiologies of chronic liver diseases.

Pretreatment Studies:

The pretreatment assessment of each patient included complete history, physical examination, complete blood count (CBC), renal and liver function tests, upper endoscope, abdominal US, and tri-phasic multislices computed tomography (CT) of the abdomen. Diagnosis of cirrhosis was based on histology (or) clinical, laboratory, and US with child –Pugh assessment of liver function status. Portal hypertension was diagnosed by the presence of esophageal varices or splenomegaly with platelets count $<100 \times 10^9/l$.

Tumor ablation:

Radiofrequency ablation (RFA) was used under guidance, using the commercial RFA system valley lab cool tip model (manufactured in USA) with needle of 15-25 cm long creating thermal ablation of zone up to 5 cm in diameter.

Percutaneous ethanol injection (PEI) was used in lesions less than 3 cm; a 22-gauge needle was introduced percutaneous after local xylocaine infiltration, the needle was introduced into the tumor or its marginal area under ultrasonography guidance. Absolute ethanol was slowly injected with careful attention to avoid passage of ethanol into the vein adjacent to the lesion. The amount of ethanol injected each time was 5-10 ml, depending on ethanol diffusion, which was checked in real time on ultrasound. The injection was done once a week for four to six sessions.^[24]

In both techniques successful ablation was denoted by absence of characteristic enhanced pattern in contrast enhanced CT imaging done four weeks after the procedure, PEI was restricted to lesions less than 3 cm in nearby vessels, viscous, liver capsule or common bile duct.

Follow-up Studies:

The protocol included abdominal US, AFP assays, and Child-Pugh related tests every 4 months (more frequently when needed) and CT every 6 months in the first year after treatment and yearly thereafter (more frequently if US, or AFP suggested recurrence). All ablated patients could not be followed up by alpha-fetoprotein as it is not elevated. Recurrence was diagnosed when enhancement reappeared within the ablation zone or 2.0 cm from its margins or appearance of new lesions.^[25] End points were recurrence appearance or study termination with follow up period of up to 39 months.

Biochemical and virological Assessment:

HCV infection was first assayed in all patients by third-generation enzyme-linked immunosorbent assay (Abbott anti-HCV ELISA, Abbott Lab, IL, and USA). The HCV viral load was measured by Real time PCR, Strata gene Mx3000P Real-Time PCR System with a sensitivity of approximately 15 IU/ml.

Routine laboratory tests included liver function tests (albumin, total bilirubin, ALT, AST) and serum creatinine (Dimension RXL MAX DADE BEHRING, Inc., Newark, DE, 19714-6101-USA.) were done for all subjects.

Analysis of CK-18 M30 apoptosis:

Serum samples were taken at diagnosis of HCC and used for quantitative measurement of the apoptosis-associated neo-epitope in the C-terminal domain of CK-18 using the M30-Apoptosense ELISA kit [Glory Science Co., Ltd, Del Rio, TX78840, and USA. ELISA assay kit].

Statistical analysis: Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 20.0. Qualitative variables were presented as number and percent. Quantitative variables were presented as mean \pm SD. The Kolmogorov-Smirnov (K-S) Test was done to test the normality of data distribution. Comparisons for parametric data were carried out by unpaired t test for two different groups and analysis of variance (ANOVA) for more than two groups. Comparisons for non parametric data were carried out by Mann-Whitney for two different groups and kruskal-wallis H test for more than two groups. The χ^2 -test and Fisher's exact probability test were used to compare independence. Spearman correlation coefficient test was used to assess correlation between different parameters. Cumulative disease-free survival was estimated using the Kaplan–Meier method and the significance of the hazard ratio for recurrence was evaluated using the log-rank test. Multivariate stepwise forward Cox proportional hazard regression model to search for independently significant risk factors. The results were reported as ratios with 95% CI. P-value 0.05 was considered statistically significant.

RESULTS

Demographic and tumor characteristic data

The baseline characteristics of the 91 hepatocellular carcinoma patients, 80 cirrhotic and 30 healthy subjects are shown in table 1. Patients with HCC were classified according to Child Pugh into 72 Child A and 36 Child B; moreover they were classified according to BCLC staging into BCLC- A. Among 91 patients without radiological evidence of vascular invasion, 30 (33%) underwent PEI and 61 (67%) underwent to RFA. The follow up period ranged from 3-39 months. Serum alpha fetoprotein in HCC patients were 22.15 ± 11.1 ng/ml

The aim of healthy and cirrhotic groups to reveal the pattern of CK-18 levels in these groups, cytokeratin 18 (CK-18 caspase M-30) showed a wide variability between different groups as demonstrated in (figure...) p value ...

Analysis of CK-18 M30 apoptosis in pre and post ablation stage:

Serum CK18 was analyzed pre- and post-ablation in 30 cases of BCLC-A. There was no significant difference in CK18 levels pre and post successful local ablation (percutaneous ethanol injection or radiofrequency

ablation) ($p= 0.15$), whereas the basal CK18 showed significantly increased levels in patients with recurrent lesion versus those with no recurrence ($p< 0.001$)(data not shown).

Relation of serum Ck-18 with HCC characteristics and recurrence

CK-18 levels showed highly significant differences as regard size of the lesion (<3, 3-5, >5cm) (137.6 ± 94.7 , 265.5 ± 207.5 , 576.5 ± 280.9 respectively, $p< 0.001$).

Analysis of CK18 in HCC group revealed that CK-18 was significantly increased in recurrent HCC versus non-recurrent (284.03 ± 187.8 versus 105.6 ± 29.1 ng/mL respectively, $p<0.001$). Recurrent hepatocellular carcinomas had higher basal levels of CK18 in earlier recurrence <12 months (306.3 ± 212.4) versus late recurrence 12 - 24 months (218.9 ± 42.06) with p value <0.001. Basal serum CK-18 level was significantly increased in patients suffering from multiple hepatic lesions when compared to those having single focal lesions (238.7 ± 168.8 versus 198.5 ± 166.9 respectively, $p<0.04$) (table 2).

Correlations of basal serum CK-18 with other laboratory parameters revealed positive correlations with serum bilirubin and alpha fetoproteins levels . (figures 5 and 6).

The median ck-18 level in studied ablated hepatocellular carcinoma patients was 166 ng/ml, this median was used to subgroup HCC patients below and above; [Analysis of the data in using kaplan-meier estimates of the survival probability for patients with CK-18 caspase cleaved levels in which the censored cases were non-recurrent cases, Patients with basal serum CK18 <166ng/mL showed significantly longer recurrent free survival and higher cumulative survival probability when compared to those with CK18>166ng/mL](#) (p value <0.001). This denotes for two points firstly patients with higher caspase level had higher association for early recurrence, secondly suggested median level could be used for prognosis.

Prognostic risk factors for HCC recurrence:

Analysis of risk factors for tumor recurrence ; recurrence rate was compared by the following possible prognostic factors: patient age, sex, serum levels of ALT, AST, albumin, bilirubin, modified Child-Pugh classification score , number of tumors, size of tumor nodule, serum alpha-fetoprotein level, platelet count, in addition to basal CK18 level

Univariate analysis of multiple risk variables between recurrent and nonrecurrent HCC denotes five significant risk factors include gender, tumor size, nodules number, cytokeratine category and aspartate transaminases .

Prediction of first recurrence using stepwise multivariate cox proportional hazard models revealed that three independent significant risk factors as following: larger lesion size 3 to 5 cm (odds ratio 34.633, 95% confidence interval 7.143 to 167.910, $P<0.001$), older age >60 years (odds ratio 46.728, 95% confidence interval 10.471 to 208.531, $P<0.001$), cytokeratin 18 caspase level >166pg/ml (odds ratio 38.855, 95% confidence interval 12.215 to 123.592, $P<0.001$) (table 4).

Table (1) Clinical, biochemical and demographic characteristics of the studied population.

	Healthy	Cirrhotic	HCC without macro vascular invasion	
Number	30 (27.27%)	80 (72.73%)	91 (84.26%)	
Age (years)	49.47 ± 7.5^a	50 ± 6.7^a	51.66 ± 8.6^a	0.25
Sex				
male	17 (56.6%)	45 (56.3%)	42 (46.1%)	0.37
female	13 (43.4%)	35 (43.7%)	49 (53.9%)	
ALT (IU/L)	16 ± 3.7^a	64 ± 17.2^b	62.46 ± 21.15^b	<0.0001
AST (IU/L)	19 ± 4.5^a	78 ± 15.6^b	69.7 ± 43^b	<0.0001
Alpha-feto proteins (ng/mL)	5.14 ± 2.34^a	21 ± 9.3^b	22.15 ± 11.1^b	<0.0001

	0.92 ± 0.12 ^a	1.2±0.37 ^b	1.12 ± 0.14 ^b	<0.0001
INR				
S. Albumin (mg/dL)	4.12 ± 0.52 ^a	3.9±0.4 ^b	3.25 ± 0.36 ^c	<0.0001
S. Bilirubin (mg/dL)	0.61 ± 0.22 ^a	0.7±0.2 ^a	1.19 ± 0.36 ^c	<0.0001
Platelet counts (×10⁹/L)	195.56 ± 36.41 ^a	175.23±56.5 ^a	80.05 ± 28.95 ^c	<0.0001
Ascites	-	-	24 (26.3 %)	-
Child class				
A			63 (69.2%)	
B			28(30.8%)	
Maximal tumor diameter				
< 3cm	-	-	21 (23.1%)	-
3-5 cm			70 (76.9%)	

Similar letters indicate non significance

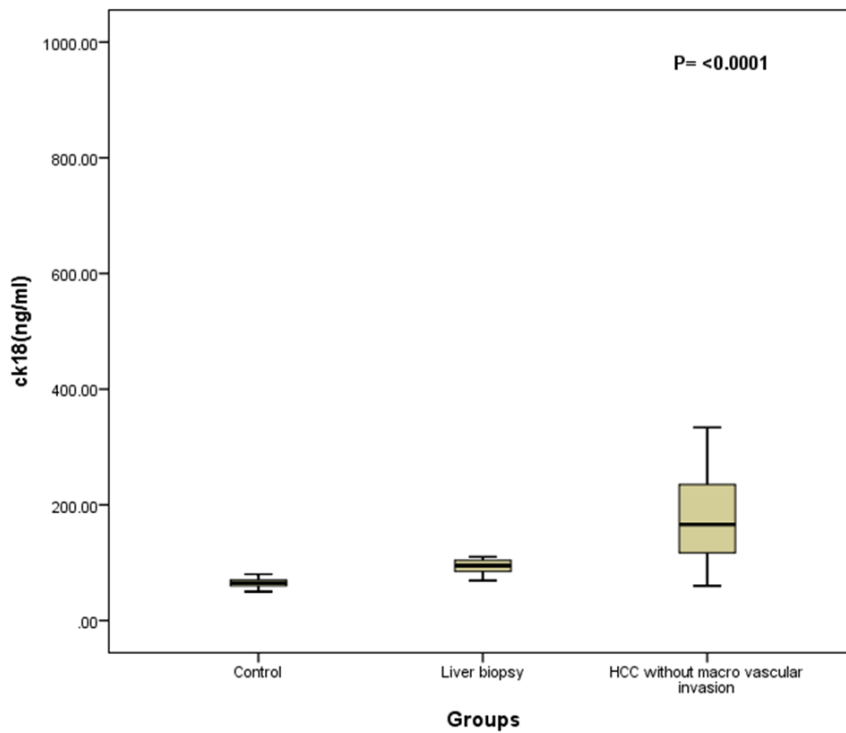


Figure 1: comparison of ck -18 in control healthy group, cirrhotic by biopsy and HCC patients.

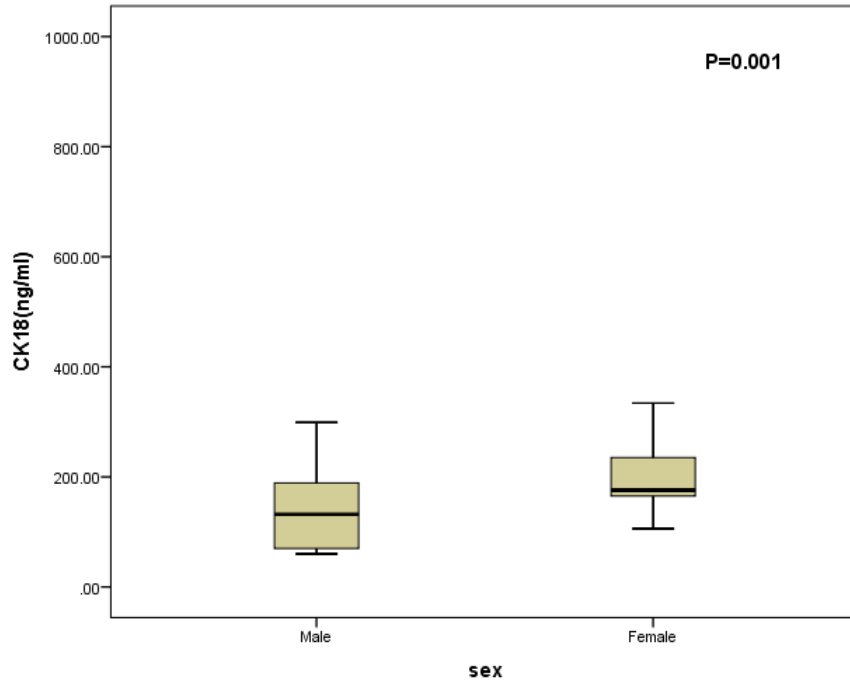


Figure 2: ck-18 among different gender in HCC patients .

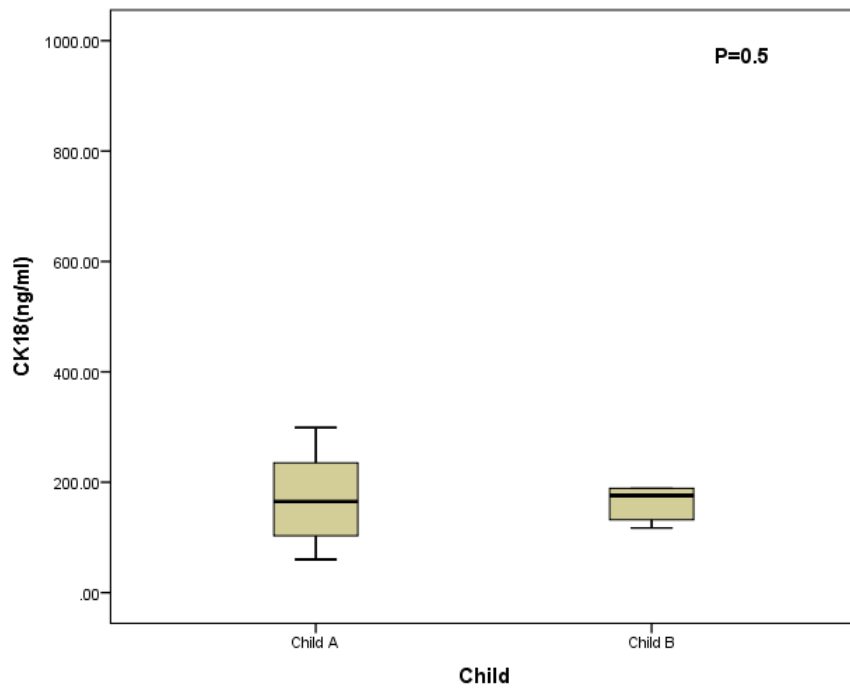


Figure 3: ck-18 difference in different child-puch class, child –A versus child-B among HCC patients.

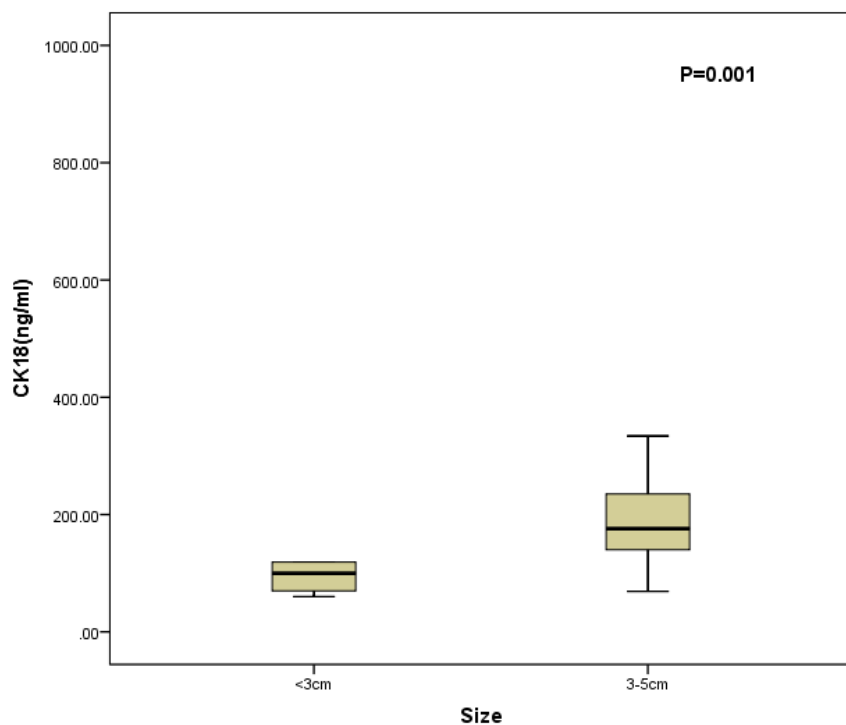


Figure 4: ck-18 difference among among different HCC size <3cm versus 3-5 cm .

Table (2): Serum cytokeatin-18 level in patients suffering from hepatocellular carcinoma without vascular invasion (at diagnosis?).

	No	Ck18 (ng/mL)	P value
Recurrent free Survival after ablation (months)			
< 12m	38(41.7%)	306.3 ± 212.4	< 0.001
12-24m	13(14.3%)	218.9 ± 42.06	
> 24m	40(44%)	105.6 ± 29.1	
Recurrent	51 (56%)	284.03 ± 187.8	< 0.001
Non-recurrent	40 (44%)	105.6 ± 29.1	
Ascites			
Present	24(26.3%)	192.6 ± 68.2	0.51
Absent	67(73.7%)	210.2 ± 190.6	
Multiplicity			
Single lesion	79 (86.8%)	198.5 ± 166.9	0.04
Double lesion	12 (13.2%)	238.7 ± 168.8	

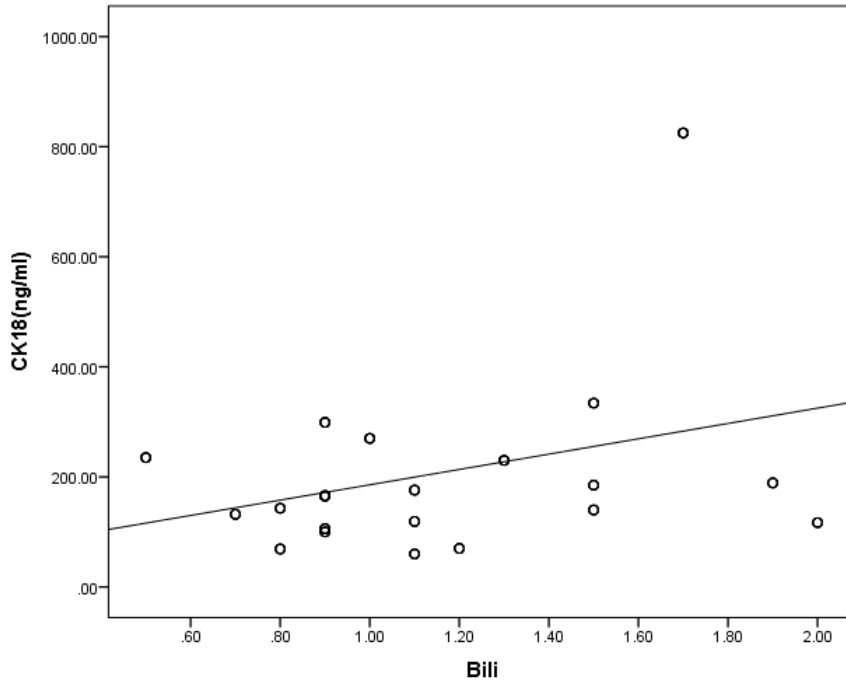


Figure 5: correlation of ck-18 with bilirubin level in HCC patients .

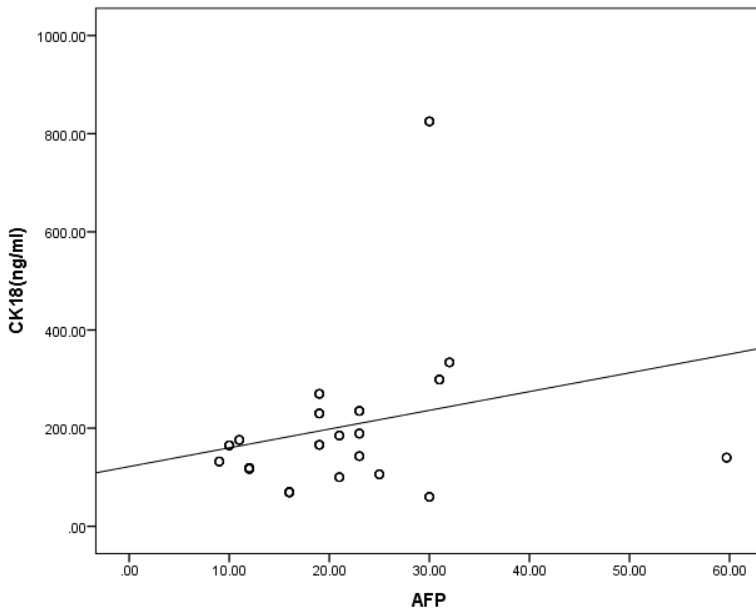


Figure 6: correlation of ck-18 with alpha-feto protein level in HCC patients.

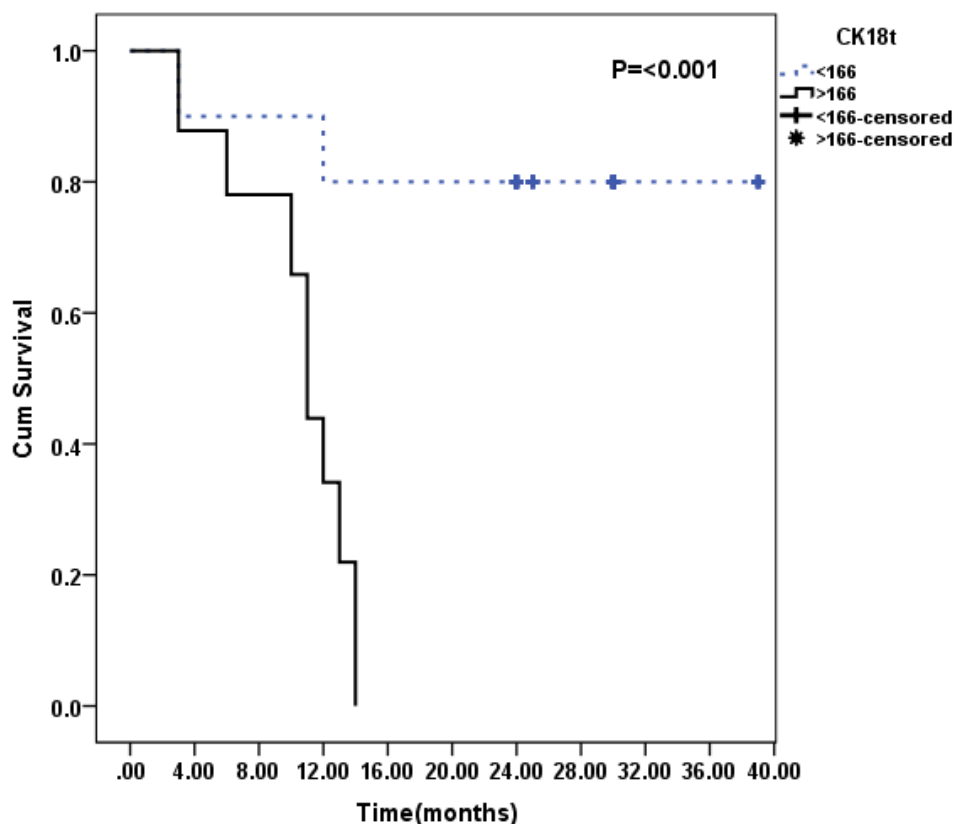


Figure7: Kaplan-Meier estimates survival probability in relation to time as regards Ck-18 levels subgrouping.

Table 3 Univariate analysis of risk factors of first recurrence after curative ablation of HCC

			Recurrence		P
			Non-recurrence	Recurrence	
Gender	Male	No	28	14	<0.001
		%	70.0%	27.5%	
	Female	No	12	37	0.2
		%	30.0%	72.5%	
Age subgroup (years)	< 60y	No	36	41	0.27
		%	90.0%	80.4%	
	>= 60y	No	4	10	0.03
		%	10.0%	19.6%	
Alanine transaminase (ALT)	Median		55.00	60.00	0.07
	Minimum		25.00	44.00	
	Maximum		85.00	110.00	
Aspartate transaminase (AST)	Median		60.00	72.00	0.35
	Minimum		34.00	26.00	
	Maximum		100.00	110.00	
Albumin level (gm/ml)	Mean		3.53	3.35	0.07
	Standard Deviation		.45	.49	
Bilirubin level (mg/ml)	Mean		1.10	1.18	0.9
	Standard Deviation		.38	.40	
Child-puch class	Child A	No	28	35	0.9
		%	70.0%	68.6%	

Nodule number of HCC	Child B	No	12	16	0.005
		%	30.0%	31.4%	
	Single	No	38	37	
		%	95.0%	72.5%	
Longest diameter of HCC	Multiple	No	2	14	0.001
		%	5.0%	27.5%	
	<3cm	No	16	5	
		%	40.0%	9.8%	
Cytokeratine 18 level subcategory(pg/ml)	3-5cm	No	24	46	<0.0001
		%	60.0%	90.2%	
	<166	No	40	10	
		%	100.0%	19.6%	
Alfa- feto protein level	>166	No	0	41	0.7
		%	0.0%	80.4%	
	Mean		22.37	21.63	
Platelet count	Standard Deviation		14.09	7.03	0.9
	Mean		82.70	81.96	
Prothrombin time (INR level)	Standard Deviation		33.66	29.27	0.2
	Mean		1.09	1.13	
Ascites	Standard Deviation		.12	.15	0.2
	absent	No	32	35	
		%	80.0%	68.6%	
	present	No	8	16	
		%	20.0%	31.4%	

Table 4: Stepwise multivariate Cox Proportional Hazard Models Showing Predictors of First Recurrence.

variables	P	OR	95.0% CI for OR	
			Lower	Upper
Age: >60/<60	<0.001	46.728	10.471	208.531
AST	<0.001	.966	.948	.983
Bilirubin	.008	.268	.101	.713
Size 3-5/<3	<0.001	34.633	7.143	167.910
CK18 >166/<166	<0.001	38.855	12.215	123.592

[HR<1=protective; HR>1=risky](#)

HR: hazard ratio CI: confidence interval

Discussion:

HCC is multi-factor process that acts in combinations including viral infection, oncogene activation, apoptosis, cirrhosis development and inactivation of tumor suppressor genes. This study evaluated the apoptosis in HCC patients in different BCLC stages and prediction of recurrence risk factors in HCC patients, as well as we studied the association of apoptosis marker (CK-18 caspase cleaved serum level M 30) with the recurrence rate.

Many studies showed evidence that CK18 plays an important role in tumor genesis of hepatocellular carcinoma. Ckawai et al., 2009^[9] showed that Ck-18 may become a useful immune-histochemical marker for detecting hepatocellular proliferative lesions while Gonzalez-Quintela and co-workers^[26] found a fragment of CK-18 with highest level in patients with hepatocellular carcinoma. The results of experimental studies suggested that CK8/18 immunohistochemistry could be used as a marker for detecting liver pre-neoplastic and neoplastic lesions in

mice^[9]. In accordance with these studies, our results indicate a highly significant CK18-m30 levels in the hepatocellular carcinoma group versus both control groups either healthy control or cirrhotic control (fibrosis score 6/6 by liver biopsy).

Results of the present study demonstrated highly significant basal CK-18m30 levels in recurrent lesions versus non recurrent and with shorter versus longer recurrent free survival; Also denotes strong relation to nodules number, tumor size, bilirubin and alpha_feto protein levels.

The strong relation of recurrent HCC to CK-18 could be explained by its high serum level in apparent vascular invasion, thus recurrent HCC patients may have microvascular affection.

Previous studies denote increases in CK-18 fragments in serum from patients with breast, liver and lung cancers, reflecting spontaneous apoptosis of tumor cells,^[27, 28] this is consistent with the present study which shows a significant increase in CK-18-m30 levels in HCC patients in comparison to HCV related liver cirrhosis. In the current study, not all HCC stages were similar; larger size and more nodules demonstrates marked elevation. This could be explained by increase in the spontaneous apoptosis of HCC cells released into blood vessels. It will be interesting to study serum CK-18 levels changes in vascular invasion of HCC in larger sample sizes.

This study demonstrated a significant association between CK-18 levels and tumor size, this could be explained by increase in the microvascular invasion. McHugh and co-workers found microvascular invasion is strongly associated with tumor size and AFP >100 ng/ml and greatly increase the risk of recurrence after transplantation for HCC.^[29]

Microscopic vascular invasion was an independent risk factor for both recurrence and poor survival even after liver transplantation.^[30, 31] Many studies have demonstrated that microvascular invasion is considered as a risk factor for early hepatocellular carcinoma recurrence after resection.^[32, 33] various studies reveals that most common cause of recurrence is presence of microvascular invasion. Chen and co-workers found that high preoperative serum alpha-fetoprotein level, larger tumor size and micro vascular invasion are independent risk factors associated with residual tumor and early recurrence after resection.^[34]

Carcinogenesis may arise when the homeostatic balance between cell survival and apoptosis is disrupted. Hepatocellular carcinoma depends largely on angiogenesis. Our study reveals a significant relation between apoptotic marker CK-18 and HCC recurrence as well as recurrence free survival. This marker could be used for diagnosis of HCC recurrence. Conversely, serum AFP levels did not show significant difference between early recurrent (less than 12 months) and late recurrence (from 12 to 24 months) in our studied patients.

It is important for hepatologists to assess patients prognosis for the purpose of determining the treatment options and improving the long term outcome, thus knowledge the risk factors of recurrence could be helpful for hepatic diseased patients which were detected in the present study by cox regression model; older age more than 60years, increasing maximal tumor diameter (3-5 cm), higher CK-18 serum levels (>166pg/ml) were independent risk factors that predict HCC recurrence. Lai *et al.*,^[35] showed strong correlation between AFP level, tumor dimensions, and microvascular invasion, all are well-known predictors of HCC recurrence. In contrast to the present study, Ng *et al.*,^[36] reported that the age and sex of patients had no influence on the risk of recurrence.

The data in our study revealed that HCC recurrence free survival could be influenced by tumor dimension, older age, and elevated CK-18. In other words, CK-18 apoptosis m-30 serum level could be used for prognostic tool.

Limitations of the study include no comparison to chemo-embolization (TACE), no tissue correlation of ck-18 with its serum level. However, patients who express high levels of this marker require careful screening.

In conclusion serum CK18 M30 could be used as a prognostic marker to predict early recurrence of HCC and to suspect vascular invasion, in addition to others two independent risk variables :older age and larger tumour size.

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