Measurement of Some biochemical markers in hepatitis C virus cirrhotic patients complicated by hepatocellular carcinoma before and after liver transplantation

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Abstract: (HCC) is one of the most common cancers worldwide with the characteristics of high mortality and the overall poor prognosis. (HCC) grows rapidly and frequently associates with vascular invasion, metastasis, recurrence, and poor prognosis. HCC is the fifth most common human cancer and the third leading cause of cancer death worldwide One of the main causes of this increase is the increased infection with HCV and its complication cirrhosis which is the most powerful risk factor for development of HCC. About HCV there are 170 million chronic carriers worldwide. Chronic hepatitis C frequently exhibits an insidious course of disease marked by progressive liver injuries that progress, often over several decades, from fibrosis to cirrhosis and, ultimately, hepatocellular carcinoma (HCC). Screening of each patient with cirrhosis of the liver regardless of etiology is of primordial importance for the detection of tumors in the initial stages of development. Tumor depend on angiogenesis for growth and metastasis in a hostile environment. At early stages of carcinogenesis, VEGF acts as an important tumor angiogenesis signal. VEGF is the best investigated angiogenic factor in HCC. Concentration of circulating VEGF increases with advancing HCC stage, the highest levels being in patients with metastasis. liver transplantation provides life-saving therapy for patients with end-stage organ disease. Cancer risk is elevated in transplant recipients, largely due to loss of immune control of oncogenic viruses arising from immunosuppressive medications administered to prevent organ rejection. In this study, the plasma levels of VEGF were assayed in fifty individuals classified into three groups: HCC patients (group I) which comprised twenty HCV infected patients with localized HCC who will undergo liver transplantation VEGF assayed in these patients three times (a) before transplantation, (b) six months after transplantation (c) twelve months after transplantation (c) subgroup divided into two categories (c1) patients who develop no recurrences & (c2) patients who develop recurrence, group II which involved twenty HCV infected patients with (cirrhosis), group III involved ten apparently healthy volunteers. The obtained results of plasma level of VEGF, a significant increase was detected in localized (group Ia) as compared with group II and group III. Also, a significance was detected in VEGF levels in (group Ic2) as compared with (group Ic1), group II, and group III. also, a significant decrease in VEGF level was detected (group Ib) as compared with (group Ia) No other significant differences were detected between the studied groups. Regarding the correlation matrix, a positive correlation between VEGF and AFP in all groups with exception of group Ib whereas non-significant correlations were detected in. In conclusion, detection of serum VEGF and AFP has different significances; VEGF could be used as an indicator of the development of HCC in patients with liver cirrhosis during follow-up, to reflect the disease's potential activity of vascular invasion and metastasis and predict HCC recurrence after treatment. Whereas, AFP is suggested to be used as a supplementary marker which may help early diagnosis of HCC, but not to detect circulating HCC cells. Therefore*, combination of multiple markers may be more valuable in the diagnosis, prognosis and recurrence of HCC.*

1. Introduction

Primary liver cancers include HCC, cholangiocarcinoma, angiosarcoma and hepatoblastoma *[1].* (HCC) is one of the most common cancers worldwide with the characteristics of high mortality and the overall poor prognosis *[2].* (HCC) grows rapidly and frequently associates with vascular invasion, metastasis, recurrence, and poor prognosis. HCC is the fifth most common human cancer and the third leading cause of cancer death worldwide *[3]* One of the main causes of this increase is associated with the increased infection with HCV *[4].* Extensive and

convincing epidemiologic evidence suggest that chronic infection with the hepatitis C virus is responsible for a significant proportion of HCC *[5].*

Hepatitis C virus (HCV) is a major cause of chronic viral hepatitis with prevalence about 2.3% all over the world. About170 million chronic carriers worldwide. Chronic hepatitis C frequently exhibits an insidious course of disease marked by progressive liver injuries that progress, often over several decades, from fibrosis to cirrhosis and, ultimately, hepatocellular carcinoma (HCC) *[6].* Liver transplantation can be considered as an appropriate treatment option for patients with end stage liver disease and earlier stage HCC. A major disadvantage with OLT (in addition to the need for lifelong immunosuppression with its attendant risks) is the long waiting time for donor organs. *[7].* Vasculo-genesis and angiogenesis are mechanisms responsible for the development of blood vessels. Angiogenesis is a physiological phenomenon leading to the establishment of the vascular tree during development *[8],* while vasculo-genesis is development of novel micro-vessels by stem and progenitor cells which is crucial in tissue engineering and regeneration as well as pathological aspects such as tumor growth as a result of pathological vessel development *[9].* The abnormal growth of blood vessels is a key pathophysiological feature of numerous disorders, including tumorigenesis, arthritis, endometriosis, and retinopathies. Despite substantial progress from studies of patients and animal models, abnormal neovascularization remains a common threat to health and well-being *[10].* The best factor known by its angiogenic effect is VEGF. This molecule has been implicated in virtually every type of angiogenic disorder, including those associated with cancer *[11].* VEGF is likely the most important angiogenic factor because it is expressed abundantly by a wide variety of human and animal tumours and because of its potency, selectivity for ECs and ability to regulate most and perhaps all of steps in the angiogenic cascade *[12].* Moreover, a number of other angiogenic cytokines act, at least in part, by up-regulating VEGF expression *[13].* Solid tumors depend on angiogenesis for growth and metastasis in a hostile environment. In the perivascular phase, the tumor is rarely larger than 2 to 3 mm^3 and may contain a million or more cells. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supplies required for growth and survival by simple passive diffusion. The properties of tumors to release and induce several angiogenic and anti-angiogenic factors which play crucial roles in regulating endothelial cell proliferation, migration, apoptosis or survival, cell-cell and cell-matrix adhesion through different intracellular signaling are

thought to be the essential mechanisms during tumor-induced angiogenesis *[14].*

Aim of the work:

The aim of this work is to use non-invasive technique in prediction of vascular invasion and early HCC recurrence in HCV patients complicated by HCC before and after liver transplantation.

2. Subjects and Methods

This study was carried out in the Medical Biochemistry department faculty of Medicine Al-Azhar university and liver transplantation unit and Internal Medicine outpatient clinics in Kasr Al-einy hospital, Faculty of Medicine, Cairo University.

40 patients with chronic hepatitis C and 10 controls (age and BMI matched).

The patients were divided into 3 groups

- **1-Group I of HCC patients:**
- **Ia-HCC patients before liver transplantation:** twenty patients with hepatocellular carcinoma diagnosed by sonography, CT and MRI before liver transplantation.
- **Ib-HCC patients six months after liver transplantation:** previous patients 6 months after liver transplantation *(eighteen patients as two patients died from graft rejection).*
- **Ic-HCC patients twelve months after liver transplantation:** previous patients 12 months after liver transplantation *(seventeen patients as one patient died from heart attack).*

They are further subdivided into two subunits:

- ● Ic 1-patients who show no recurrence: they were fourteen patients.
- ●Ic 2-patients who show HCC recurrence: they were three patients.
- **2- Group II Cirrhotic patients:** twenty subject showing signs and symptom of cirrhosis and they give positive HCV Ab.
- **3-Group III Control:** ten healthy volunteers showing negative HCV Ab were selected and assigned as a control group.

Exclusion criteria:

- 1-subjects suffering from any systemic disease like hypertension, cardiovascular system diseases, and renal dys-function.
- 2-obese subjects with BMI >30 kg/M^2.
- 3-smokers, alcoholics and drug addicts.
- 4-subjects who are known to have any auto-immune disease or those taking any medication that affect the immune system.

Only patients with HCV hepatitis were included in this study.

Both patients and controls were subjected to the followings:

- Full history taking.

- Full clinical examination.
- Pelviabdominal ultrasound to assess the echogenicity of the liver, peri-portal thickening, and diagnose malignancy.
  - CT scan and MRI.
  - Ten mL venous blood were withdrawn as follow.

-2mL was added to polypropylene tubes with stopper, left to clot for 20 min at 37 ˚C centrifuged at 3000 xg for 10 min it was used for estimation of ALT, AST, alkaline phosphatase, albumin, anti HCV Ab and HBsAg.

-2mL in a dry clean tube containing sodium citrate as an anticoagulant for Prothrombin time.

-2mL in a dry clean tube containing EDTA as an anticoagulant for CBC.

-2mL in a dry clean tube containing EDTA as an anticoagulant for VEGF level. Blood was centrifuged for 20minutes at 3000 xg immediately after collection and plasma was removed by Pipetting off the top yellow layer without disturbing the white buffy layer. Plasma was stored at <-800C till assay the following laboratory investigations were done:

- anti HCV Ab and HBsAg by ELISA.
- Liver function tests (liver enzymes, bilirubin, albumin) by Beckman CX5 auto-analyser.
- Coagulation profile (PT, PTT, INR) by auto-mated blood coagulation analyzer Sysmex CA1 500 (Siemens AG, Erlangen, Germany).

- Complete blood count (hemoglobin, WBC count, platelet count) by Dyn 1700.
- alfa fetoprotein Beckman CX5 auto-analyser.
- vascular endothelial growth factor (VEGF) by ELISA.

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics Version 24 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation as appropriate. Qualitative data were expressed as percentage. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). and between two reading for the same group (paired sample t-test)

3. Results

Table (1) show means and standard deviation of weight, height and body mass index in all studied groups and there was no significant difference between them.

Table (2) show percentage of male and female in all studied groups the participant in this study were 50 subjects (20 female that represent 40% and 30 male that represent 60%).

Table (3) show means and standard deviation of Total Bilirubin (mg/dl), Albumin (g/dl), ALT (U/ml), AST (U/ml), Alkaline phosphatase (U/ml), Haemoglobin (g/dl): White blood cells (cells X 103/ml), Platelets (cells X 103/ml), alfa fetoprotein (ng/ml) and VEGF (pg./mL).

Table (1)

	Weight	Height	BMI
Group I	Mean 83.3	Mean 177.80	Mean 26.265
	SD 11.581	SD 9.065	SD 2.683
Group II	Mean 48.8	Mean 177.50	Mean 26.945
	SD 9.663	SD 9.811	SD 2.54
Group III	Mean 84.2	Mean 178.68	Mean 26.36
	SD 9.394	SD 9.257	SD 2.448

Table (2):

	Group I	Group II	Group III
Male	No 11	No 13	No 6
	Percent 55 %	Percent 65 %	Percent 60 %
Female	No 9	No 7	No 4
	percent 45 %	Percent 35 %	Percent 40 %
No	20	20	10

Table (4): show means and standard deviation of AFP in all studied groups and there was significant decrease in means of AFP in patients of (Group **I**b) compared to (Group **I**a) and significance increase of this level compared to (Group **I**c2) theirs were also

significant differences in means of AFP in (Group **I**a) compared to to (group **II**) and (Group **III**).

Table (5): show means and standard deviation of VEGF in all studied groups and there was significant decrease in means of VEGF in patients of (Group **I**b) compared to (Group **I**a) and significance increase of

this level compared to (Group **Ic2**) theirs were also significant differences in means of VEGF in (Group **Ia**) compared to to (group **II**) and (Group **III**).

Table (3)

| Variants	Ia	Ib	Ic1	Ic2	II	III
T. Bilirubin (mg/dl)	2.493	1.1	0.99	4.33	4.275	0.43
Mean ± SD	±0.418	±0.396	±0.314	±0.862	±1.047	±0.148
Albumin (g/dl):	2.66	3.92	4.22	1.9	1.93	4
Mean ± SD	±0.422	±0.434	±0.317	±0.436	±0.391	±0.294
ALT (U/m l):	164.3	29.67	24.86	179	23.85	31
Mean ± SD	±61.149	±9.935	±8.725	±11.790	±8.845	±7.242
AST (U/m l):	140.5	25.67	25.79	204	26.9	26.1
Mean ± SD	±39.264	±7.956	±9.04	±7.937	±8.341	±9.826
Alk phos (U/m l):	529.9	77.83	67	606	123.75	48.2
Mean ± SD	±65.839	±20.118	±12.191	±106.165	±175.192	±18.097
Hb (g/dl):	11.435	10.361	11.06	7.53	9.24	13.88
Mean ± SD	±1.456	±0.860	±1.077	±1.4	±1.947	±0.950
WBCs (cells X 103/ml):	3.931	4.188	4.66	1.47	1.91	6.68
Mean ± SD	±1.488	±0.847	±1.444	±0.651	±0.891	±1.48
Plat (cells X 103/ml):	102.25	130.72	123.07	36	78.9	322.1
Mean ± SD	±44.128	±21.54	±21.857	±13.454	±28.024	±65.526
AFP (ng/m l):	686.65	20.83	16.79	726	71.25	12.9
Mean ± SD	±185.034	±13.857	±11.470	±58.643	±47.47	±4.383
VEGF (pg/mL)	657.1	263.28	242.86	689	252.9	239.112.9
Mean ± SD	±45.238	±88.8	±21.136	V32	±29.672	±32.313
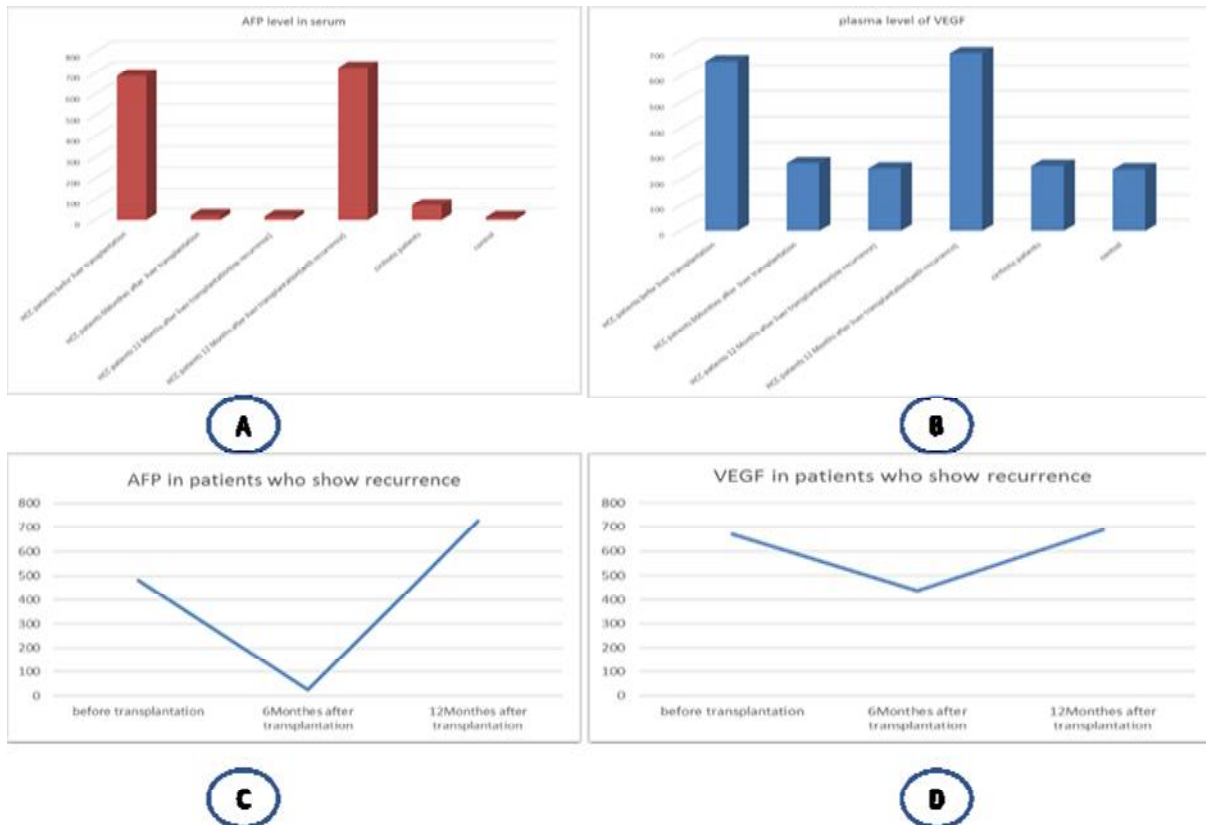
Figure (1)

Table (4)

Groups	AFP (ng/mL)			
Ia	Mean	686.65		
	SD	185.034	P <0.0001	
Ib	Mean	20.83		
	SD	13.857		
Ia	Mean	686.65		
	SD	185.034	P<0.0001	
Ic1	Mean	16.79		
	SD	11.47		
Ia	Mean	686.65		
	SD	185.034	P<0.0001	
II	Mean	71.25		
	SD	47.47		
Ia	Mean	686.65		
	SD	185.034	P<0.0001	
III	Mean	12.9		
	SD	4.383		
Ib	Mean	20.83		
	SD	13.857	P 0.092	
III	Mean	12.9		
	SD	4.383		
Ic1	Mean	16.79		
	SD	11.47	P<0.0001	
Ic2	Mean	726		
	SD	58.643		
Ic1	Mean	16.79		
	SD	11.47	P 0.321	
III	Mean	12.9		
	SD	4.383		
Ic2	Mean	726		
	SD	58.643	P<0.0001	
III	Mean	12.9		
	SD	4.383		
II	Mean	71.25		
	SD	47.47	P0.001	
III	Mean	12.9		
	SD	4.383		

Table (5)

Groups	VEGF (pg/mL)			
Ia	Mean	657.1		
	SD	45.238		
Ib	Mean	263.28	P <0.0001	
	SD	88.8		
Ia	Mean	657.1		
	SD	45.238		
Ic1	Mean	242.86	P<0.0001	
	SD	21.36		
Ia	Mean	657.1		
	SD	45.238		
II	Mean	252.9	P<0.0001	
	SD	29.672		
Ia	Mean	657.1		
	SD	45.238		
III	Mean	239.1	P<0.0001	

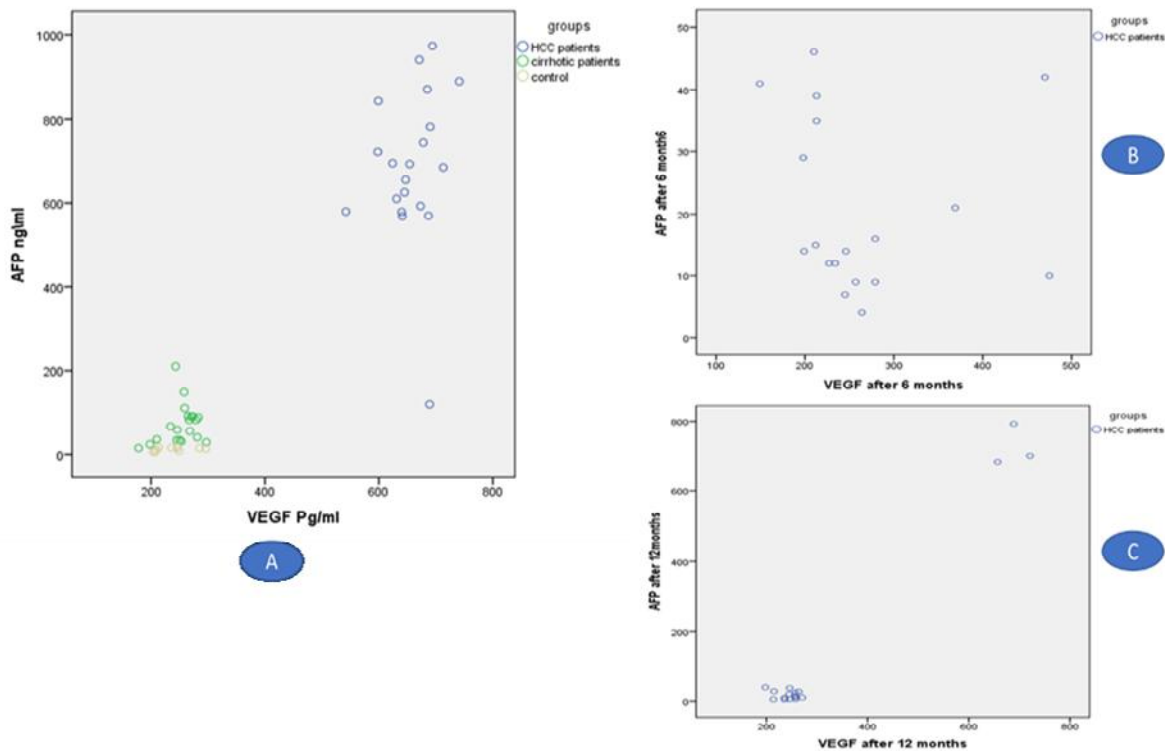|			
	SD	32.313	
Ib	Mean	263.28	
	SD	88.8	
III	Mean	239.1	P 0.417
	SD	32.313	
Ic1	Mean	242.86	
	SD	21.36	
Ic2	Mean	689	P<0.0001
	SD	32	
Ic1	Mean	242.86	
	SD	21.36	
III	Mean	239.1	P 0.733
	SD	32.313	
Ic2	Mean	689	
	SD	32	
III	Mean	239.1	P<0.0001
	SD	32.313	
II	Mean	252.9	
	SD	29.672	
III	Mean	239.1	P 0.253
	SD	32.313	

Figure (2)

Figure (1) A: Mean of serum level of alfa fetoprotein (AFP) in ng/dL in all studied groups, ***Figure (1) B:*** Mean of plasma levels of vascular endothelial growth factors (VEGF) in pg/dl in all studied groups, ***Figure (1) C:*** change in level of AFP in patients of HCC pre-liver transplantation and 6 & 12 Months after liver transplantation in those who show recurrence and it show that the level of serum AFP return to normal level after transplantation even if the patient will develop recurrence and ***Figure (1) D:*** change in level of VEGF in patients of HCC pre-liver transplantation and 6 & 12 Months after liver transplantation in those and who show recurrence

plasma level of VEGF didn't return to normal level in cases who developed recurrence.

Figure (2) A: show that there was a positive correlation between VEGF and AFP in group Ia, II and III (r=0.93, P< 0.0001) and also *figure (2) C* in group there were a positive correlation between VEGF and AFP in group Ic1Ic2 (r=0.988, P< 0.0001) while in *figure (2) B* non-significant correlation detected in group Ib.

4. Discussion

This study was designed to test two hypotheses:

1-Plasma level of VEGF increase in HCC patients but not in cirrhosis.

2-the VEGF level decrease after transplantation and it can serve as predictor for HCC recurrence.

The obtained results of age and BMI of all groups shows no significance differences between all studied groups. Serum levels of aminotransferases in HCC patients were high (AST; 140.5 IU/L, ALT; 164.3 IU/L) before liver transplantation. These high levels decreased significantly after transplantation (25.7 IU/L for AST and 29.7 IU/L for ALT). In cirrhotic group aminotransferases were around normal values (26.9IU/L and 23.85IU/L for AST and ALT respectively) and this is explained as the patients in this group are end stage liver cirrhosis with ascites. Serum albumin decreased in HCC and cirrhotic compared with other groups serum bilirubin increased in HCC and cirrhotic compared with other groups. As regard the obtained results of serum AFP, a significant increase was detected in localized HCC group (686.65ng/mL) as compared with cirrhotic group (71.25ng/mL) and healthy control groups (12.9ng/mL) (P< 0.0001). Also, a significant increase was detected in recurrent HCC group after twelve months as compared with non-recurrent HCC, cirrhosis, and healthy control groups (P< 0.0001). Also, a significant decrease in AFP level was detected in HCC patients after (20.83ng/mL) transplantation as compared with HCC patients before transplantation (686.65ng/mL) (P< 0.0001) No other significant differences were detected between the studied groups. As regard the obtained results of plasma level of VEGF, a significant increase was detected in localized HCC group (657.1 pg./mL) as compared with cirrhosis group (252.9 pg./mL) and healthy control groups (239.1 pg./mL) (P< 0.0001). Also, a significance was detected in VEGF levels in recurrent HCC group after twelve months (689 pg./mL) as compared with non-recurrent HCC (242.86 pg./mL), cirrhosis (252.9 pg./mL), and healthy control groups (239.1 pg./mL) (P< 0.0001). also, a significant decrease in VEGF level was detected in HCC patients after transplantation (263.28 pg./mL) as compared with HCC patients before transplantation (657.1 pg./mL) (P< 0.0001) No other

significant differences were detected between the studied groups. About hypothesis (1) we stated that the plasma level of VEGF is highly sensitive to HCC development Jinno et al., (1998) [15] found a significant difference in VEGF level between the HCC and other patient groups, but not among hepatitis patients, cirrhosis patients and normal controls. The plasma VEGF level is an effective marker for the determination of HCC metastasis because its specificity, sensitivity and precision satisfy clinical requirements. Li et al., (2004) [16] found that, plasma VEGF was markedly elevated in the majority of patients with HCC and the increase was closely related to a more advanced stage of diseases. Also, the VEGF levels in HCC patients overlapped considerably with those in normal controls. Schmitt et al., (2004) [17] reported that, expression of VEGF in human HCC correlates with the proliferative activity and the neo-angiogenesis of the tumor. Li et al. (1999) [18] and Yao et al., (2005) [19] concluded that, the high expression of VEGF is a useful predictor for vascular invasion and metastasis of HCC. Thelen et al., (2008) [20] concluded that, there is an important role for VEGF-D which is subtype of VEGF in HCC progression. Zhang et al., (2012) [21] reported that, VEGF is likely to promote HCC migration invasion and adhesion. Zhao et al., (2013) [22] reported that, the level of VEGF in patients without metastasis was significantly lower than that in patients with metastasis. Also, no difference was observed between the benign hepatic diseases group and the controls and between benign hepatic disease patients and cirrhotic patients. Zhan et al., (2013) [23] concluded that, serum high VEGF level was associated with poor overall survival and disease-free survival. Guo et al., (2016) [24] reported that, the patients with positive VEGF expression had poorer prognosis compared to those with negative VEGF expression. As a result, the positive expression VEGF implied poor prognosis.

About hypothesis (2) we stated that assaying the plasma level of VEGF will be of great value in predicting the recurrence of HCC as the more VEGF levels after transplant the more probability of recurrence Poon et al., (2001) [25] found a significant correlation between the serum level of VEGF and tumor stage, postoperative recurrence of HCC, absence of tumor capsule, presence of intrahepatic metastasis and microscopic venous invasion. chen et al., (2014) [26] who use percutaneous microwave coagulation therapy (PMCT) instead of liver transplantation in treatment of HCC found that monitoring the serum VEGF can provide some evidences for judging the efficacy of PMCT. We give support to Engels et al., (2015) [27] who stated that his study does not support a role for VEGF in causing cancer among transplant recipients. Wu et al., (2017)

[28] indicated that overexpression of VEGFA which is subtype of VEGF are potential risk factors that may induce tumor angiogenesis and recurrence.

From the consideration of our results, we suggest a possible role for serum VEGF as an indicator of the development of HCC in patients with liver cirrhosis during follow-up and the possibility to use it as an indicator to reflect the disease's recurrence after liver transplantation.

Interestingly Regarding the correlation matrix, a positive correlation between VEGF and AFP in all studied individuals (r=0.93, P< 0.0001) and patients twelve months after liver transplantation group (r=0.988, P< 0.0001) were obtained, whereas non-significant correlations were detected in patients six months after liver transplantation.

With respect to the non-significant correlation between VEGF and AFP that were detected in patients six months after liver transplantation, it was helpful to prove that plasma level of VEGF is of great value in predicting HCC recurrence after treatment (Liver transplantation in our study).

Conclusion

In conclusion, detection of serum VEGF and AFP has different significances; VEGF could be used as an indicator of the development of HCC in patients with liver cirrhosis during follow-up, to reflect the disease's potential activity of vascular invasion and metastasis and predict HCC recurrence after treatment. Whereas, AFP is suggested to be used as a supplementary marker which may help early diagnosis of HCC, but not to detect circulating HCC cells. Therefore, combination of multiple markers may be more valuable in the diagnosis and prognosis of HCC.

Recommendation

Although, some of our data were statistically significant, we acknowledge that the findings presented here are preliminary because of the small number of subjects and that the study requires confirmation in a separate larger cohort.

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