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Serum Alfa Fetoprotein versusNitric Oxide after Direct - Acting Antiviral Therapy to Predict Incident Hepatocellular Carcinoma in Chronic Hepatitis C Patients

OSAMA MOHAMMED BASHA[1], HANY MOHAMED EL-SADEK[2],
HANAA HOSNY EL-SAID[3], MOHAMMAD MOSTAFA SHOKRY KAMEL[4]

Abstract

Background: *In the last decade, a great success was achieved in eradication of hepatitis C virus (HCV) infection owing to treatment with direct-acting antiviral agents (DAAs). However, an increased incidenceof hepatocellular carcinoma (HCC) in treated patients was also noticed. In this study, a suggested link between altered serum nitric oxide (sNO) level after DAAs therapy and new development of HCC was investigatedas compared to serum alfa fetoprotein (AFP).* ***Methods****: We prospectively followed 180treatment naïve chronic hepatitis C (CHC) patients while receiving 12 weeks course ofDAAs therapy (sofosbuvir/daclatasvir±ribavirin)and for two years thereafter.In addition to routine laboratory evaluation, serum AFP and sNO levels were assessed before and after treatment. At the end of the study, all participants were classified into HCC group (who developed new HCC during study period) and non-HCC group.****Results****: Throughout the study, incident HCC was diagnosed in 7.8% of all participants (14/180). HCC developed only in cirrhotic patients, being more with advanced than early cirrhosis (P<0.001).As compared to baseline levels, serum AFP and sNOrise significantly after DAAs therapy (P=0.005 and P<0.001, respectively).Post treatment levels of AFP and sNO were significantly higher in HCC group than non-HCC group (P<0.001 for both).The only independent predictor of incident HCC after DAAs therapy was elevated sNO (P<0.001).Using receiver operating characteristic statistics, the sensitivity and specificity of post-DAAs sNO (100% and 99.4%, respectively), at a cut-off level ≥ 290 μmol/L, for HCC prediction (area under the curve=0.998, P<0.001), were much higher than that of AFP.* ***Conclusion****:Marked increase ofsNO level in CHC patients after DAAs therapy significantly predictsnew HCC development, which is predisposed by underlying cirrhosis.*

Keywords: *direct-acting antiviral agents (DAAs), hepatocellular carcinoma (HCC), serum nitric oxide (sNO).*

[1] *Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt*
[2] *Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt*
[3] *Clinical Pathology Department, Faculty of Medicine, Zagazig University, Egypt*
[4] *Internal Medicine Department, Health insurance hospital, El-Mahalla El-Kobra, Egypt*

I. Introduction:

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseaseand hepatocellular carcinoma (HCC). It affects nearly 170 million people globally [1]. Thanks to treatment with oral direct-acting antiviral agents (DAAs) that led to a great success towards HCV infection eradication worldwide and in Egypt [2].

A national HCV treatment program was developed in Egypt in the last years aiming to eliminate HCV infection [3]. The commonest regimen widely used in this national program was the combination therapy sofosbuvir (SOF), daclatasvir (DCV) ± ribavirin (RBV), as it is effective against all viral genotypes, inexpensive, and is allowed even if cirrhosis is present [4]. SOF is a nucleotide nonstructural protein 5B (NS5B) polymerase inhibitor that has a good tolerability and a high barrier to resistance. DCV is a nonstructural protein 5A (NS5A) replication complex inhibitor which has a good tolerability and limited drug-drug interactions [5].

HCC is the most feared complication of long-term HCV infection. Globally, more than one quarter of HCC cases are related to HCV infection [6]. There is a great debate between proofing or denying the role of DAAs in HCC development.It seems that following DAAs therapy in chronic hepatitis C (CHC) patients, it is crucial to use reliable markers that could predict HCC development [7].

Nitric oxide (NO) is a small free radical lipophilic gas that is implicated in numerous functional and patho-physiologic pathways in different organs including the liver. It contributes to blood pressure control, neurotransmission, regulation of tumoricidal and bactericidal actions of macrophages, and hepatic regeneration [8]. The biogenesis of NO is catalyzed by nitric oxide synthases (NOS), among them the inducible type (iNOS) is an important pathologic mediator. In the setting of HCV infection, NO and iNOS provoke cell injury,genetic mutations, DNA destruction and carcinogenesis which may lead to HCC [9-10].The aim of this study was evaluating the predictive value of serum AFP and sNO in HCC development after receiving DAAs therapy in CHC patients.

II. Methods:

This prospective study was conducted on 180treatment naïve chronic hepatitis C (CHC) patients (with or without cirrhosis) atInternal Medicine Department, Zagazig University Hospitals, during the period from March 2018 till September 2020. Each participant received 12 weeks course of DAAs therapy, in the form of SOF 400 mg daily and DCV 60 mg daily with or without ribavirin 1200 mg daily. Exclusion criteria included pregnancy, extremes of age (<18 years old or >60 years), previously diagnosed HCC or any detectable hepatic focal lesionand end stage renal disease. Patients who failed to achieve sustained virologic response at end of therapy (after 12 weeks) or relapsed during study period, advanced cirrhotic patients with Child-Pugh class (CPC) C [11],and cardiac patients who receive nitrates were also excluded.

All studied patients underwent baseline clinical examination, abdominal ultrasonography, fibroscan as well as laboratory investigations including complete blood count (CBC), liver function tests, kidney function tests, coagulation profile,HCV polymerase chain reaction (PCR), serum AFP level and sNO. At the end of

DAAs treatment course, serum AFP and sNO were reevaluated. AFP level was measured on Cobas E411 immunoassay analyzer, while measurement of sNO was performed by Enzyme-linked Immunosorbent Assay (ELISA) kit provided by Bioassay Technology LaboratoryCompany.

All participants were followed for two years after end of treatment for possible new development of HCC which was diagnosed according to characteristic radiologic features on quarter-yearly triphasic computerized tomography (TCT). HCV PCR was repeated at treatment end and six-monthly thereafter. Liver cirrhosis was determined by clinical, laboratory and radiological methods including fibroscan (F4 reading). Severity of liver disease was graded by CPC system [11].

Statistical Analysis

The quantitative variables were expressed as means ± standard deviation (SD) and the categorical variables as count numbers and proportions. Statistical analysis was performed with SPSS package version 25 (SPSS Inc., Chicago, IL) using the suitable test e.g. Chi- square ($\chi 2$) test,independent student (t) test, Mann-Whitney U (MW) test, paired (t) test and logistic regression analysis. The result was considered statistically significant if $P \leq 0.05$.To test the predictive accuracy (sensitivity and specificity) of different markers in HCC prediction, receiver operating curve (ROC) statistics was used.

III. Results:

Mean age of study population (n=180) was 50.3±7.7 years old, 101 patients (56.1%) were males and 75 patients (41.7%) had cirrhosis. During the two years follow up period of the study, incident HCC was diagnosed in 14 patients (7.8% of all participants), and they were referred to a multidisciplinary team consultation and further management accordingly. HCC developed only in cirrhotic patients, being more in those with advanced cirrhosis (CPC B) than early cirrhosis (CPC A) (P<0.001). Apart from the significant differences in liver functions between HCC and non-HCC patients' groups, there were no significant differences regarding, age, sex, BMI, comorbidities, CBC, or other routine biochemical tests(table 1).

As compared to baseline levels, serum AFP and sNO rise significantly after DAAs therapy (11.1±7.4 versus 13.0±10.4 ng/mL, P=0.005, and 17.1±9.3 versus 73.7±207.2 µmol/L, P<0.001; respectively). Post treatment levels of AFP and sNO were significantly higher in HCC group than non-HCC group (23.0±10.5 versus 12.1±10.0 ng/mL, P<0.001, and 721.4±235.8 versus 16.8±52.0 µmol/L, P<0.001; respectively)(table 1).

Logisticregression analysis model was used to find out significant independent predictors of incident HCC after DAAs therapy.The only independent predictor of HCC was elevated sNO (P<0.001) (table 2).

Using receiver operating characteristic statistics, the sensitivity and specificity of post-DAAs treatment level of sNO (100% and 99.4%, respectively), at a cut-off level ≥ 290 µmol/L, for HCC prediction (area under the curve [AUC] =0.998, P<0.001), were much higher than that of post-DAAs treatment serum level of AFP

(71.4%% and 75.3%, respectively), at a cut-off level ≥ 20 ng/mL, for HCC prediction (AUC =0.786, P<0.001) (table 3 & figure 1).

Table 1: Clinical and laboratory differences between HCC group and non-HCC group.

	All participants (n=180)	**HCC group (n=14)**	**Non-HCC group (n=166)**	**P**
Age (mean±SD, years)	50.3±7.7	52.1±6.2	50.2±7.8	0.355
Male patients (No, %)	101 (56.1%)	9 (64.3%)	92 (55.4%)	0.521
BMI (mean±SD)	25.3±4.6	26.9±4.6	25.2±4.2	0.202
Diabetic (No, %)	31 (17.2%)	2 (14.3%)	29 (17.5%)	0.762
Hypertensive (No, %)	22 (12.2%)	1 (7.1%)	21 (12.7%)	0.546
Hemoglobin (mean±SD, g/dL)	11.9±1.3	11.6±0.7	12.0±1.4	0.297
Platelets count (mean±SD, $x10^3/mm^3$)	177.8±54.1	152.4±36.9	180.0±54.9	0.067
WBCs (mean±SD, $x10^3/mm^3$)	6.7±2.7	6.4±2.9	6.7±2.6	0.656
Serum creatinine(mean±SD, mg/dL)	1.0±0.6	0.98±0.17	1.04±0.6	0.702
INR (mean±SD)	1.1±0.18	1.2±0.12	1.1±0.19	0.086
Serum albumin (mean±SD, g/dL)	3.6±0.5	3.3±0.3	3.6±0.5	**0.011**
Serum total bilirubin (mean±SD, mg/dL)	1.2±0.2	1.3±0.2	1.2±0.2	**0.042**
ALT (mean±SD, IU/L)	33.1±23.0	28.9±23.1	33.5±23.0	0.473
AST (mean±SD, IU/L)	56.1±38.5	49.6±44.1	56.7±38.1	0.507
HCV RNA (mean±SD,	1189075± 1076285	1308610± 887860	1178994± 1092361	0.666

copy/mL)							
Severity of liver disease (No, %)							
Chronic hepatitis without cirrhosis	105 (58.3%)		0 (0.0%)		105 (58.3%)		**<0.001**
CPC A of cirrhosis	54 (30%)		6 (42.9%)		48 (28.9%)		
CPC B of cirrhosis	21 (11.7%)		8 (57.1%)		13 (7.8%)		
AFP-pre (mean±SD, ng/mL)	P **0.005**	11.1±7.4	P **0.003**	13.9±7.1	P 0.059	10.8±7.4	0.143
AFP-post (mean±SD, ng/mL)		13.0±10.4		23.0±10.5		12.1±10.0	**<0.001**
sNO-pre (mean±SD, µmol/L)	P **<0.001**	17.1±9.3	P **<0.001**	21.6±8.5	P 0.973	16.7±9.3	0.058
sNO-post (mean±SD, µmol/L)		73.7±207.2		721.4±235.8		16.8±52.0	**<0.001**

SD: standard deviation, *P*< 0.05 is significant (in Bold). HCC: hepatocellular carcinoma, BMI: body mass index, ALT: alanine aminotransferase, AST: aspartate aminotransferase, INR: international normalized ratio, IU: international unit, CPC: Child Pugh class, HCV: Hepatitis C virus, RNA: ribonucleic acid. AFP-pre: Pre-DAAs therapy alpha fetoprotein, AFP-post: Post-DAAs therapy alpha fetoprotein, s.NO-pre: Pre-DAAs therapy serum nitric oxide, s.NO-post: Post-DAAs therapy serum nitric oxide.

Table 2: Logistic regression analysis of HCC predictors.

Predictor	**Unstandardized Coefficients**		**Standardized Coefficients**	***P***	**95% CI of B**	
	B	**Std. Error**	**Beta**		**Lower bound**	**Upper bound**
Serum albumin	0.000	0.014	-0.001	0.976	-0.028	0.027
Serum total bilirubin	-0.067	0.037	-0.052	0.075	-0.140	0.007
AFP-post	-0.001	0.001	-0.055	0.053	-0.003	0.000

sNO-post	0.001	0.000	0.980	**<0.001**	0.001	0.001

CI: confidence interval, P < 0.05 is significant (in Bold). AFP-post: Post-DAAs therapy alpha fetoprotein, s.NO-post: Post-DAAs therapy serum nitric oxide.

Table 3: Validity of Post-DAAs therapy levels of sNo& AFP in prediction of HCC, using ROC statistics.

Serum markers	Cutoff values	Sensitivity	Specificity	AUC	(95% CI)	*P*
AFP-post	20 ng/mL	71.4%	75.3%	0.786	0.679 – 0.893	**<0.001**
sNO-post	290 µmol/L	100%	99.4%	0.998	0.995 – 1.000	**<0.001**

AUC: area under the curve, CI: confidence interval, P < 0.05 is significant (in Bold), ROC: receiver operating characteristic. AFP-post: Post-DAAs therapy alpha fetoprotein, s.NO-post: Post-DAAs therapy serum nitric oxide.

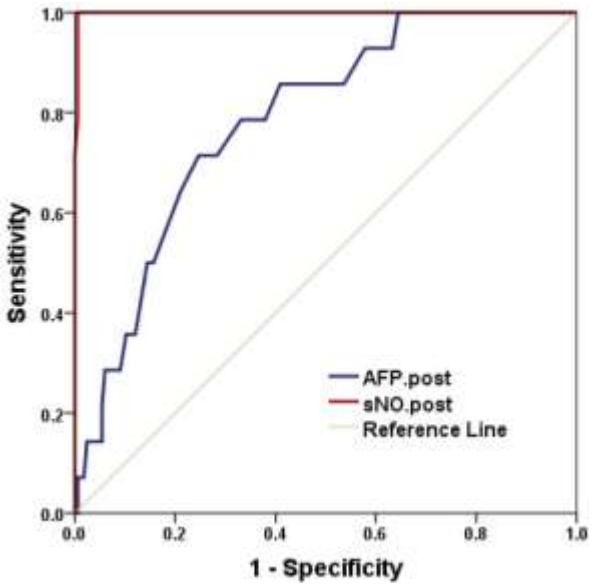

Figure 2: ROC (receiver operating characteristic) curve showing validity of Post-DAAs therapy levels of sNO& AFP in prediction of HCC among the studied cases.

IV. Discussion:

The commonest cause of HCC in Egypt is CHC, with a prevalence exceeding 20% among untreated CHC cases [12]. This two-years follow up study was conducted on 180 CHC patients to comparethe valuesof serum AFP and sNO to predict new development of HCC after receiving DAAs therapy.During follow up in the current study, 14 patients (7.8%) developed HCC after DAAs therapy who all had underlying cirrhosis; 6 out of 54 cases of CPC A cirrhosis and 8 out of 21 cases of CPC B cirrhosis (P<0.001).

In contrast to our results, several published data showed considerably lower incidence of incident HCC after DAAs therapy ranging from 0.5 to 3.5% [7,13-16].The higher incidence of HCC in our study might be due to the large proportion of cases with underlying cirrhosis (41.7%) and the relatively long follow up duration of two years. Whereas, in concordance to our study, the presence of baseline cirrhosis was also a significant risk factor for development of HCC in the study of Romano et al. [17] who studied 3917 CHC patients receiving DAAs therapy and found that incident HCC rate was 3.61% in cirrhotic patients, while it was 0.46% in non-cirrhotic patients.

It is very important to find reliable markers that could predict new HCC development following DAAs therapy in CHC patients. In this study, there was a statistically significant difference in serum AFP levels before and after therapy (P=0.005). Regarding post-DAAs therapy serum AFP, there was a statistically significant highermean level among patients with HCC than those without (P<0.001).

The above results were correlated with findings byGambarian et al. [18] who informed that serum AFP wassatisfactory for detection of HCC in cirrhotic patients. Likewise, Nguyen et al.[19] determined that serum AFP level >200 ng/ml was diagnostic for HCC in patients with HCV cirrhosis and hepatic focal lesions. However, with the accumulated evidence nowadays of the presence of AFP-negative cases of HCC in addition to its low sensitivity and specificity, AFP becameno longer acceptable for HCC diagnosis and prognosis [20-21].

Hence, there is still a need for a new biomarker that should have fair accuracy for prediction and diagnosis of HCC, especially in the setting of HCV induced liver cirrhosis. Based on the previously recognized role of NO in hepatic carcinogenesis [8-10], we postulated a fair value of sNO in that regard.

Ourstudy showed a significant rise of sNOafter DAAs treatment as compared to pretreatment level (17.1±9.3versus 73.7±207.2 µmol/L, P<0.001). More interestingly, the post-DAAs therapy mean sNO level among patients with HCC (n=14) showed marked elevation that was significantly too much higher than that among non-HCC patients (n=166) (721.4±235.8 versus 16.8±52.0 µmol/L, P<0.001). By logistic regression analysis, sNO was the only independent predictor of HCC in our study, (P<0.001). Moreover, the HCC predictive accuracy of post-DAAs treatment level of sNO at a cut-off level ≥ 290 µmol/L (sensitivity 100% and specificity 99.4%, AUC=0.998, P<0.001) was much better than that of post-DAAs treatment serum level of AFP at a cut-off level ≥ 20 ng/mL (sensitivity 71.4% and specificity 75.3%,AUC =0.786, P<0.001).

In agreement to our results, Moussa et al. [22] displayed higher levels of plasma nitrates (comprising NO) in CHC patients who had HCC than those who had not. More recently, Zhang et al. [23] reported that sNO level increases in HCC patients, and attributed that rise to over-expressionof iNOS in hepatoma tissue. On the other hand, Zhou et al. [24] assessed tissue levels of NO,both in hepatoma tissueand in normal liver tissue, in patients with HCC, and stated that NO level was significantly higher in non-malignant tissue than in cancer tissue. This apparent discrepancy is attributed to sampling difference between serum and malignant tissue, and to the extremely short half-life of NO.

Among strengths of the current study wereitsprospective nature with two years of follow up and the exclusion of patients with DAAs treatment failure or relapse to minimize the impact of HCV virulence disparity on HCC development. Whereas,the limitations of this study involved its relatively small number of participants and the lack of liver tissue NO measurement.

V. Conclusion:

After DAAs therapy, sNO and serum AFP levels rise significantly in CHC patients, and markedincrease of sNO (but not AFP)significantly predicts new HCC development.Baseline liver cirrhosis is a significant risk factor for HCC development in these patients,being more frequent with more advanced disease.

Recommendations:

When DAAs therapy is planned in CHC patients with advanced cirrhosis, great cautions and close monitoring must be given for prompt detection and management of HCC. Further larger studies are needed to validate the significance of sNO in HCC prediction and diagnosis in CHC patients with or without DAAs therapy.

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