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RESEARCH ARTICLE

Study the Role of Cell Free DNA in the Diagnosis of Hepatocellular Carcinoma, an Egyptian Study

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Abstract

Background and objective: Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. Since the conventional tissue biopsy and AFP have limited value, a new promising diagnostic method" liquid biopsy" has emerged. Cell free DNA is one of the liquid biopsy corner stones along with circulating tumor cells. **Aim of the work:** The study aims to evaluate the role of cf-DNA in the prediction of HCC. **Subjects and methods:** Eighty newly diagnosed HCC cases and seventy seven apparently healthy individuals were recruited from the National Cancer Institute, Cairo University. Cf-DNA level is measured by Qubit fluorometer assay and AFP was measured by ELISA for control. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallisand Mann-Whitney. Correlation between quantitative variables were done using Spearman correlation coefficient and a ROC curve was constructed with area under curve analysis performed to detect best cut off value of cf-DNA and AFP for detection of HCC. **Results:** The median cf-DNA and AFP levels were statistically significant higher in HCC patients (0,11ng/µl and 160.9 ng/ml respectively) than in control group (0.04 ng/ µl and 1.30 ng/ml respectively). Upon plotting ROC curve, cf-DNA and AFP gave a sensitivity of 78% and 93.7% respectively, a specificity of 59.7% and 92.2% respectively. The diagnostic value of cf-DNA in combination with AFP level has slightly improved the specificity (96.1%) on the expense of the sensitivity which was decreased (69.5%). **Conclusion:** Cf-DNA plays a role in the prediction of HCC but still AFP has the upper hand in the diagnosis of HCC in Egyptian population. Liquid biopsy still hasits own limitations. The techniques of colleting' liquid", and detection of cf-DNA must be standardized.

Keywords: HCC- cell free DNA- Liquid biopsy- AFP

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, more often in males than females (2.4:1) [1].

In the latest decade, a considerable increase was observed among Egyptian patients with chronic liver disease related HCC (from 4.0% to 7.2%), [2]. Hepatitis C and hepatitis B are the main causes of liver disease [3].

The diagnosis of liver cancer is generally performed by imaging techniques, such as ultrasonography, computed tomography and magnetic resonance tomography, in combination with the dosage of plasma alphafetoprotein (AFP) and the histological analysis of tissue biopsy [4].

AFP is not a precise marker since it provides low sensitivity and specificity and not sufficient for early diagnosis [5]. Also imaging and pathological examinations still have their limitations in diagnostic accuracy and sensitivity [6].

There are serious limitations with AFP, such as low sensitivity, false-negatives as in small HCC tumor with AFP under the detectable level and false-positives owing to conditions such as pregnancy and certain gastrointestinal tumors. In addition, the unequivocal diagnosis of a nodule detected using ultrasonography remains clinically challenging with unsatisfactory diagnostic accuracy (eg,

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~60%–80% sensitivity) [6].

A new diagnostic concept known as "liquid biopsy" has emerged with significant attention over the past years. A liquid biopsy collects the sample of body nonsolid biological tissue, such as blood for different analyses and other several body fluids. Circulating tumor cells (CTCs) and circulating tumor DNA (ct-DNA) are the cornerstones of liquid biopsy [7].

Liquid biopsy which is a simple noninvasive alternative to surgical biopsies and investigate molecular features of solid tumors through the body fluids especially blood could be a future alternative strategy. In cancer research, it has developed rapidly as a diagnostic and monitoring tool, which can be easily collected and analyzed in non-solid biological tissue. The term "liquid biopsy" include circulating tumor DNA (ct-DNA)/cell free DNA (cf-DNA), circulating tumor cells (CTCs), circulating miRNAs, and exosomes [8].

Liquid biopsy analysis of serum and plasma can reliably detect genetic and epigenetic alterations present in HCC tumor tissue, providing a less invasive alternative to the current gold standard of liver biopsy [9].

Circulating tumor DNA (ct-DNA) is a portion from the total circulating cell-free DNA (cf-DNA) in the bloodstream. Cf-DNA derived from apoptotic and necrotic cells but the majority of cf-DNA research is focused on DNA originating from cancer (ct-DNA) which is released from cancer cells by cell-death, secretion or other mechanisms still not clear [10].

Qualitative and quantitative analysis of cf-DNA as a diagnostic and prognostic parameter in cancer patients has been studied and showed a higher levels of cf-DNA among patients affected by HCC, cirrhosis and HCV-related chronic hepatitis compared to healthy subjects, and the increase was directly correlated to the disease status and reduced patients' survival [11].

The study aims at assessing plasma cf-DNA as a potential biomarker screening tool for HCC patients and comparing it with AFP as one of the currently established serum markers for diagnosis of hepatocellular carcinoma.

Materials and Methods

This is a cross sectional study conducted on a total number of 157 subjects to detect the role of cell free DNA in the prediction of HCC patients. Samples were collected from the medical oncology outpatient clinics at National Cancer Institute (NCI) hospital, Cairo University from March 2019 until November 2019.

Ethical approval was obtained from the Institutional Review Boards, and informed consent for the use of blood samples was given to all individuals.(Institutional Review Board Decision; Approval No: 201516014.2). The studied subjects were divided into two groups:

Group 1: It included 80 newly diagnosed liver cancer patients. Their ages ranged from 47 to 85 years with a mean age of 61.9 years.

Group 2: It included 77 apparently healthy volunteers as a normal control group recruited from blood bank donors, their ages ranged from 18 to 52 years with a mean age of 36.3 years.

Inclusion criteria

Patients newly diagnosed with liver cancers by clinical examination, imaging technique and serum AFP level.

Exclusion criteria

-Relapsed HCC patients

-Patients who received treatment of liver cancer as chemotherapy, radiotherapy, or immunotherapy or liver transplantation.

Serum samples from all patients and control subjects were subjected to the following laboratory investigations:

1- Tumor marker AFP was done using Axsym based on the microparticle enzyme immunoassay (MEIA) technology (Abbott USA).

2- Quantitation of cf-DNA was done for group 1 and group 2 on whole blood collected on EDTA ,the blood samples were centrifuged at 3000 rpm for 10 minutes, the supernatants was centrifuged for another 10 minutes. The plasma samples were stored at – 20 ºC until the cf-DNA extraction was performed.

Detection of cf-DNA level by quantitation

A. Extraction of DNA from peripheral blood:

DNA extraction was done using QIAamp DNA blood mini Kit (Qiagen, Hilden, Germany).

B. The measurement of cf-DNA level by quantitation:

The Qubit 2.0 Fluorometer was used for the quantitation of DNA, RNA, micro-RNA, and protein using the highly sensitive and accurate fluorescence-based Qubit quantitation assays [12].

For preparation of Qubit working solution, the Qubit buffer 199× n µl (n= number of standards plus number of samples) and the Qubit reagent 1×n were added. 190 µl from the Qubit working solution and 10 µl from standard 1were added to specific Qubit assay eppendorf (Final volume 200 µl). 190 µl from the Qubit working solution and 10 µl from standard 2 were added to specific Qubit assay eppendorf (Final volume 200 µl). 195 µl from the Qubit working solution and 5 µl from samples were added to specific Qubit assay eppendorf (Final volume 200 µl). Followed by vortexing all assay eppendorfs for 2-3 seconds then incubated them at room temperature for 2 minutes. Finally the prepared standards and samples were applied to read concentration of the extracted cf-DNA in Qubit 2.0 fluorometer.

C-Statistical Methods

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Data were summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical

data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests [13]. For comparing categorical data, Chi square test was performed. Exact test was used instead when the expected frequency is less than 5 [14]. Correlations between quantitative variables were done using Spearman correlation coefficient [15]. ROC curve was constructed with area under curve analysis performed to detect best cut off value of cf- DNA and AFP for detection of HCC. P-values less than 0.05 were considered as statistically significant.

Results

Patients' characteristics are mentioned in Table 1.

Table 2 shows the median level of AFP and cf-DNA in HCC patients and healthy controls where the median level of AFP was 1.30 ng/ml in control group vs.160.90 ng/ml in HCC group with p value <0.001 and the median level of CF-DNA was 0.04 ng/µl in control group vs.0.11 ng/µl in HCC group with p value <0.001, both were statistically significantly higher in HCC patients compared to the normal control group.

ROC curve was plotted showing a cut off value for AFP of 4.71 ng/ ml and cut off value for cf-DNA of 0.056ng/ul discriminating HCC group from normal control group (Figure 1).

The sensitivity and specificity of AFP were 93.7% and 92.2% compared to cf-DNA sensitivity and specificity were78.5% and 59.7% respectively Table 3.

The diagnostic value of cf-DNA in combination with AFP level were evaluated, the sensitivity, specificity, PPV, and NPV accuracy were 69.5%, 96.1%, 82.7%, 94.8%, and 75.5% respectively in Table 4.

A statistically significant difference in the cf-DNA median level in the HCC patients with different number of hepatic lesions (p value 0.048) (Table 5). The difference of cf-DNA level between single and two hepatic focal lesions with p value 0.026 (Table 6)

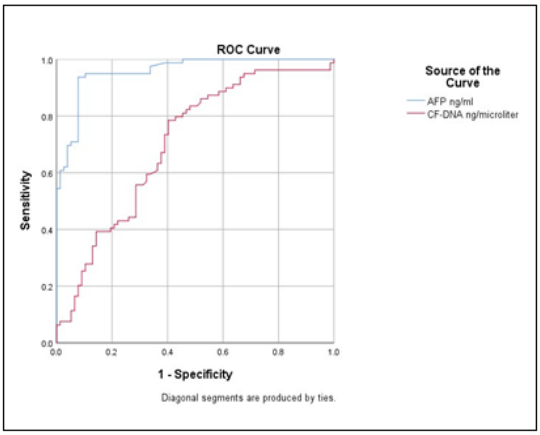

Figure 1. ROC Curve for HCC Diagnosis Using AFP and CF-DNA

The results of the present study showed no significant association observed between cf-DNA levels and any other clinical, biological and histological characteristics (age, gender, AFP levels, metastasis, HCV/HBV, clinical disorders).

No statistically significant difference in median level of cf-DNA was found in patients with and without lymph node metastasis, distal metastasis.

No statistically significant difference in median level of cf-DNA was found in patients with infection with HCV or HBV, receiving or not treatment of HCV.

There was no statistically significant difference in the cf-DNA median level in the HCC patients with size of lesions < and > 5 cm.

Discussion

The study is conducted on 157 subjects, 80 patients with primary hepatocellular carcinoma (group 1) and 77 apparently healthy individuals served a normal control group (group 2).

Table 1. Patients' characteristics

		Cases (80)	
		Count	%
Age (median)	63.18 (+or-8.63)		
Sex	Male	58	72.50
	female	22	27.50
Cirrhosis	Present	80	100.00
Lymph nodes Metastasis	Present	21	26.30
	Absent	59	73.80
Distal Metastasis	Present	6	7.50
	Absent	74	92.50
HCV /HBV	HCV	67	83.80
	HBV	2	2.50
	HCV and HBV	1	1.30
	Negative	10	12.50
Received treatment to HCV or Not	Received treatment	43	63.20
	Didn't receive treatment	25	36.80

Table 2. Median Level of AFP and cf-DNA in HCC Patients Versus Healthy Controls

	Controls			Cases			
	Median	1st quartile	3rd quartile	Median	1st quartile	3rd quartile	P value
AFP ng/ml	1.3	0.6	2.57	160.9	13.43	1834.75	< 0.001
CF-DNA ng/µl	0.04	0.03	0.14	0.11	0.06	0.28	< 0.001

Table 3. cf-DNA and AFP AUC, Sensitivity, Specificity, Accuracy, PPV and NPV

	Area Under the Curve	P value	95% Confidence Interval		Cut off	Sensitivity %	Specificity %
			Lower Bound	Upper Bound			
AFP ng/ml	0.957	< 0.001	0.929	0.986	4.71	93.7	92.2
cf-DNA ng/µl	0.702	< 0.001	0.619	0.784	0.056	78.5	59.7

Table 4. Diagnostic Value of cf-DNA Level in Combination with AFP Level for HCC Patients

	Cut off	Sensitivity %	Specificity %	Accuracy %	PPV %	NPV %
AFP ng/ml	4.71	93.7	92.2	92.99	92.59	93.42
cf-DNA ng/µl	0.056	78.5	59.7	69.43	67.02	73.02
cf-DNA + AFP level		69.5	96.1	82.7	94.8	75.5

The findings of the present work showed that 100% of the HCC patients have an established background of cirrhosis and 83.8% of them have chronic infection with HCV, 2.5% with HBV and 1.3% with both HCV and HBV which is similar to the results of a study performed in Egypt by Gomaa [16] who reported that about 99.07% of HCC patients had cirrhotic liver and the majority of cases (89.96%) were HCV positive while only 3.15% of patients were positive HBV results. Moreover, El-Sherbiny [17] reported that in a study at Egypt, 95.2% of patients had cirrhotic liver and the majority of cases 79.8% were HCV positive, 15.4% were HBV positive, and 3.5% were combined HCV&HBV positivity leaving only 1.1% negative for both HCV and HBV.

The findings of the present work showed that the median serum AFP levels were statistically significantly higher in HCC patients than in control group (160.9 ng/ml, 1.30 ng/ml, respectively with p<0.001). In a trial to find the diagnostic accuracy of AFP in HCC cases a ROC curve was plotted. It yielded an AUC of 0.957, 95% CI:0.929-0.986, at a cut off value for AFP of 4.71 ng/ml, a sensitivity 93.7%, specificity 92.2%, PPV 92.95%, NPV 93.42% and diagnostic accuracy of 92.99% were found.

However Abdelfattah [18] found that in a study of Egyptian patients, the best cut off of AFP for HCC diagnosis was 9.5 ng/ml with sensitivity, specificity, PPV, NPV of 75%, 62.5%, 74.4% and 64.4 respectively. A study performed by Hanno [19] in Egypt on 2 groups, group with HCV without HCC and group with HCV and early HCC, the study reported that best cut off point of AFP for HCC diagnosis was 6.0 ng/ml with sensitivity of 77.50%, specificity of 82.50%, PPV of 81.60%, NPV of 78.6%, and accuracy of 80%.

The median levels of cf- DNA in HCC patients were significantly higher than that of healthy controls (0.11 ng/µl, 0.04 ng/µl, with p <0.001, respectively) this was in concordance with Yan L [20] and Kunadirek [21]. However Huang [22] found that cf-DNA integrity decreased in HCC patients and has the potential as promising biomarker for HCC diagnosis and treatment surveillance.

In a trial to find a diagnostic accuracy of cf-DNA in the HCC cases a ROC curve was plotted, it yielded an AUC of 0.702, 95% CI: 0.619-0.784, the best cut off value for cf-DNA discriminating HCC group from control group was 0.056 ng/µl with sensitivity 78.5%, specificity 59.7 %, PPV 67.02%, NPV 73.02% and diagnostic accuracy of 69.43.

Iizuka [23] reported that cf-DNA assay had a sensitivity of 69.2% and a specificity of 93.3% in discriminating HCC and HCV carriers at the optimal cut-off value of 0.073 ng/µl, with an area of 0.90 (95% CI, 0.83-0.96) under the ROC curve. Huang [24] showed that plasma cf-DNA detection was able to discriminate HCC from normal controls at the cut off value of 0.0182 ng/µl with 90.2% sensitivity and 90.3% specificity and AUC was 0.949. Huang [22] found that AUCs for detecting HCC by cf-DNA integrity and AFP were 0.705 and 0.605 respectively.

In another study done by Piciocchi [11] reported that

Table 5. The Median Level of cf-DNA in HCC Patients with Different Number of Hepatic Lesions

		cf-DNA ng/µl				
		Count	Median	1st quartile	3rd quartile	P value
NO of lesions	Single	37	0.20[a]	0.06	0.48	0.048
	2 HFLs	12	0.07[b]	0.06	0.09	
	Multiple HFLs	31	0.11[ab]	0.06	0.2	

• Median level carrying different initials are statistically significant different at P <0.05

Table 6. The Difference of cf-DNA between Different Numbers of Hepatic Lesion

	P-value
Single lesion vs 2 HFLs	0.026
Single vs Multiple HFLs	0.08
2 HFLs vs Multiple HFLs	0.358

according to the ROC curve of cf-DNA in HCC diagnosis, at cut off value of 1 ng/µl sensitivity was 91%, specificity was 43% and AUC was 0.69 considering HCC versus chronic hepatitis and cirrhosis.

Although liquid biopsy is a promising tool, we identified several limitations in the literature. First, many studies did not include appropriate study populations. For early detection, the relevant comparison is between early stage HCC and patients at risk of HCC (ie, those with cirrhosis or selected patients with chronic hepatitis B). However, many diagnostic studies included CLD patients without cirrhosis or advanced fibrosis, or even healthy controls for comparisons. Furthermore, results for early stage HCC were not always reported separately. Finally, most studies have not separately examined the accuracy of liquid biopsy for detection of HCC owing to viral vs nonviral etiologies [25].

The techniques of collecting "liquid", and the isolation, detection of ctDNA must be standardized. Most of the current studies used different technologies or assays resulting in diverse sensitivity and specificity [26].

In the present study we evaluated the diagnostic value of cf-DNA in combination with AFP level in HCC diagnosis, which found that the specificity of combined detection was slightly improved (96.1%), while the sensitivity was decreased (69.5%).

Yan [20] evaluated the diagnostic power of the HCC index which is a combination model including age, cf-DNA, and AFP with 87.0% sensitivity and 100% specificity. The diagnostic power of the HCC index was superior to that of cf-DNA alone and AFP alone.

In the current study, the diagnostic value of cf-DNA in combination with AFP level in HCC diagnosis, found that the specificity of combined detection was improved (96.1%), while the sensitivity was decreased (69.5%).

While currently used biomarkers (AFP) are measured with unexpensive and simple methods, cfDNA mutational profiling and epigenetic analysis, and CTCs enrichment methods require devoted personnel and are all costly and time consuming. Nevertheless, such limitations will likely be overcome by advances in technology that will make these determinations easier and accessible to most laboratories [27].

The results of the present study showed no significant association observed between cf-DNA levels and any other clinical, biological and histological characteristics (age, gender, AFP levels, metastasis, HCV/HBV, clinical disorders),these findings were in concordance with [11].

The results of the present study showed a statistically significant difference in the cf-DNA median level in HCC patients with different number of hepatic lesions p value 0.048. The difference of cf-DNA level between single and two hepatic focal lesions with p- value 0.026.

In contrast to our results, a study in Italian population, in which HCV is the predominant etiological factor for HCC, Piciocchi [11] found that patients with multinodular disease had significantly higher cf-DNA levels. In particular, patients with >3 nodules, showed significantly elevated cf-DNA concentrations in comparison with those with 13 nodules with p = 0.05.

In conclusion, circulating tumor DNA (ct-DNA) is considered the cornerstones of liquid biopsy. Ct-DNA is a part from the total cf-DNA a double stranded and highly fragmented. Qualitative and quantitative analysis of cf-DNA in HCC diagnosis showed higher levels of cf-DNA in HCC patients compared to healthy subjects.

The diagnostic value of cf-DNA in combination with AFP level in HCC diagnosis, found that the specificity of combined detection was improved by (96.1%), while the sensitivity was decreased (69.5%).

Therefore, more researches are expected to take further exploration of more accurate biomarkers of ct-DNA or different combinations of ct-DNA with other effective markers to anticipate the diagnosis of disease recurrence or tumor progression in patients receiving either a systemic or a local treatment.

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