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## RESEARCH ARTICLE

### ASSESSMENT OF CELL FREE DNA LEVELS IN PATIENTS WITH HCV-ASSOCIATED HEPATOCELLULAR CARCINOMA.

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#### Abstract

**Background:-** HCC being a main complication of cirrhosis and representing more than 70% of all hepatic tumors among Egyptians with growing incidence. Quantitative analysis of cfDNA is a newly potential method for the detection of HCC.

**Objective:-** to evaluate the potential and clinical value of cfDNA in patients with HCV-associated HCC

**Methods:-** Blood samples were collected from 16 HCV associated HCC patients ,16 HCV related cirrhosis and 16 healthy controls. The AFP was measured and cfDNA was extracted and quantified by a real-time PCR, the survival of HCC group were followed up for two years.

**Results:-** The validity of cf DNA(ng/ml) as a diagnostic marker for HCC showed that the sensitivity and specificity of cfDNA for selective detection of the HCC over the cirrhotic liver disease were 100% and 75% respectively, while over the controls were 100% , 93.75% ,while that of AFP of the HCC over the cirrhotic group were 75% and 81.25% respectively .The patients with a high cfDNA level  $\geq 140$  ng/ml had significantly shorter OS than those with a low level of cfDNA.

**Conclusions:-** CfDNA, detected by real time PCR, is more sensitive and specific for detection of HCC than AFP and is more clinically significant.

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#### Introduction:-

Hepatocellular carcinoma (HCC) is one of the most common human cancers, being the fifth most prevalent tumor type and the third leading cause of cancer-related deaths worldwide (Shlomai et al., 2014).

Approximately 80% of hepatocellular carcinoma patients have been associated with liver cirrhosis, and even after comprehensive therapies with surgical excision, radiofrequency, or cryotherapy, this tumor shows a high percentage of recurrence and metastasis and the mean survival of the patients is still short compared to other major solid tumors, leading to death within 6 to 20 months (Scottenfeld et al., 2008).

Early diagnosis is crucial for potentially successful curative treatment, therefore, continuous HCC surveillance is recommended for high-risk patients, including those with cirrhosis of any etiology, chronic HCV patients with advanced liver disease and chronic HBV patients even without cirrhosis (Shlomai et al., 2014).

Currently, the most commonly used methods for screening and diagnosing HCC are ultrasound imaging and serum  $\alpha$ -fetoprotein (AFP) concentration measurements. AFP has been used worldwide as the golden

standard compared to other serum markers, especially in poor, remote areas. However, the diagnostic value of AFP is still controversial given that its sensitivity and specificity are unstable (Liu et al, 2013).

As a result, the clinical application of HCC diagnosis by AFP assessment is limited (Johnson et al., 2006).

Circulating cell-free DNA (cfDNA) is defined as extracellular DNA. Despite its identification over 60 years ago, research on cfDNA has lagged considerably due to the lack of sensitive quantitative methods (**Huang et al.,2012**).

The possibility of detecting and measuring tumor-derived cfDNA has opened a new method in predictive oncology, provides a non-invasive and easy-to-use tool for screening for malignancy and predicting cancer outcomes (**Tokuhsa et al,2007**).

Recently, advances on isolation methods of cfDNA and quantitative procedures using novel fluorescent dyes or quantitative PCR (qPCR) techniques have enabled progression in this research area. The potential diagnostic, prognostic, and monitoring significance of cfDNA has been clearly demonstrated in many malignancies (**Huang et al.,2012**).

## **Subjects and methods:-**

### **Study design and sample selection:**

This is a case control study carried out in Clinical Pathology and tropical medicine Departments, Zagazig University Hospitals. The study included 48 subjects classified into;

- 16 HCV associated hepatocellular carcinoma diagnosed by imaging studies (abdominal sonography, computed tomography or magnetic resonance imaging) and/or histopathological examination.
- 16 HCV related cirrhosis patients
- 16 matched healthy controls .

The three groups are matched in age and sex. Patients with Prior history of exposure to radiation, chemotherapy, or carcinogens, with other malignancy and Hepatitis B virus infection were excluded from the study. The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all participants.

All members of this study were subjected to the following Liver and kidney function tests, complete blood count, PT(prothrombin time) and INR(international normalization ratio),AFP and cfDNA by real-time PCR technique.

Follow up of survival of the patients of HCC group for 2 years were done.

### **cfDNA extraction:-**

DNA was purified from serum samples using the QIAamp® UltraSens virus® extraction kit (Qiagen)USA (**Pinzani et al,2010**) according to manufacture protocol. The extracted cfDNA was stored at -20°C till analysis.

### **Real time PCR amplification:-**

The cfDNA was amplified with quantitative Real-time PCR for human beta actin gene were performed on a Stratagene Mx3005P qPCR System (Agilent Technologies, Germany) using the qPCR SYBR® Green PCR Master Mix (Applied Biosystem by Life Technologies ,USA). PCR reaction with 20 µl final volume was prepared by adding 10 µl qPCR SYBR® Green PCR Master Mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 5 µl extracted cfDNA and 4 µl PCR grade water into real time PCR wells.

The cycling conditions were 95 ° C for 10 min, 40 cycles (95 ° C for 15 sec, 58 ° C for 1 min). For the melting "dissociation" curve one cycle was done started with 95 ° C for 1 minute then 55 ° C for 30 seconds followed by 95 ° C for 30 seconds.

### **Beta actin gene Primers:-**

**Forward primer** (5'AGCGAGCATCCCCCAAAGTT-3') ,

**Reverse primer**(5'-GGGCACGAGGGCTCATCATT-3' )

Obtained from the BLAST program (<http://www.ncbi.nlm.nih.gov>)

### **Interpretation of results:-**

Genomic DNA (gDNA) containing cloned target sequences were used as standard in quantitative PCR. A standard curve in which the cloned β-actin sequence present was prepared. For construction of the calibration curve for each amplification, we generated a standard curve using 5-fold serial dilutions of genomic DNA (16, 80, 400, 2000, 10000pg). The concentration of plasma DNA was calculated using the following equation (**Huang et al,2006**) :

$$C = Q \times (VDNA / VPCR) / Vext.$$

As  $C$  = target concentration in plasma (nanogram per millilitre),  $Q$  = target quantity (pg) determined by the qPCR,  $VDNA$  = total volume of extraction (60  $\mu$ L),  $VPCR$  = volume of DNA solution used for PCR reaction (5  $\mu$ L); and  $Vext$  = volume of plasma extracted (1000  $\mu$ L).

### Statistical analysis:-

The sample size was calculated with 80% statistical power and 95% confidence interval (CI). All data were collected, tabulated and statistically analyzed using SPSS 19.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba, Ostend, Belgium). ROC curve analysis was used to identify optimal cut-off values, sensitivity and specificity. Overall survival (OS) was determined by the Kaplan–Meier method and analyzed by log-rank test. The effect of clinicopathological factors on OS by means of the Cox proportional hazards model, hazard ratios (HRs) and 95% (CIs) were calculated. Statistical significance was considered at  $p < 0.05$ .

### Results:-

In the present study we compared the three groups as regarding laboratory findings and we founded that there was highly statistical significant difference in levels of hemoglobin, platelet count and INR. Regarding liver function tests there was highly statistical significant difference in levels of albumin, total and direct bilirubin, ALT, AST and ALP. Regarding kidney function tests there was highly statistical significant difference in levels of creatinine and BUN "data not shown".

We analyzed the level of AFP and we founded a highly statistical significance among the three studied groups ( $p < 0.001$ ) as shown in table (1), fig (1), comparison between each 2 groups showed significant difference increase in HCC group in comparison with cirrhotic group, between HCC and control groups and HCC and control groups. According to level of cfDNA levels comparison between the three groups revealed a highly statistical significant difference between the three groups ( $p < 0.001$ ) table (2). There was statistical significant increase between the HCC and cirrhotic groups, between the HCC and controls groups and between the HCC and cirrhotic groups fig (2).

**Table (1):** Alpha fetoprotein among the three studied groups

AFP (ng/ml)	HCC group* (N=16)	Cirrhotic group* (N=16)	Control group* (N=16)	Test•	p-value
Mean $\pm$ SD	6596.6 $\pm$ 14935	23.06 $\pm$ 15.7	3.24 $\pm$ 3.26	24.807	<0.001 (HS)
Median (Range)	1371.5 (0.9-60517)	18.5 (3 – 60)	2.15 (0.9 – 13)		

• Kraskall Wallis H test.

$p < 0.05$  is significant.

\*Comparison between each 2 groups with Mann Whitney U test showed significant increase between HCC and cirrhotic ( $p < 0.004$ ), between HCC and control groups ( $p < 0.001$ ) and HCC and control groups ( $p < 0.001$ ).

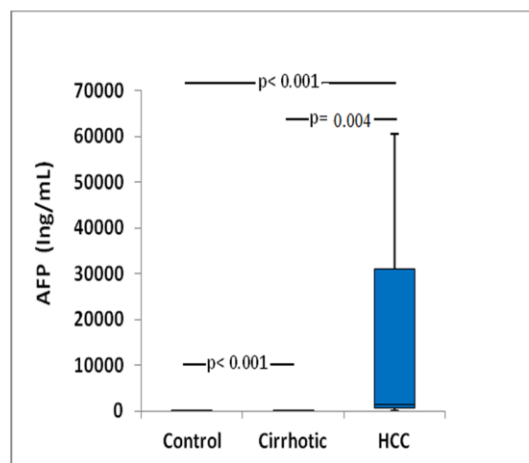
**Table (2):** Circulating free DNA among the three studied groups

CfDNA (ng/ml)	HCC group* (N=16)	Cirrhotic group* (N=16)	Control group* (N=16)	Test•	p-value
Mean $\pm$ SD	312.22 $\pm$ 348.28	46.97 $\pm$ 46.78	14.67 $\pm$ 14.08	32.030	<0.001 (HS)
Median (Range)	168.55 (48.93 – 1135.9)	33.4615 (9.40 – 199.84)	10.1250 (2.40 – 55.25)		

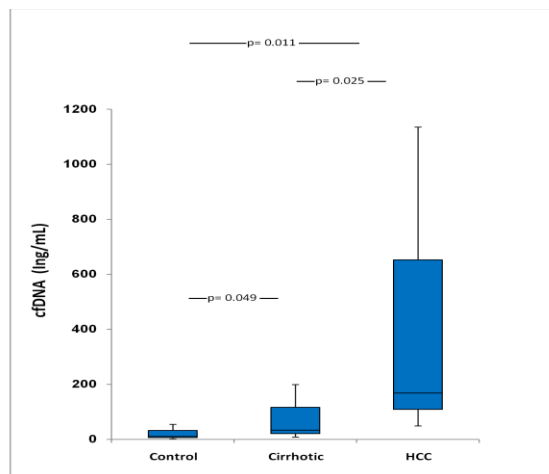
• Kraskall Wallis H test.

$p < 0.05$  is significant.

\*Comparison between each 2 groups with Mann Whitney U test showed significant increase between HCC, cirrhotic groups ( $p < 0.025$ ) and HCC, control groups ( $p < 0.011$ ) and between cirrhotic and control ( $p < 0.049$ ).



**Fig (1):** comparison of the three groups according to AFP level.

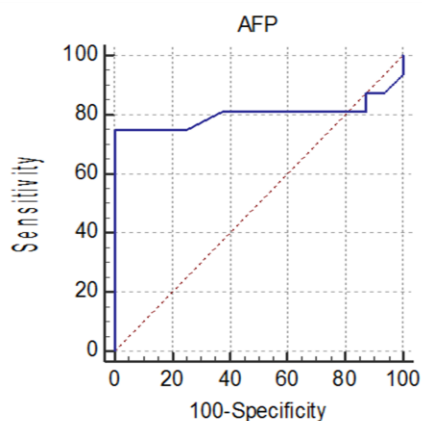


**Fig (2):** comparison of the three groups according to cfDNA level.

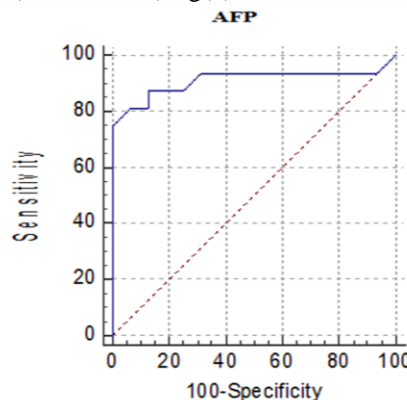
#### Diagnostic performance criteria of the tumor markers:-

The validity of alpha fetoprotein (ng/ml) as a diagnostic marker for HCC showed an area under the curve of 0.813 with 95% confidence interval (CI) (0.636 -0.928) and sensitivity, specificity, PPV, NPV, accuracy of AFP for selective detection of the HCC group over the cirrhotic liver disease group were 75%, 81.25%, 80%, 76.47% and 78.13% respectively, at a cut-off value of **13ng/ml**.fig(3)

The sensitivity, specificity, PPV, NPV, accuracy of AFP for selective detection of the HCC group over the control group were 87.5%, 87.5%, 87.5%, 87.5% and 87.5% respectively, at a cut-off value of **4.5 ng/ml** and area under the curve of 0.912 with 95% confidence interval (CI) (0.757 -0.983).fig(4)



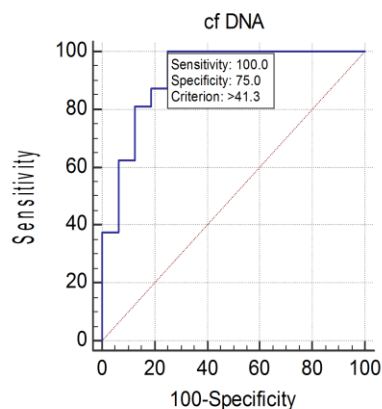
**Fig(3):**ROC curve of AFP between HCC and cirrhotic groups



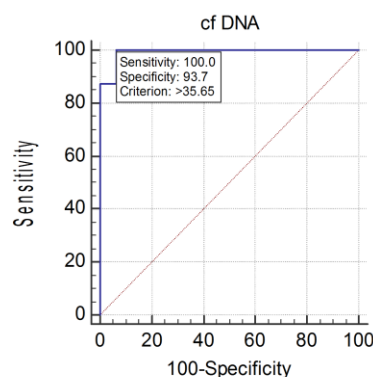
**Fig(4):**ROC curve of AFP between HCC and control groups

The validity of cfDNA (ng/ml) as a diagnostic marker for HCC showed that area under the curve of 0.918 with 95% confidence interval (CI) (0.765 -0.985), the sensitivity, specificity, PPV, NPV, accuracy of cfDNA for selective detection of the HCC group over the cirrhotic liver disease group were 100% and 75%, 80%, 100% and 87.5% respectively, at a cut-off value of **41.3ng/ml**.fig(5)

The sensitivity, specificity, PPV, NPV, accuracy of cfDNA for selective detection of the HCC group over the control group were 100%, 93.75%, 94.12%, 100% and 96.88% respectively, at a cut-off value of **35.65 ng/ml**, area under the curve of 0.992 with 95% confidence interval (CI) (0.689 -0.998) fig(6).



Fig(5):ROC curve of cfDNA between HCC and cirrhotic groups



Fig(6):ROC curve of cfDNA between HCC and control groups

The comparison of epidemiological , clinical and laboratory finding according to cfDNA level showed that the characteristics of patients with HCCs per cfDNA level [Cut off value of cfDNA was 140 ng/ml which equal to mean + 2 SD of the cirrhotic group , low <140 ng/ml and high  $\geq$ 140 ng/ml] revealed that there was no statistical significance between the two groups as regards the age , tumor size, AFP, child pugh score . There was a statistical significance difference between patients according to cfDNA levels as regards number of primary lesions (  $p=0.0063$ ) and vascular invasion as ( $p=0.009$ ).

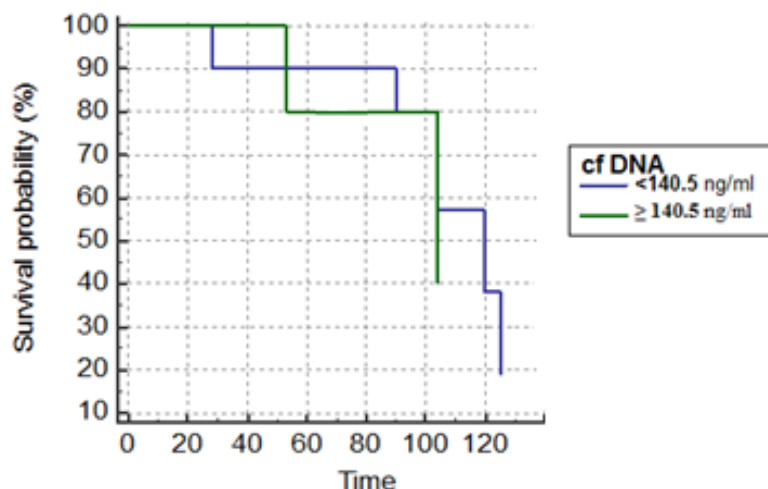
Table(3) shows the characteristics of patients with HCCs per cfDNA level " Cut off value of cfDNA was 140 ng/ml which equal to mean + 2 SD of the cirrhotic group" ( low <140 ng/ml and high  $\geq$ 140 ng/ml) revealed that there was no statistical significance between the two groups as regards the age , tumor size, AFP, child pugh score and there was a statistical significance difference between them as regards number of primary lesions (  $p=0.0063$ ) and vascular invasion as ( $p=0.009$ ).

**Table (3): Comparison of epidemiological , clinical and laboratory finding according to cfDNA level**

	cf DNA level		P-value
	Low <140ng/ml	High $\geq$ 140ng/ml	
<b>Age (year)</b> <b>&lt;60</b> <b><math>\geq</math>60</b>	4 3	3 6	$P = 0.3409$
<b>Tumour size</b> <b>&lt;5 cm</b> <b><math>\geq</math> 5cm</b>	0 7	3 6	$P = 0.0901$
<b>Number of primary lesion</b> <b>Single</b> <b>Multiple</b>	7 0	3 6	<b><math>P = 0.0063</math></b>
<b>Vascular invasion</b> <b>Absent</b> <b>Present</b>	7 0	6 3	<b><math>P = 0.009</math></b>
<b>AFP</b> <b>&lt;45.5 ng/ml</b> <b><math>\geq</math>45.5 ng/ml</b>	3 4	1 8	$P = 0.1457$
<b>Child pugh score</b> <b>A,b</b> <b>C</b>	2 5	1 8	$P = 0.3747$

According to survival analysis we studied the overall survival by follow up of patients for 2 years and overall survival was determined by the Kaplan–Meier method and analyzed by log-rank test .

The relation between cfDNA levels and overall survival in HCC patients revealed that patients with a high cfDNA level had significantly shorter OS than did those with a low level of cfDNA ( $P=0.012$  by log-rank test). Follow up of patients of HCC for 2 years revealed that the survival ratio was 37.5% (6/16).



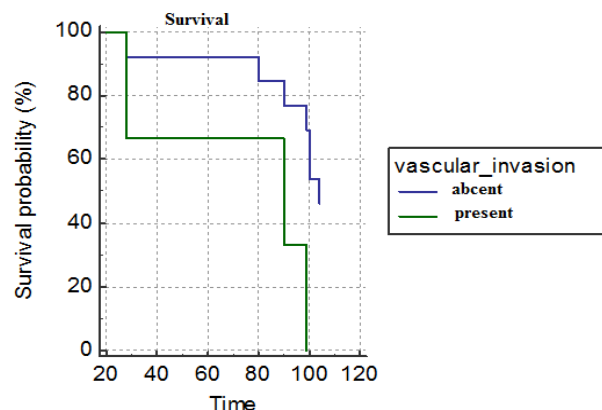
**Fig (7):**cfDNA levels and overall survival in (HCC) patients.

We studied the independent risk factors for over all survival as regarding level of cfDNA ,vascular invasion and tumor size and we revealed that the hazard ratio of cf DNA levels  $\geq 140.5$  ng/ml was 6.3 times more than cfDNA levels  $< 140.5$  ng/ml with  $p=0.0035$ . And the hazard ratio of patients with vascular invasion was 3.8 times more than those without vascular invasion with  $p=0.02$  and tumor size did not show any significance with OS and HR.table (4),fig(8)

**Table(4):Independent risk factors for OS:**

Variable	HR (CI:95%)	P value
Cf DNA		P = 0.0035
<140.5 ng/ml	1	
$\geq 140.5$ ng/ml	6.3556 (1.7795-22.6989)	
Vascular invasion		P = 0.0254
0: no	1	
1:present	3.8258 (0.4880 -29.9960)	
Tumor size		P = 0.3444
< 5 cm	1	
$\geq 5$ cm	0.5634 (0.1401 - 2.2645)	

cfDNA=cell-free DNA; CI=confidence interval; HCC=hepatocellular carcinoma; HR=hazard ratio; OS=overall survival. Cut off value of cfDNA was 140.5 ng/ml which equal to mean + 2 SD of the cirrhotic group.



**Fig (8):**cfDNA and the HR of patients with vascular invasion

### Discussion:-

In this study, we tried to evaluate the value of cfDNA as a diagnostic and prognostic marker in patients with HCC compared to AFP.

The obtained significant in AFP level in HCC patient than that of cirrhotic patients and controls goes in harmony with that reported by **Chen et al,2013** who reported that AFP in HCC was significantly higher than controls ( $p < 0.05$ ) in their study.

In our study, there was a significant increase in the level of cfDNA in HCC patients compared with cirrhotic group and with the controls, also in cirrhotic patients when compared to controls.

In agreement with our findings **Iizuka et al,2006** and **Tokuhiya et al,2007**. reported that cfDNA level was significantly higher in HCC patients than in HCV patients.

Also **Yan et al,2012** revealed that cfDNA level was in the HCC higher than the compensated liver cirrhosis and decompensated liver cirrhosis.

In agreement with our finding **Huang et al,2012**. founded that the HCC cfDNA were significantly higher than in the healthy controls and control benign patients. Also, **Chen et al.,2013** revealed that cfDNA level was in HCC patients significantly higher than in normal controls.

In the current study, the receiver operating characteristics (ROC) curve of AFP to differentiate HCC patients from cirrhotic patients revealed sensitivity and specificity 75% and 81.25% respectively, PPV 80%, NPV 76.47% and accuracy 78.13% at a cut-off value of 13ng/ml and the ROC curve of AFP to differentiate HCC patients from controls revealed sensitivity and specificity were 87.5% and 87.5% PPV 87.5%, NPV 87.5% and accuracy 87.5% respectively, at a cut-off value of 4.5 ng/ml.

At previously established cutoff value of 20 ng/ml (**Romeo et al,2010**), the sensitivity of AFP was 75%, the specificity was 90% for differentiating HCC from cirrhosis.

**Iizuka et al,2006** found AFP sensitivity of 69.2% and specificity of 72.7% at the optimal cut-off value of 10.20 ng/ml to differentiate HCC from HCV positive patients. A study by **Lok et al.,2010** demonstrated that at cut off 20 ng/ml, the sensitivity and specificity for AFP was 61% and 81% respectively for differentiating HCC from cirrhosis.

**Yagmur et al.,2007** reported that AFP at cutoff 7.7 ng/ml, has sensitivity 42.2% and specificity 84.2% to differentiate HCC patients from cirrhotic patients. Also, **Chen et al,2013** found the AFP in HCC has sensitivity of 53.8% and specificity of 91.9% for differentiating HCC from normal controls at cutoff 400 ng/ml.

**Farinati et al.,2006** who assessed in a large multicentric survey, the diagnostic reability of AFP in HCC patients who were analyzed for serum AFP. When using the ROC curve, AFP has limited sensitivity (54%) in diagnosis of HCC at the cutoff 20 ng/ml.



All these finding regarding the diagnostic performance of AFP in diagnosis of HCC revealed that wide variations in the cutoff among large number of studies referred to that AFP not sufficient alone as a marker for diagnosis of HCC due to its poor sensitivity and specificity and its limited role in diagnosis of HCC.

In that way, **Arrieta et al.,2007** demonstrated that progressive elevation of AFP>7 ng/ml/month in patients with liver cirrhosis was useful for diagnosis of HCC in patients that didn't reach AFP level >200 ng/ml. These cirrhotic patients may also present with higher AFP values than healthy individuals and with abnormal pattern of AFP release because of processes of hepatic necrosis and regeneration. They also considered AFP level <20 ng/ml to be normal, with this value as the upper level, this diagnostic cutoff had a sensitivity of 64% and a specificity of 89% to diagnose HCC.

In our study, the ROC curve of cf DNA to differentiate HCC patients from cirrhotic patients revealed that sensitivity 100%, specificity 75% , PPV 80% ,NPV 100% and accuracy 87.5% respectively, at a cut-off value of 41.3ng/ml and the ROC curve of cf DNA to differentiate HCC patients from controls revealed that sensitivity and specificity were 100% and 93.75% and % PPV 94.12% ,NPV 100% and accuracy 96.88% respectively, at a cut-off value of 35.65 ng/ml.

These finding were in agreement with **Iizuka et al.,2006** who founded the cf DNA with the cut-off value of 73.0 ng/ml, yielding a sensitivity of 69.2% and a specificity of 93.3% to differentiate HCC patients from HCV patients.

According to **Huang et al.,2012** in discriminating HCC from normal control the cutoff value of 18.2 ng/ml, showed sensitivity, specificity, PPV, and NPV were 90.3%, 90.2%, 94.2%, and 84.1%, respectively , for discriminating HCC from benign control at the cutoff of 143.0 ng/ml showed 59.7% sensitivity, 78.4% specificity, 82.7% PPV, and 52.3% NPV.

In addition ,**Chen et al.,2013** reported that cf DNA had 56.4% sensitivity, 95.6% specificity and accuracy 77.4% at cutoff 509.9ng/ml between HCC patients and normal controls.Also, **Piciocchi et al .,2013** defined a cutoff value of 1000 ng/mL with 91% sensitivity, 43% specificity ,**Chen et al . ,2012** reported a cut off 213.8 ng/mL with 86% sensitivity, 79% specificity between HCC patients and controls.

Dispute difference between various studies in value and/or cutoff of cfDNA but all agree with our data in that the level of cfDNA in HCC patients is higher than cirrhotic group which also higher than normal controls which can be explained by difference in sample size taken by each study ,difference in methodology used for cfDNA detection ,also difference in ethnic population of each study.

The levels of cfDNA reflected the effect of the progression of the cancer .CfDNA might be a better and more sensitive biomarker for HCC than the conventional tumor markers AFP in predicting recurrence and determining the necessity of further chemotherapy (**Ono et al,2015**) .

A meta analysis study performed by **Liao et al .,2015** included a total of 2424 subjects which included 1280 HCC patients in 22 studies were recruited in this meta-analysis. Pooled sensitivity and specificity and AUC of quantitative analysis of cfDNA were 74.1% , 85.1%, and 0.86 respectively. After combining quantitative analysis of cfDNA with AFP assay, the values were 81.8% , 96% and 0.96 respectively. HCC diagnostic sensitivity in the quantitative analysis subgroup and combined cfDNA and AFP analysis was superior to AFP assay alone(**Liao et al,2015**) .

The results in this meta-analysis suggested that circulating cfDNA have potential value for HCC diagnosis. After combining with AFP, the diagnostic performance of AFP will be improved (**Liao et al,2015**) .

In the present study the relation between cfDNA levels and overall survival (OS) in HCC patients revealed that patients with a high cfDNA level  $\geq 140$  ng/ml had significantly shorter OS than did those with a low level of cfDNA. Our results were in agreement with **Tokuhisa et al .,2007** who founded that patients with a high cfDNA level  $\geq 117.8$  ng/ml had significantly shorter OS than those with a low cfDNA level. And by contrast, cfDNA levels were not associated with disease free survival.



On the other hand **Huang et al.,2012.** reported that patients with low cf DNA concentration ( $\leq 173$  ng/mL) showed a trend of prolonged survival when compared with patients with high cf DNA levels ( $\geq 173$  ng/mL), but no statistical significant difference was found .

In our study the independent risk factors for overall survival (OS)revealed that the hazard ratio(HR) of cf DNA levels  $\geq 140.5$  ng/ml was 6.3folds more than cfDNA levels  $< 140.5$  ng/ml. And the HR of patients with vascular invasion was 3.8 more than those without vascular invasion.

**Tokuhisa et al.,2007** reported that according to the multivariate Cox proportional hazards model, cfDNA (HR, 3.4) and tumour size (HR, 3.8) as an independent prognostic factors for OS.

**Chen et al.,2013** attributed the value of serum cfDNA in the clinical diagnosis of HCC to that ,serum cfDNA level is positively correlated with the grading of differentiation and metastasis of HCC patients, suggesting that cfDNA may be useful in monitoring the pathogenic condition of HCC. Also, serum cfDNA level is relevant to the survival of HCC patients after hepatectomy, suggesting that cfDNA may be a useful marker for surgical or non-surgical treatment decision making.

### Conclusion:-

Our study concluded that cfDNA was higher in HCC patients than in cirrhotic patients and higher in cirrhotic patients than controls and it had a high sensitivity and specificity, and the clinical significance is higher than AFP in HCC detection ..

cfDNA was correlated with bad prognostic criteria of the tumor as regarding size, multiplicity of lesions and vascular invasion. Patients with higher cfDNA had shorter OS than those with low level.

The cf DNA concentration may be a new promising non-invasive biomarker for hepatocellular carcinoma diagnosis and to differentiate between HCC , cirrhotic and normal controls.

Our present findings support the utility of monitoring cell-free DNA levels in HCV-related HCC patients by quantitative real time PCR assay .

**Conflict of interest:-** The authors have no conflict of interest to declare.

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