

RESEARCH ARTICLE

Potential Diagnostic and Prognostic Value of Lymphocytic Mitochondrial DNA Deletion in Relation to Folic Acid Status in HCV-Related Hepatocellular Carcinoma

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Abstract

Objective: We assessed the possibility of using mitochondrial (mt) DNA deletion as a molecular biomarker for disease progression in HCV-related hepatocellular carcinoma (HCC) and to identify its association with folic acid status. **Methods:** Serum folic acid and lymphocytic mtDNA deletions were assessed in 90 patients; 50 with HCC, 20 with liver cirrhosis (LC), and 20 with chronic hepatitis C (CHC) compared to 10 healthy control subjects. The diagnostic accuracy of mtDNA deletions frequency was evaluated using receiver-operating characteristic (ROC) curve analysis. Survival analysis was performed using the Kaplan-Meier method. Differences in the survival rates were compared using log-rank test. **Result:** Our data revealed a significant elevation of mtDNA deletions frequency in the HCC group compared to the other groups (P -value <0.01). Also, our data showed a significant correlation between folate deficiency and high frequency of mtDNA deletions in patients with HCV-related HCC when compared to the other groups ($r = -0.094$ and P -value <0.05). Moreover, the size of the hepatic focal lesion in the HCC patients was positively correlated with mtDNA deletions ($r = 0.09$ and P -value <0.01). The median survival time for the HCC patients with high frequency of mtDNA deletions ($\Delta Ct \geq 3.9$; 5.7+ 0.6 months) was significantly shorter than those with low mtDNA deletions frequency ($\Delta Ct < 3.9$; 11.9+ 0.04 months, P -value <0.01). **Conclusion:** Our data provided an evidence that lymphocytic mtDNA deletion could be used as non-invasive biomarker for disease progression and patients' survival in HCV-related HCC. Also, our findings implied a causal relationship between the folate deficiency and the high mtDNA deletions frequency among Egyptian patients with HCV related HCC.

Keywords: HCC- Egyptian patients-mitochondrial DNA deletions- serum folic acid- diagnosis- prognosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer related mortality worldwide (Gomaa et al., 2008). Chronic hepatitis C virus (HCV) infection is one of the risk factors for hepatocellular carcinoma (HCC) development (Sukowati et al., 2016). The incidence of HCC shows a considerable geographical variability with a very high incidence in Egypt (Lehman and Wilson, 2009). In 2015, a published Egypt Health Issues Survey (EHIS) on a nationally representative sample showed that 10% of Egyptians who their ages were between 15 – 59 years had been infected with HCV, while 7% were chronic active hepatitis C patients (El-Zanaty et al., 2009). HCV-related HCC is induced via indirect pathways as a consequence of chronic inflammation and oxidative stress followed by fibrosis and cirrhosis and

HCC (Sukowati et al., 2016). The increased oxidative stress, due to HCV infection in the hepatocytes, results in DNA changes which include mitochondrial DNA (mtDNA) deletions (Schwarz, 1996).

The human mitochondrial DNA is a closed-circular duplex molecule, 16 kb in length (Shadel and Clayton, 1997). Since the mitochondria are involved in energy metabolism, apoptosis and generation of reactive oxygen species (ROS), any changes in mtDNA may result in an intense damage of important cell function (Augenlicht and Heerdt, 2001). mtDNA is highly susceptible to oxidative damage by ROS due to its close proximity to site of ROS production (Croteau and Bohr, 1997) and lacking of histone proteins (DiMauro and Schon, 2001) which protects nuclear DNA from oxidative damage. Besides, mitochondrial polymerases lack specificity for base excision repair which is considered the major

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pathway for eliminating oxidative DNA base lesions (Kang and Hamasaki, 2002). Until now, it is unclear how mitochondria maintain its genetic information integrity in the face of the permanent exposure to ROS. mtDNA sequencing showed that 30 to 70% of tumors have mtDNA mutations (Penta et al., 2001) that present in different cancers including thyroid (Máximo et al., 2002), gastric (Máximo et al., 2001) and liver cancer (Fukushima et al., 1995).

Choi and Mason (2000) have reported that folate status in the body influence DNA stability and thus it might be involved in carcinogenesis. The relation between blood folate levels and cancers has been previously reported; supporting the important role of folate in DNA synthesis, cell division, and cancer (Stevens et al., 2006). It was found that folate deficiency for 4 weeks increased the frequency of mtDNA deletions resulting in mtDNA dysfunction and ROS generation, all of which constitute plausible mechanisms for cancer development (Chou et al., 2007).

Since data regarding the deletion of mtDNA and folate status in HCV associated HCC patients from Egypt are inadequate, we aimed to study the role of mtDNA deletion in HCV-related HCC patients and the possibility of using mtDNA deletion as a prognostic biomarker in those patients and its relation to serum folic acid levels.

Materials and Methods

Study design and grouping

This retrospective case control study was conducted on 90 adult patients with HCV related chronic liver diseases categorized into: 50 patients with hepatocellular carcinoma (HCC); 20 patients with liver cirrhosis (LC) and 20 patients with chronic hepatitis C (CHC), in addition to 10 healthy subjects who enrolled as a control group during the period from April 2014 to May 2016.

The HCC patients were diagnosed by abdominal ultrasound with triphasic computed tomography (CT) as well as histopathological examination. They showed no evidence for local invasion and distant metastasis. Whereas, the CHC patients were diagnosed by persistently elevated liver enzymes for more than 6 months and imaging evidence of chronic liver disease with and without hepatic decompensation or portal hypertension. The LC group was diagnosed by abdominal ultrasonography and histologically through examination of hematoxylin and eosin stained slides for the biopsy. The control group included individuals with no evidence of liver disease or known medical illness at the time of sample collection. For all studied groups, a detailed history, complete clinical assessment, biochemical liver and kidney profiles, AFP, blood glucose level, hepatitis markers, HCV RNA by real-time PCR, abdominal ultrasonography and triphasic CT scan were performed. The exclusion criteria were diabetic controls as well as patients with HBV infection and patients with HCC, LC, and CHC who received previous treatment or antiviral therapy for HCV. Also, diabetic controls were excluded. As diabetes mellitus is being a risk factor for HCC development. The study was performed according to ethical guidelines of the

2004 Declaration of Helsinki and it was approved by the Institutional Review Boards (IRB) of the National Cancer Institute, Cairo University. A written informed consent was obtained from all participants prior to their enrollment in the study.

Measurement of serum level of folic acid and lymphocytic mtDNA deletions

Serum and whole blood samples were collected from subjects under investigation for measurement of serum folic acid and lymphocytic mtDNA deletions. Folic acid concentration was measured using an ELISA kit (DRG vitamin Folic Acid, Bio-4886 USA) according to manufacturer's instructions. The average of the duplicate readings for each standard, control, and sample was taken and the average zero standard optical density was subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The concentration of folic acid in the samples was determined by interpolation.

The lymphocytic DNA was extracted using Qiagen DNA Blood mini kit (QIAamp, Cat. no. 13323) according to the manufacturer's protocol. mt ND1 (rarely deleted gene) and mt ND4 (commonly absent in the majority of the patients) genes were quantified according to He et al., (2002) method using (forward primer L3485-3504; reverse primer H3532-3553; probe L3506-3529) for ND1 and (forward primer L12087-12109; reverse primer H12140-12170; probe L12111-12138) for ND4. The cycling profile was as follows; 2 min at 50°C and 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 30 s at 72°C. The fluorescence spectra were monitored on 7500 Applied Biosystem. The Ct values were used to quantify the relative amount of ND4 to ND1 with the following equation: $R = 2^{-\Delta Ct}$ where $\Delta Ct = mtCt_{ND4} - mtCt_{ND1}$. A smaller value of ΔCt represents a higher relative ratio of ND4:ND1, which in turn reflects fewer mtDNA deletions and vice versa (Wu et al., 2009). HCC patients were followed up over a period of one year dating from the initial presentation and the overall survival was calculated.

Statistical analysis

Data were statistically described in terms of mean \pm standard deviation (SD) for continuous variables and percentages for categorical variables. Comparison of continuous variables between the studied groups was performed using one way analysis of variance (ANOVA) test with post hoc multiple 2-group comparisons while Chi square (χ^2) test was used for comparing categorical variables. Fischer Exact test was used instead of Chi square (χ^2) test when the expected frequency was less than 5. P value less than 0.05 was considered statistically significant. Correlation between the variables was calculated using Spearman's Correlation Coefficient. All used statistical tests were two-sided. Receiver operating characteristic (ROC) curves were plotted in order to determine the best cut-off values of the studied markers. The analysis of overall survival was calculated by the Kaplan-Meier method. Differences in the survival

rates were compared using log-rank test. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15.

Results

The demographic features and the clinical data of the studied participants were illustrated in Table 1. Disease progression was related to older age as there was a significant increase in the age of the HCC group compared to the other groups with (P -value < 0.01). A male predominance was reported in HCV-related liver disease patients; estimated to be 78% of the HCC group, 70% of the LC group and 60% of the CHC group.

Comparison with the common risk factors showed that diabetes mellitus (DM) was reported in none of the controls, 25% of the CHC patients, 40% of the LC and 46% of the HCC groups (P -value < 0.001). Cigarette smokers were reported in 30% of the CHC, 55% of the LC and 40% of the HCC groups compared to 20% of the control group with a significant difference between the studied groups (P -value < 0.01). As for the Child scoring, 6 patients (30%) of the LC group were Child's

Table 1. Clinical Data of the Studied Groups

	Control n= 10	Non- cirrhotic n=20	Cirrhotic n=20	HCC n= 50	<i>P</i> value
Age: mean \pm SD	34 \pm 9.7a	42 \pm 9.9b	49.8 \pm 9.3c	54.9 \pm 8.7d	<0.001
Gender					
Male	8 (80%)a	12 (60%)a	14 (70%)a	39(78%)a	0.67
Female	2 (20%)a	8 (40%)a	6 (30%)a	11(22%)a	
Smoker	2(20%)a	6(30%)b	11(55%)b	20(40%)c	<0.01
D.M.	0a	5(25%)b	8(40%)c	23(46%)c	<0.001
Child score					
A			6(30%)a	6(12%)b	<0.05
B			8(40%)a	13(26%)b	
C			6(30%)a	31(62%)b	
ALT: mean \pm SD	20.1 \pm 7.6a	58.4 \pm 58.54b	47.1 \pm 42.4b	80.68 \pm 66.35c	<0.05
AST: mean \pm SD	21.5 \pm 4.7a	45.35 \pm 38.11b	68.35 \pm 52.2b	132 \pm 139c	<0.01
T-Bilirubin: mean \pm SD	0.91 \pm 0.11a	1 \pm 0.22a	2.36 \pm 1.22b	4.59 \pm 4.54c	<0.01
Albumin: mean \pm SD	4.7 \pm 0.38a	4.24 \pm 0.58a	2.53 \pm 0.54b	2.57 \pm 0.56b	<0.001
AFP: mean \pm SD	2.3 \pm 1.5 a	7.69 \pm 7.87b	15.23 \pm 14.04c	7149.48 \pm 1985.23d	<0.01

Groups bearing different initials are significantly different; "ALT", alanine aminotransferase; "AST", aspartate aminotransferase; "AFP", alpha feto protein; "DM", diabetes mellietus; "T-Bilirubin", total bilirubin

Table 2. Lymphocytic Mtdna Deletions and Serum Folic Acid Levels in the Studied Participants

	Control n=10	CHC n=20	LC n=20	HCC n=50	<i>P</i> -value
mtDNA deletions(Δ Ct) Mean \pm SD	1.67 \pm 0.42a	2.47 \pm 0.6b	2.96 \pm 0.83b	3.98 \pm 1.37c	< 0.01**
folic acid (μ g/dL) Mean \pm SD	7.44 \pm 2.5a	5.29 \pm 1.93b	5.46 \pm 2.66b	4.95 \pm 1.99c	< 0.05*

** , Association is significant at the 0.01 level (2-tailed); * , Association is significant at the 0.05 level (2-tailed)

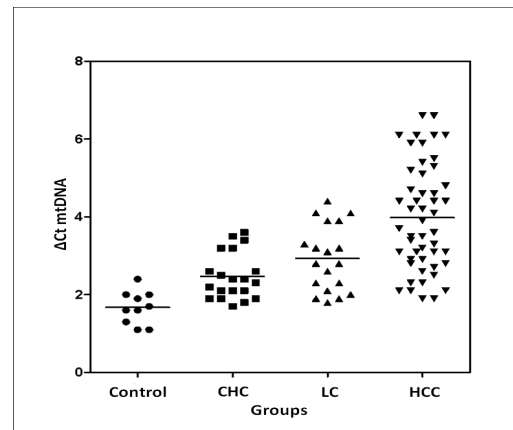


Figure 1. Scatter Dot Plot of Δ Ct of Mtdna Genes in The Different Studied Groups

class A compared to 6 patients (12%) of the HCC group. Child's class B was detected in 8 patients (40%) of the LC group and in 13 patients (26%) of the HCC group. Whereas, Child's class C was detected in 6 patients (30%) of the LC group compared to 31 of the HCC group (62%) (P -value < 0.05). Also, HCC patients were stratified according to Barcelona clinic liver cancer (BCLC) staging into 11 patients (22%) with stage A, 2 patients (4%) with stage B, 6 patients (12%) with stage C and 31 patients (62%) with stage D. Regarding focal hepatic lesion (FHL) number in the HCC group, single FHL was detected in half of the patients while multiple FHL were reported in the other half.

As for the levels of liver enzymes; the mean values

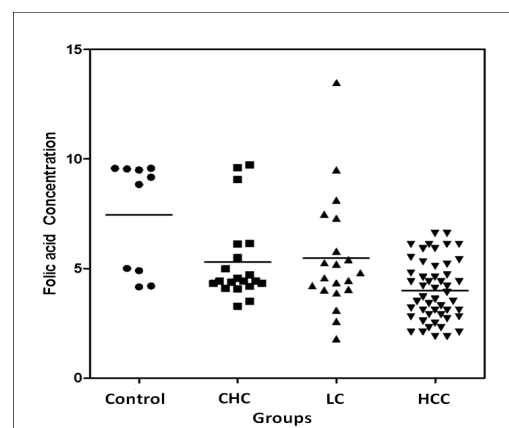


Figure 2. Scatter Dot Plot of Folic Acid Concentration in the Different Studied Groups

Table 3. Correlation Analysis of Different Studied Markers with Clinicopathological Features within HCC Group Showing Spearman's Rho Value and P-value

	Lymphocytic mtDNA deletions	Folic acid concentration	Number of focal hepatic lesion	Size of focal hepatic lesion	AFP
Lymphocytic mtDNA deletions	1	-0.94*	0.024	0.9**	0.16
		0.026	0.8	0.001	0.26
Folic acid concentration	-0.94*	1	-0.061	-0.197	-0.125
			0.61	0.17	0.39
Number of focal hepatic lesion	0.024	-0.061	1	0.18	0.37**
				0.25	0.007
Size of focal hepatic lesion	0.9**	-0.197	0.18	1	0.193
				0.25	0.18
AFP	0.16	-0.125	0.37**	0.193	1
				0.18	

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed)

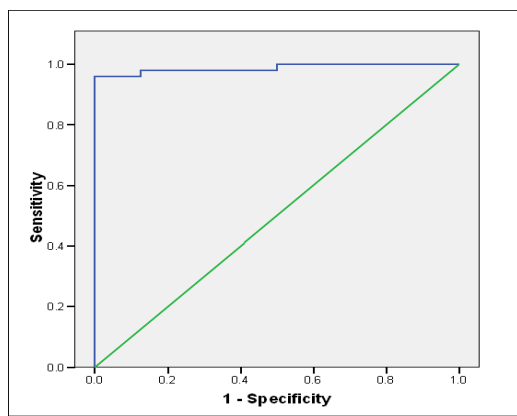


Figure 3. ROC Curve of AFP between HCC Group (Malignant) and the other Studied Groups (non-Malignant)

of serum ALT and AST were significantly elevated in the HCC group compared to the other groups (P -value<0.05, and P -value<0.01; respectively). However, there was no significant difference in the ALT or AST levels between the CHC and the LC groups. The mean value of bilirubin was significantly higher in the HCC group compared to the other studied groups (P -value<0.01), however, there was no significant difference in levels of bilirubin between the control and the CHC groups. The mean value

Table 4. ROC Curve Analysis of AFP and Lymphocytic mtDNA Deletions between HCC Group and the Other Studied Groups

Marker	AUC	Cut off	Sensitivity %	Specificity %	95% confidence interval
AFP	0.988	24.1	98	87.5	(0.97-1)
Lymphocytic mtDNA deletions	0.818	2.65	82	60	(0.73-0.9)

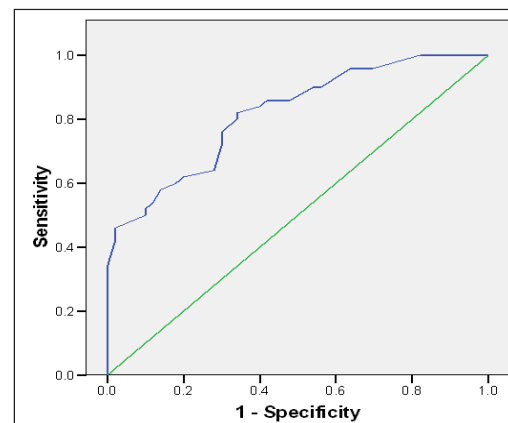


Figure 4. ROC Curve of MtdNA Deletion Frequencies between HCC Group (Malignant) and the other Studied Groups (Non-Malignant)

Table 5. Regression Analysis of Factors Associated with HCC Development

Marker	Odds Ratio	95% confidence interval	P-value
DM	1.062	(0.337-3.349)	0.918
lymphocytic mtDNA deletions ($\Delta Ct \geq 2.56$)	2.798	(1.560-5.019)	0.001**
Folic acid concentration (cut off < 5.36 $\mu\text{g/dL}$)	0.967	(0.749-1.250)	0.800

** , Correlation is significant at the 0.01 level (2-tailed); "DM", diabetes mellietus

of serum albumin was significantly lower in the LC and the HCC groups compared to the CHC and the control groups (P -value<0.01). The mean value of AFP was significantly elevated in the HCC compared to the other groups (P -value<0.01).

Our results also showed that there was significant elevation of the lymphocytic mtDNA deletions in the HCC group ($\Delta Ct=3.98 \pm 1.37$) on comparing to the control, CHC and LC group ($\Delta Ct=1.67 \pm 0.42, 2.47 \pm 0.6,$ and $2.96 \pm 0.83,$ respectively) (P -value<0.01). However, there was no significant difference in mtDNA deletions between the LC and the CHC groups (Table 2 and Figure 1). The mean level of serum folic acid was significantly lower in the HCC group (4.95 ± 1.99) compared to the control, CHC and LC ($7.44 \pm 2.5, 5.29 \pm 1.93$ and $5.46 \pm 2.66,$ respectively) (P -value < 0.05). However, there was not any significant difference was found between the LC and the CHC groups (Table 2 and Figure2).

Furthermore, lymphocytic mtDNA deletions, AFP and, folic acid did not differ significantly in the different BCLC stages of the HCC group (Supplementary Table 1).

Correlation analysis between the studied markers and the clinicopathological features of all patients using Spearman's Correlation Coefficient within the HCC group revealed that there was a significant positive correlation between serum AFP and the number of focal hepatic lesions in HCC patients ($r=0.37$ and P -value < 0.01). Also, There was a significant positive correlation between lymphocytic mtDNA deletions frequency and the size of focal hepatic lesions ($r=0.9$ and P -value < 0.01). On the other hand, there was a significant negative correlation

between lymphocytic mtDNA deletions frequency and serum folic acid level ($r = -0.94$ and P -value < 0.01) as shown in Table 3. Further analysis of the data using Receiving Operating Characteristic (ROC) analysis curves and the corresponding area under the curve were attempted to investigate the diagnostic accuracy of the lymphocytic mtDNA deletions frequency and the AFP level and to identify the optimal cut-off values for HCC diagnosis. Serum AFP level showed 98% sensitivity, 87.5% specificity at cut off value of 24.1 with AUC 0.988 for HCC diagnosis (Table 4 and Figure 3). While, the lymphocytic mtDNA deletions frequency had a sensitivity of 82% and a specificity of 60% at cut off value of 2.65 Δ Ct with AUC 0.818 for HCC diagnosis (Table 4 and Figure 4). At cut off value of 5.36; folic acid had a sensitivity of 76% and a specificity of 53% with AUC 0.655 for HCC diagnosis (Table 4 and Figure 5). Furthermore, odds ratio analysis of the factors associated with HCC development showed that patients with mtDNA deletions more than 2.65 Δ Ct were significantly susceptible for HCC development 2.798 times more than other patients with mtDNA deletions less than 2.65 Δ Ct (Table 5). After one year follow up for the HCC patients, the median survival time for patients with mtDNA deletions more than 3.98 Δ Ct was significantly shorter than those with mtDNA deletions less than 3.98 Δ Ct (5.7 ± 0.6 vs. 11.9 ± 0.04 months, P -value < 0.01) (Figure 6).

Discussion

Hepatocellular carcinoma (HCC) is a common problem worldwide which ranks the first and second most common cancer among men and women respectively in Egypt (Ibrahim et al., 2014). The progression of HCV related chronic liver disease to HCC is a multifactorial process (Omran et al., 2015). Early detection and biomarkers based on circulating DNA samples have non-invasive nature and ability of repetitive sampling (Chan and Lo, 2007). The mtDNA content from plasma or serum samples are correlated with different types of cancer (Fernandes et al., 2014; Mahmoud et al., 2015; Uzawa et al., 2015).

Many studies had shown that mtDNA mutations may be involved in apoptosis and carcinogenesis (Amuthan et al., 2001; Chinnery et al., 2002). The mtDNA deletions have been detected in cirrhotic and pre-neoplastic lesions of liver (Yin et al., 2004; Shao et al., 2004). Our results showed that there was a significant elevation of the lymphocytic mtDNA deletions frequency in the HCC group when compared to the other groups. Meanwhile, our data showed that there was no significance difference between the LC and the CHC groups. Similar recent study found a significant correlation between the lymphocytic mtDNA deletions frequency and the increased risk of HBV-related HCC, and the study was suggested that circulating mtDNA may be a non-invasive marker of HCC risk (Li et al., 2016). The mtDNA mutation are caused by a lack of protective histones, inefficient DNA repair systems, and continuous exposure to mutagenic effects of oxygen radicals (Kim et al., 2015). The deletion is the product of an intragenomic recombination between two 13 bp direct repeats (positions 8470-8482 and 13447-13459)

after a single-strand break caused by ROS (Shoffner et al., 1989).

Given the evidence that the integrity of the mitochondrial genome is related to the availability of folate and that the changes in mtDNA deletions frequency in various tissues were folate dependent (Wei and Lee 2002; Chou et al., 2007). Therefore, we assessed the frequency of mtDNA deletions in relation to folate level in HCV-related chronic liver disease with and without HCC as well as in healthy subjects. In this study, the serum level of folic acid in HCC group was significantly lower than other groups. In concordance with our data, Wu et al., (2009) reported that HCC patients with higher mtDNA deletions frequency were associated with serum folate deficiency. Meanwhile, our data showed that there was no significance difference in the serum level of folic acid between the LC and the CHC groups.

Regarding the clinicopathological features of HCC patients, we found a significant positive correlation between the mtDNA deletions frequency and the size of hepatic focal lesions. However, there was not any significant correlation between the mtDNA deletions frequency and the number of hepatic focal lesions and serum level of AFP. Similarly, Amuthan et al., (2001) showed that mtDNA damage might cause over-expression of cathepsin L, transforming growth factor, and mouse melanoma antigen, which contributes to tumor progression. Moreover, it had been reported that alterations in oxidative phosphorylation of tumor cells play a role in tumor formation, clinical phenotype and malignant potential of the tumor (Augenlicht and Heerdt, 2001).

Our results regarding the decreased serum level of folic acid in the HCV related disease patients, especially HCC patients, compared to the control group came in agreement with previous studies (Tkaczewski et al., 1971; Kotake et al., 1999) which showed that patients with viral related liver diseases commonly had low folate status. Moreover, many studies reported an increased risk of liver damage, impaired liver regeneration, and hepatocarcinogenesis in folate-deficient mice (Huang et al., 2001; Kim, 2004; Ghoshal et al., 2006). Folate deficiency results in increased uracil mis-incorporation, genomic DNA strand breaks and global DNA hypo-methylation (Pogribny et al., 1999; Duthie et al., 2000). Also, reduced folate levels cause hepatic oxidative stress resulting in ROS generation and lipid peroxidation (Huang et al., 2001; Chang et al., 2007). This causes defects in mtDNA respiratory function, and abnormal apoptotic signaling (Ghoshal et al., 2006) that contribute to hepatocarcinogenesis process (James et al., 2003).

Correlation analysis revealed that there was a significant negative correlation between the serum level of folic acid and the lymphocytic mtDNA deletion. Likewise, other studies reported an inverse correlation between folate level and the risk of liver diseases and malignancy; suggesting the possible role of folate deficiency in hepatocarcinogenesis (Welzel et al., 2007).

In an attempt to investigate the diagnostic accuracy of lymphocytic mtDNA deletion frequency; a ROC curve was plotted between HCC group (malignant) and the other studied groups (non-malignant). Our data revealed that

mtDNA deletion was able to diagnose HCC at cut off value of 2.65 Δ Ct; giving a sensitivity of 82% and a specificity of 60% with AUC 0.818. This data demonstrated that the diagnostic accuracy of mtDNA deletion for HCC diagnosis was good as AUC was in the range of 0.8-0.89. While the diagnostic accuracy of folic acid for HCC diagnosis was poor as AUC was in the range of 0.6-0.69. The odds ratio analysis revealed an increased risk of HCC development in patients with mtDNA deletions more than 2.65 Δ Ct by approximately three times more than other patients. In order to investigate prognostic accuracy of mtDNA deletion, HCC patients were followed up for a period of one year. The results showed that the median survival time for those with mtDNA deletion more than 3.98 Δ Ct was significantly shorter than those with mtDNA deletion less than 3.98 Δ Ct; suggesting the potential role of mtDNA deletion as prognostic marker for HCC patients.

In conclusion, this was the first study to our knowledge that assessed mtDNA deletion and folate status in HCV-related HCC Egyptian patients. These data broaden the knowledge about the causal relationship between mtDNA deletion and folate deficiency and also the potential role of mtDNA deletion as non-invasive biomarker for disease progression and patients' survival in HCV-related HCC.

Author contribution

AZ, HS and DO designed the experiments while YM and AZ performed the practical work. AZ, AS, RS, ZK and YM carried out interpretation of the results. AZ, YM, DO and RS conceived of the experiments. RS, SH and DS collected the clinical samples. AZ, AS, RS, ZK and YM participated in coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supplementary Table 1. AFP, Lymphocytic mtDNA Deletions and Folic Acid Levels in Relation to BCLC Staging in the HCC Group

Marker	Stage 0-A (n=11)	Stage B-D (n=39)	P-value
AFP	6267±2014	107277±1939	0.08
Lymphocytic mtDNA deletions	3.7±1	4.1±1.4	0.46
Folic acid concentration	5.1±2.1	4.1±1.6	0.14