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EVALUATION OF CLINICAL UTILITY OF DETECTING CIRCULATING TUMOR DNA MUTATION GENE LOCUS TP53 RS28934571 USING REAL TIME PCR AND DROPLET DIGITAL PCR IN EGYPTIAN HEPATOCELLULAR CARCINOMA PATIENTS

By
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Abstract

Liver cancer has been known to be the sixth cause of cancer worldwide. In Egypt, it is the fourth cause of cancer and the second -related mortality**.** The P53 is considered the guardian of the genome to prevent accumulation of oncogenic mutations that lead to malignant tumor.

The study compared the circulating tumor DNA (ctDNA) by targeting hotspot mutation gene locus TP53 rs28934571 (c.747G>T) in normal persons, chronic liver disease patients, and in hepatocellular carcinoma **(**HCC) ones to evaluate its diagnostic value and analyses the ctDNA by targeting the hotspot mutation gene locus TP53 rs28934571 (c.747G>T).

The results showed that of 100 adults, G1: Eighty (80%) patients were diagnosed as HCC on top of HCV, ten (10%) as HCV patients and ten (10%) healthy controls. All groups were matched as to age and sex. The AST was increased in HCC patients as compared to healthy control **(**P =0.018), forty patients (50%) were within Milan criteria, 12 patients (15%) within USCF, 28 (35%) were beyond all of whom 39 (48.75%) patients were child A with mean score of 12.45±5.94. Forty (50%) patients had one focal lesion, 16 patients had two focal lesions, eight patients had four focal lesions and six patients had multiple lesions. The average size of lesion in cm was (5.58±3.66). Among the HCC patients 38 (47.5%) patients developed ascites, 35 (43.75%) patients developed PVT and 9 (11.25%) patients developed encephalopathy. There was no significant difference in genetic typing between HCC patients, HCV ones and healthy controls (p =0.622)**.**

Keywords: Egyptian patients, HCC, TP53 mutation, Circulating-tumor DNA, Real time PCR.

Introduction

Liver cancer ranks the first among tumors with increased mortality in the last 20 years (Dasgupta *et al,* 2020), and the fourth most common in Egypt (Ezzat *et al,* 2021). The HCC patients typically diagnosed at intermediate or advanced stages, where potential curative treatments as surgical resection or liver transplantation were not recommended (Rashed *et al,* 2020). The TP53 mutations were the commonest found mutations in HCC tissue and cfDNA, with more than 120 non-unique alterations (Kaseb *et al,* 2019).

This study aimed to compare ctDNA by targeting hotspot mutation gene locus TP53 rs28934571 (c.747G>T) in normal persons, chronic liver disease patients, & HCC ones to evaluate its diagnostic value, to analyze ctDNA by targeting hotspot mutation gene locus TP53 rs28934571 (c.747G>T) using RT-PCR in blood sera and to identify the clinical utility of ctDNA in genetic profiling of HCC.

Patients and Methods

This study was conducted on 100 adult subjects. Patients were recruited from October 2020 to June 2022 from Ain Shams Centre for Organ Transplantation and Tropical Medicine Department and outpatient HCC clinic, Ain Shams University Hospitals. They were HCC eighty patients diagnosed with HCC on top of HCV according to the European Association for Study of the Liver Guidelines (EASL 2018). Besides, non- HCC participants were included as positive control or ten HCV-positive ones and

ten healthy controls were recruited to the study as the negative control. HCC clinical stage was determined after Barcelona Clinic Liver Cancer (BCLC) staging classification (Reig *et al,* 2022), and the Child-Pugh classification (Weerakkody *et al,* 2022).

Diagnosis of HCC in cirrhotic patients was based on non-invasive criteria and/or pathology, as well as on the identification of HCC typical hallmarks; the combination of hyper-vascularity in late arterial phase (defined as arterial phase hyperenhancement or APHE) according to LI-RADS (Liver Imaging Reporting & Data System) classification and washout on portal venous and/or delayed phases, which reflected vascular derangement that occurred during hepatocarcinogenesis.

Exclusion criteria: Patients who received prior chemotherapy or radiotherapy or locoregional treatment, without other degenerative conditions; autoimmune diseases or other malignancies no cystic liver focal lesions (abscesses, hydatid cysts), without metastatic liver focal lesions (cancer colon, and/or cancer breast).

For all participants, history taking, and clinical evaluation were accomplished. Laboratory examinations included total and direct bilirubin, liver enzymes (ALT, AST), total protein and albumin, alpha-fetoprotein, and kidney function tests (BUN and creatinine), complete blood counts (CBC) and prothrombin time with the INR as calculated by the Child score, and performed BCLC staging for HCC patients and radiological protocol (spiral triphasic CT and/or MRI) for each HCC patient.

Statistical analysis: Data were collected, tabulated and analyzed by using the Statistical Package for Social Science (SPSS 26). Data each parameter was presented as the mean, standard deviation (± SD) and range for parametric numerical data, while Median and Interquartile range (IQR) for the non-parametric numerical data, Frequency and percentage of the non-numerical data. The ANOVA test assessed significance differences between more than two groups. The Kruskal-Wallis test assessed the significance differences between more than two groups of variables. The Post Hoc test compared all possible pairs of group mean. The Chi-Square test evaluated the relationship between two qualitative variables. Fisher's exact test examined the relationship between two qualitative variables when the expected count was < 5 in more than 20% of cells. P-value of the significance was P>0.05: Non significant (NS). P< 0.05: Significant (S).

Ethics approval and consent to participate: The study was approved by the Ethical Committee of Faculty of Medicine, Ain Shams University, with an assurance number: FWA 00017585). This approval went with the Helsinki Declaration (2008) on the Human Rights. Blood samples were collected from the participants after explaining the study aim with preservation of the rights and privacy of all data.

Results

There was no significant statistical difference between the HCC group, healthy control and HCV control group as regards age ((P=.09), sex ((P=1), and comorbidities. AST was significant difference in the HCC patients as compared to the healthy control (P=.018) and with significant difference between HCC and either healthy or HCV controls as to albumin (P =.03) and INR (P <.001).

Forty patients (50%) were within Milan criteria, 12 patients (15%) were within the USCF, 28 patients (35%) were beyond all and 39 patients (48.75%) were child A, mean MELD score was 12.45 ± 5.94.

The tumor characteristics in the explant pathology picture of the HCC patients who underwent liver transplantation, the mean size of focal lesion were 3.66±2.26 and four patients had vascular invasion. There was no significant difference in the genetic typing between HCC groups (P= 0.6), the HCV and negative control (P=0.7) Of the HCC patients 38 (47.5%) had developed ascites, 35 (43.75%) developed PVT and 9 (11.25%)

developed encephalopathy. HCC male patients with were more than females. The digital PCR data were similar to RT/ PCR. Data were tabulated (1, 2, 3, 4, 5 & 6).

Table 1: Comparison between HCC group, healthy control and HCV control as regards demographic data:

Variations	Negative control	Positive control	HCC	Test of significance		
	No. (%)	No. (%)	No. (%)	Value	P value	Sig.
Age	59.5 ± 6.11	63.5 ± 9.02	57.69 ± 8.03	*f*= 2.462	0.091	NS
Male	9 (90%)	9 (90%)	66 (82.5%)	Fisher's Exact test	1.00	NS
Female	1 (10%)	1 (10%)	14 (17.5%)			

*One Way ANOVA tests of significance (f).

Table 2: Comparison between HCC group, healthy control and HCV control as to comorbidites:

Variations		Negative control	Positive control	HCC	Test of significance		
		No. (%)	No. (%)	No. (%)	Value	P value	Sig.
Hypertension	No	10 (100%)	7 (70%)	61 (76.25%)	Fisher's Exact test	0.176	NS
	Yes	0 (0%)	3 (30%)	19 (23.75%)			
Diabetes	No	10 (100%)	6 (60%)	57 (71.25%)	Fisher's Exact test	0.078	NS
	Yes	0 (0%)	4 (40%)	23 (28.75%)			
Autoimmune disease	No	10 (100%)	10 (100%)	80 (100%)			
	Yes	0 (0%)	0 (0%)	0 (0%)			

Table 3: Comparison between the HCC, healthy control and HCV control as to laboratory examinations:

Variations	Negative control	Positive control	HCC	F value	P value	Sig.
AST(U/L)	14.7 ± 4.57	26.6 ± 10.05	83.18 ± 96.32	*f*= 4.180	0.018$^{(A1)}$	S
Alt(U/L)	17 ± 5.27	29.9 ± 20.02	65.49 ± 104.37	*f*= 1.634	0.201	NS
Total billirubin(mg/dl)	0.51 ± 0.34	0.54 ± 0.32	2.61 ± 4.33	*f*= 2.261	0.110	NS
Albumin(g/dl)	3.85 ± 0.74	3.89 ± 0.8	3.26 ± 0.67	*f*= 6.296	0.003$^{(A2)}$	S
INR	0.86 ± 0.22	1.08 ± 0.19	1.34 ± 0.33	*f*= 12.550	<0.001$^{(A2)}$	S
BUN(mg/dl)	15.3 ± 6.04	20.2 ± 5.41	20.87 ± 11.74	*f*= 1.167	0.316	NS
Creatinine(mg/dl)	0.89 ± 0.19	0.96 ± 0.23	1.04 ± 0.79	*f*= 0.241	0.786	NS

*Post-hoc Bonferroni test significant between: $^{(A1)}$ Healthy controls vs. HCC, $^{(A2)}$ HCC vs. (Healthy & pathological controls).

Table 4: Staging of HCC patients:

HCC group		N (%) Mean ± SD
Milan	Within	40 (50%)
USCF staging	Within	12 (15%)
Beyond all	Yes	28 (35%)
Child -Pugh classification	A	39 (48.75%)
	B	23 (28.75%)
	C	18 (22.5%)
BCLC staging	0	1 (1.25%)
	A	18 (22.5%)
	B	26 (32.5%)
	C	18 (22.5%)
	D	17 (21.25%)
MELD Score		12.45 ± 5.94

Table 5: Tumor characteristics between all HCC patients:

Focal lesions	HCC group	N (%) Mean ± SD
Number	1	45 (56.25%)
	2	16 (20%)
	3	2 (2.5%)
	>3	17(21.25)
Site	Right lobe	44(55%)
	Left lobe	20 (25%)
	Bilobar	15 (18.75%)
	Ill defined	1 (1.25%)
Size	(cm)	5.58 ± 3.66

Table 6: Comparison between HCC patients and control (healthy, HCV Patients) as to genetic typing:

Variations	Genetic typing			Chi-Square test		
	Wild	Heterozygous	Homozygous			
Groups	N (Row %)	N (Row %)	N (Row %)	X^2	P value	Sig.
Patients	72 (90%)	7 (8.8%)	1 (1.2%)	2.627	0.622	NS
HCV control	9 (90%)	1 (10%)	0 (0%)			
Healthy control	10 (100%)	0 (0%)	0 (0%)			

Discussion

The hepatocellular carcinoma is still health challenge with a marked increasing incidence worldwide. It was estimated as by 2025, >1 million individuals would be affected by liver cancer annually (Meunier *et al,* 2021). Hepatocellular carcinoma (HCC) is the most common form of liver cancer and accounted for ~90% of cases (Llovet *et al,* 2021), immune and epigenetic mechanisms might have major consequences in understanding the onset, evolution and treatment of this malignancy (Pfister *et al*, 2021)

The present study showed that there was no significant difference between the HCC patients and the controls as regards demographic data and comorbidities. However, the AST was found statistically significant in HCC patients in comparison to healthy controls, albumin and INR showed significant between HCC patients and both healthy and HCV controls. This result agreed with Neamatallah *et al.* **(**2014), who found that there were significant increases in the activity of liver enzymes, serum total bilirubin, serum direct bilirubin and serum Alpa fetoprotein levels in the HCC patients as compared to control or with LC groups. Moreover, the result agreed with Abd-Elhameed *et al.* (2018)*,* they found that there were significant differences between HCC patients as compared to controls as to AST, total bilirubin, albumin and INR.

In the present study, showed that 40 patients without focal lesion, 16 patients had two focal lesions, eight patients had four focal lesions and six patients had multiple lesions, and the lesion mean size was averaged 5.58±3.66

In the present pathology, the HCC patients who underwent liver transplantation with the mean size of the focal lesion were 3.66±2.26 and 4 patients (33.3%) had micro-vascular invasion. The reason why most patients suffered from a single focal lesion was 48.7% of them were child A & 50% were within Milan criteria, which were diagnosed at the early stage. This result more or less agreed with Abd-Elhameed *et al.* (2018)*,* in Tanta University Hospitals, they found that among the Egyptian adults, 40 patients with HCC, another 40 with liver cirrhosis and 20 healthy controls 40 HCC patients had single focal lesions with 15 patients tumor size <3 cm and 25 patients with tumor size >3 cm, and PVT was found in 17/40(42.5%) patients. In a study done by Jeng *et al.* (2000) in Taiwan Mackay Memorial Hospital on 79 HCC patients over five years (1993 to 1997), tumor size was <3 cm in 24 (30%) patients, 3 to10 cm in 24 (30%) patients and >10 cm in 31 (39%) patients as well as vascular permeation in 56 (70.1%) patients. They reported that tumor recurrence was correlated with tumor invasiveness and tumor prognosis was determined from vascular permeation, cell differentiation grade, multi-nodular lesions, tumor free interval and positivity of TP53 gene mutation. Kudo (2004) in Japan reported that the tumor size was positively correlated with malignancy grade, except in very slowly growing, large, well-differentiated HCCs, but some of that were found in non-cirrhotic liver and were often difficult to be differentiated from hepatocellular adenoma (HCA).

In the present study, showed that of HCC patients 38 (47.5%) developed ascites, 35 (43.75%) developed PVT, and 9 (11.25%) developed encephalopathy. This result agreed with that of Kumar *et al.* (2008) in India, who reported that out of the 191 HCC patients*,* 51% suffered from the ascites and 5% showed hepatic encephalopathy.

Moreover, the present child score, BCLC and patients Milan stage of 39(48.75%) were child A, 23 (28.75%) patients were child B &18 (22.5%) patients were child C, besides, 50% were within Milan criteria, 18 (22.5%) patients were BCLC A, 26 (32.5%) patients were BCLC B. However, in contrast to a retrospective study carried out by Shaker *et al.* (2013), on 1456 Egyptian patients as to the Child classification, 519 (39.5%) were Child class A, 580 (44.2%) patients were Child class B and 214 (16.3%) patients were class

C. According to the BCLC classification system, 47.9% were class A, 24.6% were class B, which was mostly attributed to the implementation of the screening programs for the early detection of the HCC and surveillance of CLD patients on regular basis to early the demonstration of small focal lesions.

In the present study, also there was no significant difference between the HCC patients and the controls as regards genotypes and allele's distribution of TP53 gene, which may be explained by the fact that TP 53 gene mutation was more prevalent in HBV infection and genomic instability (Laurent-Puig *et al,* 2001). Di Vuolo *et al.* (2011) in Italy on 61 HCC patients (53 HCV-positive, seven HBV-positive and one HCV/HBV-positive HCC), the HCC patients compared to 122 controls were without significant difference in the TP53 gene allele distribution between patients and controls**.** In contrast to another case control study done by Ortiz-Cuaran *et al.* (2013) in Thailand, at the National Cancer Institute, proved a strong association between HBV infection and P.R249S gene mutation in the TP53 gene due to the endemic HBV there. Besides, there was an etiological link between the TP53 mutations and aflatoxin exposure, which was more prevalent in certain areas in Asia, South America and South Africa (Takeda *et al,* 2022).

In Egypt, Hifnawy *et al.* (2004) in Sharkia Governorate reported that aflatoxins, particularly aflatoxin B1 (AFB1) proved to be one of the commonest potent chemical carcinogens. They added that HCV prevalence and progressive nature of HCV-related liver diseases was influenced by many other factors. Also, Anwar *et al*. (2008) reported that aflatoxins presence and the HCV were connected to hepatic disease progression to the G3S3, which was the HCC indication. Besides, Sharaf-Eldin *et al.* (2016) in a controlled study reported that the aflatoxin levels were significantly higher in HCC patients than in the cirrhotic ones. Esmat *et al.* (2018) stated that a national screening campaign by the Egyptian Ministry of Health started in 2018 to combat high HCV prevalence by 2020. Abd El-Wahab *et al.* (2022) reported that in HCV patients with advanced cirrhosis or coexisting hepatic schistosomiasis, generic DAA-induced SVR remains robust with favorable clinical outcomes, but the risk of hepatocarcinogenesis was not eliminated.

Conclusion

There was no significant difference between HCC patients and controls as regards the TP 53 gene mutation genotypes and alleles distribution.

Mutations in theTP53 gene were detected in 12-48% of the HCC, with high frequency in advanced tumors and more common in the HBV patients and exposure to aflatoxins.

Competing interests: The authors declared that they neither have competing interests nor received any funds.

Authors' contributions: Ibrahim and Montasser designed and supervised the study, Massoud and Abdelwahed analyzed and interpreted the data, Maghraby wrote the manuscript, Montasser and Massoud revised the manuscript for important intellectual.

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