TERT C228T MUTATION IN CIRCULATING TUMOR DNA AS A CLINICAL MARKER IN HEPATOCELLULAR CARCINOMA PATIENTS ATTENDING A TERTIARY HOSPITAL IN EGYPT: A CASE-CONTROL STUDY

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ABSTRACT:

Background: Diagnosis of hepatocellular carcinoma (HCC) by liver biopsy can be challenging due to limited liver access and cirrhosis. Circulating tumor DNA (ctDNA) are genetic fragments originating from the tumor that circulate as a part of circulating free DNA and offer a representative sample of the tumor molecular profile.

Aim of work: Our study aimed to investigate the role of TERT C228T promoter mutation in ctDNA as a diagnostic biomarker in Egyptian HCC patients.

Methods: Our study is a case-control study that included 16 advanced HCC (AHCC), 12 early HCC (EHCC) cases, 10 chronic hepatitis C virus (HCV) patients as pathological control group and 10 healthy controls. Plasma was collected for genotyping of TERT C228T promoter mutation by real-time PCR.

Results: All cases in our study including AHCC and EHCC patients as well as HCV pathological controls and healthy controls carried the wild TERT genotype. There was a significant difference of AST, ALT, total bilirubin and albumin between the different groups included in the study. Our study has also shown that AFP levels were significantly higher in AHCC patients when compared to EHCC patients.

Conclusion: All studied samples in our study had the wild TERT genotype in ctDNA by real-time PCR. This could be attributed to the relatively small sample size of our study, and the relatively lower sensitivity of real-time PCR compared to other technologies such as droplet digital PCR (ddPCR) or next-generation sequencing. Future studies should include a larger sample size and utilize more sensitive technologies.

Keywords: Hepatocellular Carcinoma, Molecular Markers, TERT, ctDNA

INTRODUCTION:

Liver cancer is one of the most common cancers worldwide. In 2018, Global estimated incidence of liver cancers was 841080 cases and 781,631 deaths(1).

Hepatocellular carcinoma (HCC) is the predominant type of liver cancers which could be attributed to many risk factors. Nonetheless, hepatic viral infection is the major cause as more than 50% of worldwide HCC could be attributed to hepatitis B virus...
Hepatocellular carcinoma can be challenging due to limited liver access and cirrhosis. It is one of the few cancers that can be diagnosed without biopsy but instead, using non-invasive imaging techniques and defined radiological criteria as in European Association for the study of The Liver (EASL) (“EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma,” 2018). Therefore, there is a need for non-invasive biopsy for HCC that can help in its early detection and diagnosis.

Circulating tumor DNA (ctDNA) are genetic fragments originating from the tumor that circulate as a part of circulating free DNA and offer a representative sample of the tumor molecular profile. Furthermore, the amount of ctDNA could reflect the burden of the disease and its prognosis. In addition, ctDNA are easily accessible through a blood sample which could also allow easier follow-up at different time points for monitoring. Therefore, ctDNA could potentially be an ideal candidate for non-invasive biopsy.

Previous studies showed association between ctDNA and different cancers diagnosis, clinical characteristics, and prognosis. Telomerase reverse transcriptase (TERT) gene encodes for the reverse transcriptase subunit of the telomerase enzyme. Telomerase enzyme is responsible for the maintenance of telomeres. Normally, telomeres shorten with each replication which ultimately results in DNA damage and cell death. Telomerase enzyme functions by utilizing an RNA template to restore telomeres. Nonetheless, TERT gene is usually suppressed in most cells and thus, such cells undergo definite replication times before cell death. However, TERT upregulation could lead to uncontrolled replication in cancer cells. Two main hotspots in TERT promoter, namely C228T and C250T, have been frequently implicated in various cancers.

Single nucleotide polymorphism TERT C228T is a C>T transition occurring at chr5:1295228 genomic position within the TERT promoter region. This corresponds to a position 124 bases upstream from translation start site (c.1-124). TERT C228T has been associated with upregulated gene expression up to 4 folds. This could be attributed to the creation of new transcription factor binding sites. Subsequently, increased expression of TERT gene plays a crucial role to immortalize cancer cells.

Previous studies have shown association between TERT C228T mutation in ctDNA and HCC survival and clinical characteristics like stage, vascular invasion and a family history of cancer. However, little is known about the association between TERT C228T mutation in ctDNA and HCC among Egyptian patients.

AIM OF THE STUDY:

The aim of the present study is to investigate the clinical utility of a peripheral blood ctDNA TERT rs1242535815 (C228T) gene mutation as a clinical biomarker in Egyptian patients with early and late stages of HCC compared to chronic HCV patients and healthy controls.

SUBJECTS AND METHODS:

Patient selection: Our study included forty-eight (48) adult subjects who were divided into twenty-eight (28) cases with HCC and twenty (20) controls. Patients were diagnosed according to EASL 2018 HCC Guidelines for cirrhotic patients which are based on the identification of the typical radiological hallmarks of HCC according to LI-RADS (Liver Imaging Reporting and Data System) classification. Patients with previously treated HCC, cystic or metastatic liver focal lesions as well as patients having other degenerative conditions affecting circulating free DNA were excluded. Cases...
were divided according to their transplantation eligibility into EHCC and AHCC. Transplantation eligibility was determined based on Milan criteria (12) or University of California, San Francisco (UCSF) criteria(13). Furthermore, patients with AFP >1000 ng/ml or BCLC stage C were considered ineligible for transplantation (14-15). On the other hand, the control group was further divided into ten (10) HCV patients and ten (10) healthy controls. Informed consent was obtained from all participants.

**Laboratory Methods:** Blood samples were collected from all included subjects and tests including CBC, coagulation profile, liver function tests (AST, ALT, bilirubin and albumin), and AFP were carried out. In addition, for genotyping, 10 mL of whole blood in EDTA tube were collected and samples were transported within 2 hours after sampling using ice box to keep the temperature at 4-8°C with no direct contact between the tubes and the dry ice in the ice box to avoid unnecessary hemolysis. Plasma was then separated by centrifugation at 2 different speeds in RNase and DNase free tube. First speed was at 1600 xg for 10 minutes followed by another centrifugation at 16000 xg for another 10 minutes. Separated plasma was then transferred to RNase and DNase-free tubes and preserved at -80°C till further processing.

**DNA extraction:** Circulating DNA was extracted using QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) and eluted in 100 μl of elution buffer according to manufacturer’s instructions. Invitrogen Qubit 3.0 fluorometer was used to check purity of DNA and measure the concentration of extracted circulating DNA.

**Real-time PCR:** Genotyping for TERT C228T mutation was performed on DT-lite Real Time PCR System (DNA technology, Russia) using G40x TaqMan® SNP Genotyping Assay custom kit for rs1242535815 mutation (Applied Biosystems, CA, USA). The sequence of primers and probes used are listed in table (1). The thermal cycling program is described in table (2) and PCR reaction mix is demonstrated in table (3).

Table (1): Sequences of primers and probes

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>GTCTGTGCCCTCCTCACCTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Primer</td>
<td>CAGCGCTGCCTGAAACTC</td>
</tr>
<tr>
<td>Wildtype allele probe</td>
<td>VIC-AGCCCCCTCCGGG-MGB</td>
</tr>
<tr>
<td>Mutant allele probe</td>
<td>FAM-AGCCCCCTCCGGG-MGB</td>
</tr>
</tbody>
</table>

Table (2): Thermal Cycling Program of Real Time PCR:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase activation</td>
<td>95°C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15 seconds</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60°C</td>
<td>60 seconds</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): PCR reaction mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Genotyping PCR Master Mix</td>
<td>10 µL</td>
</tr>
<tr>
<td>20x working stock of SNP Genotyping Assay</td>
<td>1 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>4ng/reaction</td>
</tr>
<tr>
<td>RNase free water</td>
<td>To 20 uL</td>
</tr>
</tbody>
</table>
**Statistical analysis:**

Data analysis was done using Statistical Package for Special Sciences (SPSS) software computer program Version (V. 22.0, IBM Corp., USA, 2013). The Kruskal Wallis Test was used to compare continuous dependent variables with skewed distribution between three or more groups. Chi Square Test was used to compare categorical dependent variables between two or more groups. Mann–Whitney U test was used to compare continuous dependent variables with skewed distribution between two unpaired groups. Results with a P value < 0.05 were considered statistically significant.

**Ethical considerations:**

This study was approved by the Faculty of Medicine, Ain Shams University Research Ethics Committee (FMASU R 92/2020).

**RESULTS:**

There was a predominance of male gender among HCC patients in our study where they were 89.3% of total HCC patients. Similarly, 90% of HCV controls and healthy controls were males. AHCC, EHCC, HCV patients and healthy controls showed different AST, ALT, total bilirubin, and albumin levels as shown in diagrams (1 to 4). Liver enzymes AST, ALT and total bilirubin levels were significantly higher in AHCC patients with their median (IQR) being 93 (59-190) U/L, 66 (32-95) U/L and 1.4 (0.8-4.3) mg/dl respectively (p < 0.05). On the other hand, albumin was significantly lower in AHCC patients with a median (IQR) of 2.9 (2.5-3.4) g/dl (p < 0.05).

Diagram (1): Box plot showing that AHCC has highest AST levels among the studied groups.

Diagram (2): Box plot showing AHCC has highest ALT levels among the studied groups.
Our study has shown that AFP levels were significantly higher in AHCC patients when compared to EHCC patients. In addition, ascites and portal vein thrombosis were significantly more frequent in AHCC patients. AHCC patients were significantly more likely to have multiple lesions. There was also a statistically significant difference between AHCC and EHCC groups in their Child-Pugh staging. Table (4) lists various clinical parameters of AHCC and EHCC cases.

Table (4): Comparison between EHCC and AHCC demographic and laboratory parameters using Chi-square or Mann-Whitney test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EHCC (n=12)</th>
<th>AHCC (n=16)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>60 (55–63)</td>
<td>57 (52–60)</td>
<td>0.193</td>
</tr>
<tr>
<td>Male sex</td>
<td>11/12</td>
<td>14/16</td>
<td>0.724</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>11 (11-11.25)</td>
<td>11.55 (9.8-12.9)</td>
<td>0.39</td>
</tr>
<tr>
<td>Child-Pugh stage</td>
<td>A (8), B (4)</td>
<td>A (3), B (7), C (6)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>0/12</td>
<td>3/16</td>
<td>0.112</td>
</tr>
<tr>
<td>Portal vein thrombosis</td>
<td>2/12</td>
<td>11/16</td>
<td>0.006*</td>
</tr>
<tr>
<td>Ascites</td>
<td>4/12</td>
<td>13/16</td>
<td>0.01*</td>
</tr>
<tr>
<td>Multiple lesions</td>
<td>2/10</td>
<td>10/16</td>
<td>0.015*</td>
</tr>
<tr>
<td>AFP (ng/ml)</td>
<td>5.5 (3.3–136)</td>
<td>250 (65–9784)</td>
<td>0.0117*</td>
</tr>
</tbody>
</table>

Parameters are described in median (IQR) for continuous variables, *p<0.05 denotes statistical significance.
None of the HCC patients including the AHCC and EHCC cases showed the TERT C228T mutation. Similarly, both HCV controls and healthy controls did not show this TERT promoter point mutation as shown in Table (5).

Table (5): TERT C228T SNP genotyping

<table>
<thead>
<tr>
<th>TERT C228T</th>
<th>HCC</th>
<th>HCV controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>28/28</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Mutant</td>
<td>0/28</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

DISCUSSION:

HCC is one of the most common cancers where it is the sixth most common cancer worldwide (1). Recently, there has been a growing interest in the role of genetic factors in HCC and the role of different DNA mutations in hepatocarcinogenesis. Among different gene mutations, TERT promoter mutations have been frequently detected in HCC patients. Particularly, two hotspot mutations which are C228T and C250T were the most common TERT promotor mutations with C228T mutation being more common than C250T (16). The present study aimed to investigate the clinical role of TERT C228T polymorphism in ctDNA as a diagnostic biomarker in Egyptian HCC patients compared to pathological HCV-positive controls as well as healthy controls to determine its role in hepatocarcinogenesis. To our knowledge, this is the first study to investigate the presence of TERT C228T mutation in ctDNA as a potential liquid biopsy maker in Egyptian HCC patients.

Most HCC patients included in the study were males where males constituted 89.3% of total HCC patients. Previous studies have also reported increased cases of male HCC compared to females. This result agrees with a study of black south African HCC patients, where males constituted 74% of HCC patients (17). Recent studies have shown that this could be linked to higher levels of testosterone and dihydrotestosterone in male patients where both testosterone and dihydrotestosterone were shown to enhance growth and proliferation of hepatic tumor cells (18).

Our results revealed that AST, ALT and total bilirubin showed statistically significant differences between the different groups included in the study. Levels of AST, ALT and total bilirubin were highest in AHCC patients. This is in agreement with previous studies that showed that elevated bilirubin levels in HCC patients are associated with increased tumor aggressiveness, increased PVT and decreased survival (19). Similarly, another study has demonstrated that AST and ALT levels are associated with HCC stage, grade and overall survival (20). On another note, our results revealed that albumin levels were significantly lower in AHCC patients. Our results agree with previous studies reporting that lower levels of albumin in HCC patients are associated with higher Child–Pugh cirrhosis score and higher recurrence rates (21). Our study has also shown that AFP levels were significantly higher in AHCC patients when compared to EHCC patients. Our results are in accordance with the results reported by Jearth et al. (2022) who reported that high levels of AFP were associated with AHCC where such patients had higher incidence of extrahepatic metastasis and poorly differentiated tumors (22).

There was an absence of the mutant type in the genotyping results in our study where we did not detect the C228T mutation in either HCC patients, HCV pathological controls or healthy controls. The genotype of all the included subjects was wild type (CC). Schulze et al. in 2015 have shown that TERT promoter mutations were more frequent in EHCC cirrhotic patients compared to AHCC cirrhatic patients suggesting that TERT promoter mutations could be early events in
hepatocarcinogenesis\textsuperscript{(23)}. Therefore, the absence of C228T mutation in our study could also be attributed to the lower number of EHCC cases included in our study where only 12 patients had EHCC. Our results are in accordance with the results previously reported by Chae et al. (2021) who investigated the presence of mutations in ctDNA derived from HCC patients. The authors used next-generation sequencing to target TERT, TP53, and CTNNB1 genes in Korean HCC patients who had not undergone systemic therapy. None of the twenty patients included in the study had the C228T mutation or any other SNP in the TERT gene. The authors attributed the lack of TERT promoter mutations to the fact that they mainly investigated AHCC not EHCC cases \textsuperscript{(24)}. Our results do not conform with the results of Jiao et al., 2018 where this study used sanger sequencing to detect TERT promoter mutations in ctDNA of HCC patients and found that overall prevalence of TERT promoter mutations, either C228T or C250T, was 47.7\% in HCC patients. Nonetheless, this study had a sample size of 218 HCC patients which could explain the difference between our results and the results of this study \textsuperscript{(25)}. Additionally, our results disagree with Ako et al., 2020 who investigated a cohort of 36 Japanese HCC patients for the presence of TERT promoter mutations and found out that C228T mutation had a frequency of 69\% among HCC patients \textsuperscript{(23)}. Similarly, another study found out that TERT C228T promoter mutation was present in 77\% of 26 HCC patients \textsuperscript{(26)}. However, both studies used droplet digital PCR (ddPCR) to detect the mutation. The higher sensitivity of ddPCR could potentially explain the disparity between the results of these studies and our results.

Another possible explanation for the absence of C228T in our study is the relatively small size of blood samples taken from the patients. On one hand, most previous studies have used similar blood sample volume to our study (10 ml) in order to detect mutations in ctDNA \textsuperscript{(25-27)}. On another hand, few recent studies have started recommending using larger blood sample volumes where one study recently recommended drawing 10-18 ml of blood as a typical volume in order to use ctDNA sample for applications such as the identification of mutations in ctDNA\textsuperscript{(28)}. Another recent study has reported that samples of small volume might be more suitable if multiple mutations are being investigated for a certain tumor. However, in case only one mutation is being investigated for a certain tumor, they recommended a larger sample to increase the sensitivity of the test for detection of that mutation \textsuperscript{(29)}.

Our study has some limitations. First of all, our sample size was relatively small which could have potentially affected the power of our study to detect mutations in ctDNA. A second limitation is the fact that we need to use a more sensitive technique such as ddPCR or next-generation sequencing to detect the TERT C228T mutation in ctDNA. Furthermore, our study included tissue samples for HCC patients eligible for transplantation for next generation sequencing, but still under study.

\textbf{Conclusion:}

Our study demonstrated the absence of TERT C228T of ctDNA for Egyptian HCC patients using rtPCR, which needs confirmation by a more sensitive technology. Future studies should use more advanced technologies such as ddPCR or next-generation sequencing to increase the chance of detecting the mutation. In addition, future studies should aim to have a larger sample size. Ideally, more patients with EHCC should be included in investigating TERT C228T promoter mutation. In case only one mutation is being investigated, drawing larger volume of blood sample from included patients should be considered in order to increase the sensitivity of detecting this mutation. It is recommended to investigate multiple mutations especially in case only
samples of smaller volume are available. Ideally, future studies should include tissue samples from the tumors in order to confirm the absence of the mutation.

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**Conflict of interest:**

The authors declare that they have no conflict of interest.

**REFERENCES:**


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تعدد الشكل لجين TERT (C228T) في الحمض النووي المتداول للورم (ctDNA) كمؤشر حيوي كلينيكي في مرضى سرطان الكبد الثاني قسم طب المناطق الحارة، كلية الطب، جامعة عين شمس، القاهرة، مصر.

الخلفية: يمثل الحمض النووي المتداول للورم مقتطفات جينية تنشأ من الورم ذاته ثم تدور كجزء من الحمض النووي المتداول للدم. بالإضافة إلى هذا، يمكن للحمض النووي المتداول للورم أن يعكس حجم الورم ويتوافق مع مساره. إن الحمض النووي المتداول للورم سهل الحصول عليه وذلك عن طريق إجراءات ديمية. ولذا، يمكن للحمض النووي المتداول للورم أن يمثل الخيار الأمثل لخزعة غير تداخلية.

الهدف: هدفت هذه الدراسة إلى دراسة دور تعدد الشكل في الحمض النووي المتداول للورم (C228T) كمؤشر حيوي كلينيكي في مرضى سرطان الخلايا الكبدية المصريين بالمقارنة مع المرضى المصابين بالالتهاب الكبدى الفيروسي C أو الأفراد الأصحاء.

الطريقة: تم سحب عينات الدم من ثمانية وعشرون مريضاً مؤسفاً بالإضافة إلى عشرة مرضى مصابين بالالتهاب الكبدى الفيروسي C وعشرة أفراد أصحاء. تم إجراء تحاليل تعدد الشكل للحمض النووي المتداول في هذه المرضى.

النتيجة: أظهرت النتائج دراسة عدم وجود تعدد الشكل في مرضى سرطان الخلايا الكبدية المصريين، وكذلك عن عدم تواجد تعدد الشكل في مرضى الالتهاب الكبدى الفيروسي C.

الخلاصة: قد يمثل تعدد الشكل في حمض النووي المتداول للورم في حالات سرطان الخلايا الكبدية المرحلة الأخيرة من السرطان. ولكن، قد يكون استخدام هذه التقنية لتشخيص هذه الحالات أقل دقة، بسبب تعدد الشكل في حالة عدم تواجد 함ض النووي المتداول. لذلك، يمكن استخدام هذه التقنية كمؤشر لتشخيص مدى تطور السرطان وتحديد التدخلات المبكرة لعلاج المرضى.