Human TP53 gene polymorphisms among patients with hepatocellular carcinoma and chronic hepatitis B in Kenya
[version 1; peer review: awaiting peer review]

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Abstract

Background: Human TP53 is the gatekeeper for generation of human cells and is highly conserved. Any alteration/mutation to TP53 adversely affects the regulatory function of the protein, potentially resulting in cancer. This study investigated mutations in codons 7 and 249 of TP53, among patients with hepatocellular carcinoma (HCC) and chronic hepatitis B virus (HBV) infection at the Moi Teaching and Referral Hospital (MTRH), Eldoret, Kenya.

Methods: In total, 33 HBV-positive patients attending MTRH hospital between September 2013 and July 2017 were purposely selected from medical records for the study; those with HCC were confirmed from the cancer registry. The patients were aged between 25-67 years, with a male-to-female ratio of 1.1:1. Blood samples were collected from the patients. DNA was extracted, amplified and sequenced using TP53 forward and reverse primers. Gene mutation detection and analysis was done on exons 4 and 7.

Results: Of the 33 patients, 75.8% were chronically infected with HBV and had HCC; the rest were HBsAg positive without HCC. Homozygous proline was prevalent (54.5%) at exon 4 codon 72, followed by heterozygous Arg/Pro (33.3%) and lastly homozygous Arg/Arg (12.1%). Pro/Pro allele was frequent in HCC group while Arg/Arg allele was common in patients without HCC. There was no significant association between the HCC and codon polymorphisms (p=0.12). In exon 7, codon 249, 24.2% of patients had an Arg-Ser mutation of which, 75.0% had HCC and 25.0% did not. There was no significant association between HCC patients and codon 249 mutation (p=0.15).

Conclusion: TP53 is a gene gate keeper, the mutations under study may dependently play a role in HCC development. This study did not find any association or clear mutational pattern between P53 mutations and HCC.
development. Therefore, TP53 mutation is a poor indicator for prognosis and a tumor’s biological behavior among HBV-positive subjects in Kenya.

Keywords
p53 Gene mutation, Codon 249, Hepatocellular carcinoma, p53 Exon 4, p53 exon 7

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Abbreviations
HCC: Hepatocellular carcinoma; MTRH: Moi Teaching and Referral Hospital; DNA: Deoxyribonucleic acid; PCR: Polymerase Chain Reaction; G: Guanine; T: Thymine; KEMRI: Kenya Medical Research Institute; EDTA: Ethylenediaminetetraacetic acid; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information; MEGA: Molecular Evolutionary Genetics Analysis; IARC: International Agency for Research on cancer; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Introduction
Hepatocellular carcinoma (HCC) is the fourth most common malignancy according to the World Health Organization (2016). HCC is increasing in incidence and has a mortality incidence of 800,000 deaths globally per year (Stewart et al., 2016). Reported incidences of HCC vary worldwide, with the West, Asia and Africa having the highest incidence rates. According to report on the Global Burden of Disease Cancer Collaboration et al. (2017) HCC is the fifth and seventh most common cancer in men and women, respectively. There are various causes of HCC, of which the most common is chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV). In Kenya, those infected with HBV constitute 78.0% of HCC cases (Mutuma et al., 2011).

HCC is the primary liver cancer derived from uncontrolled multiplication of hepatocytes (Gomes et al., 2013). Just like in any other cancer, TP53 has a crucial role in HCC tumor suppression. The gene hampers progression of the cell cycle if DNA is damaged (Kruiswijk et al., 2015; Sasaki et al., 2011), a role that is inactivated in most cancers mainly through alteration to TP53, which can be caused by external agents (Gomes et al., 2013; Tokino & Nakamura (2000)). TP53 alterations are observed in most cancers (Kandoth et al., 2013; Levine, 2009) and they affect major regulators of various signaling pathways involved in tumor suppression.

TP53 has ten coding exons, with mutations distributed in all of them, with a strong predominance in exons 4-9, encoding the DNA-binding domain of the protein (Rivlin et al., 2015). Studies have demonstrated that mutant TP53 contributes immensely to replication of damaged DNA and to tumor progression. These mutant proteins bind to TP53 response elements thereby weakening the process of DNA repair and TP53-mediated apoptosis (Carvajal et al., 2012; Maiuri et al., 2010). In exon 7, codon 249 (AGG→AGT, arginine to serine) has been identified as a “hotspot”. Differences in ethnicity and geographical location among other factors have varied impact on TP53 codon 249 (AGG→AGT) mutation profiles (Kandoth et al., 2013; Wen et al., 2016). In exon 4, an arginine to Proline substitution at codon 72 has been investigated as risk modifier in several cancer models; however, its role in cancer progression remains uncertain.

There is paucity of information on TP53 in Kenya. In this study we evaluated the presence of TP53 gene mutations in exons 4, 6 and 7 among HCC patients attending Moi Teaching and Referral Hospital (MTRH), in western region of Kenya.

Methods
Study site and sample population
The samples were collected from jaundiced patients chronically infected with HBV attending Moi Teaching and Referral Hospital (MTRH), Eldoret, Kenya between September 2013 and July 2017. The patients were purposively selected from hospital records based on them being jaundiced and having HBV or HBV and HCC. Patients were then recruited in person. Those with HCC had their cancer status confirmed using the cancer registry of Eldoret hospital, Uasin Gishu, Kenya. A patient with HCC was defined as having liver cancer based on the patient’s medical record and cancer registry file. All patients with HCC were selected. Other patients’ medical records obtained from the hospital included gender and residential area. The MTRH was selected as it is one of the largest national referral hospitals in western Kenya, where rates of HBV infection are considered to be high (Ochwoto et al., 2016). The male-to-female ratio was 1.1:1 and the age range were from 25 to 67 years. None of the patients had received any viral HBV treatment by the time of sample collection.

Ethical consideration
The ethical approval to conduct the study was obtained from Institutional Research and Ethics Committee (IERC) of MTRH/ Moi University (approval number 001002), from Kenya Medical Research Institute Scientific Ethics Review Unit (approval number KEMRI/SERU/CVR/001/3211) and from Eldoret Cancer Registry (approval number ECR/DRA/2017/001). Further, the participants consented for the study prior to blood draw.

Collection and preparation of blood samples
Blood samples were collected in vials anti-coagulated with EDTA. Plasma was separated at MRTH and thereafter the plasma tubes were shipped on dry ice to the KEMRI Production Unit in Nairobi. The samples were then stored in aliquots at -80°C until subsequent testing.

Serological testing
Screening for hepatitis B virus surface antigen (HBsAg) and antibody to the core protein (anti-HBc) were was performed using the COBAS e411 platform (Elecsys; Roche Diagnostics, Quebec, Canada). Chronic hepatitis B (CHB) was determined by anti-HBc IgM-positive serology, as described previously (Park et al., 2015).

Extraction of DNA from plasma
Circulating DNA of Human TP53 tumor suppressor gene was extracted from 200 µl of plasma samples using QIAamp DNA mini-extraction kit (Qiagen Inc, USA) according to manufacturer’s instructions. The DNA was subsequently eluted in 60 µl of AE buffer and quantity measured by NanoDrop spectrophotometer (Thermo Scientific) and stored at -30°C until use.

PCR amplification of human TP53
Three different primers targeting TP53 gene exons 4, 6 and 7 (Table 1) were used in amplification of the extracts using conventional PCR. The PCR mix targeting the three exons was...
similar except for the primer. Each PCR tube contained a total volume of 50 µl reaction mixture, with 5µl of human genomic DNA template, 5 µl of 10X PCR buffer 5 µl of 25 mM MgCl₂, 5 µl of 1.25M dNTP mix, 0.2 µl of 5U of Taq DNA polymerase (Qiagen Inc, USA), 1.25 µl each of a 20 uM stock of forward primer and reverse primer of sequences (Table 1).

The mix was loaded to a PCR machine (ABI systems). Amplification for exon 7 the PCR profile set at 95°C for 10 minutes initial denaturation and 35 cycles of denaturation at 95°C for 45 seconds, annealing at 58°C for 30 seconds and extension at 72°C 30 seconds. Final extension was at 72°C for 10 minutes. For exon 4 the PCR was set at 94°C for 12 minutes initial denaturation and 35 cycles of denaturation at 94°C for 40 seconds, annealing at 56°C for 30 seconds and extension at 72°C 30 seconds. Final extension was at 72°C for 10 minutes.

After that, a 4-µl aliquot of PCR product was electrophoresed by heterozygous Arg/Pro (33.3%) and homozygous Arg/Arg (54.5%) of these had Pro/Pro (CCC) alleles, followed by heterozygous Arg/Pro (33.3%) and homozygous Arg/Arg (CGC) (12.1%) (Table 1). The total number of samples that were amplified with clear for those (24.2%) that did not have HCC but were HBsAg positive, of which 48.0% were female and 52.0% were male. Among those (24.2%) that did not have HCC but were HBsAg positive, half were female and the other half male.

**DNA sequencing**
All PCR-positive amplicons were purified using the Qiagen Gel purification kit according to the manufacturers recommended protocol. The purified DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and purified DNA (50 ng) were send for sequencing at Macrogen, Inc. (Netherlands) using the first primer sequences and the manufacturer’s guidelines.

**Mutation detection and analysis**
The directly amplified sequences were assembled using GENETYX version 9.1.0 (GENETYX Co., Tokyo, Japan; PCAP is an open-access alternative) DNA sequence analysis software. The sequences were aligned to TP53 gene sequences using NCBI BLAST for identity confirmation. The contigs from GENETYX were then aligned to the TP53 gene reference sequence from the International Agency for Research on Cancer (IARC) database using Biobed software version 7.2.5. Mutations to the sequences were analyzed using MEGA v.7.0 software bioinformatics editing tool.

**Statistical analysis**
Test for statistical significance of mutation profile parameters were done using the χ² test and Fisher’s exact test. P-values less than 0.05 were considered statistically significant. To examine possible associations between mutations in TP53 exons and hepatocellular carcinogenesis, we analyzed 2x2 tables using Fisher’s exact test. Odds ratios (ORs) were used to analyze two significant associations at 95% confidence interval (CI). Statistical analysis was performed using SAS version 9.4.

**Results**

**Participant demographics**
There were 33 subjects in total for whom results in exon 4, 6 and 7 were obtained. The characteristics of the subjects are shown in Table 2. The ratio of male to female was 51.5% to 48.5%. All the subjects were positive for HBsAg. Those who were chronically infected and had HCC were 75.8% (25/33), of which 48.0% were female and 52.0% were male. Among those (24.2%) that did not have HCC but were HBsAg positive, half were female and the other half male.

**TP53 exon 4, codon 72 polymorphism analysis**
The total number of samples that were amplified with clear forward and reverse sequences for exon 4 codon 72 was 33. The majority (54.5%) of these had Pro/Pro (CCC) alleles, followed by heterozygous Arg/Pro (33.3%) and homozygous Arg/Arg (CGC) (12.1%) (Figure 1). All those homozygous for Arg/Arg were male.

There was statistically significant association between the sex of the subject and the polymorphism identity (Fisher test=5.4

**Table 2. Clinical and molecular characteristics of the subjects.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
</tr>
<tr>
<td>HCC status</td>
<td></td>
</tr>
<tr>
<td>HCC</td>
<td>25</td>
</tr>
<tr>
<td>Without HCC</td>
<td>8</td>
</tr>
<tr>
<td>Codon 72 polymorphisms</td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>4</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>17</td>
</tr>
<tr>
<td>Pro/Arg</td>
<td>12</td>
</tr>
<tr>
<td>Ser 249 mutation</td>
<td></td>
</tr>
<tr>
<td>AGG (Arg)</td>
<td>25</td>
</tr>
<tr>
<td>AGT (Ser)</td>
<td>8</td>
</tr>
</tbody>
</table>
and p=0.04), with all the homozygous Arg/Arg belonging to male patients, whereas more female patients had homozygous Pro/Pro than male (64.7% vs 35.3%) and more male had the heterozygous Pro/Arg than female (58.3% vs 41.7%). On the other hand there was no statistical significance between the HCC and the polymorphisms (Fisher test=3.58 and p=0.12); however, it is important to note that at codon 72 most of the patients with HCC had Pro/Pro alleles, followed by heterozygous Pro/Arg and lastly homozygous Arg/Arg (Table 3). The Pro/Pro allele was more frequent in the HCC group, whereas all patients with Arg/Arg alleles did not have HCC (Table 4).

There was no significant association between HCC, gender and TP53 Arg72Pro/Arg when both HCC cases and the non-HCC cases were compared (P=0.57). Equally there was no significant association between HCC, gender and TP53 codon 72 Pro/Pro (P=0.40; Table 4).

Mutations at exon 7, codon 249
Out of the 33 subjects, eight (24.2%) had the Arg>Ser codon 249 mutation and the majority (75.8%) did not have the mutation. Serine 249 mutation was seen more in males (87.5%) than females (12.5%) and there was an association between the sex and mutation (Fisher’s exact test =5.47, P-value =0.039) with male at higher risk compared to female (OR=10.5, 95% CI =1.1-98.9%) (Table 5).

Codon 249 mutation and HCC
The majority (75.0%) of those with the serine 249 mutation had HCC; only 2 (25.0%) had the mutation without but not HCC. Similarly, among those without the mutation, 76.0% were had HCC and 6 (24.0%) did not have HCC. The findings showed no significant statistical association in the presence of codon 249 mutations between patients with and without HCC (p=0.15) at 95% CI (OR=0.52: 95% CI 0.054-4.773).

Figure 1. Frequency of polymorphisms in codon 72, exon 4 of TP53.

### Table 3. Association between TP53 codon 7 polymorphisms, gender and hepatocellular carcinoma (HCC).

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Female</th>
<th>Male</th>
<th>Fisher value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>0</td>
<td>4 (100%)</td>
<td>5.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>11 (64.7%)</td>
<td>6 (35.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/Arg</td>
<td>5 (41.7%)</td>
<td>7 (58.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Without HCC</th>
<th>HCC</th>
<th>Fisher value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro/Pro</td>
<td>2 (11.7%)</td>
<td>15 (88.2%)</td>
<td>3.58</td>
<td>0.12</td>
</tr>
<tr>
<td>Pro/Arg</td>
<td>4 (33.3%)</td>
<td>8 (66.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>2 (50.0%)</td>
<td>2 (50.0%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

The association between HCC and mutations at codon 72 or 249 of TP53 remains controversial. To our knowledge, this is the first information concerning TP53 exon 4, 6 and 7 mutation in Kenya. A number of studies have described two structurally different forms of wild-type p53 resulting from the substitution of a proline for an arginine at residue 72, with different biochemical and biological characteristics (Thomas et al., 1999). Different prevalence of this substitution has been reported in various studies. In this study, the HBV-positive Kenyan population, the homozygous Pro/Pro genotype is the most common (54.5%), and the least is homozygous Arg/Arg. This prevalence of allele is similar to Taiwanese (Mah et al., 2011), Egyptian (Neamatallah et al., 2014) and Chinese (Wang et al., 1999). We observed that patients with HCC had higher frequencies of Pro/Pro (88.2% Vs 11.8%) a similar observation made among Moroccan population (Ezzikouri et al., 2010) and Egyptian patients with HCV (Koushik et al., 2004; Neamatallah et al., 2014).

The association between TP53 codon 72 Pro/Arg gene polymorphism and cancer remains controversial, with some studies showing associations but others no association. Among the studies that show associations, Dong et al., 2018 found that the TP53 Pro allele and Pro/Pro genotype were associated with cancer risk, (Dong et al., 2018). In Egyptian patients with HCC, development of HCC was associated with Pro/Pro allele carriage as compared to Arg/Arg or Arg/Pr alleles (Neamatallah et al., 2014). This study did not find any association between polymorphisms and HCC among the HBV-positive patients. Other studies have shown similar findings (Eskander et al., 2014; Hu et al., 2014). The inconsistency in association and prevalence observed could be attributable to ethnic differences, since most study have been performed in Asian and Caucasian populations, while the current study was performed in an African population.

Our findings show the presence of selective guanine-to-thymine transversion mutation in the third base of codon 249 of TP53 gene.

Table 4. Association between gender, and allele polymorphism at TP53 Arg72.

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Sex</th>
<th>HCC status, n (%)</th>
<th>Total</th>
<th>Fisher value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without HCC</td>
<td>HCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>Male</td>
<td>2 (50.0%)</td>
<td>2 (50.0%)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2 (50.0%)</td>
<td>2 (50.0%)</td>
<td>4 (100%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Pro/Arg</td>
<td>Female</td>
<td>2 (40.0%)</td>
<td>3 (60.0%)</td>
<td>5 (100.0%)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2 (28.6%)</td>
<td>5 (71.4%)</td>
<td>7 (100.0%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4 (33.3%)</td>
<td>8 (66.7%)</td>
<td>12 (100.0%)</td>
<td>-</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>Female</td>
<td>2 (18.2%)</td>
<td>9 (81.9%)</td>
<td>11 (100.0%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>-</td>
<td>6 (100.0%)</td>
<td>6 (100.0%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2 (11.8%)</td>
<td>15 (88.2%)</td>
<td>17 (100.0%)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5. HCC status and Codon 249 mutation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Codon 249 mutation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGG</td>
<td>AGT</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>15 (93.8%)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>HCC status</td>
<td>Acute hepatitis</td>
<td>6 (24.0%)</td>
</tr>
<tr>
<td></td>
<td>Chronic hepatitis</td>
<td>9 (76.0%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>25 (100.0%)</td>
</tr>
</tbody>
</table>

Mutation: Guanine-to-thymine transversion in the third base of codon 249 of TP53 gene.
agents as well as study size population offer explanation to the discordance observed in the reported findings between our study and European studies (Global Burden of Disease Cancer Collaboration et al., 2017). Our study found no significant association between codon 249 mutation and hepatocellular carcinogenesis (p=0.6821) at level of significance p<0.05. However, exposure to codon 249 mutation might be considered a predisposing factor for HCC (OR=0.5278; 95% CI 0.0584-4.7736). These findings are in agreement with finite data available in Taiwan, United states, Japan, Australia, Gambian and Guangxi populations (Kirk et al., 2000; Mah et al., 2011; Montesano et al., 2010; Özdemir et al., 2010; Stern et al., 2001). Likewise, array of literature is available implicating that the presence of this very mutation in HCC patients from developed countries including the United States, China, Japan and Australia is remarkably low (Bruix et al., 2011; Montesano et al., 2010; Özdemir et al., 2010).

Males were overrepresented in the mutation positive categories in patients with and without HCC. This could be ascribed to possible occurrence of faster and more severe HCC in males than females (Li et al., 2017). However, there was there was an association between the sex and mutation (Fisher’s exact test =5.47, P-value =0.039).

Counter-intuitively, TP53 codon 249 mutations were observed not only in HCC patients but also in one the non-HCC patients, this corroborates earlier findings by Kirk et al. (2000) that reported codon 249 mutation presence in 3 of 53 control subjects (6%), and those of Ozturk et al. (1994), who reported codon 249 mutations in non-malignant liver tissues. A possible explanatory analysis for this finding is that mutations to codon 249 is generally known as a hotspot for aflatoxin B1 (AFB1)-driven modification. According to Özdemir et al. (2010), AFB1 induces codon 249 mutation among cancer patients residing in AFB1 high-risk regions, where chronic HBV and HCV infections are also endemic. Furthermore, among TP53 mutations described in human cancers and compiled in the IARC TP53 mutation database, 66% occur in patients with HCC originating from regions with a high incidence of HCC and high exposure to dietary AFB1. However, we did not perform aflatoxin exposure tests for the subjects to corroborate this. Additionally, published data from the Ministry of Health and the Gastroenterology Society of Kenya on guidelines for the treatment of HBV and HCV infections in Kenya (2015) suggested that 80% of HCC cases in the country are due to chronic infection with HBV (Ochwoto et al., 2016). This evidence perhaps indicates that the existence of the mutation in TP53 may be suggestive of an early genetic event in hepatocellular carcinogenesis. Consequently, it is argued that presence of a single mutation alone in DNA is unlikely to cause cancer, rather cumulative or multiple mutations in tumor suppressor genes are required (Adjiri, 2017).

**Recommendations and limitations**

Although this study has investigated for the presence of TP53 mutation in exon 4, 6 and 7 hepatocellular carcinoma patients, there is a need to look at the remaining exons. However, this study was a cross-sectional study that involved 33 HBV-positive patients, of whom 25 had HCC, it was hard to compare the evolution of the mutations among the patients with HCC. The use of samples that only amplified the forward and reverse fragments of exon 7 and 4 could bias the mutational prevalence. The mutations reported in this study were found in samples taken from the patients’ blood and we did not obtain tumor tissues from the patients for verification.

**Conclusion**

TP53 is a gatekeeper gene, and codon 72 and 249 mutations could play a role in HCC development. However, this study did not find any association or clear mutational pattern between TP53 mutations and HCC development. Equally the existence of multiple mutations in an individual was not associated with HCC. We therefore conclude that TP53 mutation is a poor Indicator for Prognosis and tumor’s biological behavior among HBV-positive subjects in Kenya. Other TP53 mutational sites may be considered and analysis of large numbers of cases may be an alternative option to allow specific use of the gene mutations in HCC development.

**Data availability**

**Underlying data**

TP53 sequence data obtained from this study are available from GenBank, accession numbers MN119310 to MN119350.

**Grant information**

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