**RESEARCH ARTICLE**

**Human TP53 gene polymorphisms among patients with hepatocellular carcinoma and chronic hepatitis B in Kenya**

[version 1; peer review: 2 approved with reservations]

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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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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; Maituri 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 1.25mM 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 using 2% agarose (Fisher Scientific), 2 µl of 5X Gel loading dye (Qiagen Inc, USA) together with 100-bp Track DNA ladder (Invitrogen, California, US) in 1X TBE buffer containing SYBR-safe DNA gel stain (Invitrogen, California, US) and visualized using an ultraviolet trans-illuminator gel Doc-It Imager then viewed using Vision Works LS software v.7.1.

For exon 7, 5 µl of the all negative amplicons was used in the second nested PCR (forward primer exon 7b 5'-AGGGGTCAGCGGCAAGCAGA-3' and reverse primer exon 7b 5'-TGTGCAGGGTGCGCAAGTGGC-3') and the master mix of the second round profile. The amplicons were similar to the first round profile. The amplicons were viewed following electrophoresis on a 2% agarose gel.

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 Biotedit 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 $\chi^2$ 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 1. Primers used for TP53 PCR amplification and sequencing. |
|------------------------|-----------------------|
| Primer | Sequence (5’ to 3’) |
| Exon 4 forward | ATCTACAGTCCCCCTTGCGG |
| Exon 4 reverse | GCAACTGACCGTGCAAGTCA |
| Exon 6 forward | TGGTGCCAGGTCCTCAG |
| Exon 6 reverse | TGGAGGGCCACTGACACCA |
| Exon 7 forward | CTGCGCCAGGTCTCCTC |
| Exon 7 reverse | AGGGTTCAGCGGCAAAGCA |

| Table 2. Clinical and molecular characteristics of the subjects. |
|------------------------|-----------------------|
| Characteristics | Participants |
| Gender | Male 17 51.5  |
| | Female 16 48.5  |
| HCC status | HCC 25 75.8%  |
| | Without HCC 8 24.2%  |
| Codon 72 polymorphisms | Arg/Arg 4 12.1  |
| | Pro/Pro 17 51.5  |
| | Pro/Arg 12 36.4  |
| Ser 249 mutation | AGG (Arg) 25 75.8%  |
| | AGT (Ser) 8 24.2%  |
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).

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 $=5.47$, $p=0.039$) 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).

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

<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></td>
<td>Female</td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>2 (11.7%)</td>
<td>2 (50.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>4 (33.3%)</td>
<td>3 (90.9%)</td>
<td>3.58</td>
<td>0.12</td>
</tr>
<tr>
<td>Pro/Arg</td>
<td>3 (23.1%)</td>
<td>1 (16.7%)</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>

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

This work was supported by KEMRI IRG grant from Kenya Medical Research Institute, protocol Number KEMRI/SERU/CVR/001/3211 and IRG Number L057.

Acknowledgments

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References


Carvaljal LA, Hamard PJ, Tonnesen C, et al.: E2F7, a novel target, is...


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Summary of the article by Ochwoto and colleagues

The article by Ochwoto and colleagues aimed to identify polymorphisms in the human TP53 gene among Kenyan patients with hepatocellular carcinoma (HCC) and chronic hepatitis B. TP53 is a gene that encodes tumour protein p53 that controls the cell cycle (division or proliferation), thereby suppressing tumorigenesis. Mutations in the TP53 impair the function of the tumour protein p53, causing the cells to proliferate uncontrollably. Ochwoto and colleagues examined mutations in codons 72 and 249, which exit in exons 4 and 7, correspondingly. They found that there was genetic heterogeneity in the nucleotide mutations in the TP53 gene, which resulted in either synonymous or nonsynonymous mutations. Further analyses found no association between p53 mutations and HCC, suggesting that mutations in the TP53 gene may not be good biomarkers for HCC, specifically among Kenyan patients with hepatitis B virus (HBV) infections.

There is paucity of data on the polymorphisms in the TP53 gene and their association, if there is any, with HCC on Kenyan cohort. Whereas the study by Ochwoto and colleagues adds knowledge to the existing literature, I approve it with sound reservations. First and foremost, they do not sufficiently address all their objectives. For instance, they indicate that they will investigate polymorphisms in specific codons found in exons 4, 6 and 7. However, they do not present any of the results for exon 6, yet they say in the “recommendations and limitations” section that they examined this. Besides, they detail (in the “methods” section) the primers that were used to amplify and sequence exon 6. What happened to exon 6 results? What codon positions in this exon were analysed? The authors do not consistently present their results. I mean, the way they present/tabulate their results for codon 72 (in exon 4) is totally different from codons 249 (exon 7). No PCR/sequencing and healthy controls were included in their study. There are sections in the manuscripts that have not been referenced. Ochwoto and colleagues do not adequately highlight...
findings of the literature that they use – as it is not clear what some of those studies reported. For example, in the discussion section (paragraph 1), they state: “Different prevalence of this substitution has been reported in various studies.” With this, they assume that the reader is aware of what those studies reported. To address this, they should give a range of the prevalence that these studies found, so that the reader can easily compare findings presented herein with/to what is already known. Another sentence would be in paragraph 2 (discussion), where they write, “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...” Could the authors kindly briefly highlight what some of these studies found? Apart from the aforementioned concerns, there are other concerns and a few typos and grammatical mistakes that need to be corrected. For example, the last sentence in paragraph 2 (discussion) is written as: “…since most study have been performed...” Study should be in plural.

My detailed review is as below:

**Reviewer’s comments (according to manuscript section)**

**Title and author affiliations**

Could the authors add “infection” after “chronic hepatitis B” so that it reads “chronic hepatitis B infection”? The extra comma (,) in affiliation 2 should be removed.

**Abstract**

The authors say in the background that “ANY” alteration/mutation in the TP53 gene adversely affects the regulatory function of the protein, potentially resulting in cancer. Synonymous mutations do not have this effect as the amino acids are not altered. Mutations in TP53 are not always associated with altered p53. The words “mutations to” should be changed to “mutations in”. Is “codon 7” supposed to be “codon 72”? The background does not provide motivation for the study and why it was conducted on a Kenyan cohort? Is it because there is paucity of data surrounding this topic, especially in Kenya? Is it because there are high rates of recurrent liver tumours/cancers?

Remove the semi-colon (;) in the methods and put a full stop (.). Why do the authors say exons 4 and 7 were analysed yet in the manuscript they mention exon 6 too?

In the results, “12.1%,” should be “12.1%” and “Arg-Ser” should be “Arg/Ser”. They should be consistent when they present the mutations, either use forward slash (/) or hyphen (-) throughout the manuscript.

Part of their conclusion in the abstract is not supported by their findings. The authors’ study is a cross-sectional one and test causal roles, it can only show an association between the polymorphism and HCC. It is thus inaccurate to say that the “mutations under study may dependently play a role in HCC development”. Moreover, they did not examine if other factors contributed to HCC development and whether the mutations they detected co-occurred with other mutations. The authors write, “TP53 mutation is a poor indicator for prognosis and a tumor’s biological behavior among HBV-positive subjects in Kenya.” The cross-sectional nature of their
study does not permit them to assess this. They did not use a longitudinal study to study the mutation outcomes. In addition, they did not have information on tumour staging, response to treatment, survival rates (in comparison with healthy controls), vascular invasion (blood and/or lymph vessel invasion, LBVI), and Child-Pugh score. If Ochwoto and colleagues needed to examine the role of TP53 mutation on HCC development and whether these mutations could be indicators for prognosis, then they should have i) utilized a longitudinal study with important participant information, and ii) assessed where the mutations acted dependently (synergistically with other TP53 mutations) or independently to promote carcinogenesis in HCC. My last comment on the abstract, should it be “P53” or “p53” in the conclusion?

Keywords

Why is “codon 249” included as keyword but “codon 72” is not? I am also curious why “p53 exon 6” was not included yet other exons were, despite the fact that the authors indicate that they examined mutations in codons in exon 6.

Author roles

All authors are supposed to approve the final version of the manuscript. Based on the author contribution section, it appears like Maiyo AK and Chesumbaio G did not (review and edit the manuscript); hence, did not approve the final draft of the manuscript. Could the authors clarify if this was the case?

Abbreviations

There are abbreviations in the manuscripts that need to be included in this part. For instance, “AE” buffer, “DNA”, “HBsAg”, “CHB”, “AFB1”, and so on.

Introduction

Paragraph 1: There should be a comma after “Global Burden of Disease Cancer Collaboration et al. (2017)”.

Paragraph 2: “Alteration to” should be “Alteration in”. The last sentence in this paragraph needs to be referenced as this is a fact that is being stated.

Paragraph 3: The first sentence is relatively long and needs to be split into two, that is, put as two sentences. This sentences need to be referenced: “In exon 7, codon 249 (AGG→AGT, arginine to serine) has been identified as a “hotspot”.” and “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.” In the latter sentence, “Proline” should appear as “proline”. In addition, the authors say that “codon 72 has been investigated as risk modifier in several cancer models”. What did these investigations find? Was there an association? Do their investigations suggest that mutation (Arg to Pro substitution) in codon 72 plays a role in cancer development? Or did the authors intend to say that those studies found mutation in codon 72 (Arg to Pro substitution) to be a risk factor for cancer?

Paragraph 4: there should be a comma after “In this study”. Although in paragraph the authors
write, “TP53 has ten coding exons, with mutations distributed in all of them, with a strong predominance in exons 4-9,...” it is unclear why the authors chose to study mutations in only exons 4, 6, and 7 (as they state in paragraph 4). Why did they not examine exons 8 and 9? Is there another reason besides limited information on TP53 polymorphisms in Kenya that motivated the authors to conduct their study in Eldoret, Kenya?

Methods

Study site and sample population: There is no need to write MTRH in full as this has been done before (in the last paragraph in the introduction section). The sentence “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).” should come immediately after the first sentence. Regarding this sentence, the authors should state the rates of HBV infection that have been reported at MTRH. They should not assume that anyone reading the current paper is aware of this. The second sentence needs to be rephrased. Do the authors mean that they included only jaundice patients and that these patients were HBV-positive with and without HCC? It is not clear if the study by Ochwoto and colleagues is a cross-sectional one or a longitudinal study. They mention that after the patients were selected from the medical records, they “were then recruited in person.” Were the patients called and asked to participate in this study? Was it after this that samples were collected? The design of the study (whether cross-sectional or longitudinal) is somewhat confusing. If indeed it is a cross-sectional study, they should clarify and even acknowledge the fact that this was a retrospective study that depended on stored samples. The redundancy in the section, for instance, twice-mentioning that the study selected patients with jaundice, should be addressed/eliminated. “Eldoret hospital” should be “Eldoret Hospital”, and “age range were” should be “age range was”. What were the exclusion criteria in their study? Finally, the authors mention that information on “residential area” was abstracted from the health records. In spite of this, they do not use this information in their analyses. This information is not reported in their manuscript.

Ethical consideration: “The ethical approval to conduct the study was obtained from Institutional Research and Ethics Committee (IREC) 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).” should be changed to “The ethical approval to conduct the study was obtained from the Institutional Research and Ethics Committee (IREC) of MTRH/Moi University (approval number 001002), from the Kenya Medical Research Institute Scientific Ethics Review Unit (approval number KEMRI/SERU/CVR/001/3211) and from the Eldoret Cancer Registry (approval number ECR/DRA/2017/001).” In line with my comments above (regarding the nature of the study), the authors say that “the participants consented for the study prior to blood draw.” The consent that the patient provided, was it an informed one? Was it a verbal or written consent? Were/Are the patients aware that this study in particular was being conducted? Did they receive any compensation/incentive?

Collection and preparation of blood samples: The statement “Plasma was separated at MRTH and thereafter the plasma tubes were shipped on dry ice to the KEMRI Production Unit in Nairobi.” could be written as “Plasma was separated at MRTH and then shipped in plasma tubes on dry ice to the KEMRI Production Unit in Nairobi.”
Serological testing: Remove the extra hyphen in “IgM--positive”.

Extraction of DNA from plasma: Information on the source of the reagents (e.g., DNA mini-extraction kit) or equipment (e.g., NanoDrop spectrophotometer), which is bracketed, need to be comprehensive. Apart from including the name of the company, name of the city and country is required.

PCR amplification of human TP53: The authors did not include any controls (negative and positive). How sure are they that their method is valid and that they did not get contamination during PCR? In the paragraph 1 of this section, “the extracts” should be written “the DNA extracts”. Moreover, there should be a space between “5” and “µl” so that it becomes “5 µl” instead of “5µl”. This statement “…of forward primer and reverse primer of sequences…” could be written as “…of forward and reverse primers...”. Table 1 could be put as part of the supplementary data. I am just curious, were the same primers used in PCR the same as those used for sequencing (putting into consideration that the authors mention there was an aspect of nested PCR)? Still in paragraph 1, “and” should be included between “… USA),” and “1.25 µl”. In paragraph 2, what is the model of the ABI PCR machine that was used? This sentence, “Amplification for exon 7 the PCR profile set at 95°C for 10 minutes initial denaturation and 35 cycles of denaturation...” does not make sense. It needs to be corrected. Both the statements, “Final extension was at...” should be written as “Final extension was performed at...” Still regarding this paragraph (2), why did the authors opt not to give the PCR details for exon 6? The sentence in paragraph 3 is long and needs to be written as two or more sentences. “5X Gelpilot DNA loading” should be “5X Gelpilot DNA Loading”. For paragraph 4, why did the authors use “negative amplicons” in the second nested PCR? Is “negative amplicon” not a failed experiment in their context? Why was this not performed for exons 4 and 6? Lastly, throughout this section (and the rest of the manuscript), the authors should include the information on the source of the reagents or equipment (which is bracketed) need to be comprehensive. Apart from including the name of the company, name of the city and country is required. This information should be furnished where appropriately, if possible even for the ultraviolet trans-illuminator gel Doc-It² Imager, 5X Gelpilot DNA Loading Dye.

DNA sequencing: The authors need to state that they used Sanger sequencing. There should be an apostrophe (’) in the word “manufacturers”. It should be: manufacturer’s and not manufacturers. “send” should be changed to “sent”. There should be a full stop at the end of this paragraph.

Mutation detection and analyses: Is there any reason include the sentence “PCAP is an open-access alternative”? The authors should be specific about the BLAST they used. Was it BLASTn or BLASTp or both? “Bioedit” and “mutations to the” should be changed to “BioEdit” and “Mutations in”, respectively. Delete these words, “bioinformatics editing tool”. Lastly, about this section, the authors must reference BioEdit and MEGA software.

Statistical analyses: What do the authors mean by “profile parameters”? The last sentence about SAS version 9.4 can be first sentence in this section. It is not clear what they mean by, “used to analyze two significant associations”? Did all the expected values in the 2x2 contingency tables warrant the use of Fischer's exact text? Were all the expected value >5? If this was not the case, then Chi square test should be used in such scenarios. Odds ratios are used to measure the strength/magnitude of association. “P-values less” should be “P-values of less”.

Results
Participant demographics: The authors do mention exon 6 yet these results are hugely lacking. No results regarding this are presented. Can the authors please provide the results for this? The sentence “Among those (24.2%) that did not have HCC but were HBsAg positive, half were female and the other half male.” should be written as “Among those that did not have HCC but were HBsAg positive (24.2%), half were female.” The title for Table 2 is not accurate. While the title says “clinical” and “molecular” characteristics, the variable “gender” does not fit in any of that (clinical or molecular). Gender is a demographic characteristic. The symbol % should be deleted in the numbers (e.g., 75.8%) in the last column as this (5) is already put in the column title. At the bottom of this table, ALL abbreviations (e.g., HCC, Arg, etc.) should be written in full (e.g., hepatocellular carcinoma, arginine, etc.). The same is required for Tables 3, 4 and 5. Furthermore, the numbers in Table 5 need to have spaces between them and opening brackets, e.g., it should be “6 (24.0%)” and not “6(24.0%)”. This should be done for the other numbers, where necessary. The comma in the title for Table 4 should be removed.

TP53 exon 4, codon 72 polymorphism analysis: Paragraphs 2 and 3, the authors display the Fisher test value at differing decimal places; some are at 2 decimal places while others are at 1 decimal place. They must ensure that they are consistent. The same is true for the other sections entitled: “Mutations at exon 7, codon 249” and “Codon 249 mutation and HCC”. Check the number of decimal places for the p-values and CIs, throughout the manuscript (even in the tables). Paragraph 2 (of section titled: TP53 exon 4, codon 72 polymorphism analysis), there should be a full stop after “p=0.12)”. The next sentence should then be case sensitive (“However”). The last sentence of this paragraph, “The Pro/Pro allele was more frequent in the HCC group, whereas all patients with Arg/Arg alleles did not have HCC (Table 4).” needs to be changed/revised. First, there is a typo (“with” is written as “wth”). Secondly, the statement is untrue. I see that there are HCC patients (2 males) who had Arg/Arg alleles. For Figure 1, it is not relevant to have a 3D-plot as the third dimension is meaningless. My last comments on results: In order to ensure consistency, the authors should decide if they want to write “P=value”, “p=value”, or “P-value”. Moreover, they should decide on whether to include space before/after the equal sign (=). I see in one place they have “Fisher test=3.58’ while in another they have “Fisher’s exact test =5.47”. The other thing, is it “Fisher test” or “Fisher’s exact test”. Ochwoto and colleagues do not consistently present (tabulate) their results for the mutations found in the codons that they studies. No results are presented for exon 6. Moreover, the way they presented the results for codon 72 is considerably different from those of codon 249. Why did they opt for this strategy?

Discussion

Paragraph 1: They indicate that to the best of their knowledge, this is the first time information concerning TP53 exons 4, 6, and 7 are presented in Kenya. They should say that this is among HBV-positive patients with and without HCC, as there are other investigators, who studied exons 6 and 7 (though in the context of oral squamous cell carcinoma). Ochwoto and colleagues do not present results for exon 6 yet they mention this exon in their discussion. They also have the sentence “A number of studies have described two structurally different forms of wild-type p53…”, yet they end up providing only one reference (Thomas et al., 19994).” There should be additional references then. This paragraph has a typo (“study” written as “tufy”). The prevalence values (of the specific mutations in different cohorts, e.g., Taiwanese, Chinese, etc.) should be provided. This should also be done for the part where they say, “Different prevalence of this substitution…”. A range should be provided in this case. This sentence “In this tufy, the HBV-positive Kenyan
population, the homozygous Pro/Pro genotype is...” could be written as “In our present study, the homozygous Pro/Pro genotype was...”. The last sentence of this paragraph, they say “higher frequencies”, but they do not indicate what they compared these frequencies with. Higher than which comparison group? There should be a comma after “11.8%” and the abbreviation “Vs” put as “vs”.

Paragraph 2: The comma towards the end of the second sentence (“with cancer risk,” should be removed (and he words be “with cancer risk”). The next sentence, which starts with “In Egyptian patients...”, should have a full stop at the end, not a comma. It is also not clear if Ochwoto and colleagues are referring to their present results or other’s results when they state, “This study did not find any association between polymorphisms and HCC among the HBV-positive patients.” The authors should also give a few details about the studies by Eskander et al., 2014 and Hu et al., 2014. Otherwise, the reader is left to wonder what the other studies found when they simply say, “Other studies have shown similar findings”. The last sentence of this paragraph should have the words “most study” changed to “most studies”. Just to add on some of the reasons for inconsistencies in study findings, I believe heterogeneity in study methodology, sample (population) size, and specimen type could at least partly be contributing factors.

Paragraph 3: The authors should express the 8/33 as a percentage and go ahead and give us the exact rates that were reported on the cohorts from China, Taiwan and The Gambia. This sentence “However, evidence presented from a European population, which reported no mutation, is contrary to our findings (Kirk et al., 2000).” needs to be rewritten. This study, done on an Egyptian cohort, should be included in this section and findings on rates of mutants in codon 249 reported. Another study would be this, which found no mutation in codon 249 among HCC Korean patients. In manuscript by Ochwoto and colleagues, the part written, “offer explanation to” could be written as “could explain” as it is not certain what resulted in the discordance. Delete “(p=0.6821) at level of significance p<0.05”. Personally, I disagree with the fact that the authors think that based on the odds ratio (OR=0.5278; 95% CI 0.0584-4.7736), codon 249 might be a predisposing factor for HCC. The OR value do not support that as the values range from 0 to over 1. OR >1 indicates increased occurrence of an event whereas OR <1 indicates decreased occurrence of event (protective exposure).

Paragraph 4: The sentence could begin with, “We further found that males were overrepresented...”. The last sentence (However, there was there was an association between the sex and mutation (Fisher’s exact test =5.47, P-value =0.039) needs to be rectified – as “there was” is repeated. The information on statistics (Fisher’s exact test =5.47, P-value =0.039) should be deleted as they are already in the results section.

Paragraph 5: The authors need to present the rates (prevalence) rather than numbers so that their values can be compared to what is in the literature. This sentence (“TP53 codon 249 mutations were observed not only in HCC patients but also in one the non-HCC patient”) should have the prevalence so that the rates can be easily compared with/to others. Change “this corroborates earlier findings” to “thus, corroborating earlier findings”, “mutations to” to “mutation in”, and “A possible explanatory analysis...” to “A possible explanation...”. The authors mention that mutations in codon 249 are generally known as a hotspot for aflatoxin B1 (AFB1)-driven modification, and that AFB1 induces codon 249 mutation among cancer patients residing in AFB1 high-risk regions, where chronic HBV and HCV infections are also endemic. While they acknowledge that they did not perform aflatoxin exposure tests on their subjects, what makes them speculate that AFB1
could have an impact on their cohort? Can the authors please comment on the levels of aflatoxins in the region, Eldoret? Do the authors know if there are certain foods in the region that have AFB1 levels beyond the tolerated level? Do the authors think that other factors such as alcohol and smoking could have a similar influence on TP53 as AFB1?

Recommendations and limitations

In this section, they mention exon 6, but no results were presented. The sentence “Although this study has investigated for” should be “Although this study investigated”. The sentence “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.” could be written as “The cross-sectional nature of the study limited our analysis. Thus, we were unable to perfume any analysis to determine the evolution of the TP53 mutations among the patients with HCC.”

Conclusion

I tend to disagree with the authors’ conclusion as they do not write this section based on their findings. Their study was not a longitudinal one, which could/would enable them assess the role of polymorphisms (codons 72 and 249) in HCC development. Further, their study did not show that these mutations are poor indicator for prognosis.

References

The authors need to present up-to-date information. Of all there >30 references, there is none for 2019, while there are only one, two, and three for 2018, 2017, and 2016, respectively. The rest are for the previous years, prior to 2016.

Other comments

- No PCR and sequencing controls were included in the study. Therefore, how sure are the authors that there were no contaminants in their study? How sure are they that they targeted the right gene? The authors do not mention anything about the size of the amplicon that they expected and whether they observed it.

- No healthy human controls were included. Moreover, there was no information on tumour size, histopathological features, cancer staging, and treatment outcome. Thus, it is hard to infer speculate on the role of the TP53 mutations in their study.

- The authors should not be too quick to generalize results as their sample size was relatively small, besides their study having other limitations.

- The authors did not adjust their analysis for multiple comparisons. Moreover, they do not acknowledge other confounders.

- The method they used for amplification and sequencing may have resulted in their ability to capture other mutations. They could have confirmed their findings using droplet digital PCR (ddPCR), restriction fragment length polymorphisms (RFLP), or polymerase chain reaction-
single-strand conformation polymorphism (PCR-SSCP).

- What could be the implications of the TP53 mutations that the authors identified in their study? The implications should relate to their study cohort/population.

- The study cohort/population is not sufficiently described. There is less information about the study cohort/population. Do the authors know the HIV status of the cohort? Other information that would have been relevant and important in their discussion would be HBV genotypes, other potential cancers, hereditary disorders and so on.

- The results by Ochwoto and colleagues do not have the electrophoresis results. I recommend that they provide the results for band visualization?

- The authors have information about the age of the study participants. One study on a small cohort of Hispanics\(^7\) associated TP53R249S mutation with a younger age (besides worse prognosis). The work by Ochwoto and colleagues has the potential to add more knowledge if they could stratify their participants by age (young vs old) and then examine if there is any association of the TP53 mutations with age.

- To strengthen this finding (in the discussion) where they say “Our study found no significant association between codon 249 mutation and hepatocellular carcinogenesis...”, I suggest that they include this paper\(^8\) which does not support their findings. The suggested article found a strong association between TP53R249S in plasma and HCC Qidong patients.

References

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Microbiome, Sexually Transmitted Infections, and Cancer

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 October 2019

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**General**

In this manuscript, Ochwoto *et al.* investigate mutations in TP53, among patients with hepatocellular carcinoma (HCC) and chronic hepatitis B virus (HBV) infection at the Moi Teaching and Referral Hospital (MTRH), Eldoret, Kenya. They conclude that TP53 mutation is a poor indicator
for prognosis among HBV-positive persons in Kenya, due to a lack of association between TP53 mutations and HCC development.

**Comments to Authors**

In general, the manuscript owns some degree of novelty and clarifies previously existing hypothesis on the relationship between TP53 mutations and HCC. However, the study seems to have some glaring omissions with methodology and the results. The authors aimed to evaluate TP53 mutations in exons 4, 6 and 7, but there were no results shown for exon 6. Even though, this study didn't find a significant relationship between TP53 mutations and HCC development in Kenya. It would have been ideal to include healthy subjects as part of the controls since previous studies have shown the presence of TP53 mutations in healthy cohorts albeit at much lower frequencies to HCC subjects, thus, it is difficult to make any inferences due to the lack of such controls. An interesting part of this study relies on the sequencing results, am referring to mutation detection and analysis. Accordingly, I think that the study would have greatly benefited from some experimental validation of this data e.g. Restriction endonuclease to validate the mutations. There are also no supporting results depicting the presence/lack of the said mutations i.e. Gel electrophoresis images and sequence electropherograms. Thus, making reproducibility of this work partly possible in validating the conclusions made.

**Minor Points**

Omitted results/typographical errors:
- Study site and sample population, Page 3, line 6 “HBV or HBV” delete one.
- Serology testing, Page 3, line 2 “were was” correct the sentence.
- PCR mastermix, page 4, give the genomic DNA template concentration as opposed to a generic quantity.
- DNA sequencing, page 4, line 5 “were send” edit to were sent.
- Table 1, include the expected band sizes or mention these in the methodology.
- Include Exon 6 results.
- Figure 1 legend, not detailed and descriptive.
- Results, page 5, lines 2-3 delete word “more”.
- Results, page 5, paragraph 1 last line. The authors state “....all patients wth Arg/Arg alleles did not have HCC....” But both tables 3 and 4 show the presence of 2 (50%) HCC subjects with the polymorphisms.
- Codon 249 mutation and HCC, section is not clear, check for grammatical errors.
- Discussion, Page 6, line 3, no exon 6 results.
- Discussion, Line 9, “tufy” edit to study.
- Discussion, Paragraph 2, line 9, “most study” edit to most studies.

**Summary**

This manuscript provides novel interesting data on TP53 mutation and HCC in Kenya. Subject to addressing the concerns raised, this work is scientifically sound and worthy of indexing.
Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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