MOLECULAR ANALYSIS OF *P53* GENE EXON 7 CODON 249 MUTATION IN HEPATOCELLULAR CARCINOMA PATIENTS PRESENTING AT MOI TEACHING AND REFERRAL HOSPITAL, KENYA

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

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RECOMMENDATION

This thesis has been submitted with our approval as supervisors for examination according to Egerton University regulations.

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DEDICATION

This thesis is dedicated to God Almighty, my strong pillar and source of inspiration that graciously granted me wisdom, knowledge and understanding throughout this programme.

I also dedicate this work to the memory of my greatly loved parents: Elsa Adek Nyotata and Sospeter Mirowa who together went against all odds to see me acquire education and as well gave me the vision in life and the principle of hard work and dedication.

Finally, this work is dedicated to my siblings Raymond Oduma and Quinter Oduma who offered me unflagging support and whose encouragement made sure that I gave it all it takes to finish that which I had started.

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ABSTRACT

Hepatocellular carcinoma (HCC), a common type of liver cancer arising from progressive transformation of pre-cancerous dysplasia macronodules and hepatocytes is ranked as the fourth leading cause of death globally and a fifth cause of death in Africa. Several risk factors for HCC have been identified that include dietary and life style risks, significant genetic family history and predisposition to genetic mutation. It has been hypothesized that codon 249 of *p53* gene is more susceptible to mutation induced by mutagenic and carcinogenic agents, increasing risk and/or progression of HCC. This study sought to identify mutation in codon 249 among HCC patients presenting with stage one cancer at Moi Teaching and Referral Hospital (MTRH), and to further determine the association of the mutation with the development of HCC. A total of forty six (46) archived blood samples for HCC patients and ten (10) controls were used in the study. DNA was extracted and purified from 200 uL aliquots of plasma and PCR amplified then sequenced using *p53* exon 7 forward and reverse primers. Mutation detection and analysis were done using Molecular Evolutionary Genetics Analysis v.6.0 and ESPript v.3.0 softwares. The male to female ratio for both patients and controls was 1:1. The age range for HCC patients was from 25 to 67 years with a median of 42 years, and from 24 to 64 years with a median of 41 years for the controls. Guanine (G) - to - thymine (T) transversion in the third base of codon 249 of *p53* gene was detected in plasma DNA from 8 of the 46 HCC patients and 1 of the 10 controls. There was no significant difference across gender among HCC subjects with and without mutation (p=0.4549) at 5% level of significance, however, there was a striking picture of significant existence of such mutation in a much older population in the HCC patients (p=<.0001) at 5% level of significance, suggesting that being in the old age is more susceptible to such mutation. There was no significant statistical association of codon 249 mutation between HCC patients and control (p=0.6821) at 5% level of significance. However, there was exaggerated increase in risk of acquiring codon 249 mutation among HCC patients (OR=0.5278: 95% CI 0.0584-4.7736). Consequently, although *p53* gene codon 249 mutations has been found very minimal, its existence communicates a probable role in hepatocellular carcinogenesis. Nevertheless, the present study cannot exclude the possibility that mutation in codon 249 may act at later stages of hepatocellular carcinogenesis. This study warrants supplemental cross sectional and longitudinal studies, using larger sample sizes with higher number of HCC patients presenting with different stages to observe pattern across stages.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Adenine
AD1	Transactivation Domain 1
AD2	Transactivation Domain 2
ADP	Adenosine diphosphate
AFB1	Aflatoxin B1
AJCC	American Joint Committee on Cancer
AKT	protein kinase B
Ala	Alanine
Arg	Arginine
BER	Base Excision Repair
BLAST	Basic Local Alignment Search Tool
BLCL	Barcelona Clinic Liver Cancer
Bp	Base pair
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
C	Cysteine
CI	Confidence Interval
CITI	Collaborative Institutional Training Initiative
CLIP	Cancer of the Liver Italian programme
CTD	Carboxy-terminal domain
C-ter	Carboxy-terminus domain
CTNNB1	β-catenin
Cys	Cystidine
DBD	DNA Binding Domain
DNA	Deoxy-ribonucleic Acid
DSB	Double Strand Break
DSBR	Double Strand Break Repair
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

ERK	Extracellular signal-regulated kinase
FGF	Fibroblast Growth Factor
G	Guanine
G0 phase	Dormant phase
G1 phase	Gap1 phase
G2 phase	Gap 2 phase
GLOBOCAN	Global cancer incidence, mortality and prevalence
Gly	Glycine
GOF	Gain of function
HBV	Hepatitis B Virus
HBx	Hepatitis B virus x protein
HCC	Hepatocellular Carcinoma
HCL	Hydrochloric acid
HCV	Hepatitis C Virus
HGF	Hepatocyte Growth Factor
His	Histidine
HPV	Human papilloma virus
HR	Homologous Recombination
IARC	International Agency for Research on Cancer
ICL	Interstrand crosslink
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin-like Growth Factor
IGV	Integrative Genome Viewer
JAK	Janus kinase
KCR	Kenya Cancer Report
KEMRI	Kenya Medical Research Institute
lncRNAs	Long non-coding RNAs
M phase	Mitosis phase
Mdm2	Mouse double minute-2
Mdm4	Mouse double minute-4
MDSCs	Myeloid derived suppressor cells

MEGA	Molecular Evolutionary Genetics Analysis
MEK	Mitogen activated protein kinase
MgCl2	Magnesium Chloride
miRNA	Micro ribonucleotide acid
mTOR	Mammalian target of rapamycin
MTRH	Moi Teaching and Referral Hospital
NCBI	National Center for Biotechnology Information
NCCS	National Cancer Control Strategy
NER	Nucleotide Excision Repair
NES	Nuclear Exclusion Signal
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Signal
OH	Hydroxyl group
Oli	Oligomerization
OR	Odds Ratio
p53	Functional Tumour suppressor protein 53
PAH	Polycyclic Aromatic Hydrogen
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-3-kinase
PRO	Proline
RE	Response Elements
RNA	Ribonucleic acid
RTKs	Receptor tyrosine kinases
S phase	Synthesis phase
ssDNA	Single strand deoxy-ribonucleic Acid
STAT	Signal transducer and activator of transcription
SV	Simian Virus
T	Thymine
Taq	Thermus aquaticus
TBE	Tris Borate ethylenediaminetetraacetic acid
TERT	Telomerase reverse transcriptase,

TGF	Tumor growth factor
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
TLS	Translesion synthesis
TNF	Tumour necrosis factor
TNM	Tumour Node Metastasis
Tregs	T-regulatory cells
UV	Ultraviolet rays
WCR	World Cancer Report
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background information

Cancer is a disease characterised by uncontrolled cell division, and the cells may invade other tissues based on localisation or regional spread. The disease arises from a single cell, with the transformation from pre-cancerous lesion to malignant tumours (Sasaki *et al.*, 2011). Cancer cells are very similar to the cells of the organism of origin and too have similar (but not identical) DNA and RNA (Smelser *et al.*, 2015). Due to these differences in identity, the cancerous cells are not usually detected by the weakened immune system (Quail & Joyce, 2013; Nars & Pelletier, 2012). The changes leading to manifestation of malignancies may arise from interaction between an individual's genetic factor and external agents, with ethnicity, lifestyle, gender and age being considerable risk factors for adult onset primary malignant tumours (Ferlay *et al.*, 2012). According to World Health Organisation (WHO, 2017) and International Agency for Research on Cancer (IARC, 2017), cancer is classified depending on tumour characteristics, assessment of organ involved dysfunction and patient characteristics. Generally, cancers have four stages, designated I-IV that describes the size of a cancer and how far it has grown. The staging is important in informing on treatment choice (Yim *et al.*, 2018; Zhou *et al.*, 2018; Maida *et al.*, 2014).

Cancers are among the leading causes of death worldwide. According to WHO (2017), cancer is ranked as the second leading cause of death globally. A study by GLOBOCAN (2012) showed that there were about 14.1 million new cancer cases and 8.2 million cancer deaths reported across the globe. The study further indicated that incidences of cancer increased from 12.7 million in 2008 to 14.1 million in 2012, and this trend was projected to continue with more number of new cases expected (WHO, 2017). The greatest impact of this rising trend occurs in low and middle-income countries that are ill-equipped to cope with the escalation of a number of cancer cases (Ferlay *et al.*, 2012; Sasaki *et al.*, 2011). According to World Cancer Report (2014), more than 60% of world's total new cancer cases occur in Africa, Asia, Central and South America. In Kenya, the disease is the third leading cause of deaths (NCCS, 2016).

Hepatocellular carcinoma (HCC) is a common type of liver cancer arising from progressive transformation of pre-cancerous dysplasia macronodules and hepatocytes. It is one of the most common malignancies worldwide, with limited treatment options. HCC accounts for

approximately 85% of liver cancers cases and is characterised by a highly heterogenetic pathogenesis, with an aggressive clinical course leading to poor survival (Sia *et al*., 2017; Testa *et al*., 2017). Incidences are increasing and very high fatality rate of 800,000 deaths globally per annum observed (Stewart *et al*., 2016). According to WHO (2017), it is the fourth leading cause of cancer-related deaths in the world and fifth in Africa. The disease comprises one of the most common malignancies globally, and is the fifth most common cancer in men globally, and seventh among women, with over 600,000 new cases diagnosed annually worldwide (Stewart *et al*., 2016). According to Global Burden of Disease Liver Cancer Collaboration (2015), incidence of HCC varies broadly worldwide between geographical regions, with some of the highest incidence rates found in Asia and Africa (Akinyemiju *et al.,* 2017). Notably, there is dearth of information on the overall incidences of HCC in Kenya. This is occasioned by lack of national repository for cancer cases and the few available setting-based cancer registries.

The pathogenesis of HCC is a multistep process that involves dysregulation of diverse molecular and cellular signalling pathways that varies and depends on the specific etiologic factors including alterations on genetic, epigenetic, regulation of oxidative stress, inflammation, tumour microenvironment, cancer stem cell, cancer metabolism, etiologic integration, transcriptomic and immunity (Sia *et al*., 2017; Testa *et al*., 2017; Ho *et al.,* 2016). The transformations are characterised by alteration/mutation in signalling pathways, among them is *p53* pathway (Kandoth *et al*., 2013). Other signalling pathways involved in pathogenesis include regulating growth factor signalling involving insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF). Also, cell differentiation signalling pathways such as Hedgehog and Notch pathways, and pathways related to angiogenesis such as the vascular endothelial growth factor are implicated in the pathogenesis of HCC (Sia *et al*., 2017; Testa *et al*., 2017).

Further to *p53* role in tumour suppression and early event, alteration in *p53* gene play vital role in HCC pathogenesis alongside Catenin Beta 1 (*CTNNB1*) gene (Sia *et al.,* 2017; Ho *et al.,* 2016). The gene hampers progression of cell cycle if the DNA is damaged. This it does through (i) preventing the replication of damaged DNA while activating the transcription of proteins taking part in DNA repair and (ii) triggering the cell to induce apoptosis, cell cycle arrest or senescence in the event the damage is severe and cannot be repaired (Kruiswijk *et al.,* 2015; Sasaki *et al.,* 2011).

Alteration in *p53* gene affect major regulators of various signalling pathways involved in tumour suppression. The alterations have been reported in almost all cancer types (Kandoth *et al.,* 2013; Levine & Oren, 2009), and are majorly as a result of inactivation of *p53* gene by mutation. Studies have demonstrated that mutant *p53* contribute immensely to replication of damaged DNA and successive tumour progression (Alexandrova *et al.,* 2013; Rivlin *et al.,* 2011). The mutant proteins bind to *p53* response elements thereby weakening the process of DNA repair and *p53*-mediated apoptosis (Luis *et al.,* 2012; Maiuri *et al.,* 2010).

There exists six major hotspot codons of the *p53* gene that constitute the most frequently mutated residues in majority of human cancers namely (but not limited to) codon 248 (248^{Arg}), codon 273 (273^{Arg}), codon 175 (175^{Arg}), codon 245 (245^{Gly}), codon 249 (249^{Arg}), and codon 282 (282^{Arg}) (Muller & Vousden, 2014; Kandoth *et al.,* 2013). It is established that codon 249 is an important hotspot for aflatoxin B1 (AFB1) modification and AFB1-induced mutation for patients residing in high-risk regions, where chronic hepatitis B virus (HBV) as well as hepatitis C (HCV) infections and exposure to dietary aflatoxin B1 are endemic (Özdemir *et al*., 2010; Aldona *et al*., 2009). Excessive exposure to AFB1 together with HBV infection is suggested to be a contributing factor to HCC (Hoffmann *et al*., 2018). It has been hypothesized that codon 249 arginine is more susceptible to degradation by mutagenic and carcinogenic agents, increasing risk and/or progression of HCC (Kancherla *et al*., 2018; Kandoth *et al*., 2013).

Although HCC surveillance programmes have been put in place in specific high-risk populations, early diagnosis remains a challenging task owing to low sensitivity of current screening methods (Bruix & Sherman, 2011). Unlike some other cancers, HCC has no promising molecular marker that have been incorporated into clinical management. Previous studies have shown disparities in *p53* gene exon 7 codon 249 mutation(s) in relation to cancer types and geographical locations (Wen *et al*., 2016; Kandoth *et al*., 2013). Moreover, such studies have not been explored among Kenyan patients with HCC despite its limited treatment options. Undoubtedly, differences in geographical location among other factors have varied impact on *p53* mutation profiles. Thus, this study sought to determine the mutation profile of *p53* gene exon 7 codon 249 among HCC patients presenting with stage I at Moi Teaching and Referral Hospital (MTRH), Kenya. Notably, studies have depicted *p53* gene exon 7 codon 249 to be amenable to mutational gene effects in HCC patients (Kandoth *et al*., 2013; Mah *et al*., 2011), thus warranted investigation in our study.

1.2 Statement of the problem

During phases of cancer progression, alteration of the signalling pathways involved in tumour regulation rank high among risk factors for enhanced cancer manifestation, with majority of these alterations being largely as a result of mutations of genes involved in various regulatory mechanisms including *p53* tumour suppressor genes. Initial detection, diagnosis, clinical management and prognostic evaluation of cancer is challenging because of its asymptomatic nature in the onset stages. Unlike some other cancers, HCC has no promising molecular marker for early stage detection that is crucial in its clinical management. It is known that mutations in *p53* gene contribute to the disease, and hence these changes can be good markers for the disease. This study therefore sought to investigate mutation(s) in *p53* gene exon 7 codon 249 in HCC patients from Kenya.

1.3. Main objective

To determine *p53* gene exon 7 codon 249 mutation(s) associated with HCC cases in Kenya.

1.3.1 Specific objectives

1. To identify exon 7 codon 249 mutation(s) in *p53* gene among hepatocellular carcinoma (HCC) patients presenting with stage I at Moi Teaching and Referral Hospital (MTRH) in Kenya.
2. To determine the association between the identified codon 249 mutations with stage I HCC cases at MTRH.

1.4 Hypotheses

1. There is no exon 7 codon 249 mutation(s) in *p53* gene among HCC patients presenting with stage I at MTRH in Kenya.
2. There is no association between codon 249 mutations with hepatocellular carcinogenesis in HCC patients presenting with stage I at MTRH.

1.5 Justification of the study

Despite significant recent advances in biochemical, molecular, immunohistochemistry, histopathology and imaging techniques for HCC diagnosis, detection of early stage HCC, that is asymptomatic, is still a challenge. Further, unlike some other cancers, HCC has no promising molecular marker that have been incorporated into clinical management.

Most of the HCC diagnosis are done at advanced stage that is characterised by limited treatment options. This is attributed to existence of HCC tumour heterogeneity and lack of entirely

high specific and sensitive markers for early stage HCC detection (Bruix & Sherman, 2011). For instance, use of liver biopsy specimens for diagnosis of HCC poses challenges due to heterogeneity of HCC and difficulty to confirm hepatocellular differentiation in some cases since different areas of same tumour may show varied growth patterns and levels of differentiation (Quaglia, 2018). Additionally, majority of existing immunohistochemical markers for HCC diagnosis are not highly specific nor sensitive. These markers can be expressed by other cell lineages and in other tumours presentation, and tend to wane in poorly differentiated HCCs (Wang & Thorgeirsson, 2014). Due to these hurdles, diagnosis of HCC often take longer time than expected, precluding early treatment hence resulting in poor survival and increased mortality. There is need to investigate suitable markers for early stage HCC detection to complement existing screening methods for timely management of HCC to reduce the high mortality rate resulting from HCC.

Detection of disease-specific mutations leverages great promise for cancer management (Tung & Garber, 2018; Rahman, 2014). According to HCC alliance (2017), cancer treatment is changing, with treatment becoming more personalised based on testing cancer specimen for genetic characteristic unique to the person and the cancer. This is done by testing for the type of mutation existing in cancer cells (Niravath *et al.,* 2016; Stratton, 2011; Gayther & Pharaoh, 2010).

This study was therefore centered on codon 249 because of its probable role in hepatocellular carcinogenesis, its amenability to gene mutation predisposition and suggested early event involvement in hepatocarcinogenesis (Kandoth *et al*., 2013). The study used samples from patients with stage one HCC, the initial stage of hepatocellular carcinogenesis to better understand an earlier onset picture of the codon 249 mutation profile. Noteworthy, the patients had not received any HCC treatment, this was important to arrest perturbation in the HCC molecular pathogenesis that is associated with use of drugs including sorafenib, nutlins and nexavar (Azer, 2018; Meng *et al.*, 2015).

CHAPTER TWO
LITERATURE REVIEW

2.1 Cancer epidemiology

Cancers are diseases due to uncontrolled proliferation of cells that in some cases migrate to other tissues. The diseases are leading causes of death worldwide. According to the World Cancer Report (WCR) released by WHO in 2017, cancer is ranked as the second leading cause of death globally after ischaemic heart diseases. A study by GLOBOCAN (2012) showed that there were about 14.1 million new cancer cases and 8.2 million cancer death reported across the globe. Incidences of cancer increased from 12.7 million in 2008 to 14.1 million in 2012, and this trend is projected to continue. The incidences increase is common in low and middle-income countries that are ill-equipped to cope with the management of the disease (Torre *et al.,* 2016; Ferlay *et al.,* 2012). According to World Cancer Report (2014), more than 60% of world's total new cancer cases occur in Africa, Asia and Central and South America. In Kenya, cancer disease constitute a third cause of death (NCCS, 2016).

2.2 Cancer cell biology

Cancer is generally accepted as uncontrolled proliferation of cells (Imran *et al.,* 2017). The disease generally arises from a single cell, with the transformation from pre-cancerous lesion to malignant tumours (Wang *et al.,* 2019; Feitelson *et al.,* 2015). This is manifest by various mechanisms including epithelial to mesenchymal transition and cell migration, altered expression and/or activity of cell cycle related proteins, tumour microenvironment modification, hormone signalling, altered cell metabolism, signal transduction pathways modification and the expression of telomerase enzyme that reverse the wearing down of chromosome ends that normally happens during each cell division (Feitelson *et al.,* 2015; Quail & Joyce, 2013).

Following transformation and progression to malignancy, tumour cells do not remain inert but continue to change and adapt to their local and systemic environment in order to evade death, to proliferate and to form metastases (Nars & Pelletier, 2012). Notably, the processes and dysregulation involved are diverse, resulting into a complex disease outcome. Not a single alteration or protein abnormality is involved (Sasaki *et al.,* 2011). Cancer cells are very similar to the cells of the organism of origin and have similar (but not identical) DNA and RNA (Smelser *et al.,* 2015). The changes leading to manifestation of malignancies broadly arise from interaction

between an individual's genetic factor and external agents, with ethnicity, gender and age being considerable risk factors for adult onset primary malignant tumours (Ferlay *et al.,* 2012).

Cancer cells exhibit salient features that distinguish them from normal cells (Gookin *et al.,* 2017; Smelser *et al.,* 2015). They include, first, loss of positive growth factors thus divide in the absence or presence of these factors, contrary to normal cells that require external growth factors to divide hence regulating their division. Second, whereas normal cells show contact inhibition, cancer cells lack this property therefore they continue to grow even after they touch other cells, causing a large mass of cells to form (Kosaka *et al.,* 2011). Third, whilst adult normal cells lack telomerase, an enzyme required for synthesis of telomere necessary for cell division, however, telomerase is activated in cancer cells, propagating unlimited number of divisions. Forth, cancer cells continue to divide even when there is large amount of damage to DNA or when the cell division is abnormal whereas normal cells cease to divide when there is DNA damage or when cell division is abnormal (Testa *et al*., 2017; Sever & Brugge, 2015).

Broadly, there are three pathways whose (one or all) alteration propagate cancer manifestation by causing genetic instability. These include (i) pathway involved in carrying cancer signal from cell environment, (ii) pathway involved in cells movement and mechanical interactions with its neighbors, and (iii) pathway involved in internal programmes such as cell cycle arrest, apoptosis and senescence (Sia *et al*., 2017; Testa *et al*., 2017; Sever & Brugge, 2015).

2.3 Cell cycle

Cell cycle involves duplication of genetic and organelles material and consequently segregation into two daughter identical cells (Kosaka *et al*., 2011; Clarke, 2002). The process involves various phases that also include interphase and mitosis as shown in figure 1. Duplication of genetic material, termed mitosis has various stages. DNA duplication occurs during S phase (S for synthesis). After S phase, chromosome segregation and cell division occur in M phase (M for mitosis). The M phase involves a series of events that begin with nuclear division or mitosis, equivalent to separation of daughter chromosomes, karyokinesis (Hunt *et al.,* 2011).

Briefly, mitosis begins with chromosome condensation, the duplicated DNA strands, packaged into elongated chromosomes, condense into the much more compact chromosomes required for their segregation. The nuclear envelope then breaks down, and the replicated

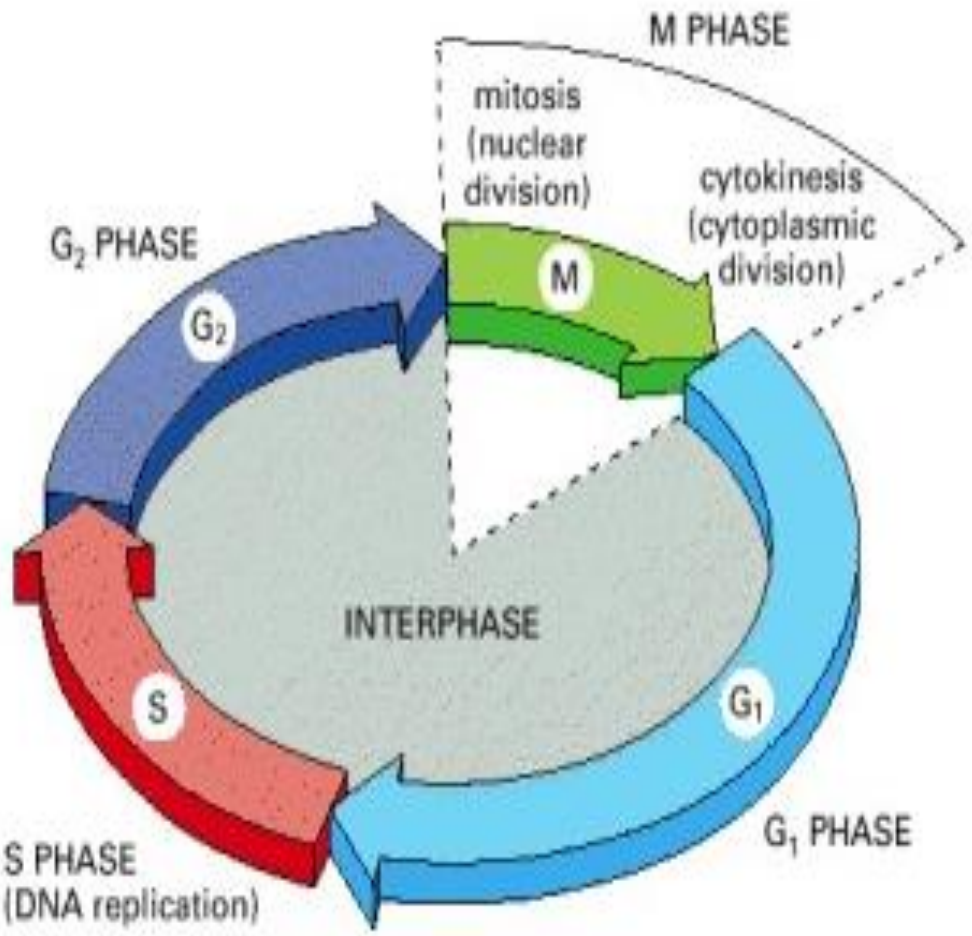

Figure 1: Phases of the cell cycle. The cell grow continuously in interphase, which comprise three phases: Synthesis phase (S phase), mitosis phase (M phase) and G1/G2 phase, the gap between M phase and S phase. DNA replication occurs in S phase. In M phase, nucleus divide and then cytoplasm divide. The G1 phase corresponds to the interval between mitosis and initiation of DNA replication. G2 phase include synthesis of more proteins, including microtubules required for mitosis, cytokinesis, division of cell membrane, synthesis and replication of organelles. The image is adopted from Alberts *et al.* (2002).

chromosomes, each consisting of a pair of sister chromatids, become attached to the microtubules of the mitotic spindle (Verdugo *et al*., 2013; Kosaka *et al*., 2011).

As mitosis, where they decondense and reform intact nuclei. The cell is then pinched into two by cytoplasmic division or cytokinesis, and cell division is completed (Edward *et al*., 2017).

The interphase is divided further into three phases including G1 phase (gap 1), S phase (synthesis) and G2 phase (gap 2). Cells that do not divide further exit G1 phase to enter an inactive stage called quiescent phase or dormant phase or senescent phase (Go) (Rombouts *et al.,* 2018). These cells remain metabolically active but no longer proliferate unless necessitated depending on the requirement of an organism (Polymenis & Aramayo, 2015; Verdugo *et al.,* 2013). Some cells re-enter G1 phase and begin dividing again under specific conditions while other cells remain in G0 and never divide. The G1 phase corresponds to the interval between mitosis and initiation of DNA replication (Edward *et al.,* 2017). This phase involves events such as synthesis of proteins required for DNA replication, synthesis of proteins required for cell's metabolism to produce energy, synthesis of ribosomes, increase of cell size growth, and each chromosome is characterised by one DNA molecule (Ingham & Gary, 2016; Hunt *et al*., 2011). Major events in S phase include replication of chromosome, formation of two sister chromatids from each chromosome, doubling of amount of DNA, synthesis of packaging of proteins (histones), synthesis of more phospholipids, molecules that make up the cell membrane and the membrane of the cell's organelles (Polymenis & Aramayo, 2015). Also notable is that the number of chromosomes remain unchanged in nucleus. Key events in G2 phase include synthesis of more proteins, including microtubules required for mitosis, cytokinesis, division of cell membrane, synthesis of organelles, including mitochondria that grow and divide, replication of organelles, and the cell assumes its normal functions (Edward *et al.,* 2017; Verdugo *et al.,* 2013).

Normal cells grow and divide in an orderly fashion, in accordance with the cell cycle events described above. Several proteins control the timing of the events in the cell cycle that is tightly regulated to ensure that cells divide only when necessary (Edward *et al.,* 2017; Feitelson *et al.,* 2015). Major switches of the cell cycle are cyclin-dependent kinases. Each of these cyclin-dependent kinases forms a complex with a particular cyclin, a protein that binds and activates the cyclin-dependent kinase (Gookin *et al.,* 2017). Briefly, the kinase part of the complex adds a phosphate to various proteins required for progression a cell through the cycle. The added phosphate may alter the structure of protein in question resulting in either activation or deactivation

of the protein, depending on its role (Hunt *et al.*, 2011). There exist specific cyclin-dependent kinase/cyclin complex at the entry points G1, S and M phases of the cell cycle (Ingham & Gary, 2016; Hunt *et al.*, 2011).

There are various body regulatory mechanisms that act as anti-cancer promotion agents through approaches including activation of DNA repair proteins when DNA has sustained damage, induce growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition, or initiate apoptosis if DNA damage is severe and proves to be irreparable as well as prevent the replication of damaged DNA while activating the transcription of proteins taking part in DNA repair (Kruiswijk *et al.*, 2015; Sasaki *et al.*, 2011).

2.4 Mechanisms associated with cancer

Most cancers are associated with genome instability that play critical role both in cancer initiation and progression (Lee *et al.*, 2016). The extent of instability is variable within and between cancer types and manifest on different levels, ranging from nucleic acid sequence to structural abnormalities at chromosome level (Ferguson *et al*., 2015). Genomic instability is brought about by several pathways including telomere damage, chromosomal instability, centrosome amplification, epigenetic modifications, and DNA damage both from exogenous and endogenous sources (Feitelson *et al.*, 2015; Ferguson *et al.*, 2015). Chromosomal instability is suggested to be a major driving force in tumourigenesis and cancer pathogenesis, actively shaping the genomes of cancer cells thus contributing to their survival advantage (Lee *et al.*, 2016).

Telomere shortening is implicated in carcinogenesis (Feitelson *et al.*, 2015; Silk *et al.*, 2009). Telomere that foster chromosomal stability in normal cells is corrupted in cancer cells. As a person ages, telomere shortening ensues, however, reverse transcriptase telomerase enzyme, that is normally repressed in normal cells, is activated, thereby conferring stability to the telomeres (Ferguson *et al*., 2015). Centrosome amplification result from mechanism including de novo assembly, centrosome overduplication and mitotic failure, inducing structural abnormalities that lead to cytokinesis failure (Pellegrino *et al.*, 2010). Cytokinesis defects generate tetraploid cells that are used in cancer as a transition state to aneuploidy, and facilitate genetic diversification within tumour to enhance cancer development (Lens & Medema, 2019). Mitochondrial genetic reprogramming and energy balance within tumourigenic cells are of importance in carcinogenesis (Hanahan & Weinberg, 2011).

Epigenetics, defined as heritable changes that may modify gene expression without changing primary DNA sequence, such as DNA methylation and chromatin remodelling, covalent histone modifications, nucleosome positioning and miRNAs are vital for normal mammalian development and regulation of gene expression (Bennett & Licht, 2017). In cancer cells, epigenetic processes are modified to modulate gene expression and affect DNA repair dynamics. For instance, posttranslational modifications such as acetylation, deacetylation, methylation, phosphorylation and ubiquitination alter function of histones (Nowsheen *et al*., 2012; Sharma *et al.,* 2009). DNA methylation which consist of addition of a methyl group at the carbon 5 position of cytosine pyrimidine ring or number 6 nitrogen of adenine purine ring, involves a group of DNA methyltransferase enzymes causing silencing of gene transcription (Ferguson *et al.,* 2015).

Receptor tyrosine kinases (RTKs) are important contributors to variety of cellular processes such as motility, growth, differentiation and metabolism. Dysregulation and abnormal activation of RTK signalling results in cancer through mechanisms including gain-of-function mutations, genomic amplification, chromosomal rearrangements and/or autocrine activation (Du & Lovly, 2018). Cancer cells also constitutively depend on activation of RAS/MAPK and PI(3)K/AKT pathways by integrin receptor signalling for their growth and survival (Yeh & Ramaswamy, 2015). Formation of metastatic cancer is characterised by different stages including local invasion, intravasation, survival in circulation, arrest at organ site and extravasation, micrometastasis formation and metastasis colonisation. The progression from each of these steps require recruitment of molecular pathways such as EGF/RAS/RAF/MEK/ERK, P13K/Akt/mTOR, HGF/Met, Wnt/β-catenin and VEGF signalling (Pachmayr *et al.,* 2017).

2.4.1 Molecular pathogenesis of hepatocellular carcinoma

Genomic landscape of HCC pathogenesis is highly complex. Its complexity is further amplified by various etiological factors that precede tumour development and signalling pathways alterations (Nasr & Pelletier, 2012; Yang & Roberts, 2010). This confers its genetic heterogeneity. Noteworthy, available literature suggest that hepatocarcinogenesis is a multi-step process as shown in figure 2, where pre-cancerous dysplasia macronodules and hepatocytes transform progressively into HCC under various factors including alterations on genetic, epigenetic, regulation of oxidative stress, inflammation, tumour microenvironment, cancer stem cell, cancer metabolism, etiologic integration, transcriptomic and immunity (Sia *et al*., 2017; Birner *et al.,* 2016). The epigenetic constitute DNA methylation, histone modification, chromatin remodeling and

microRNAs/IncRNAs (Sia *et al*., 2017; Testa *et al*., 2017; Ho *et al.,* 2016). Aberrant signalling occur in pathways involved in cell differentiation such as tyrosine kinases, Wnt-β catenin, notch and hedgehog pathways. The HCC pathogenesis also require recruitment of molecular pathways such as p53, EGF/RAS/RAF/MEK/ERK, P13K/Akt/mTOR, HGF/Met, Wnt/β-catenin and VEGF signalling. MicroRNAs (miRNAs) such as mir-34a, b, c, and mir-125b are integral to the *p53* signalling pathway and their abnormalities may have clinical implications (Robbles & Harris, 2010). The miRNAs mir34 are downstream effectors of *p53* function and are transcriptionally transactivated by the *p53* genne. Methylation on the miRNAs mir34, may affect downstream *p53* functions. The miRNA mir-125b negatively regulate *p53* expression (Testa *et al*., 2017; Robles & Harris, 2010).

The transformations involving molecular pathogenesis are characterised by alteration/mutation among other factors in signalling pathways (Kandoth *et al*., 2013; Luis *et al.,* 2012), among them is *p53* pathway that is suggested to affect major regulators of various signalling pathways involved in tumour suppression through final outcome of growth arrest, apoptosis, senescence, DNA repair (Kandoth *et al*., 2013; Luis *et al.,* 2012; Maiuri *et al.,* 2010). Further to its role in tumour suppression and early event, alteration in *p53* gene play vital role in HCC pathogenesis alongside β-catenin gene (CTNNB1 gene) (Ho *et al.,* 2016). Other pathways include regulating growth factor signalling such as the insulin-like growth factor (IGF), epidermal growth factor (EGF), PDGF, fibroblast growth factor (FGF) and hepatocyte growth factor (HGF/MET); pathways related to cell differentiation such as the WNT, Hedgehog, and Notch pathways and pathways related to angiogenesis such as the vascular endothelial growth factor (Sia *et al*., 2017; Testa *et al*., 2017). Epigenetic mechanisms including DNA methylation, covalent histone modifications, nucleosome positioning and miRNAs are vital for normal mammalian development and regulation of gene expression (Bennett & Licht, 2017). The distinct combinatorial patterns of these modifications, collectively termed the epigenome, are major contributors to determination of cell fate and gene activity (Wang & Lei, 2018). DNA methylation and histone modifications work independently and in concert to alter gene expression during tumourigenesis (Nebbioso *et al.,* 2018; Baek, 2011).

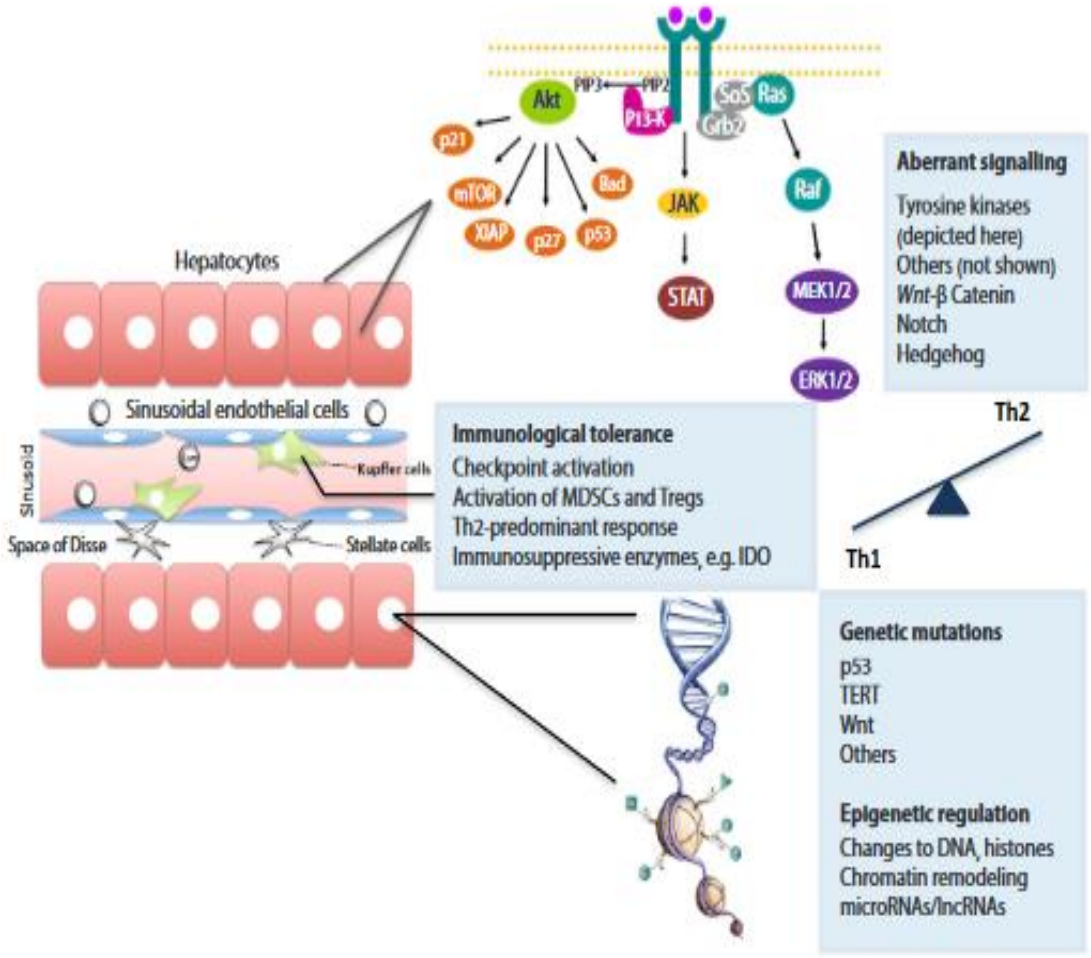

Figure 2: Molecular pathogenesis of hepatocellular carcinoma. The molecular pathogenesis of HCC involves interaction between aberrant signalling, genetic mutations, epigenetic control of gene expression and induction of immunological tolerance. MDSCs: myeloid derived suppressor cells, Tregs: T-regulatory cells, IDO: indoleamine 2,3-dioxygenase, TERT: telomerase reverse transcriptase, lncRNAs: long non-coding RNAs, JAK: Janus kinase, STAT: signal transducer and activator of transcription, Th1: type 1 T helper cell, Th2: type 2 T helper cell, ERK: extracellular signal-regulated kinase, mTOR: mammalian target of rapamycin, MEK: mitogen activated protein kinase, PI3K: phosphatidylinositol-3-kinase, AKT: protein kinase B, raf: regulatory protein involved in cellular transformation, ras: GTPase protein involved in cellular signal transduction. Figure adopted from Setshedi *et al.* (2018).

DNA methylation provides favorable stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in association with histone modifications and other chromatin associated proteins (Baek, 2011). The DNA methylation primarily occurs by the covalent modification of cytosine residues in CpG dinucleotides, which are not evenly distributed across the human genome but are instead concentrated in short CpG-rich DNA stretches (Desilet *et al.,* 2010). DNA methylation uses a variety of mechanisms to heritably silence genes and non-coding genomic regions. These mechanisms include preventing or promoting the recruitment of regulatory proteins to DNA. For instance, it can inhibit transcriptional activation by blocking transcription factors from accessing target-binding sites (Biswas & Rao, 2017; Bantscheff *et al.,* 2011). Conversely, it can provide binding sites for methyl-binding domain proteins, which can mediate gene repression through interactions with histone deacetylases (Bennett & Licht, 2017; Sharma *et al.,* 2009).

Histone proteins comprise the nucleosome core, contain a globular C-terminal domain and an unstructured N-terminal tail which can undergo a variety of posttranslational covalent modifications including methylation, acetylation, ubiquitylation, sumoylation and phosphorylation on specific residues (Flavahan *et al.,* 2017; Boumber & Issa, 2011). These modifications regulate key cellular processes such as transcription, replication and repair (Wang & Lei, 2018). Histone modifications is realized through either changing the accessibility of chromatin or by recruiting and/or occluding non-histone effector proteins, which decode the message encoded by the modification patterns (Biswas & Rao, 2017).

Tumourigenesis is characterised by widespread changes in microRNA (miRNA) expression (Nebbioso *et al.,* 2018). This is because miRNAs regulate genes that are involved in transcriptional regulation, cell proliferation and apoptosis, which collectively form the most common processes deregulated in cancer (Flavahan *et al.,* 2017). Therefore, miRNAs can function as either tumour suppressors or oncogenes depending upon their target genes. Important to note is that many tumour suppressor miRNAs that target growth-promoting genes are repressed in tumourigenesis (Stahl *et al.,* 2016; Bruix & Sherman, 2011).

There are three proposed distinct mediation mechanisms employed by chronic HBV in hepatocarcinogenesis (Stahl *et al.,* 2016). First mechanism involves integration of viral DNA into the host genome, inducing chromosome instability (Wang & Lei, 2018). The second mechanism involve multiple genetic mutations by insertion, leading to integration of HBV genome at specific

sites that can activate endogenous genes, for instance, cyclin A, TRAP1, and retinoic acetic receptor B (Boumber & Issa, 2011). The third mechanism involves modulation of cell proliferation by viral protein expression, specifically HBV X protein (HBx) which can transactivate or overexpress a variety of viral and cellular genes (Biswas & Rao, 2017). The HBx coordinate balance between cell proliferation and apoptosis through coactivation of transcription process of some important cells and viral genes. Cell promoters of genes associated with proliferation, such as interleukin 8 (IL-8), tumour necrosis factor (TNF), transforming growth factor (TGF-b1), and epidermal growth factor receptor (EGFR), as well as transcription factors, are activated with HBx transactivation (Sharma *et al.,* 2009). The HBx is also involved in the activation of signalling cascades involving Ras/Raf/MAPK pathway contributing to dysregulation of cell cycle checkpoints and activation of myriads of oncogenes including *c-myc, c-jun, c-fos* in the cytoplasm (Bennett & Licht, 2017). There are two pathways through which HBx can activate Wnt/b-catenin pathway in conjunction with proto-oncogene protein Wnt-1 namely (i) through stabilization of cytoplasmic b-catenin in HCC cells, (ii) activation of extracellular signal-regulated kinase, which leads to phosphorylation, and in turn, to inactivation of kinase-3b of glycogen synthesis (Bennett & Licht, 2017; Gomes *et al*., 2013).

2.4.2 Epidemiology of hepatocellular carcinoma

HCC arises from progressive transformation of pre-cancerous dysplasia macronodules and hepatocytes factor, is one of the most common malignancies worldwide, with limited treatment options and accounts for approximately 85% of liver cancers and is characterised by a highly heterogenetic pathogenesis with an aggressive clinical course leading to poor survival (Sia *et al*., 2017; Testa *et al*., 2017). The disease comprise one of the most common malignancies globally. Increasing incidences with very high fatality rate of 800,000 death globally per annum have been documented (Stewart *et al*., 2016). According to WHO (2017), it is the fourth leading cause of carcinoma-related death in the world and a fifth in Africa. In resource-rich countries, HCC is rare in patients under the age of 40 years and progressively increases in incidence in those in their 60s and 70s (Mak *et al.,* 2018; Torre *et al.,* 2016). The HCC cases constitute the fifth most common in men globally, and seventh among women, with over 600,000 new cases diagnosed annually worldwide. According to Global Burden of Disease Liver Cancer Collaboration (2015), incidence of HCC varies broadly worldwide between geographical regions, with some of the highest incidence rates found in the West, Asia and Africa (Akinyemiju *et al.,* 2017). Noteworthy, there

is dearth of information on the overall incidences of HCC in Kenyan population. This is occasioned by lack of national repository for cancer cases and the few available hospital based cancer registries.

2.4.3 Risk factors for hepatocellular carcinoma

The major risk factors for HCC are many. They include age, gender, inherited metabolic disease such as hereditary hemochromatosis, rare disease conditions such as tyrosinemia, alpha1-antitrypsin deficiency, porphyria cutanea tarda, glycogen storage diseases and Wilson disease (Mak *et al.,* 2018; Hoffmann *et al*., 2018. The disease risk increases with increase in age due to accumulative mutation in tumour suppressor genes and diminished immune responses (Mak *et al.,* 2018; Adjiri, 2017). HCC is more common in males than females, however, the reason remains unclear (Borzio *et al.,* 2015). Moreover, according to NCCS 2011-2016, HCC has no specific risk age groups, however, risk increases with increase in age. Conversely, GLOBOCAN (2017) and Global Burden of Disease - Liver Cancer collaboration (2016) suggest ages 20-33, 34-49, ≥50 for lower risk, medium risk and higher risk respectively. According to Genetic of cancer - National Cancer Institute (2017), occurrence of cancer is leveraged on long duration of living characterised by excessive exposure to mutagens and carcinogens thus the increased risk in the older population.

Other risk factors also due to environmental exposure and lifestyle. They include chronic infections with the hepatitis B (HBV) or C (HCV) virus, dietary aflatoxin B1 (AFB1) produced by *Aspergillus flavus,* vinyl chloride, non-alcoholic fatty liver disease, primary biliary cirrhosis, obesity, presence of lesions in the liver that are chronic with associated inflammation, necrosis of hepatocytes and fibrosis, heavy alcohol consumption tobacco smoking, (Mak *et al.,* 2018; Hoffmann *et al*., 2018; Omar *et al*., 2013).

The risk factors vary according to the geographical region, for instance, chronic hepatitis B virus (HBV) infection is most common in many Asian countries and Africa, whilst hepatitis C virus (HCV) is most common in Japan and the United States. It is important to note that these risk factors incidences continue to flare-up even among the Kenyan population (Janevska *et al*., 2015; Omar *et al*., 2013).

The main sources of exposure for aflatoxin are maize flour and milk (Senerwa *et al.,* 2016; Gong *et al.,* 2012). Whereas there are various types of aflatoxins, AFB1 is a potent carcinogen and excessive exposure to AFB1 stands out to be having an important contribution to HCC in combination with HBV infection (Hoffmann *et al*., 2018; Omar *et al.,* 2013). It is metabolised by

cytochrome P450 in the liver, and generally causes DNA damage by forming covalent and promutagenic DNA adducts. The high doses of AFB1 can lead to death from aflatoxicosis (Fujiwara *et al.*, 2018; Hoffmann *et al*., 2018). Whereas the distribution of aflatoxin in Kenya is heterogenous, high prevalence of aflatoxins have been reported in some places including Makueni and Nandi counties. Additionally, moderate prevalence of aflatoxin levels have been reported in other places including Uasin Gishu County (Kang'ethe *et al*., 2017; Gong *et al.*, 2012).

The AFB1, most potent hepotocarcinogen, is metabolized in the liver by p450 enzymes into AFB1-8, 9-exo-epoxide, which is highly reactive and forms derivatives with DNA, ribonucleotide acid (RNA) and proteins that can react with *p53* tumour suppressor gene (Hoffmann *et al*., 2018). In turn, AFB1 binds to DNA to form predominant promutagenic 8,9 dihydro-8-(N7-guanyl)-9-hydroxy AFB1 adduct (Kew, 2014). The latter can be converted into a more stable AFB1-formaminopyrimidine adduct, which causes guanine to thiamine transversion mutations. AFB1-formaminopyrimidine, incorporated into double-stranded DNA, is mutagenic, whereas the dominant species in single-stranded DNA blocks DNA replication (Janevska *et al.*, 2015; Kew, 2014; Gomes *et al*., 2013).

2.4.4 Staging for hepatocellular carcinoma

According to International Agency for Research on Cancer (2017), cancer staging broadly depends on tumour characteristics, assessment of organ dysfunction and patient characteristics. Overall, cancers have four stages, designated I-IV that describe the size of a cancer and how far it has spread (Jiang *et al*., 2018; Yim *et al*., 2018). It is important to underscore that the staging inform on treatment choice (Yim *et al*., 2018, Kinoshita *et al*., 2015). Succinctly, stage I describes cancer that is relatively small and contained within the organ of origin. Stage II describes cancer that is larger than in stage 1 but has not spread into the surrounding tissues. Stage III describes cancer that has spread into surrounding tissues and lymph nodes in the area and finally Stage IV, metastatic cancer, describes cancer that has spread to other body organs (Zhou *et al*., 2018; Nasr & Pelletier, 2012).

For HCC staging, there are three systems that are used namely American Joint Committee on Cancer (AJCC), Barcelona Clinic Liver Cancer (BLCL), and Cancer of the Liver Italian Programme (CLIP), depending on tumour characteristics, assessment of liver dysfunction and patient characteristics (Yim *et al*., 2018, Kinoshita *et al*., 2015; Marrero *et al*., 2010). The AJCC is based on the tumor, lymphnode, metastasis (TNM) stage framework that primarily addresses

tumour characteristics and spread as opposed to liver functioning status. The AJCC has classifications (I, II, IIIA, IIIB, IIIC, IVA, IVB). The BCLC takes into account overall patient performance status, using Eastern Cooperative Oncology Group's classification system. (Marrero *et al*., 2010). The BCLC has classifications (A1, A2, A3, A4, B, C, D). The CLIP takes into account the relative size of tumour to the liver, alpha fetoprotein level and a lab value (Zhou *et al.,* 2018; Yim *et al.,* 2018; Maida *et al.,* 2014). In this study, HCC staging was done using pathological diagnosis approach.

2.4.5 Hepatocellular carcinoma diagnosis

Several diagnostic approaches for HCC have been employed including computed tomography, ultrasonography, magnetic resonance imaging, digital subtraction angiography, nuclear medical imaging, liver puncture biopsy, serological molecular markers for instance alpha fetoprotein level, microscopic examination, immunohistochemical examination and pathological diagnosis (Zhou *et al*., 2018; Bruix & Sherman, 2011). These techniques complement one another for overall diagnosis and are used depending on patients' characteristics. Notably the gold standard for diagnosis of HCC is histopathological examination (Walther & Jain, 2011).

2.5 Tumour suppressor *p53* gene

Tumour suppressor *p53* gene is suggested to be the most powerful and natural cancer regulatory mechanism for tumour suppression. Mutations of this gene in individuals are associated with many cancers (Kandoth *et al.*, 2013). A study by Goldstein *et al*. (2011) showed that more than half of human cancer cells exhibit *p53* gene mutations while normal cells most often do not have mutation in the gene.

2.5.1 Structure of *p53* gene

The *p53* protein is composed of 393 amino acid residues, five structural and functional domains. These include transactivation, proline-rich, DNA binding, oligomerization and regulation domains (Rui *et al.,* 2016) as shown in figure 3a. Molecular mass of the *p53* protein's monomer form 53 kilo Dalton fraction of cell total protein thus the name, and is encoded by tumour suppressor *p53* gene which is located on chromosome 17 (17p13) (Kandoth *et al*., 2013). The gene is composed of 32, 772 base pairs spanning 11 exons and 10 introns, with a very large intron between exon 1 and 2 as shown in figure 3b. Exon 1 is a non-coding in human *p53* gene (Yael & Moshe, 2011). Therefore, the coding sequence starts in second exon and ends in eleventh exon. Further, two alternatively spliced exons exist in intron 9 (Malecka *et al.,* 2009).

The transactivation domain categorised into two namely transactivation domain 1 (AD1) and transactivation domain 2 (AD2) composed of amino acid residues 1-42 and amino acid residues 43-63 respectively. Both AD1 and AD2 are responsible for activating *p53*-inducible downstream target genes. The domain is further encoded by exons 2 and 3. It interacts with various regulatory proteins such as mdm2 and mdm4, major negative regulators to control cellular levels of *p53* protein (Rui *et al.,* 2016).

Proline-rich domain (PRO) comprise amino acid residues 64-92 and contains sulfhydryl (SH3) domain binding motifs that arbitrate *p53* response to DNA damage to induce apoptosis and growth arrest (Joerger & Fersht, 2010). The domain undergo a couple of post translational modification through phosphorylation, acetylation, methylation, ubiquitinylation, sumoylation, neddylation, adenosine diphosphate (ADP) ribosylation, and glycosylation, with acetylation and phosphorylation of multiple residues being chiefly essential for *p53* activation and DNA transcriptional activity (Rui *et al.,* 2016).The DNA binding domain (DBD) comprise amino acid residues 100-300 and forms the central core of *p53* protein structure. The domain recognizes specific DNA sequences through binding to specific double strand target DNA, thus facilitating sequence-specific binding of *p53* to *p53* response elements (RE) in DNA (Yael & Moshe, 2011). The DBD of *p53* binds to a specific DNA sequence to activate transcription, mediate apoptosis, and conduct cell cycle arrest to suppress the growth of tumour cell. However, there occurs variation in affinity with which *p53* binds its response elements and this depends on the sequence-specific properties of the RE. Notably, wild type *p53* function as a transcription factor only upon binding site-specific DNA response elements in tetrameric form in a sequence specific manner (Joerger & Fersht, 2010). This domain is further encoded by exons 4, 5, 6, 7 and 8, and it dictates the stability of the *p53* full length (Yael & Moshe, 2011).

Oligomerization (Oli) or tetramerization domain is composed of amino acid residues 326-356. It facilitates the interaction of *p53* monomers to form dimers which further interact to form tetramers through tetramization. The formation of tetramers allows the binding of *p53* to its response elements (RE) hence facilitate events such as transcriptional activation, protein-protein interaction, DNA binding and post-translational modification. The domain is further encoded by exons 9 and 10 (Rui *et al.,* 2016; Joerger & Fersht, 2010).

Regulation domain composed of amino acid residues 364-393 and is encoded by exon 11. The domain undergoes a number of post translational modification through phosphorylation,

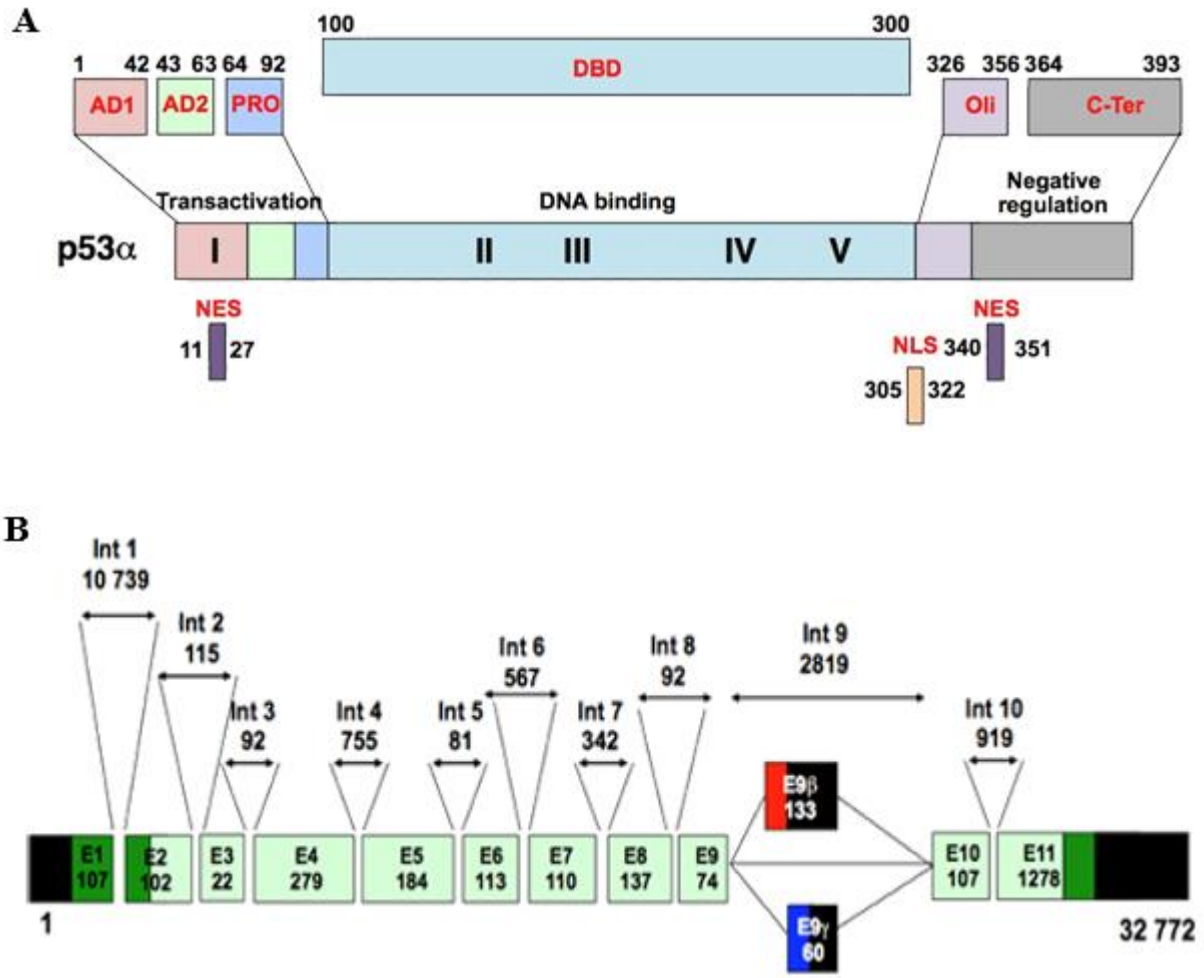

Figure 3: Structure of *p53*. Panel A. Domain architecture of p53 protein. The protein comprise 393 amino acid residues existing within its five domains including transactivation, proline-rich, DNA binding, oligomerization and regulation domains. Transactivation domain is further classified into two AD1 and AD2. AD1: transactivation domain 1, AD2: transactivation domain 2, PRO: proline-rich domain, NES: nuclear exclusion signal, NLS: nuclear localization signal, Oli: oligomerization domain, C-ter- Carboxy-terminus. Panel B. The *p53* gene. The gene is of 20 kb found in chromosome 17. It has 11 exons and 10 introns spanning. Two alternatively spliced exons, E9β and E9γ, exist in intron 9. Letters 'E' in boxes represent exons with their sizes inside. Introns - 'Int' with their amino acid sizes. The light green, red and blue colors represent translated exons for canonical *p53* protein, isoforms beta and isoforms gamma respectively. Figure adopted from Huret *et al*. (2016).

acetylation, methylation, ubiquitinylation and sumoylation to stabilize the *p53* tetramers through making subunit contacts with DNA binding (Retzlaff *et al.,* 2013).

2.5.2 Isoforms of *p53* proteins

There are twelve different *p53* proteins (*p53* isoforms) normally expressed in human but the full-length protein, composed of 393 amino acid residues is suggested as *p53*α and is often the chief *p53* isoform species (Khoury & Bourdon, 2010). The isoforms are due to alternative splicing, alternative initiation of translation, and alternative promoter usage (Khoury & Bourdon, 2010). These isoforms are shown in figure 4. They can interact with wildtype *p53* through either dominant – negative or cotransactivating functional modulations (Robles & Harris, 2010).

2.5.3 Functions of *p53* gene

The cellular tumour antigen *p53* protein encoded by *p53* gene is helpful both in normal and cancerous cells (Robles & Harris, 2010). The protein acts as a checkpoint control following DNA damage, thus referred to as 'guardian of genome' (Sever & Brugge, 2015). The primary function of *p53* is to modulate the transcription programme of cell growth regulation at G1 check point and control numerous genes involved in expression of cellular outcomes (Robles & Harris, 2010). In response to plethora of oncogenic stresses such as activated oncogenes expression, hypoxia, deoxyribonucleic acid (DNA) damage, viral infection, ribonucleotide depletion and antioxidants exposure, the protein binds specific DNA targets and modulates the expression of genes taking part in the tumour suppressive functions such as DNA repair, cell cycle arrest, senescence and apoptosis (Kruiswijk *et al.,* 2015; Nakash *et al.,* 2014). The gene inhibits the replication of damaged DNA while activating the transcription of proteins participating in DNA repair (Kruiswijk*et al.,* 2015; Sasaki *et al.,* 2011). The protein induces antitumour cytotoxic-T-cell activity against carcinoma cells. Interestingly, the protein has also been found to function in mitochondria, where it binds to Bcl-XL proteins and induce mitochondrial outer membrane permeabilization and apoptosis (Follis *et al.,* 2013).

The contribution of *p53* gene in tumour suppression is largely attributed to cell fate functions on exposure to stresses with the ability to eliminate cancerous cells without affecting organismal integrity (Sasaki *et al.,* 2011; Wu, 2004). The *p53* gene has many mechanisms of an anti-cancer promotion agent which broadly include activation of DNA repair proteins when DNA has sustained damage, induce growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition, or initiate apoptosis if DNA damage is severe and proves to be

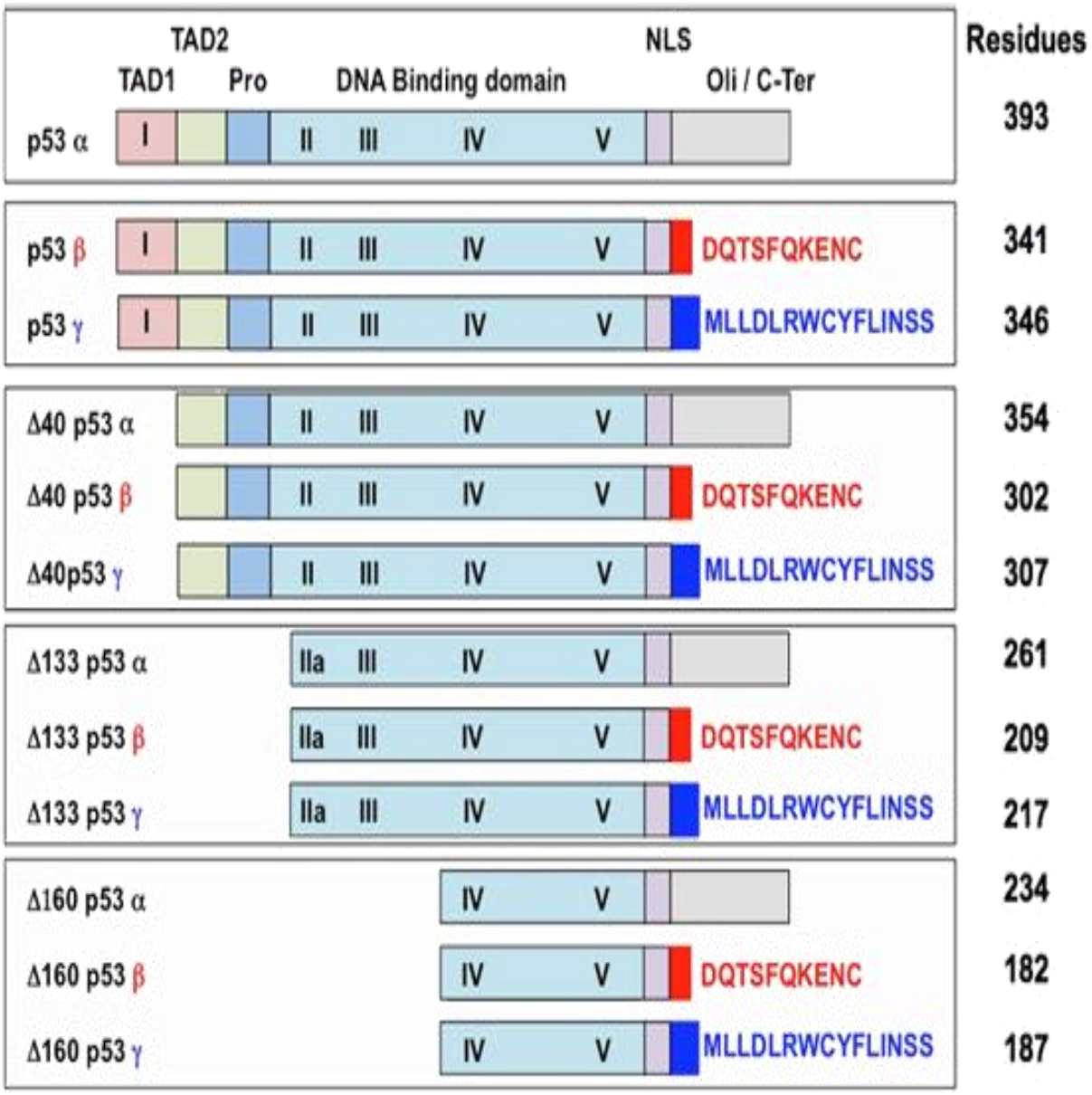

Figure 4*:* Isoforms of *p53*. The *p53*γ, *p53*β, *p53*α represent various isoforms of *p53* protein. Numerical show amino acid residues length of various isoforms. AD1: transactivation domain 1, AD2: transactivation domain 2, PRO: proline-rich domain, NES: nuclear exclusion signal, NLS: nuclear localization signal, Oli: oligomerization domain, C-ter- Carboxy-terminus. Figure adopted from Huret *et al*. (2016).

irreparable (Nasr & Pelletier, 2012; Olivier *et al.,* 2010). The *p53* gene prevent the replication of damaged DNA while activating the transcription of proteins taking part in DNA repair (Kruiswijk *et al.,* 2015; Sasaki *et al.,* 2011).

2.5.4 Signalling pathway of *p53*

The *p53* signalling pathway is mainly located in the nucleus and to a lesser extent in the mitochondria (Yael & Moshe, 2011). The pathway is categorized into three including upstream signalling, core regulation and downstream signalling a shown in figure 5. The upstream pathway signalling involves activation of *p53* pathway by stress signals, and subsequent detection and interpretation of upstream signals by upstream mediators (Sever & Brugge, 2015; Vousden & Prives, 2009). Core regulation pathway is vital in maintenance of *p53* protein levels in the cell. whereas downstream events mainly involves transcriptional activation of inducible proteins involved in final outcome of *p53* functions such as growth arrest, apoptosis, senescence, DNA repair, angiogenesis and autophagy (Sousa *et al.,* 2013; Luis *et al.,* 2012; Maiuri *et al.,* 2010)

The pathway signalling involves activation of *p53* pathway by stress signals, and subsequent detection and interpretation of upstream signals by upstream mediators (Mirzayans *et al.,* 2012). The *p53* is activated by plethora of stress signals including DNA damage, hypoxia, oncogenes activation, adhesion, nucleotide pool depletion, viral infection, nitric oxide, spindle damage or oxidative stress ultraviolet rays, heat or cold shock, γ-irradiation, genotoxic drugs, and nutrition deprivation (Sever & Brugge, 2015). For individual stress, different panel of mediators are recruited (Mirzayans & Murray, 2012; Beckerman & Prives, 2010).

In response to UV, gamma irradiation and genotoxic drugs, which make single or double stranded breaks in DNA, the ATM protein kinase CHK2 is activated and in the absence of this kinase *p53* activation is delayed or reduced (Vousden & Prives, 2009). Similarly after UV irradiation the ATR kinase CHK1 is induced and its absence alters the *p53* response (Maiuri *et al.,* 2010). Similarly, this leads to disruption of *p53* and corresponding negative regulator (mdm2 or mdm4) interaction which is essential for ensuring accumulation and activation of *p53* protein (Sever & Brugge, 2015). The activity of *p53* is modulated through subsequent post translation modifications (majorly phosphorylation and acetylation) depending on the intensity and type of damage, and the cellular context (Luis *et al.,* 2012; Maiuri *et al.,* 2010). The gene mdm2 is a product of a *p53*-activated gene. The resulting increase of *p53* activity leads to upregulation of mdm2 (Luis *et al.,* 2012).

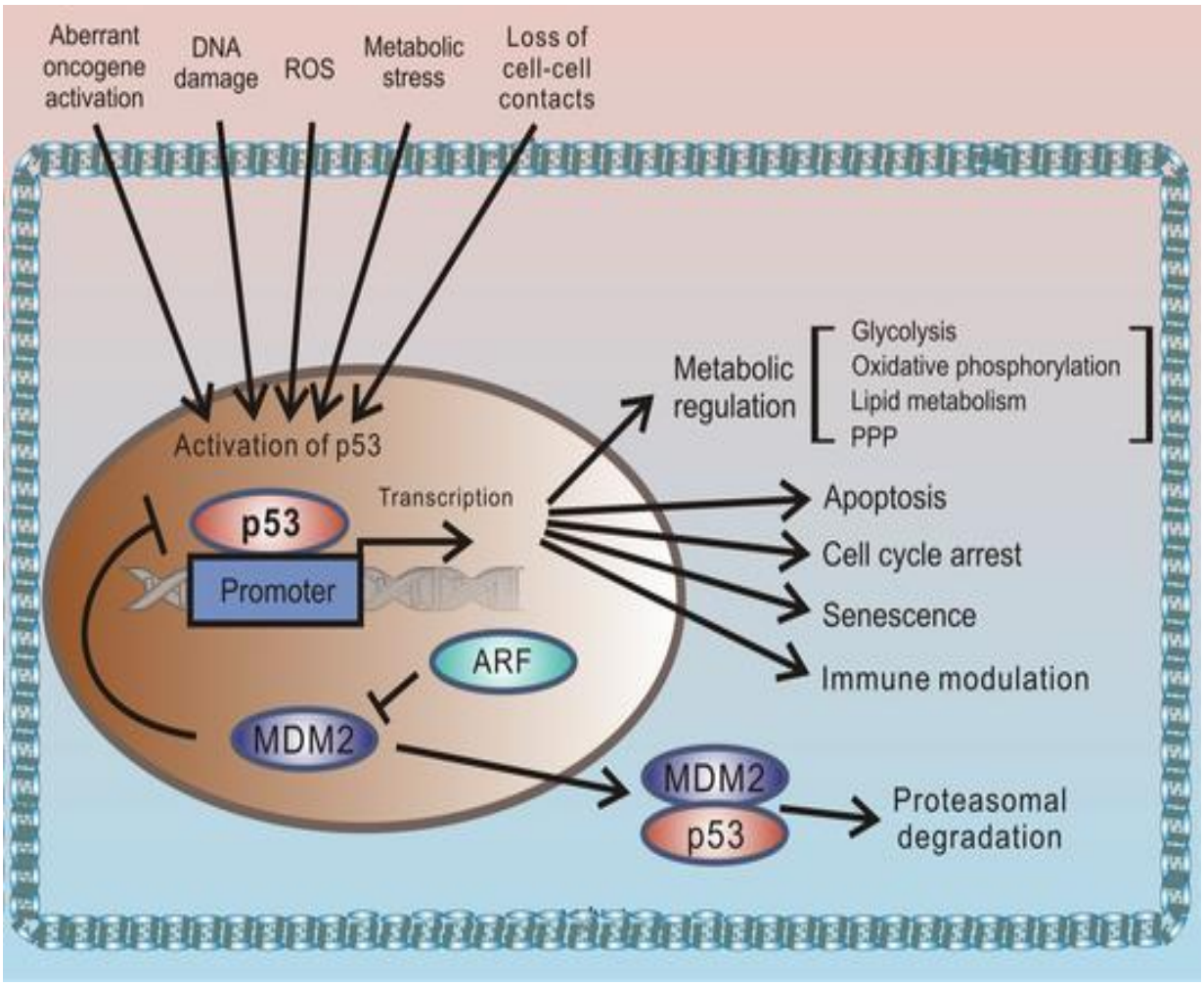

Figure 5: The *p53* signalling pathway. The pathway is classified into upstream signalling, core regulation and downstream signalling. Upstream signalling constitute *p53* activation by stress signals. Core regulation constitute *p53* negative regulators activities including mdm2 and ARF. Downstream signalling constitute transcriptional activation of inducible proteins involved in *p53* responses including apoptosis, metabolic regulation, cell cycle arrest, senescence, DNA repair, immune modulation. Under normal (unstressed) conditions, levels of *p53* protein are tightly controlled by MDM2, E3 ubiquitin ligase, which ubiquitinates *p53* leading to its proteasomal degradation. The ARF tumor suppressor, which functions upstream of MDM2 and *p53*, is required for accumulation of *p53* under oncogenic stress. The ARF inhibit proteasomal degradation of *p53*. ARF: alternative reading frame. Image adopted from Zaika *et al*. (2015).

The protein product of mdm2 binds to *p53* and acts as an E-3 ubiquitin ligase that adds ubiquitin to *p53* and results in its degradation. This produces an auto-regulatory loop where *p53* results in the synthesis of mdm2, which in turn degrades *p53* (Meek, 2015; Joerger & Fersht, 2010; Luis *et al.,* 2012). Very high levels of *p53* may accelerate aging process thus its level should be maintained as very low as in normal cells and tissues (Wang & Sun, 2016). Evidence suggests that the *in vivo* functions of *p53* seem to balance the cell-fate choice with the type and severity of damage that occurs. This is majorly achieved through the actions of core proteins which include specific E3 ligases such as mdm2 and mdm4. These proteins usually target *p53* and ensure its down regulation through proteasomal degradation and ubiquitination (Luis *et al.,* 2012; Maiuri *et al.,* 2010). Degradation of *p5*3 through the interaction with the ubiquitin ligase mdm2 remains the most important mechanism for regulation of its activity. While some of the mechanisms are connected to regulation of mdm2, others aim modifications in its target, the *p53* (Mirzayans *et al.,* 2012; Wu, 2004). An important player that regulates the activity of mdm2 is the closely related protein mdm-x. This protein has very similar composition, although unlike mdm2 it does not have the E3 ubiquitin ligase activity (Sever & Brugge, 2015). Also, a regulator of the mdm2-dependent degradation of *p53* is p14ARF, the product of an alternative reading frame of the *CDKN2A* gene that in addition encodes the CDKs inhibitor *p16*. The protein p14ARF is known to stabilize *p53* by promoting mdm2 degradation. Transcription of the *ARF* gene is subject to positive and negative regulation by complexes that contain transcription factor *E2F-1*. The *E2F-1* recognizes a specific set of DNA sequences, which regulate a number of genes that are required for the synthesis of substrate precursors for DNA synthesis and DNA replication (Desilet *et al.,* 2010).

The downstream signalling pathway includes a chain of genes that are activated by trans-activating properties of *p53* to induce responses such as growth arrest, apoptosis, senescence, prevention of DNA damage, initiation of DNA repair in case of severe damage, metabolism, angiogenesis and autophagy (Sousa *et al.,* 2013; Sabin & Anderson, 2011). The activation of the effectors occurs through distinct DNA binding of the *p53* protein to a *p53* response element. However, there occurs variation in affinity with which *p53* binds its response elements (RE) and this depends on the sequence-specific properties of the RE (Meek, 2015; Joerger & Fersht, 2010). Several genes have been shown to be regulated by inducible transcriptional activity of the *p53* depending on the overall outcome of its functions. These genes fall into several categories based upon their functions such as growth arrest, apoptosis, senescence, DNA repair, metabolism,

angiogenesis and autophagy (Mirzayans *et al.,* 2012; Olivier *et al.,* 2010). The gene induce senescence by telomeric shortening and further regulate the expression of specific microRNAs and telomeric multiprotein complex, shelterin, that includes TRF2 and POT1 as endogenous mechanisms of replicative senescence (Mondal *et al.,* 2013).

Several of these proteins mediate cell cycle arrest by *p53* including *p21* protein binds to Cyclin E–cdk2 and blocks it from phosphorylating the Rb protein which is required for entry into S phase. The 14-3-3 sigma protein binds to Cdc25C, the 14-3-3- Cdc25C complex localizes Cdc25C in the cytoplasm where it cannot act upon Cdc2, the block at this checkpoint occurs through the inhibition of Cdc2–cyclin B complex (Mirzayans *et al.,* 2012).

The great majority of *p53* responsive genes act in the pro-apoptotic pathway (Wang & Sun, 2016). Although the role of *p53* in apoptosis is undisputed, the mechanism by which *p53* induces apoptosis is not fully understood. Studies have suggested that *p53*-mediated apoptosis involves both transcription-dependent and -independent mechanisms (Wang & Sun, 2016; Sabin & Anderson, 2011). A number of genes including *BAX, Bcl2, BID, NOXA, p53AIP1, PIGS* and *PUMA* have been implicated in *p53*-mediated apoptosis. Cytochrome-c release from mitochondria binds to apaf-1 resulting in apaf-1 cleavage and activation. This in turn cleaves caspase-9 which cleaves caspase-3 leading irreversibly to apoptosis. Thus, the *p53* pro-apoptotic pathway acts upon many fronts that collectively execute programmed cell death. (Desilet *et al.,* 2010).

The *p53*-regulated genes include an angiogenesis inhibitor thrombospondin (*TSP-1*), *GDAIF, BAI1*, inhibitors of tumour invasion and metastasis *KAI1*, Maspin, an inhibitor of the plasminogen activator protein PAI, and several secreting protein factors which inhibit proliferation of exposed cells (Wang & Sun, 2016). The genes encoding components of the global genome repair, the *p48XPE*, and *XPC* are among the targets of *p53*. Another set of *p53*-regulated genes, for example, *GADD45* and *p53R2*, a ribonucleotide reductase subunit, result in enhanced DNA repair. These genes are important for maintenance of the nucleotide pools during the DNA repair (Sabin & Anderson, 2011).

2.5.5 Mutations in *p53* gene

Mutations in the human *p53* gene manifest majorly in the DNA-binding domain, with majority (95%) of these being missense mutations, a feature that differentiate it from other cancer regulatory genes (Muller & Vousden, 2014). Inactivation of *p53* gene by mutation from mutagenic and carcinogenic agents including UV radiation, tobacco, aflatoxin, viral infections and age, is

suggested to be a major cause of its alteration (Wang & Sun, 2016). Other causes include; perturbation in pathway signalling to *p53*, allelic loss, inactivation by virally encoded proteins such as SV 40 target antigen, excessive dietary aflatoxin B1 exposure, Human Papillomavirus (HPV) E6, hepatitis B X antigen and adenovirus Ela (Zerdoumi *et al.*, 2013).. These proteins target *p53* protein for degradation and in so doing, the *p53* signalling pathway is altered (Rivlin *et al*., 2013; Benjamin *et al.*, 2008).

These effect of these mutations include unfolding of the *p53* protein and a change in amino acid critical for DNA binding, both of which alter the transactivation of *p53* targets in tumours and DNA binding function. This is because most of the mutations cluster within the central DNA binding domain (Rivlin *et al.*, 2013).

A study by Goldstein *et al*. (2011) showed that more than half of human cancer cells exhibit *p53* gene mutations whereas normal cells most often lack mutation in the *p53* gene (Olivier *et al.*, 2010). Interestingly, *p53* mutants exhibit a longer half-life than wild-type *p53*, and are unstable and accumulate majorly in the tumours (Carol & Scott, 2015; Rivlin *et al.*, 2013). A noteworthy study by Rivlin *et al*. (2011) showed that more than 75% of *p53* gene mutations leads to expression of a mutant *p53* protein that has lost wild type function and may deploy a dominant negative regulation over the rest of wild type *p53*, suppressing tumour by hetero-oligomerizing with wild-type *p53*. Therefore the mutant *p53* act as a competitive inhibitor of wild type *p53* (Rivlin *et al.*, 2011). The consequence being loss of transcriptional activity of wild type *p53*, a phenomenon referred to as loss of function. Conversely, the mutant *p53* may as well acquire a new tumour-promoting activity that is independent of wild-type *p53* through production of oncoproteins that offer cancer cells growth and survival edges, phenomenon referred to as gain of function (Alexandrova *et al.*, 2013; Rivlin *et al.*, 2011). Mutations have also been found in other domains, but their contribution to carcinogenesis is largely unknown (Leroy *et al*., 2014).

Largely, mutation in multicellular organisms may be classified as germinal and somatic mutations (Schon & Tischkowitz, 2018; Zhang *et al.*, 2017). The former occur in germline cells and the latter in somatic cells as a result of environmental exposure. Somatic mutation affects codons within the DNA binding domain and accounts for major *p53* mutations while germinal mutation is majorly associated with the rare familial Li- Fraumeni syndrome (Ballinger *et al.*, 2015; Zerdoumi *et al.*, 2013; Naccarati *et al.*, 2012). Patients harboring a *p53* missense mutation, leading to expression of a mutant *p53* protein, in the germline have a significantly earlier cancer

onset than patients with mutations in *p53* that result in loss of *p53* protein expression (Zerdoumi *et al*., 2013).

2.5.6 Effects of alteration on *p53* gene

Mutations in *p53* are suggested to be the major cause of alterations in the gene (Rivlin *et al.,* 2013). These mutations are associated with genomic instability and increased susceptibility to cancer through production of an altered version of the protein that cannot control cell proliferation and is unable to trigger apoptosis in cell with mutated or damaged DNA (Kandoth *et al.,* 2013). Such mutations actively promote pro-survival signals and tumourigenesis. The mutations in the *p53* may lead to either loss of function or gain of functions, both of which hamper the *p53* function of tumour suppression activity (Alexandrova *et al.,* 2013; Rivlin *et al.,* 2011). Moreover, mutation in *p53* have been shown to cause alterations in other cancer genes including *PIK3CA, PTEN, KRAS, APC, CDKN2A,* and *ATM* (Zhang *et al*., 2017; Zerdoumi *et al.,* 2013). A noteworthy study by Rivlin *et al*. (2011) showed that more than 75% of *p53* gene mutations leads to expression of a mutant *p53* protein that has lost wild type function and may deploy a dominant negative regulation over the rest of wild type *p53* that suppress tumour by hetero-oligomerizing with wild-type *p53*. Therefore the mutant *p53* act as a competitive inhibitor of wild type *p53* (Rivlin *et al.,* 2011; Levine & Oren, 2009).

The consequence being loss of transcriptional activity of wild type *p53*, a phenomenon referred to as loss of function (Wang & Sun, 2016). Conversely, the mutant *p53* may also well acquire a new tumour-promoting activity that is independent of wild-type *p53* through production of oncoproteins that offer cancer cells growth and survival edges, phenomenon referred to as gain of function (Alexandrova *et al.,* 2013; Rivlin *et al.,* 2011). Mutant *p53* gain of function activities impact several aspects of cellular biology. The mutant *p53* affects chromatin structure, genomic stability, and activate transcription programmes impacting cancer cell metabolism, proteasome activity and microRNA biogenesis (Naccarati *et al.,* 2012). These activities may in turn foster mutant *p53* stability through increasing glucose levels and crosstalk with other oncogenic pathways including mevalonate, thereby conferring selective advantages for tumour growth and aggressiveness (Mantovani *et al.,* 2017; Naccarati *et al.,* 2012).

2.5.7 Mutation hotspots in *p53* gene

Mutation hotspots in *p53* gene have been identified in various human cancers (Kandoth *et al.,* 2013). The distribution of the hotspots in the gene appears to be centered on cancer type and

cell growth states (Robles *et al.,* 2016; Kandoth *et al.,* 2013). However, exons 4-8 of the gene are found to harbor major mutations (Muller & Vousden, 2014). There exist six major hotspot codons of the *p53* gene that constitute the most frequently mutated residues in majority of human cancers namely (but not limited to) codon 248 (248^{Arg}), codon 273 (273^{Arg}), codon 175 (175^{Arg}), codon 245 (245^{Gly}), codon 249 (249^{Arg}), and codon 282 (282^{Arg}) (Muller & Vousden, 2014; Kandoth *et al.,* 2013). The existence of these hotspot residues could be explained both by the susceptibility of particular codons to mutagens and carcinogen induced alterations and by positive selection of mutations that render the cell with growth and survival advantages (Rivlin *et al*., 2011). Compared to other cancer types, hepatocellular carcinoma (HCC) has been proposed to harbor distinctive mutation hotspot at codon 249 of the *p53* gene (Kancherla *et al*., 2018; Kandoth *et al*., 2013).

Our study was therefore centered on codon 249 because of its probable role in hepatocellular carcinogenesis and its amenability to mutational gene effects (Kandoth *et al*., 2013; Ozdemir *et al.,* 2010; Robles & Harris, 2010). Furthermore, previous studies have suggested that differences in geographical location and varied exposure to mutation inducing agents have fluctuating impact on mutation profiles of the *p53* codon 249 gene (Souza *et al*., 2013; Mah *et al*., 2011).

2.5.8 Mutation signatures in *p53* gene

Mutation signatures for specific cancers have been identified in *p53* gene (Mah *et al*., 2011; Özdemir *et al*., 2010; Aldona *et al*., 2009). In HCC, a unique transversion at codon 249 guanine (G) to thymine (T) is highly prevalent in geographic areas in which the mycotoxin aflatoxin is a widespread contaminant of the food mostly in parts of Africa, Eastern Asia, South America (Senerwa *et al.,* 2016). Excessive aflatoxin B1 (AFB1) exposure may induce formation AFB1-N7-guanine adduct that is implicated in HCC manifestation (Olivier *et al.,* 2010). Additionally, The AFB1 metabolites from cytochrome P450 in the liver causes DNA damage by forming covalent and promutagenic DNA adducts that could cause G to T transversion at codon 249 of the *p53* gene (Wang *et al.,* 2019). Non-melanoma skin cancer (basal and squamous cell carcinoma) and melanoma show a predominance of guanine: cytosine (C) to adenine (A): thymine (T) transitions at di-pyrimidine sites, including C to T tandem mutations, which result from ultraviolet-light-induced covalent coupling of carbon double bonds at adjacent pyrimidines (Janevska *et al.,* 2015). In lung cancer, *p53* mutations are G to T transversions in smokers, but not in never-smokers. Notably, this class of mutation is the main mutagenic effect of polycyclic aromatic hydrocarbons

(PAH) from the tar fraction of cigarette smoke (Olivier *et al.,* 2010). Exposure of human bronchial cells to the diol epoxide of the PAH benzo[a]pyrene causes DNA damage at the same G:C pairs that are frequently mutated into T:A in lung cancers from smokers. The excessive exposure to smoke may induce formation of PAH-N2-guanine adduct (Hainaut & Pfeifer, 2017).

The AFB1, most potent hepotocarcinogen, is metabolized in the liver by p450 enzymes into AFB1-8, 9-exo-epoxide, which is highly reactive and forms derivatives with DNA, RNA and proteins that can react with *p53* tumour suppressor gene (Hoffmann *et al*., 2018). In turn, AFB1 binds to DNA to form predominant promutagenic 8,9 dihydro-8-(N7-guanyl)-9-hydroxy AFB1 adduct. The latter can be converted into a more stable AFB1-formaminopyrimidine adduct, which causes guanine to thiamine transversion mutations. AFB1-formaminopyrimidine, incorporated into double-stranded DNA, is mutagenic, whereas the dominant species in single-stranded DNA blocks DNA replication (Janevska *et al.,* 2015; Kew, 2014; Gomes *et al*., 2013). Oxidative stress leads to lipid peroxidation of unsaturated fatty acids in membranes of cells and organelles. Cytotoxic byproducts of lipid peroxidation, such as malondialdehyde and 4-hydroxy-2′-nonenol are released and these impair cellular function, protein synthesis and induce DNA damage (Kew, 2014).

2.5.9 Mutant *p53* signalling

The majority of cancer-specific *p53* mutations result in nuclear accumulation of mutant *p53* protein that exhibits properties such as long half-life and the ability to transcriptionally activate genes that are not activated by wild-type *p53*, leading to both *p53* gain of functions and *p53* loss of functions (Kruiswijk *et al.,* 2015; Nakash *et al.,* 2014). The mutant *p53* affects chromatin structure, genomic stability, and activate transcription programmes impacting cancer cell metabolism, proteasome activity and microRNA biogenesis (Wang & Sun, 2016). These activities may in turn foster mutant *p53* stability through increasing glucose levels and crosstalk with other oncogenic pathways including mevalonate, thereby conferring selective advantages for tumour growth and aggressiveness (Mantovani *et al.,* 2017). A large number of genes are induced by gain-of-function *p53* mutants including epidermal growth factor (EGF) receptor, c-Myc, Fos, and MDR-1. Over-expression of MDR-1could result in the selective resistance of cancer cells to drugs such as vinblastine, etoposide, and taxol, which are transported by the membrane pump.

Moreover, mutant *p53* has also been suggested to augment the promigratory, proinvasive, and prometastatic properties of TGF-β (Mirzayans *et al.,* 2012; Yin *et al*., 2010). In a heterozygous

situation, where both wildtype and mutant alleles exist, mutant *p53* may antagonize wildtype *p53* tumour suppressor functions in a dominant manner (Naccarati *et al.,* 2012). This is because the transcriptional activity of wildtype *p53* relies on the formation of tetramers, whose DNA binding function may be interfered by mutant *p53* (Rivlin *et al*., 2011). Moreover, mutation in *p53* have been shown to cause alterations in other cancer genes including *PIK3CA, PTEN, KRAS, APC, CDKN2A,* and *ATM*. (Zhang *et al*., 2017).

2.5.10 Defects of *p53* gene mutation

Many different types of cancer show a high incidence of *p53* mutations, leading to the expression of mutant *p53* proteins (Robles *et al.,* 2016; Olivier *et al.,* 2010). The *p53* mutation may occur in germline cell or somatic cell referred to as germline mutation and somatic mutation respectively (Schon & Tischkowitz, 2018). However, the frequency of the occurrence of mutation in *p53* is exaggerated in somatic mutation (Yin *et al.,* 2010; Robles & Harris, 2010). Germline mutations in the *p53* gene cause a familial cancer predisposition. The somatic mutations affect codons within the DNA binding domain and accounts for major *p53* mutations while germinal mutation is majorly associated with the rare familial Li- Fraumeni syndrome (Ballinger *et al.,* 2015). Available evidence suggest that mutant *p53* have both lost wild-type *p53* tumour suppressor activity and gained functions that help to contribute to malignant progression (Robles *et al.,* 2016; Alexandrova *et al.,* 2013; Rivlin *et al.,* 2011).

Genetic disorders are traditionally categorized into three main groups: single-gene, chromosomal, and multifactorial disorders (Robles & Harris, 2010). Errors in DNA sequence of a gene lead to single gene or Mendelian disorders result from errors and include autosomal dominant, autosomal recessive, X-linked recessive, X-linked dominant and Y-linked (holandric) disorders. Chromosomal disorders are due to chromosomal aberrations including numerical and structural damages (Leroy *et al*., 2014). In autosomal dominant disorders, damage in one allele of a pair of the gene leads to the deficiency. A parent with an autosomal dominant disorder has a 50% chance of transmitting the disease to the offspring (Yin *et al*., 2010).

2.6 Mechanisms of DNA repair

Damage to DNA may result from exposure to diverse chemical and physical agents such as reactive oxygen species, ionizing radiation, ultraviolet light and various environmental, dietary or pollutant chemical agents. Defect in DNA repair gene may lead to cancer manifestation (Wright *et al.,* 2018; Olivier *et al.,* 2010). The DNA repair is subject to age-related changes and

deterioration, with the repair systems being generally weaker in very old and very young populations (Wright *et al.,* 2018; Olivier *et al.,* 2010), however, the mechanisms have not been fully unraveled.

Maintenance of the integrity and genetic stability of a cell require collective action of different repair mechanisms. Base excision repair (BER) proteins excise and replace damaged DNA bases, majorly emanating from endogenous oxidative and hydrolytic decay of DNA (Torgovnick & Schumacher, 2015). This process is initiated by DNA glycosylases that release modified base followed by cleavage of sugar phosphate chain, excision of a basic residue and local DNA synthesis and ligation (Meng *et al.,* 2015). Nucleotide excision repair (NER) mainly removes bulky adducts arising from environmental agents. The human cells use an elaborate assembly of gene products to execute NER such as XPC complex, DDB complex, XPA and RPA proteins that bind to sites of DNA damage. DNA helicases XPB and XPD, and excision by XPG and ERCC1-XPF proteins bring about unwinding of damaged DNA (Wright *et al.,* 2018; Meng *et al.,* 2015).

Mutations in DNA repair genes could be a prerequisite of tumour occurrence or could arise due to random accumulation of mutations during cycling of cancer cells (Torgovnick & Schumacher, 2015). Existence of incorrect DNA repair in tumour cells predispose them to accumulate further genetic alterations for instance colorectal and endometrial cancers with defective DNA mismatch repair due to mutations in the *MLH1* and *MSH2* genes exhibit increased rates of acquisition of single nucleotide changes and small insertions or deletions (Wright *et al.,* 2018).

The DNA double-strand breaks may be rectified by either homologous or non- homologous recombination pathways (Torgovnick & Schumacher, 2015). Homologous recombination involves branch migration enzymes and resolvases, while non homologous end-joining recombination involves protein factors like DNA-dependant protein kinase (Meng *et al.,* 2015). These mechanisms exploit the redundancy of information inherent in the double helical DNA structure. The defective region in one strand can be returned to its original form by relying on the complementary information stored in the unaffected strand (Olivier *et al.,* 2010).

The homologous recombination (HR) constitute genetic recombination in which nucleotide sequences are exchanged between two similar or identical DNA molecules. Homologous sequence is required to guide the repair (Lumir *et al.,* 2012; Li & Heyer, 2008). The HR is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA. The HR enable the

cell to access and copy intact DNA sequence information particularly to repair DNA damage affecting both strands of DNA (Ge & Hunter, 2019). Further, HR comprises a series of interrelated pathways that function in the repair of DNA double-stranded breaks (DSB) and interstrand crosslinks (ICLs). Moreover, recombination offer critical support for DNA replication in the recovery of stalled or broken replication forks, important in tolerance of DNA damage (Lumir *et al.,* 2012).

HR takes place in the late S-G2 phase of the cell cycle and involves the generation of a single-stranded region of DNA (Weiduo *et al.,* 2010). This is followed by strand invasion, formation of a Holliday junction, DNA synthesis using the intact strand as a template, branch migration and resolution (Wright *et al.,* 2018). Various tumour suppressors such as breast cancer susceptibility protein (*BRCA*1) and (*BRCA*2), and the RecQ helicase Bloom syndrome mutated have been shown to maintain genome integrity, at least in part, through HR (Frey & Pothuri, 2017).

Pathways of homologous recombination in DBS repair in somatic cells is conceptually divided into three stages including presynapsis, synapsis, and postsynapsis (Lumir *et al.,* 2012). During the first phase (presynapsis), DBS ends are recognized and subsequently processed to a 3′-OH ending single-stranded tail (Ge & Hunter, 2019). In the subsequent phase, DNA strand is invaded by Rad51-ssDNA filament to generate a D-loop (Weiduo *et al.,* 2010). Other pathways are proposed after the D-loop intermediate. In synthesis-dependent strand annealing, the invading strand is disengaged after DNA synthesis and annealed with the second end, leading to localized conversion without crossover (Lumir *et al.,* 2012). This process is characterised by multiple rounds of invasion, synthesis, and disengagement. In break-induced replication, the D-loop is assembled into a full replication fork, resulting in copying the entire distal part of the chromosome leading to loss-of heterozygosity LOH (Moureau *et al.,* 2016). In double-strand break repair (DSBR) both ends of the DSB are engaged, either by independent strand invasion or by second end capture, leading to double Holliday junction formation (Wright *et al.,* 2018). The junction can be processed by either a resolvase into non-crossover or crossover products or dissolved by a mechanism involving Bloom syndrome mutated - mediated branch migration and TOPOIIIα-catalyzed dissolution of a hemicatenane, leading exclusively to non-crossover products (Ge & Hunter, 2019; Weiduo *et al.,* 2010).

Repair of DNA interstrand crosslinks (ICL) can also be achieved through homologous recombination by resolving stalled replication forks at the ICL. The stalled replication fork is

recognized and cleaved by a specific endonuclease, hMus81-Eme1 (Torgovnick & Schumacher, 2015). This process occur in the leading-strand template to generate a single-sided DSB. A second incision is then introduction on the other side of the ICL that allows the lesion to flip out and to be bypassed by TLS. The DSB is processed to form a 3′-OH ending single-stranded tail and to initiate DNA strand invasion (Weiduo *et al.,* 2010). The replication fork is restored and the lesion is bypassed by TLS. The lesion is eventually repaired, either after HR. The DSB can also initiate DNA strand invasion using the homolog as a template. The DNA is synthesized across the lesion, disengaged and reinvasion of the sister chromatid behind the lesion site can lead to restoration of the replication fork and tolerance of the lesion (Wright *et al.,* 2018).

Repairs in DNA double strand breaks can also be achieved though non-homologous end joining (NHEJ) mechanism (Davis & Chen, 2013). This therefore offers an alternative pathway to double strand DNA damage repair. In NHEJ, the break ends are directly ligated without the need for a homologous template, in contrast to homology directed repair, which requires a homologous sequence to guide repair (Chang *et al.,* 2017; Moureau *et al.,* 2016). This pathway constitute primary mechanism for the repair of DNA double-strand breaks throughout the cell cycle, including during S and G2 phases (Mirzayans & Murray, 2012). Notably, NHEJ utilizes proteins that recognize, resect, polymerize and ligate the DNA ends in a flexible manner. In order to thread each broken DNA end, the NHEJ relies on the Ku protein that recruits enzymes and complexes that are required to trim (nucleases) or to fill in (polymerases) the ends to make them optimally ligatable by the DNA ligase IV complex (Ge & Hunter, 2019). When DNA double strand break (DBS) is induced, ku protein quickly binds to the ends of the broken DNA molecule (Chang *et al.,* 2017). The protein serves as a scaffold to recruit the core NHEJ machinery to the DNA DSB. Other proteins such as DNA-PKcs, XRCC4, XLF, and DNA Ligase IV are independently recruited to the Ku-DNA complex resulting in a stable complex at the DSB (Shen *et al.,* 2014). The initial step in NHEJ is the recognition and binding of the Ku heterodimer to the DSB. Proteins YWA and Ku core domains are involved in the heterodimerization of the complex (Moureau *et al.,* 2016; Davis & Chen, 2013).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study population and site

The study population composed of hepatocellular carcinoma (HCC) patients who presented at Moi Teaching and Referral Hospital (MTRH) for an earlier study undertaken between November 2016 to August 2017. Medical data of the patients' whose blood samples were also collected and archived included age, ethnicity and gender. The study population was generally from Kalenjin community. The MTRH was selected because it is the largest national referral hospital in western Kenya, and technical capabilities for detection and characterisation of carcinomas at its histopathology laboratory.

3.2 Ethical approval

The study utilised previous ethical approval obtained from the Kenya Medical Research Institute Scientific Ethics Review Unit (KEMRI-SERU), approval number 001/3211/2016 (see appendix I) and was part of a larger study.

3.3 Data protection

Confidentiality was maintained on all the information obtained in the course of the study. Sensitive data was maintained in password protected personal laptop computers. Data on any other encrypted computers did not include patient's names. All hard copies of the data collection tools were coded without including the patients' names. Patients' hospital admission numbers were used as identifiers for the participants. Study research staff sat web-based Collaborative Institutional Training Initiative (CITI) (West Virginia United States of America) and examination on research with Human subjects as part of requirement for human subjects' research as shown in appendix II.

3.4 Research design

This was a retrospective research study. The study used archived samples and that were collected during the period November 2016 to August 2017. All the patients were diagnosed with HCC by pathological diagnosis, a gold standard method of HCC diagnosis, according to the system shown in Table 1, and none of them had received any treatment by the time of sample collection. The study participants were only from stage I HCC cases.

Table 1: Histopathological classification criteria for hepatocellular carcinoma (HCC)

HCC staging	Description
Stage I	Single tumour without vascular invasion
Stage II	Single tumour with vascular invasion or multiple tumours < 5 cm
Stage III	Single tumour or multiple tumours of any size involving a major branch of the portal vein or hepatic vein
Stage IV	Tumours with direct invasion of adjacent organs or perforation of visceral peritoneum, lymphnodes and has metastasised

3.4.1 Sampling and sample size

The HCC cases were sampled using a purposive sampling (Etikan *et al.,* 2016; Dolores & Tongco, 2007). Fisher's formula ($N = Z^2PQ/D^2$) was used to determine the minimum sample size (Fisher *et al.,* 1998). In the formula, N = Sample size, Z = Standard error for mean at 1.96, D = Absolute precision at 5%, Q = (1-P) and P = Prevalence of the mutation at the codon which was 3% (Petitjean *et al.,* 2007).

Substituting the values into the above formula:

$$N=\frac{1.96^2 \times 0.03 \times 0.97}{0.05^2}$$

N=44.72 (which is approximately 45 individuals)

Forty six (46) HCC subjects were retrospectively used for the study. As part of control, a further ten (10) individuals were also used for the study. The controls were selected on the basis of prior similar studies that had 1 control for about 5 test people (Mah *et al*., 2011; Özdemir *et al*., 2010).

3.4.2 Inclusion criteria for the participants

HCC patients from Kalenjin ethnic community living within Uasin Gishu county who presented with stage one and were within significant risk age group for HCC development. The patients had to be between ages 20 and 80 years for eligible for the study. Also, healthy subjects from same location and community, and within the age bracket were used as controls for the study.

They were clinically defined as individuals who had tested negative for HCC serological molecular marker- alpha fetoprotein level.

3.4.3 Exclusion criteria

These included patients with benign tumours and HCC patients with either stage II, III or IV. Other exclusion criteria included patients who had received treatment for HCC prior to the time of blood collection, and those who or whose guardians objected to the inclusion at the time of blood collection were not eligible for the study.

3.5 Molecular alteration of *p53* gene

In order to analyze profile of codon 249 of *p53* gene, molecular techniques were employed.

3.5.1 Preparation of blood samples

Archived blood specimens anti-coagulated with EDTA were centrifuged (Eppendorf AG Hamburg) at 10,00rpm for 10 minutes to allow separation to obtain plasma and stored at -20 ^{0}C in biomedical freezer (Panasonic, Japan) until testing.

3.5.2 Extraction of DNA from plasma

DNA was then extracted from 200 µl of plasma samples using QIAmp DNA mini-extraction kit (Qiagen Inc, USA). Briefly, 20 µl proteinase K was pipeted into the bottom of 1.5 ml microcentrifuge and 200 µl of sample added followed by addition of 200 µl AL buffer then mixed by vortexing for 15 seconds. 1µl RNA carrier was then added to stabilize the extraction of DNA followed by incubation at 56^{0}C for 10 minutes and centrifuged at 8000 rpm for 1 minute to remove drops from inside the lid. 230 µl of 100% ethanol then added to the sample and mixed by pulse-vortexing for 15 seconds. The mixture was then transferred carefully to QIA-amp spin column (in a 2 ml collection tube) without wetting the rim, followed by centrifugation at 8000 rpm for one minute. QIA-amp column then placed into a clean 2 ml collection tube and the tube containing the filtrate discarded. 500 µl Buffer AW1 then added and centrifuged at 800 rpm for one minute, and QIA-amp spin column placed in a 2 ml collection tube and the collection tube containing the filtrate discarded. 500 µl Buffer AW2 then added in the QIA-amp and centrifuged at 14000 rpm for 3 minutes. The QIA-amp spin column was then placed in a clean 1.5 ml tube and the collection tube discarded. This was followed by addition of 60 µl AE buffer, incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for a minute. The extracted DNA was subsequently eluted in 60 µl of AE elution buffer and quantity measured by NanoDrop

spectrophotometer (Thermo Scientific) then stored at -30^{0}C in biomedical freezer (Panasonic, Japan) until tested.

3.5.3 PCR amplification of p53 exon 7 codon 249

Amplification of the targeted exon 7 undertaken in a single stage 25 µl reaction mix composed of 2.5 µl of genomic DNA template, 2.5 µl of 10X PCR buffer (10 mMTris-HCL, pH 9.0, 50 mM KCL, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% triton X-100), 1 µl of dNTP mix, 2 µl of 25 mM stock $MgCl_2$, 0.2 µl of 5 U of Taq DNA polymerase (Qiagen Inc, USA), 0.5 µl each of a 20 uM stock of forward primer and reverse primer of sequences (5'–CTTGCCACAGGTCTCCCCAA-3') and (5'–AGGGGTCAGCGGCAAGCAGA-3') respectively, and amplified in a Veritti-96-well thermal cycler (Applied Biosystems, Foster city, USA) for 35 cycles. Each cycle entailed initial denaturation at 95^{0}C for 10 minutes, denaturation at 95^{0}C for 45 seconds, primer annealing at 58^{0}C for 30 seconds and extension at 72^{0}C for 7 minutes. After that, a 4 µl aliquot of PCR product was electrophoresed by using 1% agarose (Fisher Scientific), 2 µl of 5X Gelpilot DNA loading Dye (Qiagen Inc, USA) together with 100 bp Tracklt 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^2 Imager then viewed using Vision Works LS software v.7.1. The expected size of the PCR product was 249 base pairs (bp).

3.5.4 DNA sequencing

Purification of the amplicons and sequencing were outsourced from Macrogen, Korea. All positive amplicons were purified and sequenced using forward and reverse primers (5'–CTTGCCACAGGTCTCCCCAA-3') and (5'–AGGGGTCAGCGGCAAGCAGA-3') respectively.

3.6 Mutation detection and analysis

The obtained sequences were aligned to the *p53* gene sequences by uploading the sequences onto National Center for Biotechnology Information (NCBI) – Basic Local Alignment Search Tool (BLAST) programme (www.ncbi.org) for identity confirmation. Mutations on the sequences were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) v.6.0 software bioinformatics editing tool. Consensuses of both forward and reverse sequences were built using GENETYX software version 9.0 that finds homology and create contigs. The contigs were then aligned to the *p53* gene reference sequence from International Agency for Research on Cancer (IARC) database using ClustalW alignment tool on MEGA v.6.0. The output of the aligned

sequences and the reference sequence was then viewed using MEGA v.6.0. The aligned sequences were then uploaded into T-coffee and subsequently submitted to ESPript v.3.0 software for analysis.

3.7 Data analysis

Test for statistical significance of mutation profile parameters were done using Chi-square test, and determination of association between the identified codon 249 mutations with hepatocellular carcinogenesis in HCC patients conducted using Fisher's exact test in Statistical Analysis Software version 9.4. Test for association between parameters were considered statistically significant at p values <0.05.

CHAPTER FOUR

RESULTS

4.1 Study participants' demographic characteristics

A total of forty six (46) stage I HCC subjects and ten (10) control subjects were considered as shown in Table 2. The ages for HCC cases and controls were within the bracket of significant risk for HCC development. This included ages 20-33 for low risk, 34-49 for medium risk and ≥50 for high risk as shown in Table 2. The ratio of male to female was 1:1 for both HCC subjects and control subjects. The age range for HCC subjects was from 25 to 67 years, and from 24 to 64 years for the controls. The median and mean ages for HCC subjects (42 and 44.35 respectively) and the control subjects (41 and 42.7 respectively) were not equal**.**

Table 2: Demographic characteristics of study participants

Characteristic	HCC	Control
Median age in years (range)	42 (25 – 67)	41 (24-64)
Age groups in years		
20-33	8	2
34- 49	23	5
≥ 50 years	15	3
Male: Female ratio	1:1	1:1

4.2 Mutation profile in hepatocellular carcinoma (HCC) and control subjects

Polymerase chain reaction amplicons for exon 7 of *p53* gene were visualized using ultraviolet trans-illuminator gel Doc-It^2 Imager using Vision Works LS software as shown in Figure 6. Nucleotide sequences alignment and protein sequences alignment of codon 249 of *p53* gene in HCC patients and controls are shown in figure 7. A selective guanine (G) - to - thymine (T) transversion mutation in the third base of codon 249 of *p53* gene, corresponding to arginine - to -serine amino acid substitution at codon codon 249 was present in eight (8) of the forty six patients with HCC (Table 3). Among the HCC patients with the mutation, there were more males than females (Table 3). There was no significant difference across gender among HCC patients with and without the selective mutation ($p=0.4549$) at 5% level of significance. However, there was statistically significant difference in median age among HCC patients with and without the selective mutation ($p=0.0010$). There was also a striking picture of significant existence of codon 249 mutation in a much older population in the HCC patients ($p<.0001$) at 5% level of significance (Table 3), suggesting that being in the old age is more susceptible to such mutation.

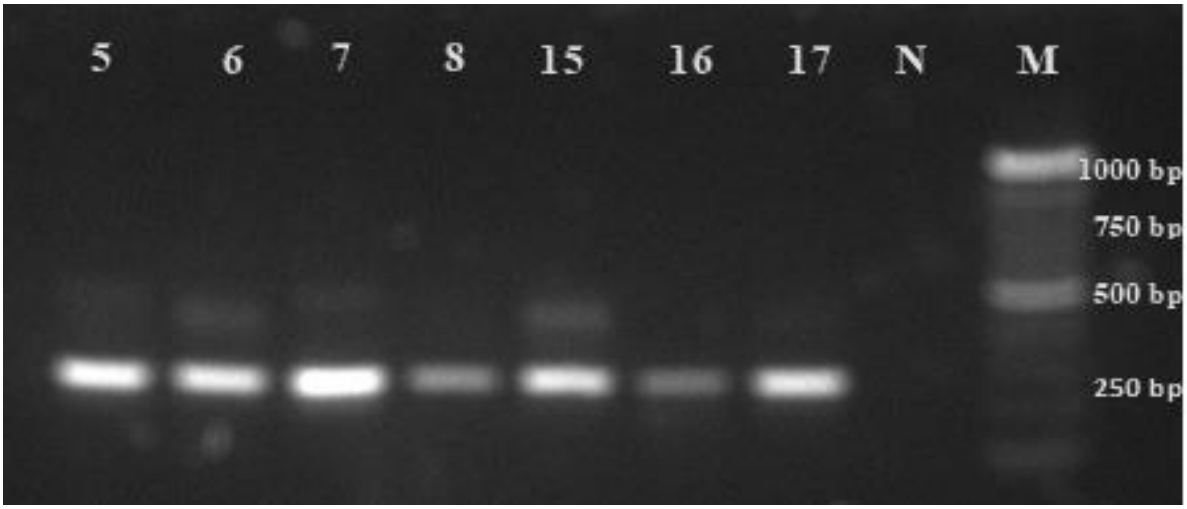

Figure 6: SYBR-safe DNA gel electrophoresis image of amplified products of exon 7 of *p53* gene. Lanes 5, 6, 7 and 8 represent amplified products of exon 7 from hepatocellular carcinoma samples while lanes 15, 16 and 17 represent amplified products of exon 7 from control subjects samples, lane N is a negative control for PCR, Lane M is a 1 kb DNA size molecular marker for amplification. Molecular grade water was used as negative control for the PCR amplification. The bands show positive amplification of exon 7 of *p53* gene.

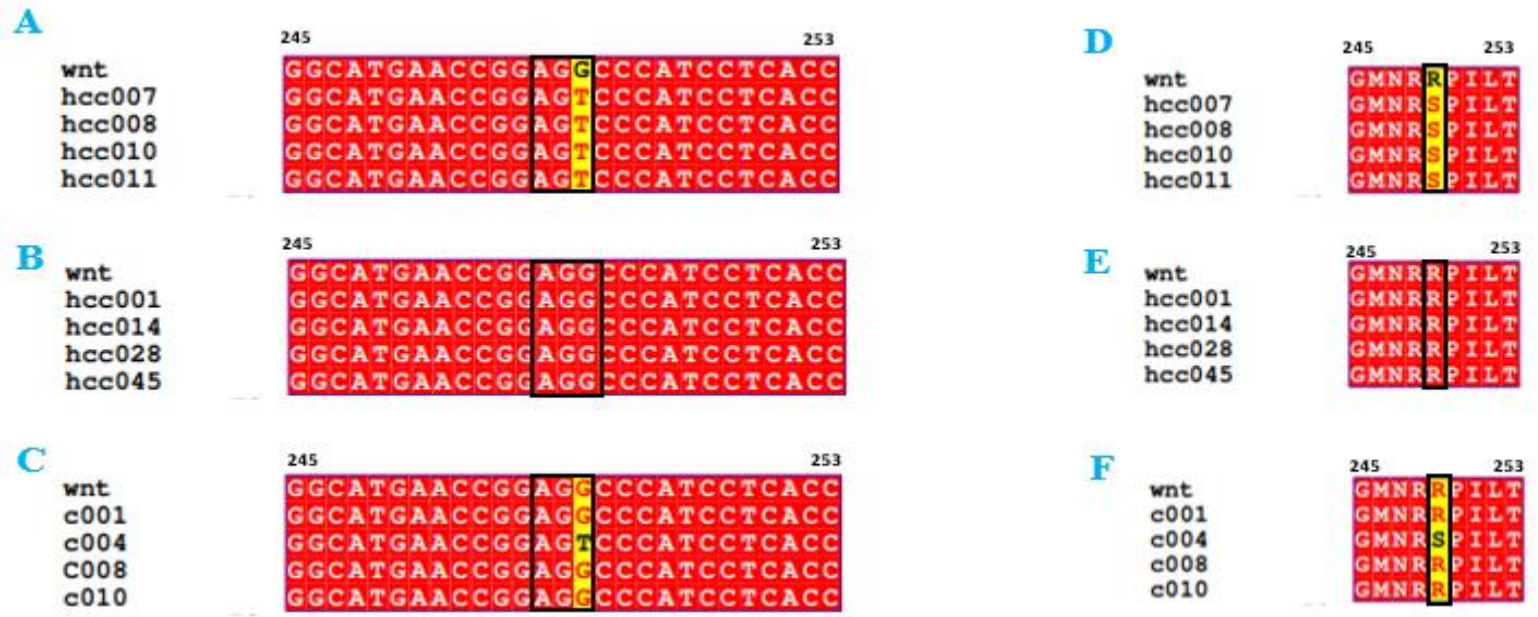

Figure 7: Multiple sequences alignment of exon 7 of *p53* gene (codons 245-253). Panel A. Multiple nucleotide sequences alignment of hepatocellular carcinoma cases with mutation at codon 249 (boxed) represented by nucleotide base T (yellow background). Panel B. Multiple nucleotide sequences alignment of hepatocellular carcinoma cases without mutation at codon 249 (boxed). Panel C. Multiple sequences alignment of controls. Mutation in one of the controls at codon 249 (boxed) is represented by nucleotide base T (yellow background). Panel D. Multiple protein sequences alignment of hepatocellular carcinoma cases with mutation at codon 249 (boxed) represented by amino acid residue S (yellow background). Panel E. Multiple protein sequences alignment of hepatocellular carcinoma cases without mutation at codon 249 (boxed). Panel F. Multiple protein sequences alignment of controls. Mutation in one of the controls at codon 249 (boxed) is represented by S (yellow background). Panels A-C, T: thymine nucleotide, G: guanine nucleotide, C: cytosine nucleotide, A: adenine nucleotide. Panels D-F, G: glycine residue, M: methionine residue, N: asparagine residue, R: arginine residue, P: proline residue, I: isoleucine residue, L: leucine residue, T: threonine residue, S: serine. wnt: wild type.

Table 3: Number and proportion of individuals with hepatocellular carcinoma with and without mutation at codon 249 by gender and age groups

Characteristic	Presence of mutation, n (%)	Absence of mutation n (%)	P value
Gender			
Male	5 (21.7)	18 (78.3)	0.4549
Female	3 (13.0)	20 (87.0)	
Age group			
20 – 33	0 (0)	8 (21.1)	<**0.0001**[a]
34 – 49	1 (12.5)	22 (57.9)	
≥ 50	7 (87.5)	8 (21.1)	

Specific mutation: Guanine (G) - to - thymine (T) transversion in the third base of codon 249 of *p53* gene corresponding to arginine - to -serine amino acid substitution. [a]Statistical significance determined by Chi-square Test. *p<0.05 was considered statistically significant. All values in bold are statistically significant at $p<0.05$.

A selective guanine (G) - to - thymine (T) transversion mutation in the third base of codon 249 of *p53* gene, corresponding to arginine - to -serine amino acid substitution at codon 249 was present in one of the ten control subjects (Table 4). Also notable was significant existence of such mutation in a much older population in the controls (p=0.0003) at 5% level of significance, suggesting that being in the old age is more susceptible to codon 249 mutation. The difference across gender was however statistically significant among the controls with and without codon 249 mutation (p=<.0001) at level of significance p<0.05. There was statistically insignificant difference for median age among the control (p=0.3851) at level of significance p<0.05 (Table 4).

Table 4: Number and proportion of control individuals with and without mutation at codon 249 by gender and age group

Characteristic	Presence of mutation, n (%)	Absence of mutation, n (%)	P value
Gender			
Male	1 (20.0)	4 (80.0)	< **0. 0001**[a]
Female	0 (0)	5 (100.0)	
Age group			
20 – 33	0 (0)	2 (22.2)	**0.0003**[a]
34 – 49	0 (0)	5 (55.6)	
≥ 50	1 (100.0)	2 (22.2)	

Specific mutation: Guanine (G) - to - thymine (T) transversion in the third base of codon 249 of *p53* gene corresponding to arginine - to - serine substitution. [a]Statistical significance determined by Chi-square Test. *p<0.05 was considered statistically significant. All values in bold are statistically significant at $p<0.05$.

4.3 Determination of association between mutation and HCC

To further understand the contribution of codon 249 mutation to hepatocellular carcinogenesis, we determined the association of the mutation with HCC using Fisher's exact test. We report no significant statistical association of codon 249 mutation between HCC and control (p=0.6821). The odds ratio of acquiring codon 249 mutation among HCC patients was 0.5278 at 95% confidence interval with a range of 0.0584 to 4.7736.

CHAPTER FIVE
DISCUSSION

In this study, we first sought to identify the profile of *p53* gene exon 7 codon 249 gene mutation(s) among HCC patients presenting with stage I cancer disease. To the best of our knowledge, we report the first, albeit partial picture of *p53* exon 7 codon 249 mutation profile in HCC patients in a proportion of Kenyan population. We report the presence of selective guanine (G) - to - thymine (T) transversion mutation in the third base of codon 249 in DNA isolated from eight out of forty six HCC patients. This mutation corresponds to arginine-to-serine substitution at the codon 249. Our findings corroborate limited data available among Guangxi, Taiwan and Gambian populations where similar mutations were reported (Mah *et al*., 2011; Özdemir *et al*., 2010; Aldona *et al*., 2009).

Notwithstanding, males were overrepresented in the mutation positive categories in HCC subjects. However, there was no significant difference across gender among hepatocellular carcinoma subjects with and without mutation (p=0.4549) at 5% level of significance. This could be attributed to possible occurrence of faster and more severe hepatocellular carcinogenesis in males than females (Li *et al.,* 2016). Also noticeable is the trend in age among those with the mutation whereby there was a striking picture of significant existence of such mutation in a much older population in both the HCC patients (p=<.0001) and the controls (p=0.0003) at 5% level of significance, implying that order individuals were more susceptible to the selective G - to - T transversion mutation in the third base of codon 249 as compared to the younger individuals who are less vulnerable. A probable explanation would be that, according to National Cancer Institute (2017), occurrence of mutation is leveraged on long duration of living characterised by excessive exposure to mutagens and carcinogens since the process of mutation occurrence is a gradual process (Janevska *et al*., 2015). The prolonged duration of excessive exposure to mutagenic and carcinogenic agents due to aging result in accumulative DNA damage in cells (Rivlin *et al.,* 2011). Consequently, it is argued that presence of a single mutation alone in DNA is unlikely to cause cancer, rather accumulative or multiple mutations in *p53* tumour suppressor gene characterised by long life span (Adjiri, 2017).

Mutations in DNA repair genes could be a prerequisite of tumour occurrence or could arise due to random accumulation of mutations during cycling of cancer cells (Torgovnick & Schumacher, 2015). Existence of incorrect DNA repair in tumour cells predispose them to

accumulate further genetic alterations (Wright *et al.,* 2018). Arguably, the present study may not confirm or rule out the possibility that mutation may show up at later stages of hepatocellular carcinogenesis with increase in age among other confounding factors.

Mutation in *p53* gene may lead to expression of a mutant *p53* protein that has lost wild type function and may deploy a dominant negative regulation over the rest of wild type *p53* that suppress tumour through hetero-oligomerizing with wild-type *p53*, thus acting as a competitive inhibitor of wild type *p53* (Rivlin *et al.,* 2011). The consequence of this is loss of transcriptional activity of wild type *p53,* a phenomenon referred to as loss of function (Wang & Sun, 2016). Mutations in *p53* is mostly followed by loss of heterozygosity during cancer progression implying that the dominant negative regulation activity of mutant *p53* is not sufficient to completely inactivate wildtype *p53,* nevertheless, the dominant regulation activity may in rare events present in late stage of cancer (Kew, 2014; Rivlin *et al*., 2011).

Conversely, the mutant *p53* may also well acquire a new tumour-promoting activity that is independent of wild-type *p53* through production of oncoproteins that offer cancer cells growth and survival edges, phenomenon referred to as gain of function (GOF) (Alexandrova *et al.,* 2013; Rivlin *et al.,* 2011). Mutant *p53* GOF activities, the most wedging oncogenic activity of *p53* protein, impact several aspects of cellular biology including affect chromatin structure, genomic stability, and activate transcription programmes wedging cancer cell metabolism, proteasome activity and microRNA biogenesis (Wang *et al.,* 2019; Naccarati *et al.,* 2012). These activities may in turn foster mutant *p53* stability through increasing glucose levels and crosstalk with other oncogenic pathways, thereby conferring selective advantages for tumour growth and aggressiveness (Mantovani *et al.,* 2017; Naccarati *et al.,* 2012).

Existence of guanine to thymine transversion mutation, corresponding to arginine-to-serine substitution, at the codon 249 leads to *p53* protein GOF activity in HCC (Liao *et al.,* 2017). It is still an open question how mutant *p53* GOF activities are enabled by the unique blend of HCC related cues. Nevertheless, it is suggested that the *p53* protein GOF activity is achieved through CDK4/cyclin D1-Pin1-*p53*-R249S-c-Myc signalling pathway (Wang *et al.,* 2019; Muller & Vousden, 2014). Residues in the *p53* protein are critical to its DNA binding function of tumour suppression realized through activation of DNA repair proteins when DNA has sustained damage, inducing growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition, or initiating apoptosis if DNA damage is severe and proves to be irreparable (Nasr &

Pelletier, 2012; Olivier *et al.,* 2010). The change in amino acid arginine to serine substitution at codon 249 result in non-conservative mutation that affects *p53* biochemical properties or pathways through interactions with other proteins involved in cell signalling including Pin1, c-Myc, CDK4 proteins (Wang *et al.,* 2019; Özdemir *et al*., 2010; Aldona *et al*., 2009). Furthermore, the non-conservative mutation may lead to disruption of hydrogen bonding causing incorrect folding of *p53* protein, altering transactivation of *p53* targets in tumours (Muller & Vousden, 2014; Rivlin *et al.,* 2013; Andrew *et al.,* 2002).

The Pin1, a phosphorylation-dependent prolyl-isomerase is an oncoprotein highly expressed in human cancer responsible for conversion of inactive proteins into active oncoproteins such as *p53* mutants or c-Myc (Girardini *et al.,* 2011; Farrell *et al.,* 2013). The protein binds to a phosphorylated resultant serine motif and through this, the protein is suggested to modulate multiple cellular events, including gene regulation, RNA processing, cell proliferation, nuclear import, and differentiation thus enhancing cell proliferation, survival and tumourigenesis (Wang *et al*., 2019). Codon 249 mutation result in the substitution of arginine residue with serine residue, which offers platform for Pin1 phosphorylation activity to induce HCC tumourigenesis (Girardini *et al.,* 2011).

The c-Myc, a nuclear transcription factor, highly expressed in majority of human cancers is important in regulation of gene transcription required in cell proliferation, survival and tumourigenesis, with the overall aim being activation of expression of genes involved in ribosomal biogenesis and protein synthesis (Lin *et al.,* 2012). This oncoprotein is essential for growth and progression of HCC (Wang *et al.,* 2019).

The CDK4 protein is of importance in the G1/S phase of the cell cycle by forming a complex with cyclin D, which is utilized by tumour cells for their survival and growth advantages (Asghar *et al.,* 2015). The CDK4/cyclin D1 complex phosphorylate *p53* mutant protein to inhibit cell senescence and apoptosis hence can perpetuate tumourigenic events, including hepatocellular carcinogenesis (Asghar *et al.,* 2015; Sheppard & McArthur, 2013).

Various risk factors associated with HCC development such as AFB1 and HBV perpetuate the GOF activity of *p53* protein by creating microenvironment or molecular environment and reprograms oncogenic signalling pathways leading to a rare posttranslational modifications, such as methylation at K370 by SETDB1 (Fei *et al.,* 2015), and phosphorylation at codon 249 by CDK4/cyclin D1 (Liao *et al.,* 2017).

The *p53* inactivation due to mutation occur at late stages of cancer, that is characterised by shortening of telomeres and loss of short arm of chromosome 17, which contains *p53* gene, rather than early stage (Rivlin *et al*., 2011). This partially explain the absence of mutation for the thirty eight HCC cases who were all stage one HCC subjects. Still, the presence of mutation does not unequivocally indicate that *p53* is fully inactive, similarly, the absence of mutation does not necessarily indicate that *p53* is functionally proficient (Robles & Harris, 2010). Thus, assessing functional activity of *p53* mutants is essential for an accurate indication of clinical relevance.

Secondly, we also sought to determine association of *p53* gene exon 7 codon 249 mutation(s) with hepatocellular carcinogenesis in HCC patients. Our study findings show no statistically significant association of codon 249 mutation with 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). This seamlessly implied that there was exaggerated increase in risk of acquiring codon 249 mutation among HCC patients. These findings are in agreement with finite data available in Taiwan, United States, Japan, Australia, Gambian and Guangxi populations (Mah *et al*., 2011; Özdemir *et al*., 2010; Stern *et al*., 2001). Likewise, accumulative evidence 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 (Imran *et al*., 2017; Bruix & Sherman, 2011; Özdemir *et al*., 2010).

Counter-intuitively, the specific codon 249 mutation was not only evident in HCC patients but also in one out of the ten controls. This corroborates earlier findings by Kirk *et al.* (2000) that reported codon 249 mutation presence in 3 of 53 control subjects, and that by Ozturk (1994) that reported codon 249 mutation in non-malignant liver tissues. A possible explanatory analysis for this intriguing finding would be that generally, it is established that codon 249 is an important hotspot for AFB1 modification and AFB1-induced mutation for patients residing in high-risk regions, where chronic HBV as well as hepatitis C (HCV) infections and exposure to dietary AFB1 are endemic (Özdemir *et al*., 2010; Aldona *et al*., 2009).

Furthermore, among *p53* mutations signatures for HCC compiled in the International Agency for Research on cancer (2018) *p53* mutation database, 66% occur in patients with HCC originating from regions with a high incidence of HCC and high exposure to dietary AFB1 (Mah *et al*., 2011; Özdemir *et al*., 2010; Aldona *et al*., 2009). Additionally, in HCC, a unique transversion at codon 249 (G:C.T:A) is highly prevalent in geographic areas in which the

mycotoxin aflatoxin is a widespread contaminant of the food mostly in parts of Africa, Eastern Asia, South America (Senerwa *et al.*, 2016). The excessive AFB1 exposure may induce formation of AFB1-N7-guanine adduct that is implicated in HCC manifestation (Mah *et al*., 2011). Moreover, AFB1 metabolites from cytochrome P450 in the liver causes DNA damage by forming covalent and promutagenic DNA adducts that could cause G to T transversion at codon 249 of the *p53* gene (Wang *et al.,* 2019). This study, however, did not perform aflatoxin exposure level test 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 array of probable analysis may perhaps implicate that the existence of the mutation in one of the controls could have paralleled excessive exposure to aflatoxin B1 and/or integration of viral DNA of chronic HBV infection into the host genome which may induce chromosome instability (Stahl *et al.,* 2016). Alternatively, we hypothesize that it may be suggestive of an early genetic event in hepatocellular carcinogenesis as seen in some of our study HCC subjects.

This study acknowledge that the samples were from patients presenting with only stage one HCC thus the small sample size, and that the study did not obtain tumour tissues from the patients for verification of concordant or discordant mutation status.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study conclude that though there is existence of a specific mutation in codon 249, our findings found no statistically significant association between *p53* exon 7 codon 249 mutations and hepatocellular carcinoma from patients presenting with only stage I cancer. However, there seems to be exaggerated increase in risk of acquiring codon 249 of *p53* gene mutation among hepatocellular carcinoma patients. Consequently, although codon 249 of *p53* mutations has been found very minimal in HCC patients, its existence communicates a probable role in hepatocellular carcinogenesis. Nevertheless, the present study cannot exclude the possibility that mutation in codon 249 in HCC patients may show up and become statistically significant at later stages of hepatocellular carcinogenesis as suggested elsewhere. Also evident from this study is that there is increased risk acquiring codon 249 mutation of *p53* gene in HCC patients in older population compared to younger individuals irrespective of gender. Additionally, we anticipate that there could be confounding factors that drive mutation manifestation in codon 249 of *p53* gene in HCC patients.

6.2 Recommendations

Although this study has investigated the presence of mutation in codon 249 of *p53* gene and its subsequent analysis in stage one HCC patients, the following recommendations are necessary:

i. Patients with HCC should be monitored for codon 249 *p53* gene mutations as part of assessment for disease prognosis.
ii. There is need for supplementary cross sectional studies on codon 249 of *p53* gene mutations in HCC patients presenting with different stages to observe pattern across the stages.
iii. We recommend studies on the whole length of *p53* gene for mutation investigation to identify other HCC mutation hotspot(s) ant to better ameliorate the mechanisms involving hepatocellular carcinoma pathogenesis.
iv. There is need to investigate the contribution of HCC multi-etiologic factors in codon 249 of *p53* gene mutation patterns.

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APPENDICES

Appendix I: Ethical approval certificate

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **March 31, 2016**

TO: **OCHWOTO MISSIANI,**
PRINCIPAL INVESTIGATOR

FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. BOX 54628
NAIROBI.
1st APRIL 2016

THROUGH: **DR. ROSEMARY SANG,**
ACTING DIRECTOR, CVR,
NAIROBI

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CVR/001/3211 (*RESUBMISSION2 OF INITIAL SUBMISSION*): AFLATOXINS AND ALTERATION OF TP53 TUMOR SUPPRESSOR GENE IN HUMAN CANCERS; THEIR PREVALANCE AND POTENTIAL USE A CANCER MARKERS FOR DIAGNOSIS OR TREATMENT IN KENYA-(*VERSION 3.0 DATED 24TH MARCH, 2016*)**

Reference is made to your letter dated 24th March, 2016. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised protocol on the 29th March, 2016.

This is to inform you that the Committee notes that the issues raised by the Expedited Review Team have been adequately addressed.

Consequently, the study is granted approval for implementation effective on **31st March, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **March 30, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **February 13, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

pp **DR. EVANS AMUKOYE,**
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

FOR VIRUS RESEARCH
01 APR 2016
P.O. Box 54628 - NAIROBI

In Search of Better Health

Appendix II: CITI certificate

CITI PROGRAM

Completion Date 13-Dec-2017
Expiration Date 13-Dec-2018
Record ID 25309940

This is to certify that:

colins oduma

Has completed the following CITI Program course:

Biomedical Research - Basic/Refresher (Curriculum Group)
Biomedical Research - Basic/Refresher (Course Learner Group)
1 - Basic Course (Stage)

Under requirements set by:

Kenya Medical Research Institute

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?wdd5bdd6b-e346-46e2-8c9d-cb425ba79740-25309940