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CHARACTERISATION OF HEPATOCELLULAR CARCINOMA AND OTHER HEPATIC TUMOURS DIAGNOSED AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, ZAMBIA.

By

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A Research Dissertation Submitted to the University of Zambia in partial fulfilment of the Requirements for the Degree of Master of Science in Pathology (Clinical Pathology)

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Declaration

I, **STEWARD MUDENDA** this 11th day of January 2017, declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references. I further declare that I followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Masters of Science in Pathology (Clinical Pathology), University of Zambia guidelines.

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Certification of completion of dissertation

I, Prof. Trevor Kaile, having supervised and read this dissertation is satisfied that this is the original work of the author under whose name is being presented. I confirm that the work has been completed satisfactorily and is ready to the examiners.

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Certification of Approval

DISSERTATION TITLE: CHARACTERISATION OF HEPATOCELLULAR CARCINOMA AND OTHER HEPATIC TUMOURS DIAGNOSED AT THE UNIVERSITY TEACHING HOSPITAL

This dissertation for Steward Mudenda has been approved as partial fulfilment of the requirements for the award of the Master of Science degree in Pathology (Clinical Pathology) at the University of Zambia.

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Abstract

Introduction: Hepatic tumour is a joint term for a diverse group of liver tumours which are caused by a variety of agents and fall into primary or secondary hepatic tumours. A tumour is an abnormal benign or malignant new growth of tissue that may or may not possess physiological functions and arises from uncontrolled usually rapid cellular proliferation. There is little information on the distribution of hepatic tumours in Zambia. Histopathology and immunohistochemistry is cardinal in the classification of hepatic tumours.

Aim: To determine the phenotypic distribution and prevalence of hepatic tumours at the University Teaching Hospital, Lusaka.

Materials/Methods: This was a retrospective cross-sectional study that was conducted at the University Teaching Hospital (UTH) from February 2016 to July 2016. Archival formalin-fixed paraffin embedded 34 liver biopsy specimen blocks were obtained, sectioned, re-stained and examined histologically. These tissues were diagnosed as hepatic tumours from 2012 to 2015. Tissues were cut for histological analysis using a microtome. Haematoxylin and Eosin staining were done on the slides to confirm hepatic tumor status. Immunohistochemical staining was done to detect HBV using hepatitis surface antigen (HBsAg). Data was analyzed using IBM SPSS version 20.0 for windows. Chi-square and Fisher's exact test with a p-value of less than 0.05 was used to indicate statistical significance of the results.

Results: From the examined 34 cases, patient age distribution of hepatic tumours was as follows; <10 years (8.8%), 10-20 years (8.8%), 21-30 years (20.6%), 31-40 years (14.7%), 41-50 years (14.7%), 51-60 years (17.6%), 61-70 years (11.8%), and >70 years (2.9%). Hepatic tumours affected males 19/34 (55.88%) and females 15/34 (44.12%) with a p-value of 0.03 as statistical significant. The male to female ratio of patients diagnosed with hepatic tumors was found to be 1.3:1. The phenotypes of hepatic tumours included 17/34 (50%) hepatocellular carcinomas (HCC), 6/34 (17.6%) metastatic adenocarcinomas, 4/34 (11.8%) intrahepatic cholangiocarcinomas (ICC), 3/34 (8.8%) hepatoblastomas, 2/34 (5.9%) metastatic small cell carcinomas, and 2/34 (5.9 %) hepatic adenomas. The histological subtypes of HCC diagnosed included trabecular pattern 9/17 (52.9%), pseudoglandular pattern 4/17 (23.5%), solid pattern 2/17 (11.8%), diffuse pattern 1/17 (5.9%), and fibrolamellar pattern 1/17 (5.9%). HCC was graded into well-differentiated 7/17 (41.2%), moderately differentiated 8/17 (47.0%), and poorly differentiated 2/17 (8.8%).

Conclusion: Hepatic tumours affect patients mostly in the age range of 21-30 years and more males than females. HCC is the most common histologically diagnosed hepatic tumour at UTH followed by metastatic adenocarcinomas. The most diagnosed grade of HCC is moderately differentiated followed by well differentiated grade.

Key words: Hepatic tumour, hepatocellular carcinoma, histological subtype, Zambia.

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Dedication

This work is dedicated to my loving wife Memory Muleya Mudenda and our beautiful daughter Esnart Luyando Mudenda. I further dedicate this dissertation to all my family members and classmates.

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Steward Mudenda (September, 2016)

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Acronyms and Abbreviations

CMV	–	Cytomegalovirus
dsDNA	–	double stranded Deoxyribonucleic Acid
EBV	–	Epstein - Barr Virus
FFPE	–	Formalin-Fixed Paraffin Embedded
H & E	–	Hematoxylin and Eosin
HAV	–	Hepatitis A Virus
HBV	–	Hepatitis B Virus
HBsAg	–	Hepatic B surface Antigen
HCC	–	Hepatocellular Carcinoma
HCV	–	Hepatitis C Virus
HDV	–	Hepatitis D Virus
HEV	–	Hepatitis E Virus
HPB	–	Hepatoblastoma
IHC	–	Immunohistochemistry
ICC	–	Intrahepatic cholangiocarcinoma
PCR	–	Polymerase Chain Reaction
RNA	–	Ribonucleic Acid
UNZA	–	University of Zambia
UNZABREC	–	University of Zambia Biomedical Research Ethics Committee
UTH	–	University Teaching Hospital
WHO	–	World Health Organisation

Chapter 1: Introduction

1.1 Background

A hepatic tumour is a joint term for a diverse group of liver tumours which are caused by a variety of agents and fall into primary or secondary hepatic tumours. A tumour is an abnormal benign or malignant new growth of tissue that may or may not possess physiological functions and arises from uncontrolled usually rapid cellular proliferation. There is little information on the distribution of hepatic tumours in Zambia. Histopathology and immunohistochemistry is cardinal in the classification of hepatic tumours.

1.1.1 Incidence, morbidity and mortality of hepatic tumours

Hepatocellular carcinoma (HCC) is the commonest type of primary hepatic tumour. Its yearly incidence is approximately 2-7/100 000 inhabitant's worldwide (Schuppan *et al.*, 2008) and it is expected to increase in the future. According to Gomaa *et al.*, 2008, HCC is the 5th commonest malignancy worldwide and the third leading cause of cancer-related death (Gomaa *et al.*, 2008). Although more rarely detected, cholangiocarcinoma (CC) either intrahepatic cholangiocarcinoma (ICC) or extrahepatic cholangiocarcinoma (ECC) is the second most common primary hepatic tumour (Park *et al.*, 2009). In less than 1% of the cases, ICC occurs simultaneously with HCC (Bosman *et al.*, 2010). Most cases of primary hepatic tumours occur in Sub-Saharan Africa, East Asia and Malaysia and low incidence of the disease has been recorded in North and South America, Europe, Australia and New Zealand (Boyle *et al.*, 2008). According to data provided by the GLOBOCAN project of the IARC (International Agency for Research on Cancer), in 2012 the global prevalence of primary hepatic tumours in males was 7.5% and 3.5% in females, a number of 782, 000 cases, ranking sixth among all cancer types and mortality was 10/100, 000, respectively 746, 000 death cases, ranking second regarding global cancer mortality rate. Thus, in 2012 there were 554, 000 male patients diagnosed with primary malignant hepatic tumours worldwide, out of whom 521, 000 died. In case of female patients, there were 224, 000 death cases out of the 228, 000 new cases diagnosed as primary malignant hepatic tumours (Ferlay *et al.*, 2012).

Romania occupies its place among countries with average incidence of hepatic tumours, thus according to data provided by GLOBOCAN in 2008, the incidence of hepatic tumours for both genders was of 5.3/100 000, respectively 1971 cases, ranking twelfth among all tumour types. The patients' mortality was 6.9/100 000, respectively 2686 death cases were recorded, ranking seventh regarding national cancer mortality rate. In case of male patients, the incidence of hepatic tumours in Romania in 2008 was 8.1/100 000, respectively 1279 cases and mortality was 10.5/100 000, respectively 1722 death cases. In case of female patients, the incidence of hepatic tumours in Romania in 2008 was 3/100 000, respectively 692 cases and mortality was 4/100 000, respectively 964 death cases (Ferlay *et al.*, 2008).

Globally, as of 2010, hepatic tumours resulted in 754,000 deaths, up from 460,000 in 1990, making it the third leading cause of cancer deaths after lung and stomach cancers. In 2012, it represented 7% of cancers diagnoses in men, the fifth diagnosed cancer that year (World Cancer Report, 2014). Of these deaths, 340, 000 were secondary to HBV, 196,000 were secondary to HCV, and 150,000 were secondary to alcohol (Lozano *et al.*, 2012). In 2013, 300,000 deaths from hepatic tumours were due to hepatitis B, 343,000 to hepatitis C and 92,000 to alcohol (Wang *et al.*, 2013). HCC is the most common hepatic tumour showing a striking geographical distribution. China has 50% of HCC cases globally, and more than 80% of total cases occur in sub-Saharan Africa or East Asia due to HBV (Jemal *et al.*, 2011). The most frequent hepatic tumour, accounting for approximately 75% of all primary hepatic tumours, is HCC (also named hepatoma, which is a misnomer because adenomas are usually benign). HCC is a cancer formed by liver cells, known as hepatocytes, which become malignant. Another type of cancer formed by liver cells is hepatoblastoma, which is specifically formed by immature liver cells (Ahmed *et al.*, 2009). It is a rare malignant tumour that primarily develops in children, and accounts for approximately 1% of all cancers in children and 79% of all primary liver cancers under the age of 15. Most hepatoblastomas occurs in the right lobe (Emre *et al.*, 2004).

1.1.2 Causes of hepatic tumours

Hepatic tumours can be caused by a number of agents such as hepatotropic viruses, haemochromatosis, tyrosinemia, excessive alcohol intake, and exposure to aflatoxin B1 (Canadian Liver Foundation, 2015). Risk factors for developing hepatic tumours include male gender, age, smoking, race, cirrhosis, fatty liver disease, obesity, diabetes, alpha-1 antitrypsin deficiency, anabolic steroids, oral contraceptives, Schistosomiasis, and arsenic. The viruses that cause hepatic tumours include Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Hepatitis D Virus (HDV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), and Herpes Simplex Virus (HPV). These hepatotropic viruses and other agents are involved in the aetiology of hepatic tumours (Roger *et al.*, 2007).

1.1.2.1 HBV involvement in aetiology of hepatic tumours

This study concentrated on HBV as a possible causative agent of hepatic tumours. The HBV genome is a double stranded Deoxyribonucleic Acid (dsDNA) which can be defined as a molecule of DNA consisting of two parallel strands joined by hydrogen bonds between complimentary purines and pyrimidines; a double helix, the form in which DNA occurs in chromosomes. The HBV genome is made of circular dsDNA of which one end of the full length strand is linked to the DNA polymerase and can be detected in tumor cells or tissues (Kumar *et al*., 2007).

The transmission of HBV is mainly caused by infected blood or its products. However, other risk factors have been demonstrated for HBV infection, which are mainly represented by the intravenous use of illicit drugs, sexual intercourse, working in a healthcare, living with an infected person, blood transfusions, and hemodialysis (Buddeberg *et al.*, 2008). Patients with long-standing HBV and HCV infections are at increased risk for the development of HCC, even in the absence of established cirrhosis (Arzumanyan *et al*., 2013). Hepatic neoplasia can come about due to destruction of hepatocytes by a variety of substances such as hepatocyte destruction in liver cirrhosis, drug or toxin-induced hepatic tumours, and hepatocyte injury due to chronic hepatitis (Kew *et al*., 2013).

1.1.3 Classification of hepatic tumours

The World Health Organization (WHO) classifies hepatic tumours into primary or secondary tumours, benign or malignant tumours, epithelial or non-epithelial tumours. Primary hepatic tumours originate from the liver cells and hepatic ducts. Secondary hepatic tumours are tumours which spreads from the primary tumour site and lodge in the liver by a process of metastasis (Ananthakrishnan *et al*., 2006).

WHO histological classification of hepatic tumours is as follows; benign epithelial tumours include hepatocellular adenoma, focal nodular hyperplasia, intrahepatic bile duct adenoma, and intrahepatic bile duct cystadenoma. Malignant epithelial tumours include HCC, ICC, bile duct cystadenocarcinoma, combined HCC-ICC, hepatoblastoma, and undifferentiated carcinoma. Benign non-epithelial tumours include angiomyolipoma, lymphangioma, hemangioma, and infantile hemangioendothelioma (WHO, 2010).

Malignant non-epithelial tumours include epithelioid hemangioendothelioma, angiosarcoma, embryonal sarcoma, and rhabdomyosarcoma. Miscellaneous tumours include solitary fibrous tumours, teratomas, York sac tumour, carsino-sarcoma, and rhabdoid tumour. Hematopoietic and lymphoid tumours include secondary tumours such as liver cell dysplasia, epithelial abnormalities, and bile duct abnormalities. Miscellaneous lesions include mesenchymal hamartoma, nodular transformation, and inflammatory pseudotumor (Bosman *et al*., 2010).

The sub-classification of HCC include trabecular pattern, pseudoglandular pattern, solid pattern, fibrolamellar pattern, clear cell pattern, scirrhous pattern, diffuse pattern, steatohepatitic pattern, transitional liver cell tumor pattern, sarcomatoid pattern, undifferentiated carcinoma pattern, combined HCC-ICC pattern, and lymphoepithelioma-like HCC pattern. Trabecular pattern of HCC is composed of thickened trabeculae of large polygonal eosinophilic tumor cells, which are separated by a network of vascular channels lined by epithelium. There is abundant eosinophilic and mild atypia in well-differentiated HCC (WHO, 2010).

Pseudoglandular (acinar) pattern of HCC is less common compared to trabecular pattern of HCC. It is characterized by acini (gland-like spaces) lined by tumour cells, resembling cholangiocarcinoma (Bosman *et al*., 2010).

The solid pattern of HCC is relatively uncommon and is comprised of sheets of tumour cells lacking endothelial cell-wrapped trabeculae or cell plates. Bile staining is prominent and portal tracts are absent. Tumour cells resemble non-neoplastic hepatocytes with varying degrees of nuclear atypia. The cytoplasm is more basophilic compared to non-neoplastic hepatocytes. Solid pattern of HCC is usually associated with HBV (Ferlay *et al*, 2012).

Fibrolamellar pattern of HCC occurs in young male and female adults (20 to 40 years of age) with equal incidence, has no association with HBV or cirrhosis risk factors, and often has a better prognosis. It usually presents as single large, hard "scirrhous" tumour with fibrous bands coursing through it. On microscopic examination, it is composed of well-differentiated polygonal cells growing in nests or cords and separated by parallel lamellae of dense collagen bundles. Clear cell pattern of HCC has cellular variants of HCC which include giant cell forms with multinucleated tumor cells, spindle cells, sarcomatoid, and clear-cell types. In clear cell HCC, cytoplasm clearing may be due to fat, glycogen, or water. Clear cell HCC pattern of HCC should be differentiated from metastatic renal cell carcinoma.

Scirrhous pattern of HCC contains focal or diffuse fibrosis which may be associated with any of the other forms of HCC such as trabecular, pseudoglandular, or solid. The tumour cells are separated by fibrous connective tissue. Differentiation from fibrolamellar pattern of HCC is based on the absence of abundant granular cytoplasm and the appropriate clinical setting for each tumor. Higher magnification of scirrhous pattern of HCC shows bands of fibrosis, separate cords, and nests of large, pleomorphic neoplastic hepatocytes. These subtypes of HCC affect the treatment outcomes (WHO, 2010).

The other factor that affects prognostic outcome is the grading of HCC which is subdivided into well differentiated (Grade I), moderately differentiated (Grade II), poorly differentiated (Grade III), and undifferentiated (Grade IV). Tumour grade is very important; high-grade tumours will have a poor prognosis, while low-grade tumors may go unnoticed for many years. The well differentiated type of HCC is composed of cells that resemble the normal mature hepatocytes (Pawlik *et al.*, 2007).

In well differentiated and moderately differentiated hepatic tumours, cells that are recognizable as hepatocytic in origin are disposed either in a trabecular pattern (recapitulating liver cell plates), or in an acinar, pseudoglandular pattern, cords, nests, and may contain bile pigment in the cytoplasm. On the other hand, undifferentiated and poorly differentiated types of HCC are composed of primitive-appearing cells which do not resemble the normal mature hepatocytes and are unspecialized. In poorly differentiated forms, tumor cells can take on a pleomorphic appearance with numerous anaplastic giant cells, can become small and completely undifferentiated cells, or may even resemble a spindle cell sarcoma (WHO, 2010).

1.1.4 Pathogenesis of hepatic tumours

Pathogenesis of hepatic tumours is mostly due to vasculogenesis and angiogenesis. Most causative agents of hepatic tumours primarily interfere with the functions of the liver by injuring hepatocytes. During HBV infection, the host immune response causes both hepatocellular damage and viral clearance. HBV, HCV, and chronic alcohol consumption causes hepatic tumours by causing massive inflammation, fibrosis, and eventually cirrhosis which can further lead to hepatic tumours such as HCC (Rosen *et al.*, 2011).

In terms of an immune response, T-lymphocytes, contribute most of the injury associated and lead to hepatocyte death by apoptosis. Aflatoxins cause hepatic tumours by damaging DNA which leads to mutations. Oral contraceptives cause hepatic tumours through the interaction of estrogen on estrogen receptors which lead to genetic mutations (Iannacone *et al.*, 2007).

Repeated cycles of cell death and regeneration, as occurs in chronic hepatitis from any cause, are important in the pathogenesis of hepatic tumours. Preneoplastic changes such as hepatocyte dysplasia can result from point mutations in selected cellular genes, loss of heterozygosity in tumour suppressor genes, DNA methylation changes, and constitutive expression of hepatocyte growth factor (HGF) and transforming growth factor alpha (TGF-a). These changes and possibly the effect of some viral proteins act to further stimulate the replication of hepatocytes. The accumulation of mutations during continuous cycles of cell division may damage DNA repair mechanisms and eventually transform hepatocytes (Kumar *et al.*, 2005).

Indeed, molecular analysis of tumour cells in HBV-infected individuals reveals that each case is clonal with respect to HBV DNA integration pattern, suggesting that viral integration precedes or accompanies a transforming event. For reasons that are not clear, genomic instability is more likely in the presence of integrated HBV DNA, giving rise to chromosomal aberrations such as deletions, translocations, and duplications (Kumar *et al*., 2005).

1.1.5 Clinical manifestations of hepatic tumours

Hepatic tumours clinically present in a variety of ways depending on the size, stage, and type of the hepatic tumor (Shirine *et al.,* 2010). Hepatic tumours are discovered on medical imaging equipment (often by accident) or present themselves symptomatically as an abdominal mass causing hepatomegaly, ascites, abdominal pain, back pain, itching, jaundice, nausea, weight loss, fever, or liver dysfunction (Tanaka *et al.,* 2011). However, some hepatic tumours may remain silent and asymptomatic. Hepatic tumors may be formed from either the liver itself or from structures within the liver, including blood vessels or the bile duct (Global Burden of Disease, 2013).

1.1.6 Clinical investigations

A number of investigations can be performed for the diagnosis of hepatic tumours. These investigative techniques include imaging modalities such as sonography (ultrasound), Computed Tomography (CT), and Magnetic Resonance Imaging (MRI) (Malaguarnera *et al.*, 2013). Other investigations that can be done include biochemical tests to check for tumor markers which are chemicals that are found in blood of people with cancer; such as alpha-fetoprotein (AFP) (Zhao *et al.,* 2013). Elevated levels of serum AFP are found in 50% to 75% of patients with HCC. A fine needle aspiration (FNA) can also be done to investigate hepatic tumors. Tru cut liver biopsies may be used to identify or exclude possible aetiologies for physical or laboratory abnormalities. Tru cut biopsies provide a better insight for diagnosis of suspected hepatic neoplasm (Ingram *et al*., 2015).

1.1.7 Management of hepatic tumours

Management of hepatic tumours usually depends on the stage of hepatic tumours. It can be divided into preventive and treatment measures. Prevention of causes can be separated into primary, secondary, and tertiary prevention. Primary prevention preemptively reduces exposure to risk factors of hepatic tumours.

One of the most successful primary hepatic tumour prevention is vaccination against HBV. Universal vaccination of children against HBV in endemic areas may dramatically decrease the incidence of hepatocellular carcinoma. Such a program was begun in Taiwan in 1984 and reduced HBV infection rates from 10% to 1.3% in 10 years (Kumar *et al.*, 2005).

Secondary prevention includes both cure of the agent involved in the formation of hepatic tumors (carcinogenesis) and the prevention of carcinogenesis if cure is not possible. Usually, cure of virus-infected individuals not possible, but treatment with antiviral drugs such as interferon can decrease the risk of hepatic tumours.

Tertiary prevention includes treatments to prevent recurrence of hepatic tumours. These include the use of chemotherapy drugs such as doxorubicin, cisplatin, vincristine, and antiviral drugs. Other treatment modalities include surgical resection, liver transplantation, radiotherapy, and percutaneous ablation (Hoshida *et al.*, 2012).

Percutaneous ablation is the only non-surgical treatment that can offer cure. There are many forms of percutaneous ablation, which consist of either injecting chemicals into the liver (ethanol or acetic acid) or producing extremes of temperature using radio frequency ablation, microwaves, lasers or cryotherapy. Radiotherapy is only beneficial when used together with chemotherapy drugs or cytotoxic drugs (Feng *et al.*, 2011).

For intrahepatic cholangiocarcinoma, aggressive surgery remains the only treatment offering hope for long-term survival because it is usually diagnosed or detected in its late course. Management of hepatic tumours is very efficient when the tumours are detected at an early stage (Kumar *et al.*, 2005).

1.1.8 Prognosis of hepatic tumours

The prognosis of hepatic tumours is dependent on the size of the tumour and the stage. High-grade tumours have a poor prognosis, while low-grade tumours have a good a prognosis and may go unnoticed for many years. The prognosis of hepatic tumours is good when the hepatic tumour is diagnosed in its early stage.

Unfortunately, the prognosis is poor when the hepatic tumour is diagnosed in its late stage because even the treatment modalities are limited. The usual outcome is poor, for instance only 10-20% of HCC can be removed completely using surgery (Gorayski *et al.*, 2007). Intrahepatic cholangiocarcinoma is not usually detected until late in its course, either as the result of obstruction to bile flow through the hilum of the liver or as a symptomatic liver mass. The clinical outlook is dismal, with 1- and 2-year survival rates of 25% and 13%, respectively. The median time from diagnosis to death is 6 months. This therefore means that the prognosis of intrahepatic cholangiocarcinoma is very poor (Kumar *et al.*, 2005).

In this study, immunohistochemistry (IHC) was used in the detection of the HBV in hepatic tumour samples and later used to characterize hepatic tumours specifically in HCC which may be due to HBV. This study was therefore helpful in determining the proportion of hepatic tumour phenotypes and HCC possibly associated with HBV.

1.2 Statement of the problem

Globally, as of 2012, there were 554, 000 male patients newly diagnosed with primary malignant hepatic tumours worldwide, out of whom 521, 000 died. In the case of female patients, there were 228, 000 new cases diagnosed as primary malignant hepatic tumours death cases of whom 224, 000 died. This mortality rate shows how fatal hepatic tumours can be and hence are a global problem. Hepatic tumours of viral origin are one of the commonest tumours worldwide and hence the need to conduct studies to associate viruses with hepatic tumours. Hepatic cancer is the fifth most common cancer in the world (Ferlay *et al*., 2012).

In Africa, there is little information that has been published on the histological phenotypes, subtypes, and grades of hepatic tumours. About 80% of hepatic tumour cases occurred in low-income countries. Studies done in sub-Sahara African countries have shown the burden and mortality of hepatic tumours which affects someone's quality of life and also affects the economy of the country (Serigne *et al.*, 2013).

In Zambia, there is paucity of data on the histological phenotypes, subtypes, and grades of hepatic tumours. No literature has been published on the histological phenotypes and subtypes of hepatic tumours in Zambia. Hence, this necessitated the conduction of this study to provide evidence for future studies.

According to the registry book in histopathology laboratory at UTH in Zambia, hepatic tumours are usually isolated from patients who usually present with abdominal mass and have been detected by abdominal scan. This gave an insight that hepatic tumours are actually diagnosed at UTH and prompted the candidate to conduct a study to determine the distribution of phenotypes of the hepatic tumours diagnosed at UTH across all age groups and sex.

1.3 Justification of the study

In Zambia, the main diagnostic criterion for hepatic tumours is histology. However, it may not provide adequate information on the aetiology of hepatic tumours. It is very important to know the phenotypes and histological subtypes of hepatic tumours and the possible causative agents involved in the aetiology of hepatic tumours. These tumours can be identified in many cases by immunohistochemistry (IHC) and other molecular methods like polymerase chain reaction (PCR).

There is no information that was published on the histological phenotypes of hepatic tumours in patients that present with hepatic tumours at UTH and hence this study is of benefit because it gives an insight of the common and rare hepatic tumours diagnosed at UTH. This is significant because it can help treatment to be tailored at the specific histological phenotypes and subtypes of hepatic tumours.

Control of viral hepatitis infection and better treatments for chronic hepatitis could prevent about half of liver cancer cases worldwide (World Cancer Report, 2014). The study is significant because it was meant to determine the proportion of HCC associated with HBV, but unfortunately HBV was not detected.

Publications of the results that were obtained from this study may be used as reference by the general public and scholars on the phenotypes and histological subtypes of hepatic tumours diagnosed at UTH. Importantly, treatment of hepatic tumours can be improved because once the tumours have been characterized; treatment can be tailored directly on the specific histological phenotypes and subtypes.

Furthermore, this study helped gather information that would act as a baseline for further studies and establish evidence of the histological phenotypes of hepatic tumours in Zambia as presented at the University Teaching Hospital, Lusaka.

1.4 Literature review

1.4.1 Prevalence, age and sex distribution of hepatic tumors

Many studies have been conducted concerning primary and secondary hepatic tumours. According to the American Cancer Society (2015), the biggest risk factor for hepatic tumours is infection with HBV. In a study which was carried out in Canada, it was found that the most risk factors for developing liver tumours was infection with hepatitis B and hepatitis C viruses (Irena *et al.,* 2009). Primary liver cancer is globally the sixth most frequent cancer, and the second leading cause of cancer death. In 2012 it occurred in 782,000 people and resulted in 746,000 deaths. Higher rates of liver cancer occur where hepatitis B and C are common, including East-Asia and sub-Saharan Africa. Five year survival rates are 17% in the United States (World Cancer Report, 2014).

Primary liver cancer account for less than 1% of all cancers in North America whereas in Africa, Southeast Asia, and China, they may account for up to 50% of cancers. The high prevalence of people carrying the HBV and having liver cirrhosis may account for this geographic discrepancy (Canadian Liver Foundation, 2015). As infectious virus is not retained in tumours, searches for viral associations with different tumor types will rest on the application of molecular methods which are not dependent on the presence of an intact virus in test samples such as PCR (Janet *et al.*, 2000). About 15% to 25% percent of people with chronic hepatitis B die of liver disease (Nettleman, 2014). Among Chinese, very close association between HCC and chronic HBV infection has been reported. The estimate of the fraction of HCC due to chronic HBV is 60-90% in high-risk regions (Tanaka *et al.*, 2009).

Data according to Surveillance, Epidemiology and End Results (SEER) estimated that in the United States of America (U.S.A), during 2001 and 2005 the median age of people diagnosed with primary liver cancer and intrahepatic bile duct cancer was 65 years. Approximately 1.1% were diagnosed under the age of 20 years, 1.1% between the ages of 20 and 34 years, 3.9% between 35 and 44 years, 20% between 45 and 54 years, 23.2% between 55 and 64 years, 24.2% between 65 and 74 years, 20.3% between 75 to 84 years, and 6.2% over the age of 85 years (Ries *et al.*, 2005).

In European Union countries, the incidence of primary liver cancer for both genders in 2008 was 4.2/100 000 respectively 65 644 cases, being the 18th among all cancer types, and mortality was 4.1/100 000, respectively 66 319 death cases, ranking 8th regarding mortality in Europe due to cancer (Ferlay *et al.*, 2008).

Over 80% of hepatic tumours occur in developing countries and the risk factors include infections with HBV and HCV, and exposure to aflatoxins (Serigne *et al.*, 2013). In Dakar, Senegal, 8% of tumours were hepatic tumours in the year 2000, of which 11.3% were men while 5.0% were female (Serigne *et al.*, 2013). HCC is one of the most malignant tumours with high mortality, aggressive growth behavior and a high recurrence rate. Egypt has the highest prevalence of HCV in the world and up to 90% HCC cases in the Egyptian population were due to HCV. Therefore, HCC represents an important public health problem in Egypt and the rest of the world (Osama *et al.*, 2011).

Studies have been done to show an association between HBV, HCV, and HCC. A prospective study performed to establish whether infection with specific HCV genotypes was associated with an increased risk of development of HCC in cirrhosis, showed that cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC than patients infected by other HCV types (Bruno *et al.*, 2007). There are suggestions that the presence of HBV gene in patients with chronic HCV-associated liver injury appears to promote hepatocarcinogenesis (Fujioka *et al.*, 2009). Similar to most types of cancer, hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to the malignant transformation of the hepatocyte (Tan *et al.*, 2008).

As the HIV epidemic in Zambia moves forward in the Anti-Retroviral Therapy (ART) era, new data on hepatitis co-infection are clearly needed to guide health policy. In a study which was done at UTH, it was found that active HBV co-infection (HBsAg seropositivity) occurred in 9.9% of ART-naive HIV-infected patients. In contrast, HCV occurred in only 1.2% of HIV-infected persons (Kapembwa *et al.*, 2008). It is a well-known fact that an HIV-positive patient co-infected with HBV or and HCV has increased mortality due to liver failure (Chiluba, 2009).

No documented studies were done on histological phenotypes, subtypes, grading of hepatic tumours and on association of HBV with HCC in Zambia. Hence, most documented literature is on HBV infections and HBV/HIV co-infections.

1.4.2 Histological phenotypes of hepatic tumours

Several studies have been done to classify and characterize hepatic tumours. In a certain study in Romania, the following phenotypes of hepatic tumours were found; malignant hepatic tumors-HCC (33.33%), intrahepatic cholangiocarcinoma (6.06%), combined hepatocellular and cholangiocarcinoma (0.379%), hepatoblastoma (0.379%), angiosarcoma (0.379%), bile duct cystadenocarcinoma (0.379%), while benign tumors included hemangioma (30.68%), simple serous cyst (20.07%), hamartomatous hepatic lesions (4.16%), hepatocellular adenoma (2.65%), biliary cystadenoma (0.75%), hepatic pseudolipoma (0.0379%), and hepatic lymphangioma representing 0.0379% respectively (Turdean *et al.*, 2012). This study indicated that the most common hepatic tumour is HCC, followed by hemangioma. Another study conducted in Nigeria by Vritehire *et al.*, 2016, reported HCC as the commonest histologically diagnosed hepatic tumour, followed by metastatic tumors, hepatoblastoma, ICC, and hemangioendothelioma respectively.

1.4.2.1 Hepatocellular carcinoma

It is a widely accepted fact that cirrhosis, which occurs due to chronic infection with HBV and HCV, is an important risk factor for HCC. However, this disease can also develop in a non-cirrhotic liver, especially in those countries where the infection with hepatitis B is endemic (Ishikawa *et al.*, 2010). Attention should also be paid to other risk factors such as diabetes and increased body mass index with subsequent development of non-alcoholic steatohepatitis (El-Serag *et al.*, 2007), mainly due to the epidemic increase of obesity in adults and children during the last 25 years. Other non-viral causes of HCC include iron storage disorder, alcohol abuse, smoking, oral contraceptives, exposure to aflatoxin and habitual consumption of betel. HCCs consist of tumour cells that resemble hepatocytes. The stroma is composed of sinusoid-like blood spaces lined by a single layer of endothelial cells.

Unlike the sinusoidal endothelial cells in normal liver tissue, those of HCC are immunohistochemically positive for CD34 and factor-VIII-related antigen. Ultrastructural observation shows a basement- membrane-like structure between the endothelial cells and tumour cell trabeculae, and basement-membrane-like materials are immunohistochemically positive with antibodies for laminin and type IV collagen. Thus, the sinusoid-like blood spaces resemble capillary vessels. This phenotypic change of sinusoids is called capillarization. In the sinusoidal blood spaces, varying numbers of macrophages, which show immunohistochemical positivity with antilysozyme and CD68, are also present and resemble Kupffer cells in well-differentiated tumours. HCCs vary architecturally and cytologically (James *et al.*, 2008). In a certain study, the grading of HCC was done as follows; well-differentiated or Grade I (13.63%) moderately differentiated or Grade II (53.4%) poorly differentiated or Grade III, (30.68%) and undifferentiated or Grade IV (2.27%) (Turdean *et al*, 2012). In this case, most HCC was found to be moderately differentiated, followed by poorly differentiated. In another study conducted in Romania on 879 cases of HCC, the authors found that 57.7% of the cases were associated with cirrhosis and 33.2% with vascular invasion and the ratio of Grade I and Grade II/Grade III and Grade IV was 1:1.04 (Cheng *et al.*, 2011). In another study by Turdean *et al.*, 2012, in the Emergency Hospital of Mures County, Romania, they found that 40.9% of the cases were associated with cirrhosis and 51.13% with vascular invasion and the ratio of Grade I and Grade II/Grade III and Grade IV was 2.04:1. Although the patients studied in study above presented a better differentiation compared to Ishikawa *et al*., 2010, the rate of vascular invasion was significant higher.

1.4.2.2 Cholangiocarcinoma

Cholangiocarcinoma represents about 10% of the primary hepatic tumours worldwide (Yang *et al.*, 2008). It seems to be more frequent in females (12%) than males (5%) of all the primary hepatic tumours. The literature data shows that the male to female ratio is 1:1.5. The highest incidence of cholangiocarcinoma is found in the regions of Laos, North and North-East Thailand which are affected by endemic infections with liver parasites called Opisthorchis viverrini.

Most ICCs are adenocarcinomas showing tubular and/or papillary structures with a variable fibrous stroma. There is no dominant histological type of ICC in cases associated with liver flukes or hepatolithiasis when compared to those in non-endemic areas. ICC can arise from any portion of the intrahepatic bile duct epithelium. Lesions are gray to gray-white, firm and solid, although some tumours show intraductal growth, sometimes with polyp formation. Typical tumours consist of variably sized nodules, usually coalescent. Central necrosis or scarring is common, and mucin may be visible on the cut surfaces. ICC cases involving the hepatic hilum are hardly distinguishable from hilar cholangiocarcinoma, and such cases show cholestasis, biliary fibrosis, and cholangitis with abscess formation. ICC is not often noted in a non-cirrhotic liver (Parkin *et al*., 1991).

1.4.2.3 Combined hepatocellular and cholangiocarcinoma

Combined hepatocellular and cholangiocarcinoma is the term preferred for a tumour containing both hepatocellular and distinct or separate cholangiocarcinoma. The presence of both bile and mucus should be sought in the combined tumour. This category should not be used for tumours in which either form of growth is insufficiently differentiated for positive identification. Hepatocytes preferentially express cytokeratins 8 and 18 and, like duct epithelial cells, cytokeratins 7 and 19. However, the different patterns of expression are not as clear-cut in these tumours. For practical purposes, demonstration of bile canaliculi by polyclonal CEA (mixed biliary glycoproteins) combined with Hep Par immunoexpression is sufficient for the diagnosis of a hepatocellular carcinomatous component, and that of neutral epithelial mucin by the PAS-diastase reaction for the diagnosis of a cholangiocarcinomatous component (Turdean *et al.*, 2012).

1.4.2.4 Bile duct cystadenoma and cystadenocarcinoma

Bile duct cystadenoma and cystadenocarcinoma are rare tumours. Cystadenomas appear exclusively in females Qu *et al.*, 2009 while cystadenocarcinomas appear equally in both males and females (Ren *et al.*, 2010). The mean age of patients affected with these diseases is 50-60 years.

Cystadenomas are usually multi-locular and are well defined by a fibrous capsule, which may contain smooth muscle fibres. The contents of the locules are either thin, opalescent or glairy fluid, or mucinous semisolid material. Two histological variants are recognized. The mucinous type is more common and is lined by columnar, cuboidal, or flattened mucus-secreting epithelial cells resting on a basement membrane; polypoid or papillary projections may be present. The serous type consists of multiple, small locules lined by a single layer of cuboidal cells with clear cytoplasm containing glycogen. The cells rest on a basement membrane but are not surrounded by the mesenchymal stroma typical of the mucinous variety. Squamous metaplasia may also occur.

Cystadenocarcinomas are usually multi-locular and contain mucoid fluid. Malignant change may not involve all of the epithelium lining the cyst; it is usually multifocal. The tumours are so well defined that complete removal can usually be achieved with good prognosis. Differentiation from intrahepatic bile duct cystadenoma depends on the demonstration of cytological (particularly nuclear) atypia, mitosis, and invasion of the underlying stroma (Qu *et al*., 2009).

1.4.2.5 Hepatoblastoma

Hepatoblastoma is the most common hepatic tumour in children, the incidence of the disease under the age of 15 years in western countries is 1.5/1 000 000. About 4% of hepatoblastomas are present at birth, 68% in the first two years of life and 90% by five years of age. Only 3% are seen in patients over 15 years of age. There is a male predominance of 1.5:1 to 2:1, but no racial predilection. Hepatoblastomas vary in size from 5 to 22 cm in diameter and from 150 to 1,400 g in weight. Single and multiple lesions may be well circumscribed, the edge of the lesion being separated from the normal liver by an irregular pseudocapsule. Pure fetal hepatoblastomas have the tan-brown colour of normal liver, while mixed hepatoblastomas display a variety of colours from brown to green to white. The lesions are often nodular and bulge from the cut surface. Areas of necrosis and haemorrhage are usually present and may appear as soft or gelatinous, brown to red tissue.

Hepatoblastomas display a distinct variety of histological patterns that may be present in varying proportions. Some tumours are composed entirely of uniform fetal epithelial cells or small undifferentiated cells, while others contain a variety of tissue types including hepatic fetal epithelial and embryonal cells, fibrous connective tissue, osteoid-like material, skeletal muscle fibers, nests of squamous epithelial cells, and cells with melanin pigment (Roebuck *et al.*, 2006).

1.4.2.6 Hepatic angiosarcoma

Hepatic angiosarcoma is a rare disease, representing 2% of primary hepatic tumours. In the United States, the incidence of this disease is estimated to be 25 new cases a year. The male: female ratio is 3:1, most of the patients being diagnosed in the sixth decade of their life and approximately 60% of the cases present metastasis when diagnosed (Mani *et al.*, 2001).

1.4.2.7 Cavernous hepatic hemangioma

Cavernous hepatic hemangioma is the most common benign tumour that occurs in the liver. The incidence of this disease varies from 0.4 to 20% (Brancatelli *et al*, 2001). It is more common in females, it may increase in size or relapse in patients undergoing estrogen therapy (Glinkova *et al.*, 2004).

1.4.2.8 Hepatocellular adenoma

Hepatocellular adenoma is a rare benign hepatic tumour, whose clinical significance increases due to the use of oral contraceptives by young women. The incidence of this lesion is about 3/1 000 000 per year (Barthelmes *et al.*, 2005).

1.4.2.9 Metastatic carcinomas

In a study of randomly selected liver biopsies from England and Wales, the commonest histological type of metastatic tumour was adenocarcinoma (39%), followed by carcinoma not otherwise specified (36%); the rest were undifferentiated small cell carcinoma, other special types of carcinoma, and lymphomas. The liver has a rich systemic and portal blood supply, providing a potentially abundant source of circulating neoplastic cells.

Circulating tumour cell arrest is controlled by Kupffer cells in the sinusoids and may be enhanced by growth factors such as transforming growth factor alpha (TGFa) , tumour necrosis factor (TNF), and insulin-like growth factor-1 (IGF-1). As tumour deposits enlarge, they induce angiogenesis using native sinusoidal endothelium; this enhances their chances of survival and is often macroscopically evident. Most metastases from unpaired abdominal organs reach the liver via the portal vein, and from other sites via the systemic arterial circulation. Lymphatic spread is less common and extension to the liver via the peritoneal fluid is rare (Kumar *et al.*, 2005). Adenocarcinomas show tubular and/or papillary structures with a variable fibrous stroma.

Molecular characterization is important as a diagnostic method for indicating the presence or absence of a specific microorganism in a clinical specimen. For instance, molecular diagnostic methods were used to detect HBV DNA in hepatic tumour samples. Molecular characterization is very beneficial as the pathogens such as viruses can be detected and characterized rapidly. This helps in tailoring of medication therapy onto the target causative organism (Hui *et al.*, 2006).

Therefore, this research was meant to investigate and characterize hepatic tumours by age and sex of the patient, and also associated HCC with HBV at the University Teaching Hospital in Lusaka, Zambia.

1.5 Research question

What are the histological phenotypes of hepatic tumours diagnosed at the University Teaching Hospital?

1.6 Objectives

1.6.1 General objective

To characterize hepatocellular carcinoma and other hepatic tumours diagnosed at the University Teaching Hospital, Lusaka.

1.6.2 Specific objectives

1.6.2.1 To determine the age and sex distribution of patients presenting with hepatic tumours.

1.6.2.2 To determine the histological phenotypes of hepatic tumours.

1.6.2.3 To determine the histological subtypes and grades of HCC.

1.6.2.4 To determine the proportion of HCC associated with HBV.

Chapter 2: Materials and methods

2.1 Study design and period

This study was a laboratory based retrospective cross-sectional study. The study was conducted from January 2016 to July 2016 on archived Formalin-Fixed Paraffin Embedded (FFPE) hepatic tumour tissue blocks.

2.2 Study site

The study was carried out at the University Teaching Hospital, Lusaka, in the Histopathology and Immunohistochemistry Laboratory, Department of Pathology and Microbiology.

2.3 Sampling frame

In this study, a convenience sampling method was used on 34 FFPE archival tissue blocks which were diagnosed with hepatic tumours between 1st January, 2012 and 31st December, 2015. Convenience sampling method was used on all the hepatic tumour tissue blocks which were available, accessible, and eligible during the period of the study. From the available hepatic tissue blocks, convenience sampling method was used because of the small sample size.

2.4 Study population and sample collection

Archival hepatic tumour tissue blocks were retrieved from the archives, de-waxed and prepared for H & E stain, reticulin stain, mucin stain, Periodic Acid Schiff (PAS) stain, and immunohistochemical staining to determine the hepatic tumour phenotypes.

2.5 Inclusion criteria

All FFPE specimens laboratory confirmed as hepatic tumours between 1st January, 2012 and 31st December, 2015 irrespective of age or gender.

2.6 Exclusion criteria

All FFPE specimens laboratory confirmed as non-hepatic tumours between 1st January, 2012 to 31st December, 2015.

All diagnosed hepatic tumours which did not fall in the period within 1st January, 2012 to 31st December, 2015.

All case specimens that had biopsies that were less than 1mm in diameter or inadequate were excluded from the sampling frame.

2.7 Determination of hepatic tumour phenotypes

2.7.1 Specimen labeling

The labeling was done for each hepatic tumour specimen to which a new identification code was given for confidentiality and easier identification. The letter "H" represented hepatic tumour specimen plus a four digit numbering system that is (0000) were used. Therefore, the specimen numbers such as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 were labeled as H0001, H0002, H0003, H0004, H0005, H0006, H0007, H0008, H0009 and H0010, respectively.

2.7.2 Specimen sectioning

In the histopathology laboratory, the specimens to be sectioned on the microtome machine were placed face down on an ice-cold plate for about 20 minutes. Then the water bath was turned on and temperature was set at 37 ^{o}C. The paraffin block was placed in the block holder of the microtome machine. The paraffin block advanced closer to the microtome blade. The dial was set to cut approximately 10µm sections and the blade was set at an angle of 6º. The rough trimming of the paraffin section was done until a complete section was seen in the tissue block. A ribbon was obtained by using a clean section of the blade and was picked by the forceps and then it was transferred to the water bath. To allow the sections to stretch, the ribbon was laid on the water bath for a few seconds. The cut sections were then gently separated and each section was picked on a glass slide. To allow the water to exit the slide and section, the sections were picked at an angle.

Before putting the specimen slide on the hot plate, the slide sections were allowed to drain just for a few minutes. The tissue sections were put on the hot plate so as to remove some more water from the tissue section. The glass slides were finally placed in a warm plate for about 15 minutes to help the tissue section adhere to each slide.

2.7.3 Ehrlich's Haematoxylin and Eosin (H and E Stain) Staining

Mounted specimen sections were softened using ethanol and rehydrated so as to facilitate the penetration of the water based H and E stain. The sections were then taken into the water, and were dewaxed in two washes of xylene for 2 minutes each. The sections were then washed in 2 changes of absolute alcohol for 2 minutes each. After that, the sections were washed in water for 2 minutes. Finally, the slides were then stained with Ehrlich's Haematoxylin solution for about 30 minutes. The stained slides were washed in running tap water for about 5 minutes and then differentiated in 1% hydrochloric acid in 70% alcohol (1% acid alcohol) for 15-30 seconds. The stained slides were blued in running tap water for 10 minutes and later counter stained in 1% aqueous eosin for 5 minutes. Lastly, the stained slides were then rinsed in 95% alcohol, dehydrated in 2 changes of absolute alcohol for 2 minutes each, and then cleared in 2 changes of xylene for 2 minutes each.

2.7.4 Cover slipping

In this procedure, the slides were placed on a clean horizontal bench surface and a drop of DPX Mountant was applied to each tissue section at the far end of the slide away from the frosted end in order to preserve the stain. The cover-slip was then gently applied by placing one end of the slip on top of the drop of DPX Mountant at the far end of the slide. Slowly, gently, and carefully, the cover slip was rolled down the side, only using minimal pressure to allow the medium to spread evenly. The slides were later placed flat onto the clean horizontal surface bench for 120 minutes to eliminate bubbles under the cover slip. The slides were removed from the bench after they became dry. At this point, the slides were ready for microscopic examination.

2.7.5 Microscopic Examination

The World Health Organization standard guidelines of 2010 were used to histologically classify hepatic tumours. This was done in the histopathology laboratory at UTH.

2.7.6 Immunostaining Protocol

The retrieved tissue blocks were re-cut and placed on slides in readiness for the immunostaining in the immunohistochemistry laboratory. The cutting of the specimens was done using the procedure mentioned above on specimen sectioning. After sectioning, the specimens were hydrated in two changes of xylene for 5 minutes each and three changes of ethanol for 5 minutes each; 2 absolute alcohols, 1 in 95% alcohol for facilitation of penetration of the water based primary antibodies (HBsAg).

2.7.7 Antigen Retrieval

The tissue blocks which were re-cut sections were deparaffinized using xylene. The tissue sections were then hydrated using alcohol as was done in the preparation for the H & E staining. After that, the Envision FLEX Target Retrieval Solution was pre-heated to 95°C in an incubator. The sections were immersed in the pre-heated Retrieval Solution for about 20 minutes. The slides were thoroughly covered with the Retrieval Solution throughout the process. The slides were then allowed to cool thoroughly in the Retrieval Solution for 20 minutes at room temperature. The slide rack was then removed from the container and the sections were rinsed with cold running tap water immediately in order to prevent drying out of the tissues. The rack that contained the rinsed sections was then placed in Wash Buffer working solution at room temperature for 5 minutes. This process was done in preparation of the staining of the tissues.

2.7.8 Staining procedure

In the first place, a humidified staining chamber for staining was prepared. The slides were immersed into a 0.3% H_2O_2 and 100% methanol for about 10 minutes at room temperature to quench endogenous peroxidase. The slides were then rinsed in cold wash buffer for 1 minute. The slides were then placed in the prepared humidifying chamber and a pap pen was used to mark the edges of the tissue on each slide so as to provide a heat-stable, water-repellent barrier that keeps reagents localized on tissue specimens. Drops of the primary antibody (HBsAg) were then placed on the tissues on each slide and left in the humidifier for 60 minutes. The HBsAg antibody was then rinsed off from the slides with TBS buffer for 1 minute. After that, the slides were again placed back in the humidifier and the streptavidin biotin (LSAB) link was then placed carefully on each slide. The slides were then left in the humidifier for 20 minutes.

The slides were again rinsed in deionized (DI) water for 1 minute and were placed back in the humidifier and LSAB Streptavidin Peroxidase was poured on the slides, newly prepared chromogen was then added and the slides will be left in the humidifier for 30 minutes. Thereafter, the slides were again rinsed in cold wash buffer for 1 minute and then placed in DAB (3,3'-diaminobenzidine) working solution for 5 minutes, the DAB gets oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a brown, alcohol-insoluble precipitate which gives some colour contrast on the slides. To create a light counterstain, the slides were placed in haematoxylin for about 1 minute. The slides were then placed under running tap water for 1 minute until they turned blue. The sections were then dehydrated through 2 changes of alcohol (3 minutes each in 95% and absolute alcohol respectively) and were cleared in 3 changes of xylene (5 minutes each). The slides were then cover slipped as was done in the procedure above on cover slipping. The mounting medium used at this point was cryoseal. 2 drops of cryoseal permanent mounting medium were applied on each slide and then a cover glass was applied. The slides were allowed to air dry and later viewed under a light microscope and the results were determined to be either positive or negative for hepatitis B antibody.

2.7.9 Primary Antibodies used to detect HBV

The primary antibodies used in this study were purchased from Dako, Glostrup, Denmark 2016. The antibodies that were used were the hepatitis B surface antigens (HBsAg).

2.7.10 Interpretation of Immunohistochemistry results

The interpretation of the results for IHC was done based on the staining of the sample either positive or negative, each specimen created a pattern which when analysed was placed in a particular hepatic tumour type whether associated with HBV or not. The staining using HBsAg is both cytoplasmic and membranous that indicates the presence of HBV.

2.7.11 Controls used for Immunohistochemistry

The sections cut for IHC were viewed with both positive and negative controls. The negative controls were from the cases under this study while the positive controls came from previously positively diagnosed hepatic tumour specimens.

2.8 Data processing

Data from this study was analyzed using Statistical Package of Social Sciences (SPSS) Version 20.0. The data was presented in form of frequency tables and charts. Chi-square was used to determine association between HBV and hepatic tumours, the histological subtypes and sex of the patient. Fisher's exact test was used to evaluate statistical significance between age of patients versus hepatic tumour phenotypes, sex versus hepatic tumour phenotypes, age of patients versus HCC subtypes, sex versus HCC subtypes, age versus grades of HCC, and sex versus grades of HCC. HBV detection was categorized into either present or absent from hepatic tumour tissues. All statistical tests were performed at 5% significance level or 95% confidence interval with a p-value < 0.05 to determine statistical significance.

2.9 Ethical considerations

The research was done under ethical approval from the University of Zambia Biomedical Research Ethics Committee (UNZABREC). Permission to conduct the study was sought from the University Teaching Hospital Senior Medical Superintendent. Permission to conduct the study in the histopathology and immunohistochemistry laboratory was sought from the head of department of Pathology and Microbiology. The samples which were used were archival tissues which came from human beings and hence required ethical approval because of confidentiality. The information about where the hepatic tumour tissues were isolated from remained confidential.

2.10 Utilization of findings

The findings of this study were used to determine the age and sex distribution of hepatic tumours, phenotypes and histological subtypes of hepatic tumours. These results can be used for reference by scholars and as a baseline for future studies in pathology and other health-related disciplines.

Chapter 3: Results

3.1 General characteristics of study population

3.1.1 Age distribution

The results showed that the majority of the patients were from the ages of 21 to 30 years.

From figure 1 above, it was established that the most age range of patients diagnosed with hepatic tumours was 21-30 years with a percentage of 20.6%. This was followed by age group of 51-60 years old. The least age range diagnosed with hepatic tumours was found to be patients above the age of 70 years with a percentage of 2.9%.

3.1.2 Sex distribution

The sex distribution showed male predominance as is seen in figure 2.

Figure 2 above, male patients represented a percentage of 55.88% (19 cases) while females represented 44.12% (15 cases) of the study population. Hepatic tumours at UTH affect more males than females with a male to female ratio of 1.3:1.

3.2.0 Phenotypic distribution of hepatic tumours

The phenotypic distribution of hepatic tumours showed that HCC was the most commonly diagnosed tumour as seen in figure 3.

Key: HCC: Hepatocellular carcinoma, M.ADC: Metastatic adenocarcinoma, ICC: Intrahepatic cholangiocarcinoma, HPB: Hepatoblastoma, M.SCC: Metastatic small cell carcinoma.

The phenotypic distribution of hepatic tumours included HCC 17/34 (50%), metastatic adenocarcinoma 6/34 (17.6%), ICC 4/34 (11.8%), hepatoblastoma 3/34 (8.8%), metastatic small cell carcinoma 2/34 (5.9%), and hepatic adenoma 2/34 (5.9%). There was a statistical significance between age of patients and hepatic tumour phenotypes (p-value = 0.03). HCC development is significantly affected by age and sex of the patient (p-value = 0.04).

3.3.0 Histological subtypes of hepatocellular carcinoma

The histological subtypes showed that the trabecular subtype was the most commonly diagnosed subtype of HCC as shown in figure 4.

HCC occurred in a number of histological subtypes which included trabecular 9/17 (52.9%), pseudoglandular 4/17 (23.5%), solid 2/17 (11.8%), diffuse 1/17 (5.9%), and fibrolamellar 1/17 (5.9%) respectively. There was no statistical significance between age of patients and the subtypes of HCC (p-value = 0.4), and similarly between sex of patients and the subtypes of HCC (p-value=0.4).

3.4.0 Grades of hepatocellular carcinoma

The most commonly diagnosed grade of HCC was the moderately differentiated grade as shown in figure 5.

The grades of HCC which were diagnosed included the moderately differentiated grade (Grade II) representing 8/17 (47.0%), well differentiated grade (Grade I) representing 7/17 (41.2%), and the poorly differentiated (Grade III) representing 2/17 (11.8%) respectively. The most affected age range with regards to grading of HCC was 21-30 years which had 2 well-differentiated and 3 moderately differentiated grades. There was a statistical significance between age of the patients and the grades of HCC (p-value 0.01). There was no statistical significance between sex of the patients and the grades of HCC (p-value 1.0).

3.5.0 Hepatocellular carcinoma associated with hepatitis B virus

Figure 6: Table 1 displaying the immunohistochemical staining of HCC

Antigen	HCC subtype	Cytoplasmic staining -/+	Membrane staining -/+
HBsAg	Trabecular	-	-
HBsAg	Trabecular	-	-
HBsAg	Trabecular	-	-
HBsAg	Trabecular	-	-
HBsAg	Trabecular	-	-
HBsAg	Diffused-pattern	-	-
HBsAg	Pseudoglandular	-	-
HBsAg	Pseudoglandular	-	-
HBsAg	Pseudoglandular	-	-
HBsAg	Solid-pattern	-	-
HBsAg	Solid-pattern	-	-
HBsAg	Fibrolamellar	-	-

Key: HBsAg: Hepatitis B surface Antigen, HCC: Hepatocellular carcinoma, (-): negative staining, (+): positive staining

The results in the table above shows that all the subtypes of HCC stained negative for HBsAg. Since there was no positive staining of the cytoplasm or cell membrane, the percentage of HCC associated with HBV was found to be 0%. The HBV antigens were not detected possibly due to a small sample size of HCC which was available in this study. The other reason would have been that the hepatic tumour samples could have been obtained from patients with no chronic exposure to HBV.

Chapter 4: Discussion

Hepatic tumours are of global concern due to increased morbidity and mortality which occurs as a result of these tumours. Due to the heterogeneity of hepatic tumors, diagnosis is very challenging and requires careful morphological evaluation, immunophenotyping, genotyping, and use of modern techniques for accuracy of diagnosis (Ananthakrishnan *et al*., 2006). This study provided the following information; age and sex distribution of patients affected with hepatic tumors, histologic phenotypes of hepatic tumours, histologic subtypes of HCC, grading of HCC, and the proportion of HCC associated with HBV.

4.1 General characteristics of the patients

The hepatic tumour tissue blocks that were used in this study came from patients with ages ranging from 1 to 79 years (mean age of 37.97 years, median age of 38.50 years, and mode of 23 years). The majority of the patients diagnosed with hepatic tumours in this study were from the age range between 21-30 years. Hepatic tumours seem to affect a much younger age group as compared to those which are found in the more developed countries (Kew, 2013). The results of this study are in contrast with the findings of Wabinga *et al*., 2000, who recorded that hepatic tumours affect mostly patients in the age range of 30 to 50 years with a mean age of 39.8 years. With regard to the age distribution, the results are consistent with studies that have been done in West Africa (Akinyinka *et al*., 2001). In this study, 64.7% of patients were younger than 50 years of age while 35.3% of patients were older than 50 years of age. The sex distribution was male predominant, with males being 55.9% and females at 44.1%. This gave a male to female ratio of 1.3: 1. Though the male to female ratio was slightly lower than the studies done in most of the sub-Sahara African countries, it was close though still lower than that found in Romania 1.7:1 (Turdean *et al*., 2012). With respect to HCC in this study, most patients with HCC fall in the following age ranges; 21-30 years, 31-40 years, and 51-60 years. This is very much in agreement with the studies done in Nigeria by Vhriterhire *et al*., 2016.

Globally, the male to female ratio of HCC was found to be 2.3:1 (Kew, 2013). The male to female ratio of patients affected with HCC in this study was recorded to be 2.4:1 which was the similar as the one recorded in sub-Saharan resource-constrained regions (Jemal *et al.*, 2011).

Other studies reported the male to female ratio of 1.8:1 to 28.0:1.0 respectively (Parkin *et al.*, 2003) and (McGlashan *et al.*, 2003). A similar evaluation in Nigeria recorded a male to female ratio of patients affected with HCC as 1.9:1 (Vhriterhire *et al.*, 2016). In South Africa, HCC showed a male to female ratio of 4.4:1 (Fujita *et al.*, 2014). These differences, at both a sub-continental and at a more local level, are in keeping with environmental factors playing an important role, but one which varies in extent geographically, in the aetiology of HCC in sub-Saharan Africa. The increased risk of HCC development in Black African males reflects in part, their higher rate of chronic infection with HBV, approximately twice that of females. Moreover, males generally have a greater intake of food and hence dietary carcinogens such as aflatoxin B1 than do females. There are also differences between the two sexes in the rates and efficiency with which ingested chemical carcinogens are metabolized. Chronic HBV infection and dietary exposure to aflatoxin B1 are known to have a synergistic hepatogenic interaction (Kew *et al*, 2003). Furthermore, dietary iron overload in the Africa, another cause of HCC in Black Africans, is more common in males than in females because they consume far larger volumes of the iron-rich home-brewed alcohol and they do not menstruate. For the other hepatic tumours, the male to female ratios were as follows; metastatic adenocarcinoma 1:6, ICC 3:1, hepatoblastoma 1:2, metastatic small cell carcinoma 1:1, and hepatic adenoma 1:1. The importance of knowing the most affected age group and gender of the patients is that it helps in planning for treatment options for the patients. Age group identification can give an idea of risk factors that could be associated with certain hepatic tumours and the best therapy for a particular age group. It is important to know the gender of the patient because treatment may differ in females and males. When considering treatment options, the patient characteristics may be taken into consideration such as in pregnant or lactating women.

4.2 Phenotypes of hepatic tumours

From the total number of 34 cases of histologically diagnosed hepatic tumours, 17 cases (50%) were HCC, 6 cases (17.6%) were metastatic adenocarcinomas, 4 cases (11.8%) were ICC, 3 cases (8.8%) were hepatoblastomas, 2 cases (5.9%) were metastatic small cell carcinomas, and 2 cases (5.9%) were hepatic adenomas respectively in descending order of their occurrence. The prevalence of HCC found in this study is lower but comparable with what was found in other studies; a study done in Romania by Turdean *et al.*, 2012, HCC showed a prevalence of 81.5%, ICC was 14.8%, and the rest contributed 3.8%. Another study in Nigeria by Vhriterhire *et al.*, 2016, reported HCC as the commonest histologically diagnosed primary liver cell carcinoma. These results are comparable because all studies recorded that HCC is the commonest histologically diagnosed hepatic tumour. A study in China by Yang *et al.*, 2008, showed that ICC represents about 10% of the primary hepatic tumours worldwide which was almost comparable with the 11.8% that was found in this study. The high result which was found in this study might be attributed to the small sample size. Primary hepatic tumours included HCC, ICC, hepatoblastomas, and hepatic adenomas. Secondary hepatic tumors included metastatic adenocarcinoma and metastatic small cell carcinoma. Primary hepatic tumours represented 26 (76.5%) of all diagnosed tumours at UTH as compared to 8 (23.5%) of secondary hepatic tumors. Hence, most of the hepatic tumours which were diagnosed at UTH are primary hepatic tumours. These results were different from a study which was carried out in Romania by Turdean *et al.*, 2012, where the results indicated primary hepatic tumours to be (35.3%) compared to secondary hepatic tumours (64.7%). The results show a variation of the hepatic tumour phenotypes in different geographical locations, but in all instances the prevalence is quite high. It is important to know the hepatic tumour phenotypes because each phenotype is treated differently from the others. Some hepatic tumours such as benign tumours do not cause any symptoms and hence require no treatment. Other hepatic tumours such as malignant tumours are very aggressive and may require special care and aggressive treatment to improve the patients' quality of life and survival rate (American Cancer Society, 2016).

4.3 Histological subtypes of hepatocellular carcinoma

Five HCC subtypes that were identified in decreasing order of occurrence include trabecular pattern (52.9%), pseudoglandular pattern (23.5%), solid pattern (11.8%), diffuse pattern (5.9%), and fibrolamellar pattern (5.9%). The most common subtype of HCC recorded in this study was the trabecular pattern. The results of histology showing that trabecular pattern of HCC as a predominant histologically diagnosed subtype are comparable with studies done in West Africa in Southern Nigeria and in East Africa in Tanzania (Seleye-Fubara *et al*., 2007) (Jaka *et al*., 2014).

Other studies which are consistent with the findings of this study showing the trabecular pattern of HCC as the commonest subtype includes studies done in the USA (Chedid *et al*, 1999), in Southern Thai (Sooklim *et al*., 2003), in Malaysia (Cheah *et al*., 2003), and a study done in France (Bralet *et al*., 2000) respectively. These subtypes of HCC are very important when considering treatment options for the patients. Other studies such as in Bangladesh showed different outcomes, Khan *et al*., 1997, showed pseudoglandular pattern of HCC as the predominant subtype while in this study it is the second most common histologically diagnosed subtype. The distribution of the other subtypes is however different in various studies except for fibrolamellar pattern which is consistently rare. The distribution of HCC seems to be according to geographical locations (Sergi, 2014). Furthermore, the HCC variants demonstrate distinct differences in presentation, treatment, and prognosis. Some subtypes of HCC have better prognosis than others. Fibrolamellar subtype has a better outlook than other subtypes (Jernigan *et al*., 2015). Clinicians can use the information of HCC subtypes as a reference when considering treatment options.

4.4 Grades of hepatocellular carcinoma

Three grades of HCC were histologically diagnosed and recorded in this study that included well differentiated (41.2%), moderately differentiated (47.0%), and poorly differentiated (11.8%). No HCC was found to be undifferentiated. This showed that most of HCC which were histologically diagnosed at UTH were moderately differentiated and this keeps in line with the results of Pawlik *et al*., 2007, who similarly stated that most of HCC presented as grade II (moderately differentiated).

These results were comparable with studies done in Romania by Turdean *et al.*, 2012, which showed that most of HCC are graded as moderately differentiated (53.4%), but differed slightly on the other grades. In this study at UTH, the second commonly occurring grade was the well differentiated grade (41.2%), followed by poorly differentiated (11.8%), with no undifferentiated grade found while in the study by Turdean *et al.*, 2012, the second most occurring grade of HCC was found to be poorly differentiated or Grade III (30.68%), thirdly was well differentiated or Grade I (13.63%), and lastly undifferentiated or Grade IV (2.27%). The ratio of Grade I and Grade II/ Grade III and Grade IV was found to be 7.5:1 in this study. This ratio was comparable with the one obtained in China by Cheng *et al.*, 2011, who found a ratio of 1.04:1. This huge difference would have been caused by the fact that no undifferentiated or Grade IV was recorded in this study at UTH.

The prognosis of solid tumours is generally related to tumour stage at presentation. Tumour stage also guides treatment decisions. However, in patients with HCC, the prediction of prognosis is more complex because the underlying liver function also affects prognosis. The grading of hepatic tumours is very important for prognostic outcomes (Stigliano *et al.*, 2007). Well differentiated HCC tend to grow and spread at a slower rate and hence has a good prognosis. Hence, treatment of well differentiated HCC produces a better outcome and higher survival rates. Moderately differentiated HCC has a moderate prognosis and moderate survival rates. Poorly differentiated HCC is high grade tumour which grows and spreads faster and may require immediate or more aggressive treatment (Tamura *et al.*, 2001). Finally, since most HCC diagnosed at UTH were moderately differentiated, much patient care is required because this is an intermediate grade of which if HCC surpasses this grade, it becomes a high grade tumour which is difficult to manage.

4.5 Hepatocellular carcinoma associated with HBV by immunohistochemistry

From a total of 17 HCC, 12 were eligible for IHC. The other 5 HCC were ineligible for IHC because the tissues were completely used up when preparing for H & E and special stains. From the 12 cases of HCC which were immune stained, none of the cases stained positive for HBsAg.

This therefore showed that from the stained HCC specimens, there was no association between HCC with HBV. This therefore could have been due to the small sample size used in this study and hence we cannot conclude that there is no HCC associated with HBV at UTH. The results obtained in this study are in agreement with the findings in Southern Thai where it was found that there was no association between the histological subtypes of HCC and the HBV (Sooklim *et al*., 2003). However, the results of this study contradicted the results obtained by Di Bisceglie *et al*., 2003 which showed that about 20.1% of HCC were associated with HBV and a study by Papatheodoridis *et al*., 2015, reported the association of HCC with chronic HBV infection. Other studies have shown that HBV is a risk factor for developing HCC (Michielsen *et al*., 2011) (Evans *et al*., 2002). The obtained results from this study may not be generalized because of the small sample size of HCC which were tested for HBV. The aetiology of many HCC cases is multifactorial including viral infection and exposure to environmental factors such as aflatoxin B1 and chronic alcoholism. Thus, this justifies that 0% prevalence of HCC associated with HBV could be due to other risk factors such chronic alcoholism, exposure to aflatoxins, chronic infection with HCV, iron storage disorder, habitual consumption of betel, oral contraceptives, and smoking (Blonski *et al*., 2010).

Therefore, the treatment of hepatic tumours must be improved by use of better treatment options (Nouhaud *et al*., 2013). Furthermore, there is need to increase usage of immunohistochemistry in the diagnosis of hepatic tumours in our country as more better therapies are being tried and researched on in clinical trials.

Chapter 5: Conclusions and Recommendations

5.1 Conclusion

Hepatic tumours affect patients mostly in the age range of 21-30 years and more males than females. HCC is the most common histologically diagnosed hepatic tumour at UTH with the trabecular subtype as the predominant variant followed by pseudoglandular. The most diagnosed grade of HCC is moderately differentiated followed by well differentiated grade. The HBV was not detected in HCC specimens. This cannot thoroughly mean that HCC diagnosed in Zambian patients at UTH in Lusaka is not associated with HBV but a small sample size that was used could have affected the results.

5.2 Strengths and Weaknesses

In Zambia, no study has been published on the histological classification of hepatic tumours. Therefore, this study may be used as a baseline study for future studies which may improve the prevention, diagnosis and treatment of hepatic tumours.

The study has weaknesses because the concentration was on the detection of HBV as a possible causative agent of HCC leaving out other prominent causative agents such HCV and aflatoxin B1.

Another notable weakness of this study was the use of a small sample size which makes it difficult to make inferential generalization.

5.3 Future works

Future studies with the use of polymerase chain reaction would give better information on the causative agents and the pathogenesis of the hepatic tumours which are histologically diagnosed at the University Teaching Hospital in Lusaka, Zambia. The association of hepatocellular carcinoma with other non-viral cofactors should also be studied.

5.4 Limitations

This study had a number of limitations such as financial constraints that caused the usage of few primary antibodies.

The other limitation was lack of availability of hepatic tumour tissues. This could be due to the fact that most patients would not consent for surgery to be performed on them maybe due to the invasiveness of this procedure.

5.5 Recommendation

A larger sample size to be used in future studies in order for the results to be generalized.

More studies to be carried out in various hospitals rather than University Teaching Hospital only so that the distribution of hepatic tumours can be generalized for the country.

A prospective study should be conducted on all patients presenting with hepatic tumours in Zambia so that the survival rate can be determined.

Genotyping of causative agents should also be performed in future studies.

The information gathered from this study can be used to advocate for the use of special stains and IHC as a routine test for all diagnosed hepatic tumour specimens.

Chapter 6: References

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Appendices

Appendix A

Research Work Plan

	January 2015	September 2015	January 2016	January 2016	July 2016	July 2016	September 2016	December 2016
Present to Department/ GPPF	■							
Submit proposal to Assistant Dean (PG) office	■							
Submit proposal to UNZABREC		■						
UNZABREC review and approval		■						
Collect data			■	■	■			
Data Analyze					■			
Write dissertation						■	■	
Submit final dissertation								■
Publish the findings								■

Appendix B

Research Budget

	Article	Quantity	Unit cost(ZMK)	Total (ZMK)
1	H & E Stains	5L	500	500
5	Microtome Blades	1 x 5	1 500 x 2	3 000
6	Xylene	10L	500	500
7	Wash buffer	1 kit	870 x 1	870
8	Specimen containers	100	150 x 100	1 500
8	IHC Microscope Slides X100	20	371 x 20	7 420
9	Cover slips	24 x 40	10 x 200	1 005
10	Special stains (reticulin, PAS, mucin)			2 000
11	Fine pasteur pipettes	1000	1000 x 1	1 000
12	Paraffin Seals		450	450
13	DPX Mountant	500mL	600 x 1	600
14	High pH antigen retrieval	1 kit	2345 x 1	2 345
15	Primary Antibodies	1 vial	6000 x 1	6 000
16	Pipette tips	200	1000	1 000
17	DAB+ 110ml	1 kit	1005	1 005
18	Frosted-End Specimen Glass Slides	3 boxes	20 x 3	60
19	Stapler and staples	1	1 x 100	100
20	Technical support			3 500
21	Printing and Binding			400
22	Medium-Sized Disposable Latex Groves	200 pairs	50 x 4	200
23	Scientific Calculator	1	1 x 130	130
24	Pens, pencils, eraser	5, 5, 1	10, 5, 4	19
25	Reams Of Paper	3	45 x 3	135
26	Refreshments and snacks		20 x 50	1 500
27	UNZABREC Clearance Fee		500	500
	GRAND TOTAL			36 734

Appendix C

World Health Organization Grading of Hepatocellular Carcinoma (2010)

Grade	**Characteristics**
Well-differentiated	Mild atypia Increased nuclear/cytoplasmic ratio Thin trabecular pattern Often pseudoglandular pattern
Moderately differentiated	Trabecular More than 3 cells thickness Tumor cells having eosinophilic cytoplasm Round nuclei Distinct nucleoli Frequent pseudoglandular
Poorly differentiated	Increased nuclear/cytoplasmic ratio Slit-like blood vessels Moderate to marked pleomorphism
Undifferentiated	Spindle or round shaped Little cytoplasm Solid growth