Characterization of Hepatic Cancer Cell Line (HCAM) Isolated Locally from Murine Digestive System Tumor

A thesis

Submitted to the Council of the College of Education for Pure Sciences (Ibn Al-Haitham), University of Baghdad in Partial Fulfillment of the Requirement for the Degree of Philosophy of Doctorate of Science in Biology/ Zoology / Tissue Culture

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We certify that this thesis (**Characterization of Hepatic Cancer Cell Line (HCAM) Isolated Locally from Murine Digestive System Tumor**) was prepared under our supervision at College of Education for Pure Sciences / Ibn- Al-Haitham, University of Baghdad as a partial fulfillment of the requirements for the Degree of Doctor of Philosophy of science in Biology.
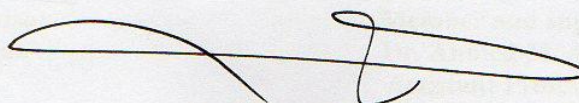
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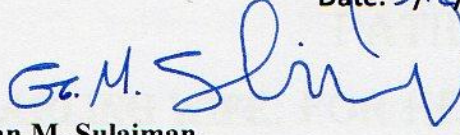**Dr. Thamer A. Muhsen**
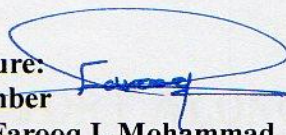**Head of Biology Department**

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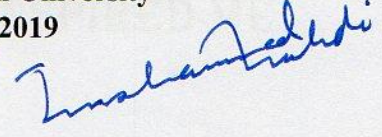
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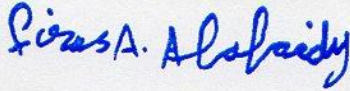
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DEDICATION

I WOULD LIKE TO DEDICATE THIS THESIS TO:

THE SOUL OF MY FATHER: WHO INSPIRED ME THE PURPOSEFULNESS AND THE PERSISTENCE

MY DEAREST MOTHER: WHO TAUGHT ME THE PATIENCE

MY DARLING WIFE: FOR HER SUPPORT ALONG THE STUDY

MY SWEETHEART CHILDREN: WHO INSPIRED ME HOPEFULNESS

MY DEAR SUPERVISORS: FOR THEIR ENCOURAGE AND SUPPORT ALONG MY STUDY

MY DEAREST SISTERS AND AUNT: WHO PRAYERS FOR ME

EVERYONE: WHO GETS BENEFIT OF THIS WORK

AYMEN R. HABEEB

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<div align="right">Aymen R. Habeeb</div>

Summary

The characterization study of murine Hepatic Cancer Ahmed Majeed (HCAM) is considers as a useful tool in research of liver cancer. This cell line was established from tumor of a white Swiss albino mouse *Mus Musculus*. The different experiments of present study targeted to characterize HCAM cancer cell line, which are:

The continuous subculture characterization of the grown cell cultured in tissue culture flask each 48- 72 hours. studying the growth kinetics for different cells number of HCAM (10,000 cells /1ml) at several passages 5, 9 and 12, and calculating population doubling time that have been 17 h. The lag phase was identical for the curves 24 hr, while the ranging of log phase from 72- 144 h, where the decline phase beginning at 168-240 h.

The cytogenetic studies of HCAM cell with passages 7, 8 and 12 revealed chromosomal variation with many numerical changes among the cancer cells along with structural changes including telocentric, abnormal length, and breaks in the arm of chromosomes.

The morphological study was carried out by using inverted microscope with crystal violet, and hematoxylin & eosin at passages (6, 9 and 14). The cells were elongated multi-polar epithelial- like with central nucleus, multi-nuclei, and high nuclear to cytoplasmic ratio.

Epidermal growth factor receptor (EGFR), Human epidermal growth factor receptor (HER2 –neu) and P53 protein were detected using immunocytochemistry in HCAM tumor cells at 13, 15 and 17 passages and the results were low positive of HER2-neu and positive of P53, EGFR compared to negative control.

HCAM cells at several passages 16, 19 and 21 were exposed to chemotherapy agents, as the following Cisplatin 50µg/ml and Docetaxel 100µg/ml for 72 h to determine the inhibition rate of cancer cells and compared

SUMMARY

with Mouse Embryonic Fibroblast (MEF) cell line after exposure to Cisplatin and Docetaxel at the same concentrations and duration.

All these concentrations of chemotherapy agent were had inhibitory effect on HCAM cells and the results revealed that HCAM cell were sensitive to Cisplatin and Docetaxel, while MEF cell after being exposed to Cisplatin and Docetaxel, showed resistance to chemotherapy compared with HCAM cells.

Analysis of changes in cell morphology was evaluated using hematoxylin and eosin staining. Many morphological changes were characterized on HCAM cancer cells after exposure to IC_{50} concentration of chemotherapy Cisplatin10.47μg & Doetaxel 12.82 μg for 72 h, the morphological changes represented as cytoplasm degeneration, large space between cells, cell membrane decomposition and Irregular shape of cells and enlarged shrink of nucleus.

In addition, the present study involved the technique PCR and DNA sequencing of some genes, The P53 gene product exhibited specific band at 500 bp and β-actin gene product exhibited band at 400 bp on gel electrophoresis whereas, the β-globin gene product showed no specific band on the gel electrophoresis. Based on P53 gene product partial sequencing NCBI blast result, it showed similarity with two partials sequenced P53. the identification percentage of p53 gene sequencing according to (NCBI), which was (99 – 94) % between Rattus norvegicus p53 tumor suppressor (p53) gene.

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List of Abbreviations

Abbreviation	Complete Name
ACTH	Adrenocorticotropic hormone
AFB1	Aflatoxin B1
AFP	Alpha-Fetoprotein
CCA	Cholangiocarcinoma
CEA	Carcinoembryonic antigen
CRC	Colorectal Cancer
DAB	Di amino benzedine
DMSO	Di Methyl Sulpha Oxide
DPX	Distyrene Plasticizer Xylene
DT	Doubling Time
DW	Distilled Water
EDTA	Ethylene Di Amine Tetra Acetic Acid
EGFR	Epidermal Growth Factor Receptors
F	Forward
FBS	Fetal Bovine Serum
FISH	Fluorescence *in situ* hybridization
FSM	Free Serum Media
GC	Gastric Cancer
GCH	Comparative Genomic hybridization
GI	Gastrointestinal Tract
GIST	Gastrointestinal Stromal Tumor
H&E	Hematoxylin And Eosin
HBV	Hepatitis B Virus
HCAM	Hepatic Cancer Ahmed Majeed
HCC	Hepatocellular Carcinoma
HCG	Human chorionic gonadotropin
HCL	Hydrochloric Acid
HCV	Hepatitis C Virus

HER2- NEU	Human Epidermal Growth Factor Receptors
ICC	Immunocytochemistry
ICCMGR	Iraqi Center Of Cancer And Medical Genetic Research
IHC	Immunohistochemistry
IR	Inhibition Rate
MD	Moderate Differentiated
MDM2	Murine Double Minute 2
MEF	Mouse Embryonic Fibroblast
MPS	Massive parallel sequence
MTT	Methyl Thiazolyl Tetrazolium
NAFLD	Non-Alcoholic Fatty Liver Disease
NCBI	National Center of Biotechnology Information
NGS	Next Generation Sequencing
PALP	placental alkaline phosphatase
PAP	Prostatic Acid Phosphatase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Poorly Differentiated
PDT	Population Doubling Time
PSA	Prostate-Specific Antigen
RPMI	Roswell Park Memorial Institute
RT-PCR	Real Time Polymerase Chain Reaction
SKY	Spectral Karyotyping
SNP	Single nucleotide Polymorphisnm
TAE	Tris, Acetic acid, EDTA
TDW	Triple Distilled Water
TNM	Tumor Size, Node Involvement, Distant Metastasis
WD	Well Differentiated

1. Introduction

The digestive system is composed of many organs and glands, and because of its complicated anatomy and physiology, numerous diseases may occur, especially tumors including the third, fourth and eighth most common cancers worldwide. There are many malignancies of the digestive system including esophagus, gastric, colorectal cancers, pancreatic and hepatocellular carcinoma (HCC), which are the most common types of cancer and a major cause of death worldwide (Chen *et al.,* 2016).Therefore it is necessary for researcher to characterize some digestive system cancer cell lines.

One of the most common gastrointestinal cancers is a liver cancer which is classified into two major categories: primary liver tumors and metastatic liver tumors. The primary tumor originates in the liver, while the metastatic tumor spreads to the liver from other organs of the body. Primary malignant epithelial tumors include hepatocellular carcinoma (HCC), hepatoblastoma and cholangiocarcinoma (CCA) (Zailaie, 2015).

Hepatocellular carcinoma is a widespread malignancy of the liver and is one of the most frequent causes of cancer related death worldwide. The major type of liver cancer is HCC, the main cause for 75-80% of liver cancer cases (Balogh *et al*., 2016). It is the fifth most prevalent carcinoma worldwide and the third cause of mortality among deaths from cancer with an annual number of 600 thousand (Castelli *et al.,* 2017). The malignant hepatocyte phenotype may be produced by the disruption of a number of genes that function in different regulatory pathways, producing several molecular variants of hepatocellular carcinoma (Thorgeirsson and Grisham, 2002). Hepatocarcinogenesis is a complex, multistep process that involves a sequential number of genetic and epigenetic alterations, ultimately leading to hepatocyte malignant transformation (Coleman, 2005).

The main risk factors for HCC are multi-factorial and consists of chronic viral hepatitis (caused by hepatitis B and C viruses), cirrhosis, aflatoxin B1, alcohol abuse, and inherited metabolic disorders (Demir, 2007).

The majority of HCC cases result from chronic liver disease of various etiologies, which leads to activation of hepatic inflammation, regeneration of necrosis, accumulation of intrahepatic lipid , oxidative stress and cirrhosis, and eventual development of HCC with accumulation of genetic mutations (Thomas and Zhu, 2005).

Cell lines have revolutionized scientific research and are being used in study of cytogenetic, testing drug metabolism and cytotoxicity, antibody testing, study of gene function, generation of artificial tissue. It is often used in place of primary cells to study biological processes (Kaur and Dufour, 2012).

The characterization study of cell culture involves the study of chromosome aberration caused by genomic alterations are the common features of cancerous cells, which may appear as chromosomal aberrations, including numerical and structural changes (Garnis *et al.,* 2004). In cytogenetic studies of tumor it is shown that large fraction of chromosomal abnormalities in many cancer types are neoplastic-specific. Such findings might serve as valuable tools for diagnosis and classification of tumors, prediction of clinical outcome, disease monitoring, and the choice of therapy (Mitelman, 2000).

The characterization of growth kinetics of cell line is an indicator of biological malignant tumor potential and provides a useful parameter for clinical decision making regarding the optimal therapeutic strategy and appropriate follow up intervals (Shingaki *et al.,* 2013).

The term chemotherapy refers to the use of drugs to kill or inhibit the growth of cancer cells as opposed to normal cells. Most chemotherapy drugs cause damage to deoxyribonucleic acid (DNA) or prevent

chromosomal replication, which leads to programmed cell death (apoptosis) (Bhosle and Hall, 2006).

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cultured cells by use of a specific antibody, thereby technique allowing visualization and examination under a microscope. Therefore it is valuable tool for the determination of cellular contents from individual cells (Dadson, 2002).

The authentication of any cell line by using sequencing method is an important tool to confirm the origin of species and identification of the lineage to which the cell belongs (Freshney, 2006).

1.1 Aim of the study:

The aim of this study is to identify the characteristics of cancer cell line (HCAM) through the following steps:

1- Maintenance the HCAM cells in tissue culture flask.

2- Calculate the growth rate of HCAM cell line.

3- Cytogenetic study of the HCAM cell line that involved observing the numerical and structural changes of chromosomes.

4- Cytological study of HCAM cells that involved morphological study.

5- Detection of the expression of some proteins expression such as P53, EGFR and HER2/neu genes by immunocytochemistry technique (ICC).

6- Cytotoxicity assay of HCAM and Mouse Embryonic Fibroblast (MEF) cell lines after exposure to cisplatin and docetaxel agents by using Methyl Thiazolyl Tetrazolium (MTT) assay.

7- Morphological changes of HCAM cells after treated with chemotherapy agents (cisplatin and docetaxel).

8- Detection of some genes (P53, β-actin and β-globin) in HCAM cancer cell line by using PCR technique and gel electrophoresis.

9- Using DNA sequencing method for the identification of cancer related gene P53.

2.1 Definition and Characterization of Tumor

The tumor is an abnormal growth of the tissue, also known as (new formation), it may benign as non-dangerous non harmful) or malignant (cancerous or fatal) and the tumor cells continue undetectably for an extended period of time in the primary tumor period and in the cells cancerous disease (patel and chen, 2012). Benign cancer differs from malignancy, malignant cancer grows more rapidly, invades surrounding tissues, spreads to other parts of the body using the bloodstream or lymphatic system, and is more lethal than a benign tumor. In addition, the tumor can be removed by surgery because it has a clearer border, and as a result, less likely to occur (Sinha, 2018). Tumor tissue consists of cancerous cells and stromal cells. As is common, cancer cells are a malignant cell that is not subject of differentiation, and stromal cells are non-malignant cells that surround cancer cells (Shiga *et al.*, 2015). For example the Gastrointestinal Stromal Tumor (GIST) are one of the rare tumors of Gastrointestinal Tract and constitute less than 1% of all digestive tract tumors, they may be benign or malignant (Cichoż-Lach *et al.*, 2008). The characteristic features of cancer cells include six biological properties acquired during the development of multiple human tumors, including maintenance of propagation signals, escape from apoptosis, resistance to cell death, unlimited multiplication potential, sustainable vessel formation, activation of invasion and metastasis (Hanahan and Weinberg, 2011).

Metastatic cancer is the cancer that has spread from the place where the primary site started to another place in the body (secondary site). There are multiple steps to the cancer cell metastases, which separate from the primary tumor and start the invasion through the endothelial barrier to enter the blood circulation and then find its way to the sites of distant members where they extravasate and establish metastatic lesions because cancer cells don't stick together as well as normal cells do, also produce substances that stimulate them to move (Sharif *et al.*, 2015).

For example, when the bowel cancer that has spread to the liver, it is called bowel cancer with liver metastasis, not called liver cancer because the cancerous cells in the liver are actually cancerous bowel cells.

The tumor cells leave the primary tumor and enter the circulating are called circulating tumor cell, which enters the distant target and continues as a spreading tumor cell, which plays an important role in maintaining its survival, regulating its growth and giving it resistance to treatment (Dasgupta *et al*., 2016) , Figure (2-1) . Therefore, Shyamala *et al*. (2014) were viewed besides reproducing uncontrollably of cancer cells, it is lose cohesiveness and orderliness of normal tissue, invade and get detached from the primary tumor to travel and set up colonies at other place.
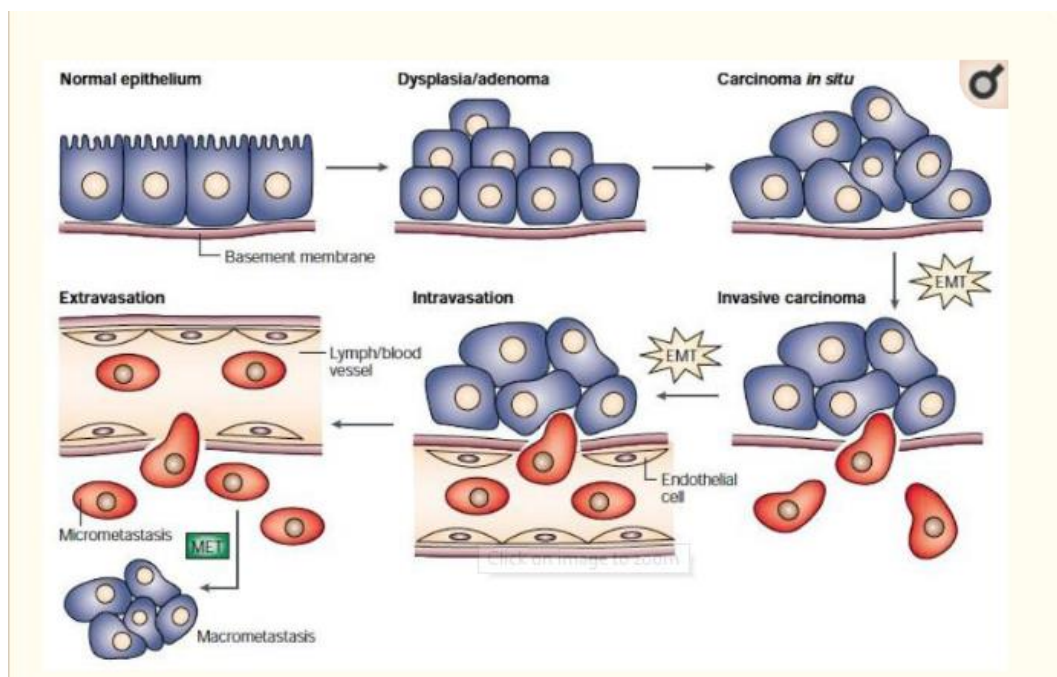
Figure (2-1): Metastasis of cancerous cell (Seyfried *et al.,* 2013)

2.1.1 The Differences between Cancer and Normal Cells

Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive so this one important difference that cancer cells are less specialized than normal cells, unlike normal cells, cancer cells continue to divide uncontrollably, and can ignore signals that normally tell cells to stop dividing or begin a process known as programmed cell death, or apoptosis, which the body uses to get rid of unneeded (Elstrom, *et al*., 2004). In addition, Cancer cells exhibit anaerobic glycolysis, which means that cancer cells derive most of their energy from glycolysis that is glucose is converted to lactate for energy followed by lactate fermentation, even when oxygen is available. Therefore, tumor cells alter their metabolism to maintain unregulated cellular proliferation and survival   (Fadaka *et al*., 2017). As well as tumor cells have undergone a set of genetic and epigenetic alterations. Often, these changes underlie cancer development, progression, and drug resistance (Goodspeed *et al*., 2016). Cancer cells has the ability for induction of new blood vessels growth (Angiogenesis), produce (G_o signals) (growth factor from oncogene) and set aside (stop signals) (anti-growth signals from tumor suppressor genes) (Ziyad and Iruela-Arispe, 2010).

2.1.2 The Classification of Tumor Tissues

A- Carcinoma: this term refers to cancer that originates from the epithelial layer of cells that form the covering of external parts of the body or the internal linings of organs within the body, carcinomas are two types : adenocarcinoma and squamous cell carcinoma such as the gastrointestinal tract, breast, lung cancers (Idikio, 2011). Barbara (2006) was published that carcinomas are the most frequent source of metastases to the liver.

B- Adenocarcinoma: It is a cancer of the epithelium that arises in the glandular tissues. Epithelial tissue includes: the superficial layer of the skin, glands and other diverse tissues that line the cavities and body organs.

C- Sarcoma: Are rare malignant tumors of mesenchyme origin, and this type of cancer originate in connective and supporting tissues including muscles, bones, cartilage and fat (Hui, 2016).

D- Myeloma: cancer that originates in the plasma cells of bone marrow, Plasma cells are capable of producing various antibodies in response to infections. Myeloma is a type of blood cancer (Bianchi and Munshi, 2015).

E-Leukemia: is a group of cancers that are grouped within blood cancers, Leukemia is a neoplastic proliferation of one particular cell type (granulocytes, monocytes, lymphocytes, or infrequently RBCs) (pokharel, 2012).

F-Lymphoma: Lymphomas comprise a group of lymph proliferative malignant diseases that originate from lymphocytes T and B cells in the lymphatic system (Alexander *et al*., 2007).

In addition, cancer tissues can be classified according to the grading into four classes: **Grade one**: well differentiated cell and slight abnormality, **Grade two**: cell is moderately differentiated and slightly more abnormal, **Grade three**: cell is poorly differentiated and very abnormal, **Grade four**: cell is immature and primitive and undifferentiated. For example, Kulesza *et al.* (2004) were found the sensitivity for accurate grading was found to be highest for well differentiated (WD) lesions and moderate differentiated (MD) , the specificity was found to be highest for poorly differentiated (PD) Hepatocellular carcinoma cells (HCC) for both cytopathologists.

Also cancer staging is another method to classify cancer cell , the most commonly approach uses classification in terms of tumor size (T), the degree of regional spread or node involvement (N), and distant metastasis (M) This is called the (TNM) staging (Patel and Shah, 2005).

2.2 The Characteristics of Normal Liver

The liver is a largest organ of the body, weighs approximately 1500 g, and is located in the upper right corner of the abdomen, it consists of four lobes: two

larger ones (right and left) and two smaller ones (quadrate and caudate), With regard to size, the liver is on average 25- 30 cm in width, 12 - 20 cm in length and 6 - 10 cm in thickness (Plaats, 2005) (Figure 2-2). This organ has a unique ability to regenerate its own tissues, where more than two-quarters of the liver can be lost, and the organ can grow back or expand to its original size within several weeks (Franciscus, 2018).

The hepatic lobules are the structural unit of the liver, each lobule consists of a hexagonal arrangement of plates named as hepatocytes, the hepatocytes are liver parenchymal cells which consist of 60-80% of liver cell community, they are arranged radially within the lobule to form cellular plates, between which the liver capillaries and the sinusoids are located. While, the another cells such as kupffer cells, stellate cells, biliary epithelial cells, sinusoidal endothelial cells and lymphocytes are non-parenchymal cells and comprise the rest 20-40% of the liver cell community (Racanelli and Rehermann, 2006). The role of these cell in the liver (hepatic sinusoidal endothelial cells, dendritic cells, hepatic stellate cells, and Kupffer cells) for regulating immune function have been demonstrated by Xing *et al.* (2016). The liver receives blood supply from two major blood vessels: the hepatic artery supplies oxygenated blood, whereas the portal vein, which provides 80% of the total blood supply, supplies nutrient-rich deoxygenated blood (Vekemans and Braet, 2005).

The main function of the liver is to filter the blood received from the digestive system, before passing it to the rest of the body, as well as detoxification of chemicals and metabolism of drugs, secretion of bile in the intestine, formation of proteins important for blood clotting and other jobs (Ramadori *et al.*, 2008).
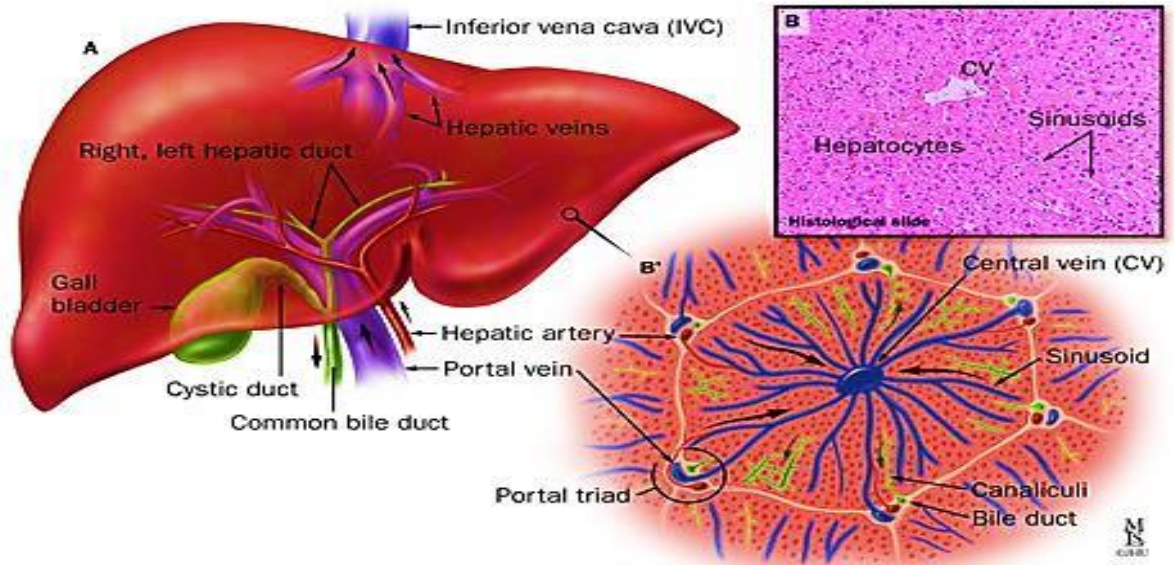
Figure (2-2): The histological structure of the liver (Plaats, 2005)

2.2.1 Liver Cancer

Liver cancer is the second most common cause of cancer-related death worldwide. Herszényi and Tulassay (2010) have been reported that liver cancer is the sixth most common cancer in the world and the third most common cause of cancer mortality. In addition, Nio *et al*. (2017) have been revealed that liver cancer is a fatal malignant tumor with a high recurrence rate and chemo resistance. Malignant liver tumors are usually classified into two categories: the primary liver cancer and the secondary liver cancer. Primary cancer refers to the first mass of cancer cells that has grown in an organ or tissue. The major forms of primary liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (Castelli *et al.,* 2017).

2.2.2 Classification of Liver Cancer

2.2.2.1 Primary Liver Cancer

2.2.2.1.1 Hepatocellular Carcinoma (HCC)

The most frequent type of liver cancer which is also called hepatoma or HCC. It accounts for 90% of all liver cancers. Hepatocellular carcinoma begins in hepatocytes which is the main cells of the liver (Befeler and Di Bisceglie, 2002). França *et al.* (2004) were noted that hepatocellular carcinomas are aggressive tumors with a high dissemination power. Also, the characteristics are rapid progression, poor prognosis and frequent recurrence (Waghray *et al.,* 2015).

2.2.2.1.1.2 Intrahepatic Cholangiocarcinoma

It is a liver primary carcinoma with increasing significance and major clinical, pathogenic and therapeutic challenges, it arises from malignant transformation of cholangiocytes bordering small portal bile duct to second-order segmental large (Ducts that carry bile to the gallbladder) within the liver. Actually most of cholangiocarcinoma are start in the bile ducts outside the liver (Buettner *et al.,* 2017).

2.2.2.1.1.3 Angiosarcoma

These tumors begin in the cells lining the blood vessels of the liver. They grow rapidly and are usually very wide so that while they are found they cannot be removed surgically. Hepatic angiosarcoma is uncommon neoplasia with an incidence between 0.5 to 2 % of primary hepatic tumors, Due to a nonspecific presentation it is very difficult to reach the diagnosis which is usually late and by that time the organ has been involved by tumor and surgical treatment is not possible (Long *et al.,* 2018).

2.2.2.2 Secondary Cancer of Liver

These types of tumors separate the cells from the primary cancer and move with the bloodstream to another part of the body, it's called a secondary cancer or a metastasis which caused by the hepatic sinusoidal endothelium that may facilitate penetration of malignant cells into the hepatic parenchyma, the liver's geographic proximity to other intra-abdominal organs or the liver's vast blood supply (Abdeldayem *et al.,* 2013). In addition, the liver site is the most common for hematogenous metastasis from colorectal cancers (CRC) due to its anatomical position regarding its portal circulation. About 14 to 18% of patients with colorectal cancer present metastasis at the first medical consultation, and 10 to 25% at the time of the resection of the primary colorectal cancer (Valderrama-Treviño *et al.,* 2017). Sheth and Clary (2005) were viewed that the vast majority of hepatic metastasis in the United States occur in patients with a primary colorectal malignancy.

Hepatic metastases occur up to 75% of patients of neurodegenerative tumors (Frilling *et al.,* 2010). Neuroendocrine tumors include those arising in the parathyroid, adrenal, and pituitary glands, and in calcitonin-producing cells of the thyroid (causing medullary thyroid carcinoma) (Kulke *et al.,* 2012).

2.2.3 TNM Classification of Liver Cancer

The tumor size, node involvement and distant metastasis (TNM) classification system includes the characteristics of tumors including: size, number, vascular invasion, as well as lymph node involvement and diffuse diseases (Table 2-1). Gress *et al.* (2017) indicated that TNM staging should applied to selection of appropriate primary and adjuvant therapy, to estimate the prognosis, and also to assist in the evaluation of the results of treatment. Several reasons explain the difficulty in classifying a world-wide system. First, the disease is very heterogeneous around the world, and this reflects different underlying epidemiological backgrounds and risk factors. Second, HCC is a complex

neoplasm inserted on a pre-neoplastic cirrhotic liver, and thus variables of both diseases leading to death should be taken into account. Third, only around 20% of the cases are currently treated by surgery (Pons *et al.,* 2005). Fourth, HCC is the sole cancer treated by transplantation in a small proportion of patients. Kinoshita *et al.* (2015) were showed that beyond classical TNM stage, the particular management of HCC led to a more suitable classification that divides patients in 4 categories: non metastatic resectable, borderline resectable, locally advanced patients and metastatic patients.

Table (2-1): Classification of hepatocellular carcinoma (TNM) (Chedid *et al.*, 2017).

Stage	Characteristics
Tumor (T)	
Tx	**Primary tumor cannot be assessed**
T0	**No evidence of primary tumor**
T1	**Solitary tumor without vascular invasion**
T2	**Solitary tumor with vascular invasion or multiple tumors, none > 5 cm**
T3	**T3a Multiple tumors > 5 cm** **T3b Single tumor or multiple tumors of any size involving a major branch of the portal or hepatic vein**
T4	**Tumor(s) with direct invasion of adjacent organs other than gallbladder or with visceral peritoneum**
Regional nodal metastasis (N)	
NX	**Regional lymph nodes cannot be assessed**
N0	**No regional lymph node metastasis**
N1	**Regional lymph node metastasis**
Distant metastasis (M)	
M0	**No distant metastasis**
M1	**Distant metastasis**

2.2.4 The Process of HCC Carcinogenesis and Risk Factor

The clonal evolution model demonstrates a multi-step process of tumor development from precancerous lesions to metastatic carcinoma, arising from the accumulation of genetic and epigenetic changes in a cell in the setting of chronic inflammation. The majority of cases do occur as a consequence of chronic inflammation, suggesting the involvement of individual genetic and environmental factors (Kumar *et al.,* 2011), therefore, the accumulation of genetic and epigenetic changes, such as the loss of tumor suppressor genes and the active of an oncogene, gives rise to a mass of primary tumor cells that are considered monoclonal in origin (Herceg and Hainaut, 2007). also, Lee *et al.* (2016) showed that genetic mutations and epigenetic alterations in hepatocellular carcinoma appear to be correlative factors that accelerate tumorigenesis.

Leong and Anthony (2005) revealed that chronic hepatitis and cirrhosis constitute the major pre-neoplastic conditions in the majority of HCC and may related to other etiologic agents such as environmental chemical carcinogens including nitrites, hydrocarbons, pesticides, chemicals in cleaning agents, processed foods, cosmetics and pharmaceuticals, as well as plant toxins such as anatoxine produced by fungi that cause spoilage of grain and food in the tropics.

Liver cancer which is a heterogeneous condition that usually develops within liver cirrhosis related to various causes: hepatitis B virus (HBV) infection, hepatitis C virus (HCV), chronic alcohol abuse or metabolic syndrome (Castelli *et al.,* 2017).

Reasons that increases a person's chance of developing cancer called risk factor, that often influence the development of liver cancer such as:

2.2.4.1 Cirrhosis

The liver condition deteriorates slowly and cannot function normally due to chronic (long-term) or injury, scar tissue replaces healthy liver tissue and partially restricts blood flow through the liver. This condition is called liver cirrhosis. Starr and Raines (2011) showed that Cirrhosis is a major risk factor for the development of hepatocellular carcinoma. Also Paradis (2013) showed that Hepatocellular Carcinoma (HCC) is the most frequent primary liver malignancy, occurring in the context of chronic liver diseases such as cirrhosis. Patients with advanced fibrosis or cirrhosis are at increased risk for carcinogenesis because chromosomal alterations that occur in fibrotic tissue are associated with tumor formation (O'Rourke *et al.,* 2018). In addition, HCC to grow and progress that it requires an immunosuppressive niche within the fibrogenic microenvironment of cirrhosis.

2.2.4.2 Chronic Viral Hepatitis (Hep-B or Hep-C)

Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) are the most common risk factors for liver cancer, these infections lead to liver cirrhosis and are responsible for making liver cancer. Hepatitis B and C virus infection can promote HCC development without prior end-stage liver disease (Ringehan *et al.,* 2017). Wong and Goh, (2006) were summarized the evidences derived from several studies including epidemiological, animal model, histopathology and molecular genetics studies leading to the establishment of hepatitis B virus as the main etiological agent for hepatocellular carcinoma. Also, HCV may have a direct or indirect role in carcinogenesis mainly through fibrosis and promote carcinogenesis through chronic inflammation or stimulation of hepatic stellate cells with subsequent fibrosis. Chronic liver inflammation has been associated with a shift in signaling from tumor growth factor beta (TGFB) resulting in a change from tumor suppression to fibrosis and carcinogenesis (Zamor *et al.,* 2017).

2.2.4.3 Chronic Alcohol

The scientific and clinical study has shown an association between chronic alcohol consumption and the occurrence of cancer in humans (Ratna and Mandrekar, 2017). Alcohol consumption has been linked to an increased risk for various types of cancer which can lead to the development of cirrhosis, in turn increases the risk for HCC (Bagnardi *et al.*, 2001).

2.2.4.4 Obesity

Obesity has become a universal and major public health problem with increasing prevalence in both adults and children in the 21 century, even in developing countries. The connection between obesity and liver cancer is particularly strong and obesity often results in liver diseases such as non-alcoholic fatty liver disease (NAFLD) and the more severe non-alcoholic steatohepatitis (Sun and Karin, 2012).

2.2.4.5 Smoking

The effect of tobacco in the development of HCC is biologically superficial, due to the carcinogenic potential of several of the ingredients in tobacco that are metabolized in the liver (Koh *et al.,* 2011). Zhang *et al.* (2014) were clarify that cigarette smoking is a potential risk factor for (HCC) initiation, partially through interaction with hepatitis B virus and continued smoking postoperatively might accelerate tumor recurrence and patient death.

2.2.4.6 Iron Overload

The liver is the main organ for storaging iron body metabolism. Therefore, abnormal iron metabolism would get started damage of liver tissue at first place (Gao *et al.,* 2012). The development of HCC as a consequence of increased dietary iron, the complicated of metabolic syndrome by the supervening of HCC and increased body iron may contribute to this complication (Kew, 2014).

2.2.4.7 Pesticides

The exposure of pesticides is one of the environmental factor hypothesized for increasing the risk of HCC because it considered as an epigenetic carcinogens through one or many mechanisms, such as spontaneous initiation of genetic changes, oxidative stress, cytotoxicity with persistent cell proliferation, inhibition of apoptosis, intracellular communication suppression and activated receptors construction (VoPham *et al.,* 2017).

2.2.4.8 Aflatoxin B1

(AFB1) is mycotoxin produced by a fungus (*Aspergillus* spp) and is found in foodstuff such as grain, corn, peanuts and legumes. There are four types of Aflatoxins (Aflatoxin B1, B2, G1 and G2) that are known to be carcinogenic to both humans and animals, of which Aflatoxin B1 is the most potent hepatotoxic and hepato-carcinogenic agent (Hamidi *et al.,* 2014). Kew, (2013) viewed that Aflatoxin B1 acts synergistically with hepatitis B virus in causing hepatocellular carcinoma these interactions between the two carcinogens may be responsible for this case including integration of hepatitis B virus x gene, as well as interference with nucleotide excision repair, altered methylation of genes and generation of DNA mutations.

2.2.4.9 Gene Mutation

Genetic alterations in specific genes lead to disruption of cellular pathways and are crucial events in the progression and instigation of hepatocellular carcinoma (Cleary *et al.,* 2013). Also, Zhan and Ji (2014) found that mutation of *p53* increased the frequency of HCC from 16% to 43%. Moreover, significant positive correlation was identified between p53 mutation and tumor size and grade II, III.

2.3 Tissue Culture

It is a general term means removal of cells, tissues or organs from the body and its subsequent placement in an artificial environment linked to growth. This environment consists of suitable glass or plastic culture containers containing a liquid medium that provides nutrients for survival and growth. Also, the tissue culture refers to cultures derived from dispersed cells taken from the original tissues (Unchern, 1999).

Cultured cells can be classified into two types:

A- Anchorage dependent cell culture (Adherent Culture):

Cells shown to require attachment for growth are set to be anchorage dependent cells. It has various biotechnological applications such as representing a great production means of vaccination purposes for viruses and with the advent of potential use of stem cells in clinics for cell therapy and regenerative medicine purposes (Merten, 2015).

B- Anchorage independent cell culture (Suspension Culture):

Cells which make not necessary attachment for growth or do not attach to the surface of the culture flask, suspension cultures are derived from cells of the blood system (Nema and Khare, 2012).The process of anchorage-independent growth of cancer cells *in vitro* as a key aspect of the tumor phenotype, particularly with relation to metastatic potential (Mori *et al*., 2009).

Tissue culture maintain the cell proliferation by the capability to manipulate the physicochemical (i.e., temperature, pH, osmotic pressure, O_2 and CO_2 tension) and the physiological environment (i.e., hormone and nutrient concentrations) (Nema and Khare, 2012). In the face of there is disadvantage of cell culture that cell line cross-contamination can be a trouble for scientists working through cultured cells, such as mycoplasma, fungi and bacteria together with instability, both genetic and phenotypic are among the problems that continue to affect cell culture (Geraphty *et al*.,2014).

Also, protection of aseptic condition is one of the most complex challenges in tissue culture, there are quite a lot of route to contamination which includes malfunction in the sterilization procedures used for glassware & pipettes, particulates cross contamination of air inside the room, weakly maintained incubation, inappropriate handling (Bykowski and Stevenson, 2008).

The tissue cultures used in several field such as environmental studies and cell development to give a clear picture of the environment of these cells and their activities within the body such as study of metabolic pathways within the cell as well as study factors that control the cell cycle. Cellular lines are used on cytotoxic studies such as the study of the chemotherapy effect in cancer cells and determine the target that is affected by the toxicity of the substances under experiment, as well as studying the metabolic changes that occur within the cell (Allen *et al*., 2001).

2.3.1 Definition and Properties of Primary and Secondary Culture

Culture prepared by removing the cells surgically from the organism and placed in suitable culture environment they will attach, divided and grow (Ryan, 2008). Primary cultures are *in vitro* cultures of cells obtained directly from the organ of interest, considered primary culture until the first sub-culture (where cells are taken into a new flask with fresh medium, often at a lower concentration). Primary cell cultures are used in areas of research such as cellular metabolism and physiology, cell morphology and genetic studies (Marquis, 2012). The problems of primary cultures are the mixed nature of each preparation, and the limited lifespan of the culture (Varga, 2011). In addition, the cellular composition of primary cultures is often very variable with hematopoietic and stromal cell types contributing to the cellular mix. Specially fibroblasts can be problematic as they attach readily to matrices and often outgrow the cancer cell colony (Macleod and Langdon, 2004).

Whereas, Subculture is a new cell culture made by transferring some or all cells from a previous culture to fresh growth medium. It gives the opportunity to expand the cell population, apply further selective pressure with a selective medium and attain a higher growth fraction and allows the generation of replicate cultures for characterization, preservation by freezing, and experimentation. In other word, subculture involves the dissociation of the cells from each other and the substrate to generate a single-cell suspension that can be quantified (Freshney, 2006).

2.3.2 Cell Line Properties

Cell line is arise from a primary culture at the time of the first successful subculture. The term cell line implies that cultures from it consist of lineages of cells originally present in the primary culture (Rodríguez-Hernández et al., 2014). One of the most important cell line categorized is to Finite cell line which encompass a restricted life span and continuous cell line which transformed under laboratory surroundings or *in vitro* culture environment give rise in the direction of continuous cell line (Nema and Khare, 2012).

The cell line (L) is the first cell line that was established by Earl in 1948, derived from the subcutaneous mouse tissue and displayed a different morphology of tissue origin (Ryan, 2008). The US embryologist Ross Granville Harrison developed the first cell line techniques in the laboratory at the first decade of the twentieth century 1970, isolated small pieces of tissue embryonic frogs and grew outside the body. Then Hela cells was the first cultured cancer cell line derived from cervical cancer, cells took from Henrietta Lacks in 1951(Kappel, 2014).

Another research was viewed that cell lines have revolutionized scientific approach and are being used in vaccine production, testing drug metabolism and cytotoxicity, production of antibodies, study of gene function, generation of artificial tissues (Kaur and Dufour, 2012).

Cancer cell lines are fundamental models that used in laboratories to study the biology of cancer, and to test the therapeutic efficacy of anticancer agents (Sharma *et al.,* 2010). Also it had been used in wide fields for research and had proved to be a useful tool in the genetic approach, and their characterization appears to be a distinctive model for the study of cancer biological mechanisms (Louzada *et al*., 2012).

The characterization of the properties of cell lines is important for a several reasons shown as the cell line relationship to the cells of origin should be established to confirm that the cell line is derived from and is representative of its origin tissue (Langdon, 2004). Also, cancer cell lines retain many genetic, epigenetic and gene expression features which the cell line should reflect the properties of the cell type from which it was derived (Sinha *et al*., 2015). For example, cell line established from a breast carcinoma it is helpful to show that the cell line has characteristics consistent with breast and epithelial origin (Van Staveren *et al*., 2009). Also, Fang and Beland (2009) showed that Hepatic cancer cell lines maintain the abnormalities of tumor-specific chromosomes in the first passages and show the same morphologic and molecular characteristics of the primary tumor. Although the genetic profile should remain constant however expression may change and features such as differentiation characteristics may be lost over time in culture.

2.4 Cancer Cell Lines of the Digestive System

Digestive system tumors that refers to malignant conditions of the gastrointestinal tract (GI tract) and accessory glands , including esophagus, stomach, biliary system, Liver, pancreas, small intestine, large intestine, rectum and anus. Esophageal cancer is the eighth most common cause of cancer death worldwide with squamous cell carcinoma and adenocarcinoma (Alema and Iva, 2014). Hu *et al*. (2000) were established new human cancer cell line from primary squamous carcinoma of the esophagus from 47 years of chines man.

Another type of Gastric cancer that is the second most common cancer world - wide and the mortality rates have remained relatively unchanged over the past 30 years, and continues to be one of the leading causes of cancer-related death (Dicken *et al.,* 2005). So, Mytar *et al.* (2017) were established three cancer cell lines as gastric cancer (GC) 1401, GC1415 and GC1436 were derived from peritoneal effusions from patients with gastric adenocarcinoma. Biliary tract cancer cell lines are very rare and an understanding of the biological nature of this neoplasm is needed to improve the prognosis of these cases. Therefore, Ku *et al.* (2002) were established six new biliary cancer cell lines from primary tumor samples of Korean patients. Also, Ma *et al.*(2007) were viewed the establishment of a new human cancer cell line from a moderate to poorly differentiated intrahepatic bile duct carcinoma from a Chinese patient.

Cancers that start in the cells that line the inside of the colon (the longest part of the large intestine) and rectum (the last few inches of the large intestine before the anus) are called colorectal cancers. Therefore ,the study of Ku *et al*. (2010) were found that characterization of colon cancer cell line should be useful in investigation of biological characteristics when established 13 human colorectal carcinoma cell line that obtained from Korean patients.

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 80% of cases and pancreas cancer, the most common type of pancreatic cancer, is even more likely than HCC to remain undetectable for extended periods of time due to the physical location of pancreas (Miller, 2013). For instant, Zhang *et al*. (2017) were established 6 novel HCC cell line derived from Chines patients which displays significant differences of cell morphology, growth rate, chromosomal number and response to 12 anti-tumor agents, and the experimental study of Kalinina *et al*. (2010) were established newly pancreatic cancer cell line (PaCa 5061) that characterized for its morphology, growth rate, chromosomal and mutational analysis.

2.4.1 Cytogenetic Studies of Digestive System Cancer Cell Lines

Cytogenetic study has been used in the study of cancer cell chromosomes from the perspective of morphology, and has provided direct evidence for early tumor research (Wang and Xia, 2008). One of the most striking features of cancer cells are abnormal numbers of chromosomes (aneuploidy) and large-scale structural rearrangements of chromosomes (Thompson and Compton, 2011). Wong *et al.* (2000) found chromosome aneuploidy ranging from a near-diploid to hyper-hexaploid karyotype with hepatocellular carcinoma.

The chromosomes of the cancer cells show structural recombination as well as a set of non-random numerical deviations (Klein *et al.*, 2006). Therefore, the identification of chromosomes aberrations or description of normal karyotype is important for patients afflicted with genetic disease such as liver cancer. In addition, the initiation and progression of solid tumors is associated with accumulation of alterations in the function of key regulatory genes (Tsafrir *et al.*, 2006). So, the increasing detection of these genetic changes allowed the description of specific tumor entities and the associated patterns of gene expression (Grade *et al.,* 2016). The telomere shortening correlates with chromosomal instability and the development of cancer. For that the researchers, Plentz *et al.* (2004) were viewed that telomeres significantly shortened in hepatocytes of HCC compared to hepatocytes in surrounding noncancerous liver tissue. Because chromosomal anomalies involve inheritance of extra genetic material or the deletion of important genetic material, the vast majority of them are lethal and result in spontaneous abortion. The chromosomal anomalies may be numerical or structural in cancer cells (Thompson and Compton, 2011), Numerical anomalies can result in either (aneuploidy or polyploidy), Aneuploidy is loss or gain of one or, rarely, two chromosomes, exemplified by trisomy 21 or monosomy X. Polyploidy is the addition of a complete haploid, as in 69 XXX or 69XYY (Zasadila *et al.,* 2013).

For example, Khandekar *et al.* (2013) were viewed several major chromosomal syndromes with altered numbers of chromosomes such as Down syndrome (trisomy21), turner syndrome (45, x) and klinefelter syndrome (47, xxy). Coward and Harding (2014) were advocated for the hypothesis that tumor cells with significantly elevated genomic content (polyploid tumor cells) facilitate rapid tumor evolution.

Structural anomalies are rearrangements of genetic material within or between chromosomes. These may be either genetically balanced, in which there is no change in the amount of essential genetic material and the phenotype is normal, or unbalanced, with a gain or loss of essential chromosome segments (Hasty and Montagna, 2014).

Other Anomalies: Marker chromosomes are extra chromosomal pieces found during karyotyping that usually derived from a structural rearrangement. They may be in the form of a ring or giant rod-shaped (Macchia *et al.,* 2017).In addition, chromosome breaks present as random visible lesions in metaphase of chromosomes. They can lead to subsequent structural changes such as deletion and translocation. Chromosome fragile sites are gaps, and breaks can be visualized with or without modification of culture conditions (Przybytkowski *et al.,* 2014).

There are several techniques for cytogenetic investigations of cancer cell lines such as spectral karyotyping (SKY), fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH).

Karyotyping is one of the few cellular genetic techniques available to allow us to identify chromosomal changes within a single cell, distinguishing both the clonal- chromosomes and non-clonal chromosomes (Rangel *et al*., 2017).

G-banding is the most common form of banding for karyotyping which G-bands can be obtained with Giemsa or Wright stains pretreated with trypsin or phosphate buffer, respectively (Bridge, 2008).

However, karyotype analysis can only observe the overall loss of chromosome, although the improvement of G - banding resolution it is fail to detect the exact locus of a specific gene on a certain chromosome (Wang and Xia, 2008).

Fluorescence *in situ* hybridization (FISH) is a powerful technique used in the detection of chromosomal abnormalities, it has provided significant advances in both the research and diagnosis of hematological malignancies and solid tumors (Bishop, 2010). But there is a problem in Fluorescence *in situ* hybridization of probes with chromosome is to obtain the tumor metaphase chromosome cells, which is difficult to prepare *in vitro* (Wang and Xia, 2008).

Houldsworth and Chaganti (1994) were viewed that comparative genomic hybridization (CGH) is a newly described molecular-cytogenetic assay that universally assays for gains and losses of chromosomal in a genomic complement. As compared with FISH, CGH solved the problem of obtaining metaphase chromosome of tumor cells *in vitro*, and it does not need to prepare probes for chromosome specific region (Darai-Ramqvist *et al.,* 2006). Chromosomal loss in both clinical tumor specimen and hepatocellular carcinoma cells was revealed by comparative genome hybridization analysis (Cheung *et al.,* 2014).

Hepatocellular carcinoma is known to exhibit highly heterogeneous molecular aberrations across the tumors, including somatic genetic and epigenetic alterations (Hirschfield *et al.,* 2018).

For instant, chromosome 4q has a high frequency of loss in HCC patients, while its aberration is relatively rare in other tumors (Wang and Xia, 2008).

2.4.2 Growth Kinetic of Digestive System Cancer Cell Line

An alteration in cellular growth can indicate a significant problem within the cell line and if undetected can have detrimental effects on experimental results, the growth curve of tumors is fundamental to understanding the genesis and consequences of cancer. In addition, the tumor volume doubling time reflects the natural rate of tumor growth and is an indicator of the biological malignant tumor potential (Shingaki *et al.,* 2013). The cancer cells passed through different phases.

Lag Phase – at this stage the cells do not divide, the cells adapt to the culture conditions and the length of this phase will depend upon the growth phase of the cell line at the time of subculture and also the seeding density. Therefore, were showed that duration of lag phase could take from a few hours up to 48 h, the time required for a cell to recover from the trypsinization, to rebuild its cytoskeleton, and to secrete an extracellular matrix that facilitates the linkage between the cells and their propagation along the substrate (Assanga *et al.,* 2013). Log growth Phase where the cells actively proliferate and an exponential increase in cell density arises and the cell population is considered to be the most viable at this phase, therefore it is recommended to assess cellular function at this stage (Mehrara, 2010). Each cell line will show different cell proliferation kinetics during the log phase and it is therefore the optimal phase for determining the population doubling time.

Rückert *et al*. (2012) were indicated that the doubling time is an important feature of each cell line, because it can be correlated with important pathophysiological parameter. There are many research used this technique to investigate the growth rate of liver cancer cells. For example, the study of Xin *et al.* (2014) were showed that seven hepatocellular cancer cell with doubling time was ranged from 24.95 hours to 110.60 hours.

Also Pan *et al*. (2015) were established and characterized primary hepatic stellate cell line (HSC) for population doubling time 8 hours.

The last period is decline phase which the cellular proliferation slows down due to the cell population becoming confluent or reduction in nutrient supplements which lead to the reduction in the number of viable cells (Yao and Asayama, 2016).

2.4.3 Molecular Study of Liver Cancer Cell Line

Cancer is caused by alterations in oncogenes, tumor-suppressor genes, and microRNA genes and most evidence points to a multistep process of sequential alterations in several oncogenes, tumor-suppressor genes, or microRNA genes in cancer cells (Croce, 2008). Determining genetic alterations in oncogenes and tumor suppressor genes in tumors may be useful for diagnosis, prognosis and determining therapeutic regimes (Spandidos *et al.,* 1993).

Molecular characterization of most liver cancer cell lines is an important tool for identifying hepatocellular tumors (Di Masi *et al.,* 2010). Through these studies, the hepatocellular carcinoma cell line can be identified depending on genetic changes , gene expression and methylation profiles because there are many somatic genetic alterations have been observed at the level of liver cancer including mutations, gene number transcription and the chromosomal alterations (Castelli *et al.,* 2017). Also, the accumulation of alterations in cancer driver genes and associated pathways are major triggers for hepatocarcinogenesis and tumor progression (Schulze *et al.,* 2016).

Therefore, numerous laboratories look for identifying always new putative markers for improving HCC diagnosis/prognosis (Qiu *et al.,* 2016). Louzada *et al.* (2012) performed a genetic and cytogenetic characterization of two rat cancer cell lines, at the levels of morphology, ploidy and identification of clonal chromosome rearrangements and breakpoint regions, as well as they analyzed the expression profile of two oncogenes (Mycn and Erbb2) and the influence of demethylation in the expression of these genes and realized that these two cell lines are a good cell model tumor *in vitro*. There is correlation of gene expression between HCC tumor

sample and HCC cancer cell line. For instant, Chen *et al.* (2015) revealed that more than 200 HCC tumor samples correlate closely with HCC cell lines in comparison to cell lines derived from other tumor types.

Liao *et al.* (2016)   determined (WTX) gene expression in different hepatocellular cancer cell line by RT-PCR and found that the value of human hepatocellular cell line (L02), WTX expression value was obviously lower in human hepatocellular cancer cell lines, MHCC-97L, MHCC-97H, HepG2 and SMMC-7721.

To define molecular pathways in mouse tumor model of hepatocellular carcinogenesis from normal liver and advanced tumor, gene sequence profiling experiments were designed to identify genes related to the hepatocellular carcinoma (Ryschich *et al.,* 2006).

2.4.4 Mechanisms of Cancer Development

2.4.4.1 Activation of Oncogenes

Oncogene is a type of cancer gene, which is unique because of mutations but does not eliminate the functions of the proteins it encode. Compared with proteins encoded by proto-oncogenes, the oncogene encoded proteins with high levels of biochemical functions (Bunz, 2016).

The Alteration of proto-oncogenes expression could led to activate them to become oncogenes which capable of inducing in susceptible cells the phenotype of neoplastic such as *ras* proto-oncogenes, which are targets for many genotoxic carcinogens (Anderson *et al.,* 1992).

The oncogenes products can be classified into six groups: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers, and apoptosis regulators (Croce, 2008). The latest studies of the interactions between the oncogene and its target cell have shown that oncogenes contribute to

the development of cancer by inducing propagation and also by developmental reprogramming of the epigenome (Vicente-Duen˜as *et al*., 2013).

Activation of oncogenes by mutations, chromosomal rearrangements and gene amplification confers growth kinetics or increased cell survival carrying such alterations. All three mechanisms cause either an alteration in the oncogene structure or an increase in or deregulation of its expression (Botezatu *et al.,* 2016)

2.4.4.2 Inhibition of Tumor Suppressor Genes

Tumor suppressor genes are the genes that encode the proteins, which are usually inhibiting the formation of tumors. Their natural function is to inhibit cell proliferation, or act as a brake for the cell cycle.

Visuttijai (2016) was added that tumor suppressor genes play a main role as a growth regulator, gatekeeper of a cell and their inactivation is often detected in malignant tumors. Identification of novel tumor suppressor gene could help to further understand tumorigenesis and discovery of a new treatment leading toward cancer cure. Mutations in tumor suppressor genes contribute to the development of cancer by inactivating that inhibitory function, mutations of this type are termed loss-of-function mutations (Lee and Muller, 2010). These are a category of genes that have a critical role in the genesis of neoplasia, when transcribed and translated, result in the synthesis of proteins that regulate the cell cycle, repair DNA mutations and the control of apoptosis but when these genes are mutated, cells lose control then leads to tumor growth. The types of tumor suppressor gene can be classified into genes that control cell division, repair DNA and cell suicide genes (Gamudi and Blundell, 2010). For example, P53 gene is responsible for destroying the cell by triggering the apoptosis and when p53 is mutated cells with DNA damage have not repaired lead to proliferate and grow and become cancerous.

The p53 gene of cell cycle signaling pathway was the second most commonly altered cascades in HCC samples (Amaddeo *et al.,* 2012). In addition, Zhao *et al.*

(2011) described novel inactivating mutations of ARID2 (AT-rich interactive domain 2) in four major subtypes of HCC, there are three types of AID2 mutations identified in the HCV associated with HCC.

2.4.4.3 Mutations in Apoptosis Genes

Apoptosis is one of the normal activities of cells, as in renewable cells such as the skin, intestine or bone marrow. Cells die every day at a rate of 50-70 million cells to eliminate the body of inflation that can occur in tissues (Gewies, 2003). This process can occur in cells that are exposed to a type of infection or defect in the process of division, so the inhibition of the genes regulating the process leads to the continued growth and multiplication of heterogeneous cells (Hongmei, 2012).

There are two types of molecules responsible for gene regulation, namely the inducer and suppressor, including Bcl-2, responsible for coding the production of a membrane protein that inhibits apoptosis, whereas Bax gene stimulates apoptosis. These genes are organized by another gene, P35 which stimulates the gene expression of Bax gene, thus reversing the effectiveness of the Bcl-2 gene for cell intervention in programmed apoptosis (Geng *et al.*, 2010).

2.4.4.4 Mutations of DNA Repair Genes

Genes that present naturally in the living cell that repair any defect in the sequence of nitrogen bases during the process of DNA cloning, or when the cell genome is exposed to mutants. Its role here is to preserve the integrity of genes from any defect that can lead to cancerous tumors. When DNA repair genes undergo mutations, it accelerates the transformation of the normal cell into a cancerous cell, especially when it is associated with a defect in the DNA repair system and mutations in one of the previously mentioned genes (Ambekar *et al.,* 2017).

The growth of the abnormal cancer cell is not due to the imbalance in the activation and inhibition pathways of tumor genes or tumor-suppressor genes. However, the cell cycle plays an important role in this process. The increased efficiency and production of special proteins called Cyclins, Cyclin A, Cyclin B, Cyclin C, Cyclin D, Cyclin E leads to the infection of certain human cancers (Qie and Diehl, 2016).

2.4.5 Genes associated with Cancer

2.4.5.1 P53 Gene

The p53 tumor suppressor gene is responsible for the cell destroying by triggering apoptosis. If the p53 gene is mutated, cells with DNA damage that have not been repaired continue to proliferate and grow and may eventually become cancerous , it is thought that p53 genes are the most commonly mutated genes in human cancer (Steele *el al.,* 1998). One of the most distinguished features of the inactive mutant p53 protein is its increased stability (half-life of several hours, compared to 20 min for wild-type p53) and its accumulation in the nucleus of neoplastic cells (Soussi, 2000). 50% of tumors are associated to the mutation of p53 and 80% of mutations are located in the DNA binding domains of the protein p53, p53 contain three major domain, N-terminal domain, DNA binding domain and C-terminal domain (Sun, 2015). Rivlin *et al.* (2011) were found that mutant p53 proteins not only lose their tumor suppressive activities but also gain additional oncogenic behavior that endow cells with growth and survival advantages, thus contributing to tumor initiation, aggressiveness, promotion, and metastasis. Therefore, Jeng *et al.* (2000) showed that mutant p53 gene has lost its tumor suppression function and is considered to be a very important step in hepatocellular carcinoma development. Graur *et al.* (2016) were found altered expression of p53 related to hepatocellular carcinoma.

2.4.5.2 Actin Gene

Actin is one of the most abundant, evolutionarily-conserved proteins in the cell. It is a member of a super family composed of three conventional actin isoforms (α, β, γ), Alpha actin is confined to skeletal muscle cells, beta-actin is present only in non-muscle cells, and gamma actin resides within both muscle and non-muscle cell types (Spencer, 2011). Beta-actin (ACTB) has been regarded as an endogenous housekeeping gene and has been widely used as a reference gene in quantifying expression levels in tumors. However, beta- actin is closely associated with a variety of tumors and accumulating evidence indicates that ACTB is de-regulated in liver, melanoma, renal, colorectal, gastric, pancreatic, esophageal, lung, breast, prostate, ovarian cancers, leukemia and lymphoma (Guo *et al.,* 2013). Khan *et al.* (2014) demonstrated by real time-PCR and western blotting a high expression of β- actin in gastric adenocarcinoma. Also, Waxman and Wurmbach (2007) found a higher expression of β- actin in Hepatocellular carcinoma.

2.4.5.3 Globin Gene

Hemoglobin molecules are made of two sets of proteins, produced by alpha and beta globin. The alpha-globin gene locus is located mainly in a region containing actively expressed housekeeping genes. In contrast, the β-globin locus is embedded in a large region of inactive olfactory receptor (OR) genes. β-globin gene located on the short arm of chromosome 11 near to insulin gene (Gibson, 1994). Polymorphism of hemoglobin beta-chain (Hbb) is widespread in natural populations of house mouse, *Mus musculus* and have been characterized of five haplotypes Hbbs ,Hbbd , Hbbp , Hbbw1 and Hbbw2. The human and mouse beta-globin loci are the most intensively studied globin loci of mammalian , they contain several globin genes, a large upstream regulatory element called the locus control region (LCR) and a number of additional regulatory elements (Noordermeer and Laat, 2008).

Zheng *et al.* (2017) showed that depletion of β-globin in circulating tumor cells (CTC)-derived cultures has minimal effects on primary growth of tumor, but it increases apoptosis following reactive oxygen species (ROS) exposure, and dramatically reduces CTC-derived lung metastases. In addition, the study of RT-PCR analysis showed that α-globin and β-globin genes expressions were significantly higher in cervical cancer tissues (Li *et al.,* 2013). Also Liu *et al.* (2011) observed the gene expression of globin in human hepatocellular carcinoma (Hep2) cell line.

2.4.5.4 EGFR Gene (epidermal growth factor receptor)

Epidermal growth factor receptor (EGFR) is a trans-membrane receptor which contributes to several processes involved in cell growth, proliferation and apoptosis inhibition , that may lead to cancer development (Abediankenari and Jeivad, 2013). Therefore, Baselga (2002) noted that the activation of the EGFR signaling pathway in cancer cells has been correlated with decreased apoptosis, increased cell proliferation, angiogenesis, and metastasis. The gene has been located on the p14-p12 region of human chromosome 7 by somatic cell hybridization and radioisotope in situ hybridization techniques (Wang *et al.,* 1993). These genes are frequently overexpressed in hepatocellular carcinoma and contributed to the aggressive growth of these tumors, so the study of Buckley *et al.* (2008) revealed that moderate to strong expression of EGFR was observed in 66% of 76 hepatocellular carcinomas by immunohistochemistry analysis.

In contrast, Kari *et al.* (2003) showed that experiences to treat cancer with EGFR antagonists have met with remarkable initial successes, especially when EGFR antagonist was used in combination with chemotherapy or radiotherapy. For example, Song *et al.* (2017) were diagnosed that anti- EGFR might serve as a therapeutic agent, particularly for patients with hepatocellular carcinoma who are unable to tolerate surgery and chemotherapy.

2.4.5.5 HER-2/neu GENE

Human epidermal growth factor receptor HER2-neu gene is a membrane tyrosine kinase and oncogene that is overexpressed and amplified in about 20% of breast cancers and in some ovarian and gastric cancers. When activated it provides the cell with potent proliferative and anti-apoptosis signals and it is the major driver of tumor development and progression for this subset of breast cancer (Gutierrez and Schiff, 2011). Azarhoosh *et al.* (2017) showed that HER-2/neu gene is more amplified in stage 4 of gastric cancer with a larger size of mass. To investigate HER-2/neu status in HCC by immunohistochemistry (IHC), HER-2/neu overexpression was detected in 21 (2.42%) of the 868 primary HCC (Xian *et al.,* 2005). Therefore, many investigations have assessed the role of HER2 in tumors of the digestive system particular in (colorectal, gastric, hepatocellular carcinoma, Pancreatic adenocarcinoma and esophageal cancers) in both prognostic and therapeutic settings, with heterogeneous results (Fusco and Bosari, 2016).

2.5 Immunocytochemistry Analysis (ICC) of Digestive   Cancer Cell Line

Immunocytochemistry (ICC) refers to immunostaining of cultured cell lines or primary cells including smears, swabs, and aspirates. ICC offers a semi-quantitative means of analyzing the relative abundance, conformation, and subcellular localization of target antigen. Also it is used to identify the location and distribution of targeted antigens in cells or tissues by staining with specific antibodies (Zhang *et al.,* 2017). Determining an exact tumor type using standard eosin and hematoxylin (H&E) staining of formalin-fixed tissues could be challenging, especially with metastatic tumor and/or tumors with poorly differentiated. IHC and ICC are dependent on several factors, including tissue and species type, duration and type of fixation, quality fresh or frozen sectioning, and antibody specificity (primary, secondry) (Painter *et al.*, 2010).

The initial approach of ICC applications of tumor diagnosis is discriminating epithelial from mesenchymal differentiation using Vimentin and Cytokeratin markers, although may occur false-negative and/or false-positive results (Schacht and Kern, 2015). Individual markers for proliferation, apoptosis, and specific tumor proteins can be used to help distinguish hyperplasia from neoplasia and determine specific tumor type. For example, Wang *et al*. (2008) were observed a positive immune-staining for Glypican-3 (GPC3) in 84 of the 111 (HCC) cases.

The history of currently used tumor markers began in the 1940s, the first discovered being alpha-fetoprotein in 1956, followed by that of carcinoembryonic antigen in 1965, since then the range of tumor markers has widened continuously, their chemical structure and genetics is now well known (Buzás, 2013). Tumor marker are biochemical substances elaborated by tumor cells either due to the cause or effect of malignant process and synthesized in excess concentration by wide variety of neoplastic cells (Malati, 2007).

Novaković (2004) classified tumor markers as Alpha-Fetoprotein (AFP), glycoproteins (CEA), placental alkaline proteins (PLAP), Adrenocorticotropic hormone hormones (ACTH and HCG), enzymes Prostatic Acid Phosphatase (PAP) Prostate-Specific Antigen (PSA) and other molecular species. For example, AFP is measured and used as a marker for a set of tumors, especially endodermal sinus tumors (yolk sac carcinoma), neuroblastoma, hepatocellular carcinoma, and germ cell tumors (Duffy and Crown, 2008). Mutation of the KRAS oncogene has emerged as a powerful negative predictive biomarker to identify patients with colon cancer (Siddique and Piperdi, 2010).

In general, the cell lines in general provide a valuable standard for gauging HER-2/neu assay sensitivity irrespective of the antibody, antigen retrieval system, detection system, or evaluation method (Rhodes *et al.,* 2002).

Assays of various tumor markers can be used for population screening, tumor detection, diagnosis, staging, prognosis, or follow up of malignant diseases (Kumar *et al.*, 2011). Velpula *et al.* (2017) explained tumor markers have wide applications in cancer care, starting from screening, choosing modality of management, assessment of prognosis to follow up after treatment.

The main advantages of IHC, ICC analysis over FISH method are that it is using more fast and economic, and the assay can easily be included as part of a routine diagnostic service using immunohistochemical analysis so infectious diseases and tumors are the main focus of ICC. Most histology tests cannot determine the source of a tumor, but ICC has different tests that can help determine the origin of tumors (Park *et al.*, 2007). Whereas, the main disadvantages of immunohistochemical assays lie in the variation of sensitivity assays between different laboratories and the different approaches of evaluation frequently used to interpret the results (Rhodes *et al.,* 2002).

2.6 The Treatment Methods of Hepatocellular Carcinoma Cell Line

Despite these scientific advances and the implementation of measures for the early detection of HCC in patients at risk, patient survival has not improved during the last three decades. This is due to the advanced stage of the disease at the time of clinical presentation and limited therapeutic options. Some researchers falls the therapeutic options into five categories: surgical interventions, radiation therapy, drugs as well as gene and immune therapies (Blum, 2005).

There are several methods to treat cancer cells such as:

2.6.1 Chemotherapy

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers, for patients with unresectable or metastatic HCC, conventional chemotherapy is for limited or no benefit (Deng *et al.*, 2015). Chemotherapy uses drugs to damage and kill cancer cells and considered as the main treatments for HCC cancer. Different

chemotherapy drugs are available to treat hepatocellular carcinoma such as Gemcitabine, Fluoropirimidines and Combination chemotherapy , (combination of gemcitabine with other chemotherapeutic agents such as Cisplatin, Oxaliplatin, Fluorouracil (5-FU), Capecitabine and Irinotecan) (Brito *et al., 2012*). They can be used in a variety of ways depending on how far the cancer has spread, and general health for patients. For that, Cao *et al.* (2012) explained that is no convincing evidence that hepatocellular cancer patients benefit from chemotherapy treatment, and doxorubicin was routinely used as a single drug for advanced HCC, but has shown inefficacy, with a response rate of about 15-20%. Other chemotherapy agents, such as cisplatin, epirubicin, 5-fluorouracil, etoposide and their combinations demonstrate even lower efficacy.

These agents can be uses before surgery to shrink the cancer so that there is a better chance of removing it after surgery to reduce the chances of the cancer coming back and to slow down the growth of cancer that has spread to nearby structures (Ruarus *et al*., 2018). Chemotherapy must create a lethal injury to DNA to produce malignant cell death which is the mechanism of apoptosis (Cheung-Ong *et al., 2013*).

Example of chemotherapy agents:

Cisplatin: Platinum complex is clinically used as adjuvant therapy of cancers targeting to induce tumor cell death, depending on cell type and concentration. It is a key drug for the standard regimens of various cancers in the respiratory, digestive and genitourinary organs (Ishikawa, 2009). Cisplatin induces cytotoxicity: by interference with transcription and replication mechanisms of DNA. Additionally, Cisplatin damages tumor tissues via induction of apoptosis, mediated by the activation of various signal transduction pathways, including calcium signaling, death receptor signaling, and the activation of mitochondrial pathways (Florea and Büsselberg, 2011). Alderden *et al*. (2006) were viewed that cisplatin anticancer activity discovered in the 1960s and its subsequent clinical success generated interest in the use of metal compounds in cancer treatment. It is

effective against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas. Its mode of action has been linked to its ability to crosslink with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells (Dosari and Tchounwou, 2014).

Docetaxel is a second-class taxane. A semisyn-thetic analogue of paclitaxel, it stabilizes and binds to tubulin, preventing physiological microtubule polymerization and disassembly, and inhibiting cell proliferation by blocking the cell cycle at the metaphase/anaphase transition (Lee *et al.*, 2013). It promotes abnormal assembly of microtubules via stabilization and it has been found to be cytotoxic against both murine and human cell lines (Tabaczar *et al.*, 2010). These drug are used to treat many types of cancer including breast, prostate, stomach, head and neck, and non-small cell lung cancer. In addition, it is used to treat hepatocellular carcinoma (Alberts *et al.*, 2012).

2.6.2 Biotherapy

Biotherapy is opposed to chemotherapy of cancer which the use of macromolecular, biological agents instead of organic chemicals or drugs to cancer treatment. It is a treatment modality that blocks the growth of cancer cells by interfering with specific, targeted molecules needed for carcinogenesis and tumor growth instead of simply interfering with rapidly dividing cells as in chemotherapy (Oldham and Dillman, 2009). Some biological therapies for cancer stimulate immune system of the body to act against cancer cells. These types of biological therapy are sometimes referred to collectively as immunotherapy, do not target cancer cells directly. Crissien and Frenette (2014) were showed that HCC can be treated by molecularly targeted therapies.

2.6.3 Immune Therapy

Cancer immunotherapy is designed to reactivate the immune response of the body to the cancer and is showing exciting promise with clear benefit in some cancers such as melanoma, lung, kidney (Lacombe, 2016). Multiple methods exist to induce tumor formation in mice, as immunotherapy is increasingly applied to HCC cancer, mouse models for these approaches are required for preclinical data (Brown *et al.,* 2018). In addition, Pisconti *et al.* (2018) showed it is easy to assert that strengthen of the immune response against cancer cells may be an incisive strategy of therapy. Zhang and Chen (2018) revealed the recent years success of cancer immunotherapy including monoclonal antibodies, cancer vaccines, adoptive cancer therapy and the immune checkpoint therapy (Immune checkpoints include stimulatory and inhibitory checkpoint molecules), for example, Xu *et al.* (2018) showed immune checkpoint therapy provides survival benefit for greater numbers of patients with two main primary liver cancers, including hepatocellular carcinoma and cholangiocarcinoma.

2.6.4 Surgical Therapy

Historically , Surgery has been the first line of defense against the tumor, the increasing use of new adjuvant therapies often shifts surgery to the second or third line, as the management of cancer is altered by increased knowledge of genetics, molecular biology, and tumor immunology (Sabel *et al.,* 2014). Manzini *et al.* (2017) viewed that all guidelines recommend resection (surgical therapy) as choice therapy in healthy liver where the transplantation of hepatocellular carcinoma is not available. Therefore, Zamora-Valdes *et al.* (2017) showed if liver transplantation is not available or contraindicated, liver resection can be offered to patients with multi-nodular of HCC, provided that the underlying liver disease is not decompensated.

2.6.5 Hormonal Therapy

Hormones are chemical messengers produced by specific organs of the endocrine system or cells in the body and act on targets distant from their site of origin (Fairchild *et al.,* 2015). Hormonal treatment of cancer involves administration of exogenous hormones in a hormone-dependent malignancy to manipulate the endocrine system by interfering either with hormone production or with the activity of receptors. In addition, certain hormones, such as corticosteroids, have general anti-proliferative effects due to their ability to down-regulate genes and induce apoptosis (Schmidt *et al.,* 2004). Also Fairchild *et al.* (2015) viewed that cancer treatment used by hormonal therapy can be classified in general as hormone analogues (naturally occurring hormones or their derivatives that have a direct anti-neoplastic effect such as corticosteroids, somatostatin , and progestins), inhibitors of hormone synthesis (Hormone-dependent cancers are sensitive to circulating levels of hormones and respond to suppression of hormone synthesis such as Gonadotropin-Releasing Hormone Agonists and Antagonists), and inhibitors of hormone receptors (Inhibitors of hormone receptors compete with physiologic hormones for binding with hormone receptors and blocking them). For example, the researchers evidenced that estrogen has been proven to exert protective effects against HCC through IL-6 restrictions, STAT3 (key regulator of macrophage function) inactivation and tumor-associated macrophage inhibition (shi *et al.,* 2014).

2.6.6 Virotherapy

Therapy targeting of cancer using oncolytic viruses has generated much interest over the past few years in the light of the limited efficacy and side effects of standard cancer therapeutics for advanced disease. Oncolytic viruses are defined as genetically engineered or naturally occurring viruses that selectively replicate in and kill cancer cells without harming the normal tissues (Fukuhara *et al.,* 2016). Also, Jebar *et al.* (2015) showed the preface of Oncolytic virotherapy lies

in their protein expression, discriminatory genomic replication and productive infection of malignant cells. There are more than 3,000 species of viruses but not all are suitable as oncolytic agents, both Russell and Peng (2018) focused on three oncolytic viruses : an echovirus, an adenovirus, and a herpes simplex-1 virus for clinical use and highlights the benefits each platform provides. Viro-therapy has shown promising results in treating HCCs and the effects can be more enhanced by adopting immune modulatory molecules (Yoo *et al.,* 2017).

2.6.7 Liver Transplantation

Among patients with unresectable tumor, the most viable surgical option is often liver transplantation (Crissien and Frenette, 2014). Liver transplant for HCC has been an accepted format of treatment and the mainstay of cure for HCC, other than surgical resection. Therefore, Xu *et al.* (2015) revealed that transplantation of liver is an optimal radical therapy for selected patients with hepatocellular carcinoma beyond the Milan criteria. Conventional Milan criteria are the reference for the selection of patient worldwide (Biolato *et al.,* 2017). The problem remains that the number of candidates awaiting transplantation far exceeds the donor population genetic pool (Ochoa and Paramesh, 2016).

2.7 Gene Sequencing

The term DNA sequencing refers to methods for determining the sequence of the nucleotides bases adenine, guanine, cytosine and thymine in a molecule of DNA. The identification of DNA sequences from genes and other parts of the genome of organisms has become essential for basic research that studies biological processes, as well as in applied fields such as diagnostic or forensic research (Munshi, 2012). These approaches reveals several repeated chromosomal rearrangements including deletions, introductions, complications, translation and more complex rearrangement in HCC. Therefore, Schulze *et al.* (2015) indicated that genomic analysis promote tumor characterization to improve personal therapy

for hepatocellular carcinoma. Several techniques used to identify the gene sequencing of cancer cells such as First-generation DNA sequencing known as Sanger sequencing was developed in the 1970s by Frederick Sanger and was the original DNA sequencing method. Conventional Sanger sequencing approach began with the obtaining of sequence data from cell line that required initial DNA fragmentation and decoupled sequencing reaction that used a molecular biology step to generate labeled dideoxyterminated sequence ladders, followed by electrophoretic separation and detection of the ladders (Johnsen *et al.,* 2018). For example, Beck *et al.* (2016) measured a validation rate of 99.965% for next generation sequence (NGS) variants using Sanger sequencing, which was higher than many existing medical tests that do not require orthogonal validation.

The development of next-generation sequencing (NGS) has changed the comprehensiveness of human genetic analysis and significantly reduced the costs associated with sequencing a genome NGS data is particularly advantageous for the study of structural variation because it offers the sensitivity to detect variants of various sizes and types, as well as the precision to characterize their breakpoints at base pair resolution (Koboldt *et al.,* 2012).

Next-generation sequencing also known as massive parallel sequence (MPS) which are not separated as they are in the Sanger sequence, technologies are revolutionize our ability to characterize cancers at the genomic, transcriptomic and epigenetic levels. one of the major differences in reading of the massive parallel sequence is that the length of sequential bases of any sequence is shorter (100-400 base pair essentially, depending on the platform) than the Sanger approach 700-1000 base pair ago). This feature of MPS has greatly enhanced our understanding of the heterogeneity of cancer cell populations and helped characterize how the genomic heterogeneity of cancers evolves during therapeutic response and resistance (Reis-Filho, 2009).

Sia *et al.* (2015) were performed RNA- and exome-sequencing analyses by (MPS), which reported a novel fusion event, FGFR2–PPHLN1 (16%), and

damaging mutations in the ARAF oncogene in intrahepatic cholangiocarcinoma. Also, Jiang *et al.* (2015) were used massively parallel sequencing to achieve plasma DNA size measurement at single-base resolution and in a genome-wide manner of hepatocellular carcinoma.

3.1 Materials:

3.1.1 Apparatus &Equipment's

Table (3-1): The apparatus and equipment were used in this study:

No	Equipment and Apparatus	Company	Origin
1	Plastic tissue culture tube (15) ml		USA
2	Plastic tissue culture tube (25) ml		
3	Pap pen	Sangyo-Tokyo	
4	Inverted microscope	Olympus	
5	Light microscope		Japan
6	Glass culture bottle	Santa Cruz	
7	Glass tissue culture petri dish		
8	Beaker		
9	Graduated cylinder		
10	Nalgene filter units, pore size 0.22 µm	Nalgene	USA
11	Nalgene syringe filter, pore size 0.22 µm		
12	Light microscope digital camera	Scopetek	
13	Liquid nitrogen container	Union Carbide	
14	Whatman filter papers No. 1	Whatman	
15	Magnetic stir bar	Science Lab	
16	Distillator	Running Water	
17	Parafilm	Bemis	

18	Disposable sterile syringe (1ml,5ml)	Medeco	UAE
19	Urine cup		China
20	Cooled centrifuge	Hettich	Germany
21	Water bath		
22	Incubator	Memmert	
23	Refrigerator	Assistent	Korea
24	Laminar air flow cabinet	K&K	S.Korea
25	Electrical oven		
26	Magnetic stirrer	Gallen kump	UK
27	PH- Meter		
28	Sensitive balance	Stanton	
29	Cover slips (22*22 mm)	Apel	China
30	Microscope glass slides	Afco	
31	Micro- pipette (2-20µl)	Lypress	Belgium
32	Micro- pipette (10-100µl)		
33	Micro- pipette (100-1000µl)		
34	Disposable tips		
35	Deep freeze (- 80 C°)		
36	Autoclave	Nuve	Turkey
37	Disposable sterile cell scraper	GSL	
38	Cryo tube	Geriner bio one	Germany
39	Vacum pump	Simport	Canada
40	Vortex mixer	Kelon	Korea

41	Refrigerated micro centrifuge	Franklin Elective	German
42	Quantus florometer	Promega	USA
43	Micro titration plate flat bottom (6-96) well	Capp	Denmark
44	Tissue culture flask (25, 50 ml)	Santa Cruz	USA
45	Thermal cycler (PCR)	Bio- Rad	USA
46	Gel imaging system	Major Science	Taiwan
47	OWL Electrophoresis System	Thremo	USA
48	Roll mixer	Local marker	China
49	Micro spin centrifuge	My Fugene	

Table (3-2): Chemical and Biological Materials

NO	Chemical and biological materials	Company	Origin
1	Streptomycin 500mg /ml	TROGE	Germany
2	Penicillin 500mg /ml		
3	DPX mount ant	Fluka	
4	Ethanol alcohol 70%	BHD	UK
5	Di Methyl Sulfa Oxide (DMSO)		
6	Di Sodium hydrogen phosphate (Na2HPO4)		
7	Potassium chloride (KCL)		
8	Sodium bicarbonate (NaHCO3)		
9	Docetaxol 20mg / 0.5ml	Miracalus	India
10	Cisplatin 50 mg / 50 ml	Miracalus	

11	Methyl thiazolyltetrazolium (MTT)	Sigma- Aldrich	
12	Fetal bovine serum	Gibco	
13	Trypsin – EDTA	US Biological	USA
14	Formaldehyde 37%	Santa Cruz	
15	Hydrogen peroxide (H_2O_2)	Santa Cruz	
16	Hematoxylin	Santa Cruz	USA
17	Eosin		
18	Glacial acetic acid	Scharlau	Spain
19	Xylene		
20	Sodium chloride (NaCl)	Thomas baker	India
21	Giemsa stain	Stain& Indicator	India
22	Hydrochloric acid	Scharlau	Spain
23	Rossel Park Memorial Institute, (RPMI -1640)	US-Biological	USA

Table (3-3): Diagnostic kits

No.	Kit	Company	Origin
1	Immuno-Cruz mouse ABC Staining	Santa Cruz	
2	P53 primary antibody	US Biological	USA
3	Epidermal growth factor receptor (EGFR)primary antibody		
4	HER$_2$- neu primary antibody		
5	Secondary antibody		
6	ReliaPrep™ Blood gDNA Miniprep	Promega	

	System, Agarose, Ethidium Bromide Solution (10mg/ml), GoTag Green Master Mix, Nuclease Free Water, TAE 40X, Quantiflor dsDNA System.		

Table (3-4): Primers used in these study:

Primers	Primers sequencing	References
P53 (F) P53 (R)	5'-GCG TAA ACG CTT CGA GAT GTT-3' 5'-TTT TTA TGG CGG GAA GTA GAC TG-3'	(Alimonti *et al*., 2010)
β-Actin (F) (R)	5'-CTACAATGAGCTGCGTGTGG-3' 5'-TAACCCTCATGTCAGGCAGA-3'	(Steube *et al*., 2008)
β-globin (F) (R)	5'-CCTGTGGGGAAAGGTGAAC-3 5'-ATACCAGATACCTGCAGGCTTAT-3'	

3.2 Cell Culture

3.2.1 Solution Preparation of Cells Cultures

3.2.1.1 Antibiotics: all solution prepared in sterile conditions as the following:

A- Streptomycin: 1gm of streptomycin was dissolved in 5 ml triple distilled water (T.D.W) and 0.5 ml from it was added to 1 litter of culture media,

B- pencillin: 0.5gm of ampicillin was dissolved in 5 ml triple distilled water, and 1 ml from it was added to 1 litter of culture media,

C- Anti-fungal**:** The solution of anti-fungal (Nystatin, Sigma) was ready to use, kept at sterile conditions in -20C°until used (Langdon, 2004).

3.2.1.2 Phosphate Buffer Saline PBS (PH 7.2)

Ready PBS powder were used by dissolving (5.4gm in 400 ml triple distilled water), PH adjusted for 7.2 and the volume was completed to one litter then filtered in Nalgene filter and stored at 4° C. Prior to any usage, solution allow to warm at 37° C (Al- Shami, 2014).

3.2.1.3 Trypsin-Versene Solution

Trypsin-versene solution was prepared by dissolving 6.2g of trypsin-versene powder in 500 ml of triple distilled water then 1.1 g of mixed solution (Na-bicarbonate, 0.5 ml of ampicillin and 0.25 ml of Streptomycin) was added, and stirred continuously on a magnetic stirrer at room temperature . prepared solution was then filtered by Nalgene filter unit 0.22 μm and stored at 4C° (AL-Shammari, 2014).

3.2.1.4 Roswell Park Memorial Institute (RPMI- 1640) Medium, 10% Serum:

The media prepared by mixing the following:

8.2 gm RPMI-1640 medium powder was dissolved in 450 ml of triple distilled water and then the following components were added:

Sodium bicarbonate powder 1.1g, Ampicillin 0.5 ml, Streptomycin 0.25 ml, fetal bovine serum 100 ml/ 900ml), then the pH was adjusted to 7.2, and the volume

was completed one litter by TDW, then the media was filtered by using Nalgene filter unit 0.2μm (AL-Shammari, 2014).

3.2.1.5 Roswell Park Memorial Institute (RPMI- 1640 Media 20% Serum:

This media was prepared as described in (3.2.1.4) with adding fetal bovine serum 200 ml/ 800ml.

3.2.1.6 Roswell Park Memorial Institute (RPMI- 1640) Serum Free Media:

This media was prepared as described in (3.2.1.4) without adding fetal bovine serum.

3.2.1.7 Fetal Bovine Serum (FBS)

It was ready to use (Gibco/ USA), Kept at sterile condition in - 20 C° before it was used.

3.2.2 Methods of Cell Culture

3.2.2.1 Obtain of Cell Lines

The cell lines used were supplied from Iraqi center of cancer and medical genetics research (ICCMGR), including Murine Hepatocellular Carcinoma Ahmed Majeed (HCAM) that isolated from primary tumor of liver cancer and mouse embryonic fibroblast cell line (MEF) which is a type of fibroblast prepared from mouse embryo.

The cell lines were preserved by cryopreservation methods that were involved in the storage in liquid nitrogen. To re- cultured freezing cells, transfer the cryo tube in water bath (37 C °) until thawing the cell solution, then transfer the solution to the tissue culture flask and added to it fresh media RPMI-1640 supplemented with 20% serum and incubate in 37 C°, after 5-6 hours of incubation re-changed the media and incubate in 37 C ° (Al-Shammari, 2003).

3.2.2.2 Maintenance of Cell Lines *in Vitro*

Cells in culture were sub-cultured when monolayer was 80-90 % confluent. Routinely passaged by trypsinization, the passages are how many times a cell line has been sub-cultured, the growth medium was decanted off and the cell washed once with 2 ml of trypsin-EDTA solution. Two to three ml of trypsin – EDTA added to cover adhering cell layer and the flask rocked gently, cells had been detached from the flask. Cells were dispensed in growth medium (RPMI-1640) and then redistributed at the required concentration into culture flask and incubated at 37 C° (Al- Shammari, 2003). The media initially appear orange when the cells grow, it become yellow thereby the reducing media should be changed. The occurrence of turbidity means that the culture is most likely contaminated and should be disposed of.

3.3 Growth Curve (Population Doubling Time PDT)

The growth curve of hepatic cancer cell line (HCAM) was studied for passages (5, 9 and 12) then:

1 - Ten of tissue culture flask 25 ml were cultured with the same number of cells 100000 cells / 1 ml, and incubated in 37 C°.

2- The following equation was used to calculate the number if viable cells per unit volume (cells / ml): $C = N \times D \times 10_4$

C is the number of viable cells per milliliter, **N** is the number of viable cells counted, and **D** is the dilution factor (Freshney, 2005).

3- Every 24 hours, the cells were suspended in 0.5 ml trypsin. Then complete cells solution to 2 ml with new media.

4- 10µl of cells suspension was transferred to ahaemocytometer slide chamber that was covered with cover slip.

5- Drive the growth curve for ten days between cells number in each 24 h.

6- The population doubling time was determined using the following equation:

Doubling time (h) = 0.063 (t-t0) / In (Nt/N0),

Where the (**t0**) is the time at which exponential growth has been, (**t**) is the time in hours, (**Nt**) is the cells number at time t, and (**N0**) is the cells number at t0 (Doyle and Griffiths, 2000).

3.4 Chromosomal Analysis

3.4.1 Preparation of Solution for Chromosome Analysis

3.4.1.1 Colsemid

Colsemid solution 10mg / 1ml is ready to use (Capricon, GMPH, German).

3.4.1.2 Hypotonic Solution

The solution was prepared by dissolving 1.39 gm from KCL in 250 ml DW then stored at 4C° and used in 37C°.

3.4.1.3 Fixative Solution

Prepared immediately by adding 20 ml of cold glacial acetic acid to 60 ml methanol.

3.4.1.4 Giemsa Stain

The stock solution prepared by dissolving 2 mg of Giemsa powder in 10 ml methanol and put it on the stirrer for one week then the solution filtered and store in dark bottle. As to stain the slide, prepare Giemsa solution by adding 1 ml of Giemsa stain to 4 ml Sorenson buffer (prepared immediately).

3.4.1.5 Sorenson Buffer Solution

Prepared by dissolving 3.54 gm of $NaHPO_4$ with 3.37 KH_2PO_4 in 500 ml of D.W and stored in 37 C° (Yassen, 1990).

3.4.2 Methods of Cytogenetic analysis of HCAM Cancer

The cytogenetic studies were carried out on the cells that grow several passages, (7, 8 and 12).

The procedure of cytogenetic applied according to (Yassen, 1990) modified by ICCMGR laboratory as the following:

3.4.2.1 Cell Harvesting

1- Cells in each flask were re-fed with fresh medium (changing media) for (5-6) hours before adding 0.1ml of Colsmaid solution for 45 min.

2- Then the growth medium was decanted off and the cells sheet washed twice with PBS. Two ml of trypsin – versene were added to the cell sheet and the flask rocked gently.

3- After approximately 30 seconds most of trypsin was poured off and the cells incubated at 37 C° until they had detached from the flask, the cells were further dispersed by gently knocking the flask with the hand for cells clumps separation and were then transferred to centrifuge tube (centrifuged at 1500 rpm for 10 min at 18 C°).

4- Afterwards, the supernatant was discarded and remaining cells pellets were re-suspended in hypotonic solution.

3.4.2.2 Hypotonic Step

Cells were re-suspended in 10 ml of pre-warmed 37C° hypotonic solution. Subsequently, the cell suspension was subjected to centrifugation at 1500 rpm for 10 min and the supernatant was discarded.

3.4.2.3 Fixation Step

Few drops of the freshly made fixative (menthol : glacial acetic acid) (3:1v/v) were added drop by drop with gentle mixing till then reaching 5ml. afterwards, centrifugation was performed for 10 min at 1500 rpm after which the fixative was decanted. The process was repeated for 4 to 5 times. Therefore, cells were re-suspended in 3 ml of the freshly made fixative and stored at -20 C°.

3.4.2.4 Slide Preparation

After the fourth time of fixation and centrifugation, the supernatant was decanted and cell re-suspended in appropriate amount to make suspension thinly cloudy. Using Pasteur pipette, 3 to 5 drops of the cell suspension were dropped evenly from appropriate distance on to wet, grease, chilled - free glass slide, and allowed to dry overnight at incubator. Subsequently, one or two of slides were stained using Giemsa stain which was applied for 2.5 min and immediately washed with Sorenson buffer. The slides were left to dry at room temperature. Microscopic examination under high magnification using oil objective lens followed to check for and count the number of metaphase and photographed by using Light microscope digital camera.

3.5 Immunocytochemistry Analysis

3.5.1 Preparation of Working Solution (Immunocytochemistry):

All these reagents prepared for immunocytochemistry studies prepared according to manufactures protocol as follows:

3.5.1.1 Hydrogen Peroxidase H_2O_2 (3%):

This reagent was prepared by mixing 12 ml of H_2O_2 with 88 ml of PBS. The reagent was used immediately or kept at 4°C until use.

3.5.1.2 Primary Antibody

In mixing tube combined 100 µl blocking reagent stock and 1 µl primary anti-body stock.

3.5.1.3 Secondary Antibody

In mixing tube combined 50 µl of blocking reagent stock and 1 µl of secondary antibody stock.

3.5.1.4 Peroxidase Substrate

In substrate mixing bottle, combines 600 µl triple distilled water (TDW), 60 µl of DAB 10x substrate buffer, then 12 µl of DAB 50 x chromagen added to peroxidase substrate , and 600 µl of peroxidase substrate 50x sufficient was used immediately for 6 slides (this preparation was done under dark conditions).

3.5.1.5 Stain: Hematoxylin was ready to use.

3.5.1.6 DPX: Ready to use solution.

3.5.1.7 Fixing Reagent

Acetone solution was ready to use.

3.5.1.8 Blocking Reagent: it was ready to use.

3.5.2 Protocol of Immunocytochemistry Analysis of HCAM Cells:

The immunocytochemistry analysis was accomplished in the cells that were grown in several passages (13, 15 and 17). After cells were dispersed with trypsin – versene and suspended in RPMI growth media the cell were re-cultured in multi-well tissue culture slide chamber (6 well) in RPMI supplemented with 10% FBS, the plate were re-incubated at 37C° to allow the cells for developing a monolayer of adherent cells within 1-2 day, after that the media was aspirated and the cell were fixed by acetone for 5 min.

The fixed cells were examined with the following (primary anti-bodies: EGFR, P53 and HER2-neu) for detection of HCAM cancer cell line, which were diluted and used according to manufactory company (Santa Cruze / USA) in next procedure:

- Cells were incubated for 10 min in hydrogen peroxidase.

- Wash cells with PBS for 2 min at (3) times.

- Cells were incubated for 45 min in blocking serum reagent, then wash in PBS at (3 times).

- Cells were incubated with primary antibody (diluted at 1:100) for 3 hours at incubator then wash in PBS for 2 min (3 times).

- Cells were incubated for 3 hours in secondary antibody (diluted 1:50), then wash in PBS twice for 2 min (3 times).

- Cells were Incubated in 50 µl of DAB substrate (were prepared in 3.5.1.4) develop staining is visible, although up to 30 min (dark condition).

- Next, the plates washed with PBS and stained in hematoxylin stain for 5-10 min, and washed with distilled water.

- Immediately add 1-2 drops of DPX and cover with cover slip glass, and examine and observe the cells by using Light microscope digital camera (Leake *et al.,* 2000).

3.5.3 Quantitative Image Test

ICC images were used for quantitative analysis protocol for the hematoxylin – DAB staining slides viewed by Leica microscope with camera (Leica microsystems, Germany), three different staining zones of immunocytochemistry images of each slide were analyzed in this study. Firstly, un-mix the DAB by color de-convolution technique, hematoxylin stained areas leaving a complimentary image. As we take three new images. First image is the hematoxylin stain, the second one is the DAB image, and then the DAB image was quantify The number of pixels of a specific intensity value vs. their respective intensity was raised using "Fiji" version of Image J from http://fiji.sc . The intensity numbers in the results window to optical density (OD) numbers with the following formula:

OD = log (max intensity/Mean intensity), where max intensity = 255 for 8-bit Images (Mustafa *et al*., 2015).

3.6 Cytotoxicity Assay

3.6.1 Preparation of Cytotoxicity Solution

3.6.1.1 The Preparation of Chemotherapy Reagents

Cisplatin and Docetaxel were obtained from the pharmacy and stored at a concentration of (Cisplatin 50 µg/ml) and (Docetaxel 100 µg/ml) at 4°C. It is dissolved in distilled water to make a stock solution. This solution was further diluted in the serum free media and used in the cell culture immediately before each experiment (AL-Shammari *et al.*, 2014).

3.6.1.2 Methyl thiazolyl tetrazolium (MTT) Solution

Methyl thiazolyl tetrazolium (MTT) is a yellow colored water soluble tetrazolium dye. Mitochondrial enzyme lactate dehydrogenase, produced by metabolically active

cells reduces MTT to water-insoluble formazan crystals. When dissolved in appropriate solvent, these formazan crystals exhibit purple color. The intensity of the purple color is directly proportional to the number of viable cells and can be measured spectrophotometrically at 584nm. 0.2g of MTT was dissolved in 100 ml of PBS in order to prepare 2 mg/ml concentration of the dye.

The solution was filtered through 0.2 μm syringe filter to remove any blue formazan product, and then stored in sterile dark, screw-capped bottles at 4°C. The solution was used within no longer than 2 weeks of preparation (Betancur-Galvis *et al.*, 2002).

3.6.1.3 Crystal Violates Stain

7 ml of Crystal violet added to 3 ml of PBS then filtered by filter paper before stain usage each time, this stain is used for staining and fixation of cell culture.

3.6.1.4 Hematoxylin

Hematoxylin stock solution was ready to use.

3.6.1.5 Eosin

Eosin stock solution was ready to use.

3.6.2 Methods of Cytotoxicity Assay of (HCAM) and (MEF) Cell Lines:

Cells from several passages (16, 19 and 21) were culture in 96 wells plate and the procedure was carried out as described by (Al-Shammari *et al.,* 2015) as following:

3.6.2.1 Cell Seeding Stage of the Cell Lines

Cell line HCAM was detached from tissue culture flask when they reached to sub-confluent monolayer by trypsinization as described previously (3.2.2.2). 20 ml of culture medium with 10% serum were add to the flasks and mixed gently with cells to prepare cell suspension. The cell suspension in culture flask was poured aseptically to a sterile beaker, then by used plate 96 well, 200μl of cell suspension was transferred to each well by using micropipette, plates were covered with a sterile

adhesive film, lid placed on, shake gently and incubated in incubator at 37 °C for 48h to allow cell attachment, proliferation and confluent monolayer achievement.

3.6.2.2 Exposure of the Cell lines to Chemotherapy (Cisplatin and Docetaxel)

From stock solution 50 µg/ ml of Cisplatin, prepare serial dilution (50, 25, 12.5, 6.25 and 3.125) µl, and 100 µg/ ml of Docetaxel, prepare serial dilution (100, 50, 25, 12.5, 6.25) µg/ ml which were diluted with RPMI-1640 serum free medium, were add to confluent monolayer cells. The plate that had cultured cell lines were examined under the inverted microscope to be sure that the confluent monolayer was formed, treated cells with Cisplatin and Docetaxol has been done by removing the medium from the micro-titration plate. Then 200 µl of each dilution of chemotherapy above was added to each well, there were five replicates used for each titer as well as the control cell which were treated with serum free media only to make sure of the validity of the assay. The plate was covered with a new sterile adhesive, lid placed on, and sealed with Para film. The plate was incubated in incubator at 37Co for 72 hours.

3.6.2.3 Cytotoxicity Test

Cell viability was measured after 72 hours of chemotherapy exposure and treatment by removing the medium, adding 100 µl of 2 mg/ml solution of MTT (1 ml of MTT added to 9 ml of SFM) , and incubating for 2-4 hours at 37°C.

After removing the MTT solution, the remaining crystals in the wells solubilized by the addition of 50 µl of Di methyl sulfa oxide (DMSO) followed by 37°C incubation for 30 min. The optical density of each well was read by using a micro-ELISA reader at a transmitting wave length on 584 nm (test wave length). The inhibiting rate of cell growth was calculated by counting the percentage of proliferation rate as **(IR)** = (A-B)/A*100. Where **A** is the mean optical density of untreated wells and **B** is the optical density of treated wells (Betancur-Galvis *et al.,* 2002).

3.6.3 Morphological Changes Study after Treatment

In this study H&E stains were used to visualize the effects of Docetaxel and Cisplatin agents on (HCAM) cells. The cells $2x10^4$ cells/2ml were seeds on 6 wells plates incubated overnight at 37°C to allow cell attachment and confluent monolayer achievement. At confluence 80%, the cells were treated with IC50 of Cisplatin and Docetaxel, concentrations which calculated with graph pad prism program for HCAM and MEF cell lines, and incubated for 72 h at 37^oc (Al-Shammari *et al.,* 2015).

At the end of experiment, media of the plates was removed and the cells were fixed in 4%formaldyhyde for 30 min followed by the following steps:

A. washed with tap water (three times)

B. Absolute alcohol was added for 1 min.

C. Rinsed in grade of series of ethanol (100%, 90% and 70%) to be dried and then washed in D.W for 1 min.

D. Hematoxylin staining was added for (1-3) min, then dip in tap water to depose of the excess dye.

E. Acid alcohol was added until the color turned to pink then rinsed in tap water.

F. Eosin was added for 3 min, then dip in distilled water.

G. Rinsed in grade of ethanol series (70%, 90% and 100%) to be dried and then washed in D.W for 1 min. then mounted in glycerine.

After H&E staining procedure the cells were examined with inverted microscope (Li *et al.*, 2018).

3.7 Genotyping analysis

3.7.1 Extraction of Genomic DNA from HCAM Cancer Cell Line

Frozen HCAM cell line thawed, genomic DNA was then extracted at passage (7) directly using the tissue DNA extraction spin kit.

3.7.1.1 The Protocol of DNA Extraction

1- Sample preparation adherent cultured animal cells (trypsinize cells prior to harvesting), remove the culture medium and wash cells in PBS. Aspirate PBS and add 2ml of trypsin. Once cells detach add the medium then transfer 1.5 ml to micro-centrifuge tube.

2- 20µl of Proteinase K solution (20 mg/ml) and 200µl of buffer BL (Blood Lysis Buffer) was added to cell pellet, then the tube mixed vigorously using vortex and incubated at 56˚C for 30 min, for farther lysis   incubated 30 min at 70˚C.

3. Add 200 µl of absolute ethanol (not provided) to the sample. Pulse-vortex to mix the sample thoroughly, and spin down briefly to remove any drops from the inside the lid. Centrifuge at 12500 rpm for 5 minutes.

4. All of the mixtures were transferred to the mini-column carefully, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replace with a new one.

5- From buffer BW (Column Wash Buffer B) 600µl was added to the mini-column, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm) and the collection tube was replaced with a new one.

6- Apply 700 µl of buffer TW (Column Wash Buffer T). Centrifuge for 1 min at 6,000 rpm, discard the pass-through and re-insert the mini column back into the collection tube.

7- The mini-column was centrifuge at full speed (>13,000 x g) for 1 min to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube.

8- Add 200 µl of buffer AE (Elution Buffer) or sterilized water. Incubate for 1 min at room temperature. Centrifuge at full speed for 1 min.

3.7.1.2 Quantitation of DNA

Quantus Florometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of

DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected (40 ng/ml).

3.7.2 Conventional PCR Protocol:

1- Kit component and other reagents thoroughly mixed before were used.

2- kit components and assemble reactions were kept on ice.

3- All the DNA samples, nuclease-free water, and each forward and reverse primer were calculate the required volumes of each component based on the table (3-5).

4- Prepare a reaction mixture in a special PCR tubes in order as follows: nuclease-free water, master mix, forward primer, reverse primer, dNTP$_S$, DNA template.

Table (3-5): The reaction step of PCR

PCR	Volume
DNA template	3 µl
Forward primer	1 µl
Reverse primer	1 µl
Master mix	12.5 µl
Nuclease-free water	7.5 µl
Total	25 µl

5- After the reaction cocktail was prepared, reaction mixture volume 25 µl then transferred to each well of a PCR tube (separated PCR tube for every sample).

6- The cycling protocol was done in thermacycler (PCR machine) as the following in the table (3-6).

Table (3-6): The cycling protocol programming

Step	Temperature	Time	Repeat cycle
Initial denaturation	95	30 sec	
Denaturation	95	30 sec	**35 Cycle**
Annealing	63	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	

3.7.2.1 Optimization:

 To examine the optimum annealing temperature of primer, the DNA template was amplified with the same primer pair, (Forward) (Reverse), by 35 cycles of denaturation at 95°C for 30 sec; annealing at 55, 58, 60, 63 or 65°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation of 5 min at 72°C was included, followed by a10 min incubation at 4°C to stop the reactions.

3.7.3 Agarose Gel Electrophoresis

 Gel was prepared by dissolving 1gm of agar in 100 ml of TAE buffer (Tris, Acetic acid and EDTA). The solution was heated to boiling instrument (using microwave) until all the gel particles were dissolved. 1μl of ethidium bromide (10mg/ml) was added and mixed to the agarose when heat was down avoiding bubbles. The solution was allowed to cool down at 50-60C°. The agars solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed and the gel was placed in the gel electrophoresis tank. The tank was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel. For PCR product, 5μl was directly

loaded to well. Electrical power was turned on at 100 volt/ 50 m Amp for 90 min. DNA moves from cathode to plus anode poles. The ethidium bromide stained bands in gel were visualized using gel imaging system (Lee *et al*., 2012). The amplified products were determined by comparison with a commercial 1,500 bp ladder.

3.7.3.1 DNA Ladder:

This marker (from KAPPA Express Ladder Kits) has four DNA fragments (in base pairs): 250, 500, 750 and 1000. It was ready to use solution, stored at -20 °C.

3.8 Sequencing Method

PCR product of p53 gene was prepared for sent to sequencing analysis. These reactions were performed to the purified PCR products in both directions by using 3730XL DNA Analyzer (Applied Biosystems Inc, USA). The complementary sequences were aligned using ApE (A plasmid Editor) software (v2.0.55, May 4, 2018). Sequencing was accomplished at the National Instrumentation Center for Environmental Management (NICEM), College of Agriculture and Life Sciences, Seoul National University (South Korea). The obtained nucleotide sequences of the P53 gene of HCAM isolate were submitted to GenBank under the submission ID: 2235321.

3.9 Statistical Analysis

All data were expressed as mean ± standard deviation. For ICC experiment, n = 5 images were used. One-way analysis of variance (ANOVA) multiple comparison was done to show variations among groups. The statistical analyses for ICC and cytotoxicity of docetaxel and cisplatin on two cell lines were performed using (GraphPad Prism version 6.07 for Windows, GraphPad Software, San Diego, CA, USA), and $p \leq 0.001$ was considered to be statistically significant.

4. Results and Discussion

4.1 The Maintenance of HCAM Cell Line

At monolayer, the cells were passaged by trypsinization, then controlling its proteolytic property by adding RPMI-1640 media to inhibited enzyme activity that might damage cells. Trypsinization is the term applied to the treatment of cells by the proteolytic enzyme trypsin to change their adhesiveness (Unchern, 1999), then preparing the cell suspension at ratio 1:4 (cell suspension: medium), re-incubated at 37°C for allowing the cells to grow and continue a new confluent monolayer of culture cells, the media was changing to offer some nutrients of the cell culture.

The choice of enzyme is critical to maximize the viable cell yield and it can be determined by the type of tissue subjected to dissociation, because different tissues had different extracellular matrix compositions (Ryu *et al*., 2016).

RPMI-1640 has demonstrated wide applicability for supporting growth of many types of cell cultures. Yang and Xiong (2012) were indicated that RPMI-1640 is suitable for most types of cells, including tumor cells, normal cells, primary culture cells, passage cell. The supplemented media with serum is very important for cell line because of serum serves as a source of amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, inorganic salts, trace elements, and other compounds. It also improves the pH buffering capacity of the medium and helps to reduce the physical damage that is caused by pipette manipulation and stirring) (Yao and Asayama, 2017).

The cells have been grown as a cluster of colony cells after 24 -48 hours of cultured in tissue culture flask and become monolayer at 72 hours (figure 4-1 A, B). Proliferated cells has become important characteristics, as the culture can now be propagated, characterized, and the potential increase in cell number and uniformity of the cells open up a much wide range of experiment of the possible, these results correspond with this study described by (McElroy *et al.,* 2009).
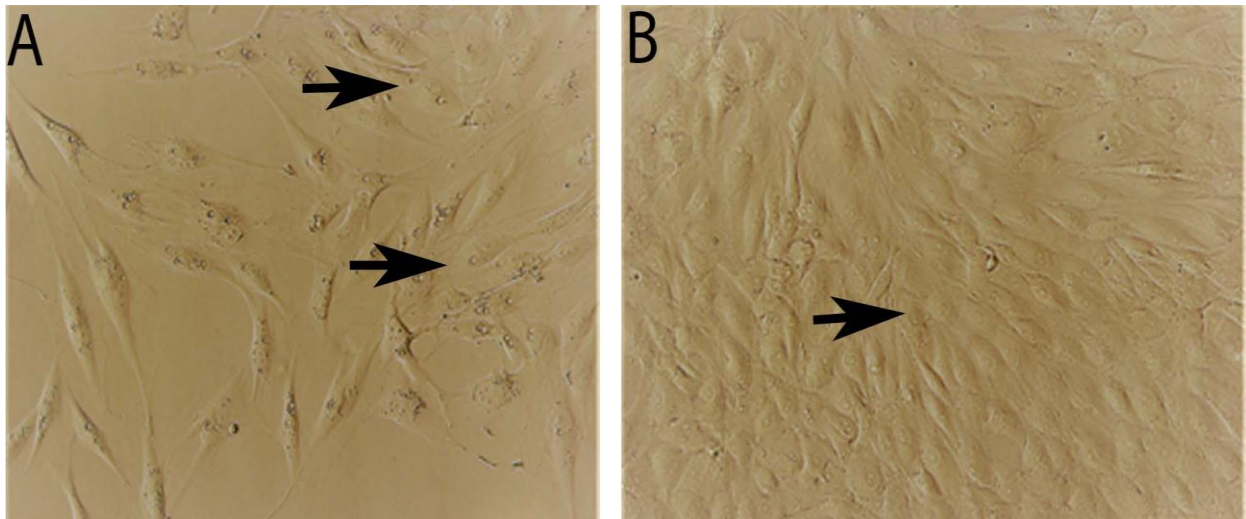
Figure (4-1): HCAM culture cells in 50 ml tissue culture flask (20X)
          (A) Cells grew as a cluster after (24-48) hours (black arrow)
          (B) Cells overgrowth and forming monolayer at more than 80% confluence
              after 72 hours (black arrow).

4.2 Growth Kinetic of HCAM Cell Line

The growth kinetics of murine hepatic cancer cell line (HCAM) at passages (**5, 9** and **12**) was obtained by seeding the cells at density of 100000 cells / 1 ml at exactly 24 h intervals for a series of 10 days and the average value of duplicates was used to calculate the population-doubling time and plot their growth curve with a Neubauer improved Haematocytometer (Table 4-1). The growth curve of HCAM cell line was shown as figure (4-2). In tissue culture flask the cancer cells undergo different phases which called cell cycle or cell division.

The growth curve of cell line was applied in the evaluation of the characteristics of cellular growth, which show immediately after reseeding a lag-phase.

Table (4-1): Growth rate of HCAM cells interval for a series of 10 days as well as growth periods

Growth rate (Mean) (cell number* 100000/ml)	Time/ Hour	Period
123.750	24	Lag phase
239250	48	
392.500	72	Log phase
660.000	96	
901.250	120	
1.112.250	144	
911.250	168	Decline phase
742.500	192	
491.250	216	
330.000	240	
Population doubling time (PDT)	17	
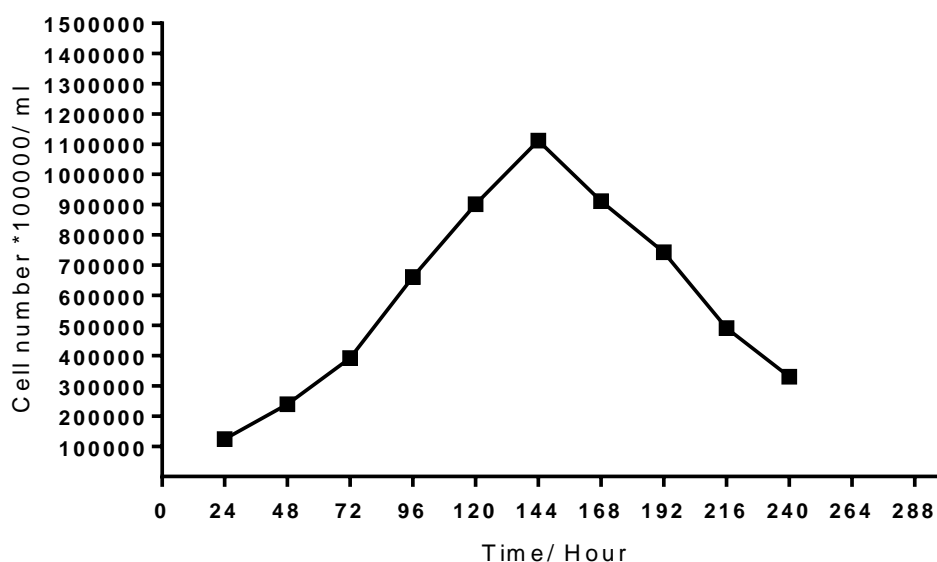
Figure (4-2): The Growth Curve of Hepatic Cancer Cell Line (HCAM), which show that HCAM cells receding lag phase at 24-48 h, log phase at 72-144h and decline phase at 168-240h.

At lag phase, there is no increase in cell counts, but cellular synthesis (Bento *et al.,* 2016). The duration of this phase could take place from a few hours up to 48 hours, but is usually around 12–24 hour, and allow the cells to recover from trypsinization, to reconstruct it is cytoskeleton, and to secret matrix that aid attachment the cell with each other (Figure 4-3 A,B). Originate the cells and form the serum become associated with the surface of substrate and help cell adhesion, when potentially propagation, all of them facilitated the linkage between the cells and their propagation along the substrate. All these performance enable the cell to enter into a new cycle (Assanga *et al.*, 2013). In addition, Al-Shammari *et al.* (2015) Found that cell adhesion increased with increased serum concentration which is the beginning of the proliferation, communication and growth of cells, whenever a little adhesion the growth rate was slightly. HCAM need 10% of the serum and when the serum is not used most of cells remain floating.

Following a "lag period" cell numbers increased exponentially and enter into "log-phase" when cell population doubles at a characteristic rate defined as doubling time (DT) between (72 hours – 144 hours) and the population doubling time become 17 hours (Table 4-1), (Figure 4-3 C, D). The growth kinetic of hepatocellular carcinoma maybe significantly associated with the external environment, nutrition supplied or liver disease such as hepatitis virus type.

An *et al.* (2015) were manifest that HCC grew faster in patients infected with HBV than in those infected with HCV and suggests that the HCC growth rate may vary depending on the individual patient, therefore the effects of drugs, chemical agents and diseases that stimulate or inhibit cell growth can be studied in this phase. In addition, Keibler *et al.* (2016) explored that oncogenic transformation rewires cellular metabolism to sustain elevated rates of growth and division. For example, the growth rates of the 6 hepatocellular cancer cell lines were significantly different: CNHCC0106 cells grow at the fastest rate, their doubling time is 28 h; CNHCC0104 cells grow slowly, their doubling time

is 77 h, While the other four cell lines grow at moderate rate, their doubling time range from 32 to 34 hours (Zhang *et al.*, 2017). Whereas, Xin *et al.* (2014) revealed that doubling time with 7 hepatocellular cancer cell line was ranged from 24 hours to 110 hours.

After 168 hours, the growth rate declined, the cells enter to the decline phase, where the medium was not changed and increase the number of dead cells at the expense of cells resulting from cell division, the cells number declined and become aging (Figure 4-3 E, F). While Sutherland *et al.* (1983) showed that a maximum cell density was reached by day 8, and thereafter, the cell numbers declined due to unchanged media. In addition, when the cell population is very dense and the substrate has practically all been occupied, the cells enter in a stationary phase, where the rate growth drops nearly to zero (Nakhjavania and Shirazia, 2017).

Many research noted the effect of environment and be one of the important risk effected on the cancers. Also the cell proliferation involved a large number of gene products that control the steps in cell cycle, programmed cell death and response of cells to external growth signal, so any mutation of this genes could be a direct reason of forming cancer cells. Borner (1996) showed that oncogene product Bcl-2 expression is associated with a retardation of mammalian cell proliferation due to a prolongation of the G1 phase of the cell cycle. Whereas, cells lacks Bcl-2 expression die from any point of the cell cycle in response to apoptotic agents. In contrast, Westwood *et al.* (2013) reported that environmental enrichment does not impact on tumor growth in mice.

Mathematical modeling is a useful tool to elucidate new mechanisms involved in tumor growth kinetics, which can be relevant to understand cancer genesis and select the suitable treatment (González *et al.,* 2017). For instant, the growth of HCC was significantly decreased by HCC-specific modulation frequencies of electromagnetic fields (Zimmerman *et al.,* 2012). Because the dose of gamma radiation (γ-IR) could inhibited DNA synthesis and cell proliferation

furthermore, γ-IR induced cell cycle arrest in the G2/M phase and the percentage of cells in the G2/M phase was increased from 15% (control) to 49% (Vučič*f et al.,* 2006).
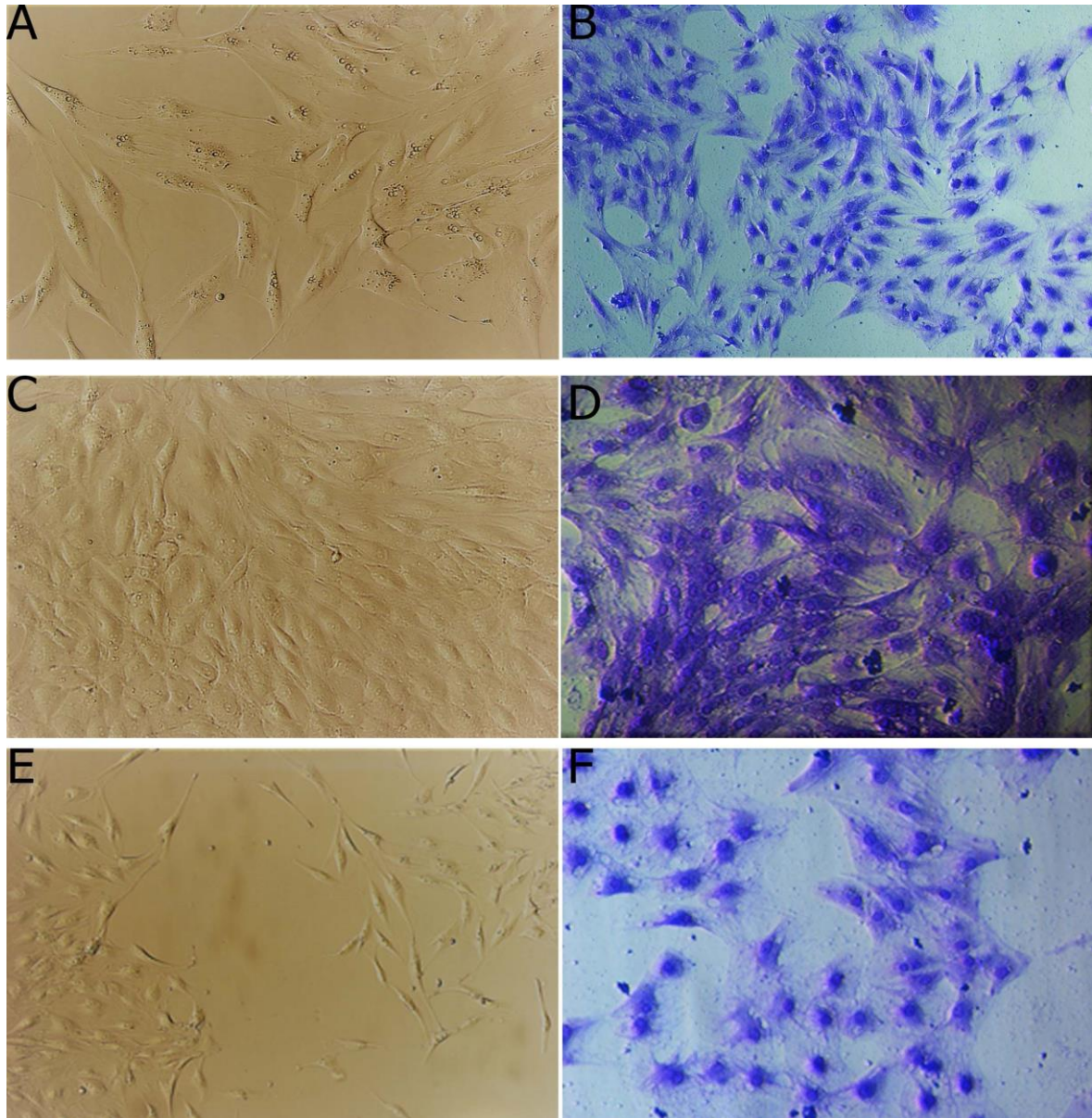
Figure (4-3): **HCAM cells staining with crystal violet dye at period types: (A-B): shows HCAM cells at lag phase, which, begin to adhered, grow and formed clones. (A) Unstaining cells 20X, (B) staining cells 20X. (C, D): shows HCAM cells grown at log phase which begin to grow more than 80% confluence and formed monolayer, (C) Unstaining cells 20X, (D) staining cells 20X, (E, F): shows HCAM cells enter to decline phase which the cells become aging and cell division arrest, (E) Unstaining cells 20X, (F) staining cells 20X.**

4.3 Cytogenetic Study of Hepatic Cancer Cell Line (HCAM)

The results of cytogenetic study for HCAM in mice with passages (7, 8 and 12) showed numerical changes in chromosomes. Normal hepatic cells of Swiss mice contain 40 chromosomes that is the original number of laboratory chromosomal mice at it is described by Shahrour *et al.* (2016) (Figure 4-4). The present results showed that HCAM cells contain duplicated number of chromosomes between (80-100) chromosomes (Figure 4-5), compared with normal cells.

In addition, the study noted multi structural changes such as appearance of breaks in the chromosomal arms (Figure 4-6), telocentric chromosomes and many chromosomes which have an abnormalities length compared with control (Figure 4-7). Our results detected that HCAM chromosomal numbers has multiple ploidy, most mammalian cells usually contains two identical chromosomal groups (Haploid)(1n) and when organisms reproduce sexually then has complete number of chromosomes (Diploid)(2n) (Gentric *et al.,* 2012). An abnormal division take place in the cell through different division phases especially metaphase leading to numerical change of ploidy (more than two set of chromosomes) which called polyploidy (Wang *et al.,* 2017).

Polyploidy results from deregulated cell division and has been considered an undesirable event leading to increased mutation rate and cancer development (Tovar *et al.,* 2010). Davoli and Lange (2011) were showed that karyotypic analysis of cancer cell lines has revealed a wide range of chromosome numbers ranging from hypodiploid to hypertetraploid.

These study is agree with present study, such as Ogawa *et al.* (1999) who indicated that gain and loss of chromosomes in 14 hepatocellular carcinoma cell line and this result is associated with carcinogenesis *in vivo* and established cell line.
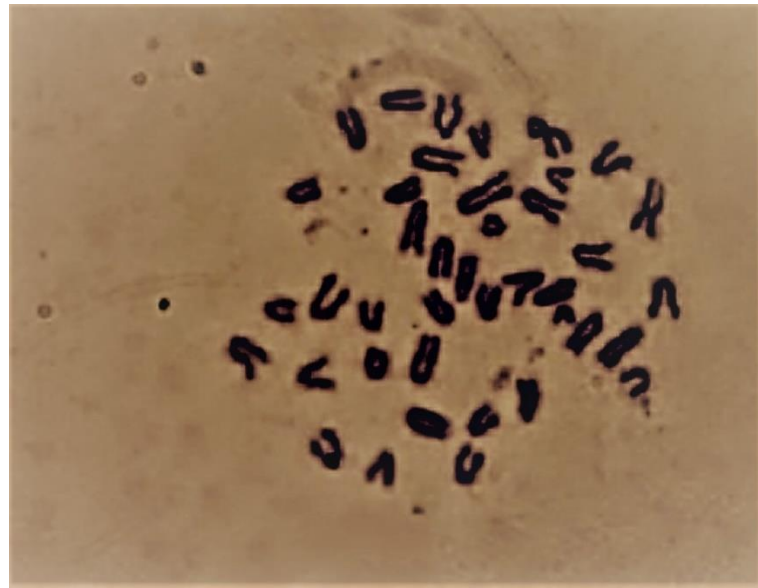
Figure (4-4): Representative photographs of cytogenetic study of HCAM cell line, Magnification 100X, Metaphase with normal chromosomes number (n=40) (Shahrour *et al.,* 2016).
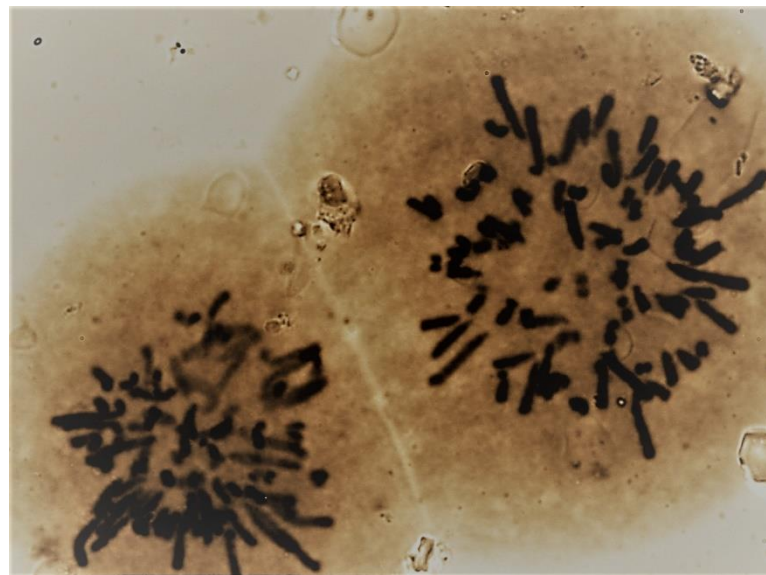
Figure (4-5): Representative photographs of cytogenetic study of HCAM cell line with passages 7, 8 and 12, 100X, Metaphase with duplicated number of chromosomes (n=80-100).
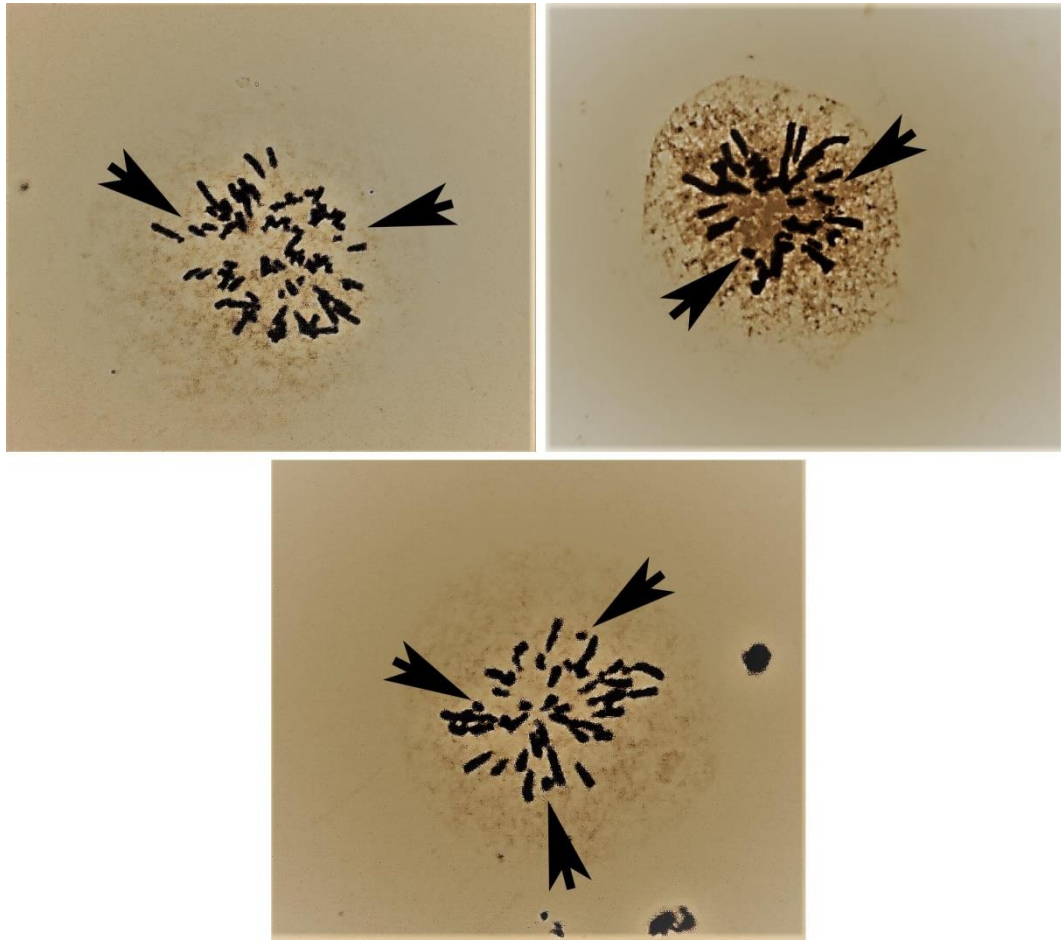
Figure (4-6): cytogenetic study of HCAM cells with passages 7, 8 and 12 showing abnormal structure of chromosomes such as break in chromosomal arms (black arrow), 100X.
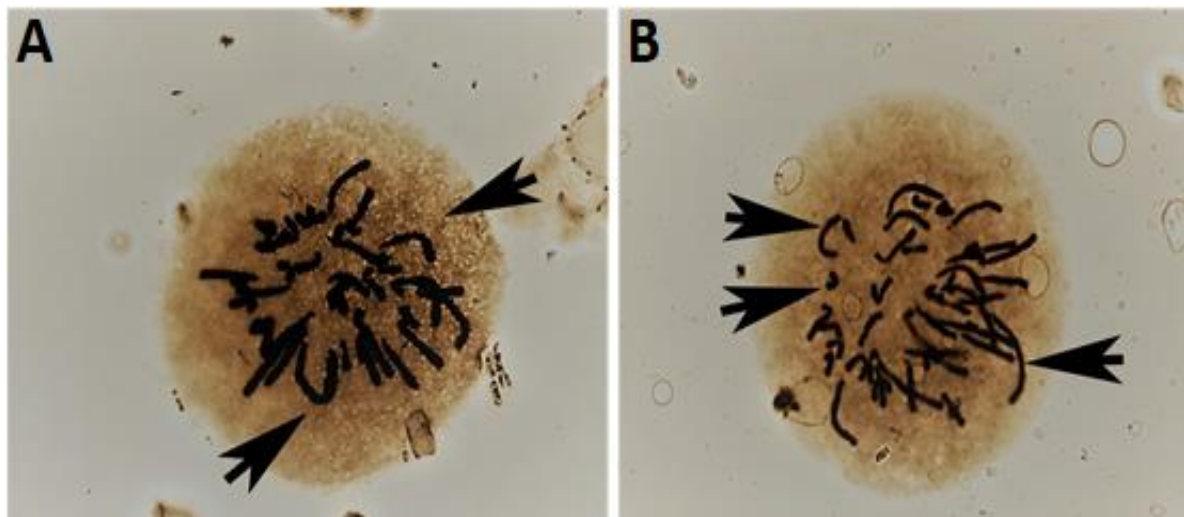
Figure (4-7): cytogenetic study of HCAM cell at several passages 7, 8 and 12 showing (A): telocentric chromosomes and (B): abnormal length of chromosomes (black arrows), 100X.

Genomic instability in mouse chromosomes may play an important role in the development and progression of Hepatocellular carcinoma in mice (Zhang *et al.,* 2004). Al- Shammari *et al.* (2015) was indicated that chromosomal instability leads to aneuploidy which is more effect than the change in the nucleotide level.

These chromosomal instability effects on a wide range of proliferation, apoptosis, and differentiation of normal cells have been transformed into cancer cells by loss of suppressor genes function or loss of oncogene inhibition.

This study illustrated many of structural changes in HCAM chromosomes such as telocentric chromosomes, chromosomes break and increased of ploidy length. The researchers, Also Sargent *et al.* (1999) were identified chromosomal alteration during tumor development of HCC by G-banding and were observed partial or complete loss of chromosome 4 in all tumors with nonrandom breakage in band C2, deletions of chromosome 1 were observed in 80% of the tumors and breakages of chromosomes 4, 9, 14, and X. Also Bilger *et al.* (2004) were mapped a potent modifier for the development of hepatocellular carcinoma to distal chromosome 1 which carries one or more genes that are sufficient to confer susceptibility to liver cancer.

Although, this study noted structural changes of chromosomal length as is it more length than normal chromosomes which indicated to increasing of copy parts due to the translocation of materials from other chromosomes. Zimonjic *et al.* (2009) established seven hepatocellular carcinoma cell line which exhibited chromosomal changes through gain of material from chromosomes 5, 6, 8, 10, 11, 15.

Oncogene or tumor suppressor gene alterations are associated with chromosomal change in hepatocellular carcinoma (Rashid *et al.,* 1999). P53 gene locus on the chromosome 17 which is the most observed anomaly in HCC (Yano *et al.,* 2004). Taylor *et al.* (2018) viewed that chromosome arm imbalance (Aneuolpoidy) correlated with TP53 mutation, somatic mutation rate, and

expression of proliferation genes. It is consistent with the expression of p53 gene in HCAM cells.

4.4 Morphological Study of Hepatic Cancer Cell Line (HCAM)

The Results were showed that HCAM cells with several passages 6, 9 and 14 after 24 hours of tissue culture adhered to the culture flask and formed single cells with clear borders. When the hepatic cancer cells stained with crystal violet dye, appeared in a polygonal shape, spherical to oval body containing more than one nuclei (central nucleus), cytoplasmic cavity and some of extension and dendritics from cells surfaces (Figure 4-8). In addition, the study noted that cancer cells began to form small clusters after 48 hours of incubation as they grew in closed position with each other to formed single colonies, cells appeared elongated, some are multipolar and epithelial like cells, appeared in spherical to oval body containing central nucleus with multi nuclei, cytoplasmic cavity and some of extension from cells surfaces (Figure 4-9). Also the cells that stained with H&E appeared elongated and integral of its internal component. The cells continued to multiply, expansion of clusters in size until reaching to confluent to form monolayer after 72 hours of incubation and show high nuclear to cytoplasm ratio (Figure 4-10).

The results are consistent with several studies that have studied phenotypic characteristics of HCC, for example, a study of 8 hepatocellular carcinoma cell lines showed that all cell lines adhesion to the tissue culture flask and reached monolayer within 24-72 hours of incubation and most of the cells maintain their original tumor characteristics (Park *et al.,* 1995). In addition, another hepatic cancer cells were characterized by an epithelial-like shape with prominent nuclei, monolayer polygonal with clear boundaries (Tian *et al.,* 1999). Also the four cell line of hepatocellular carcinoma (FLC) is characterized by an epithelial image when cultivated in the tissue culture plate and become monolayer after 72 h (Kato *et al.,* 2014).
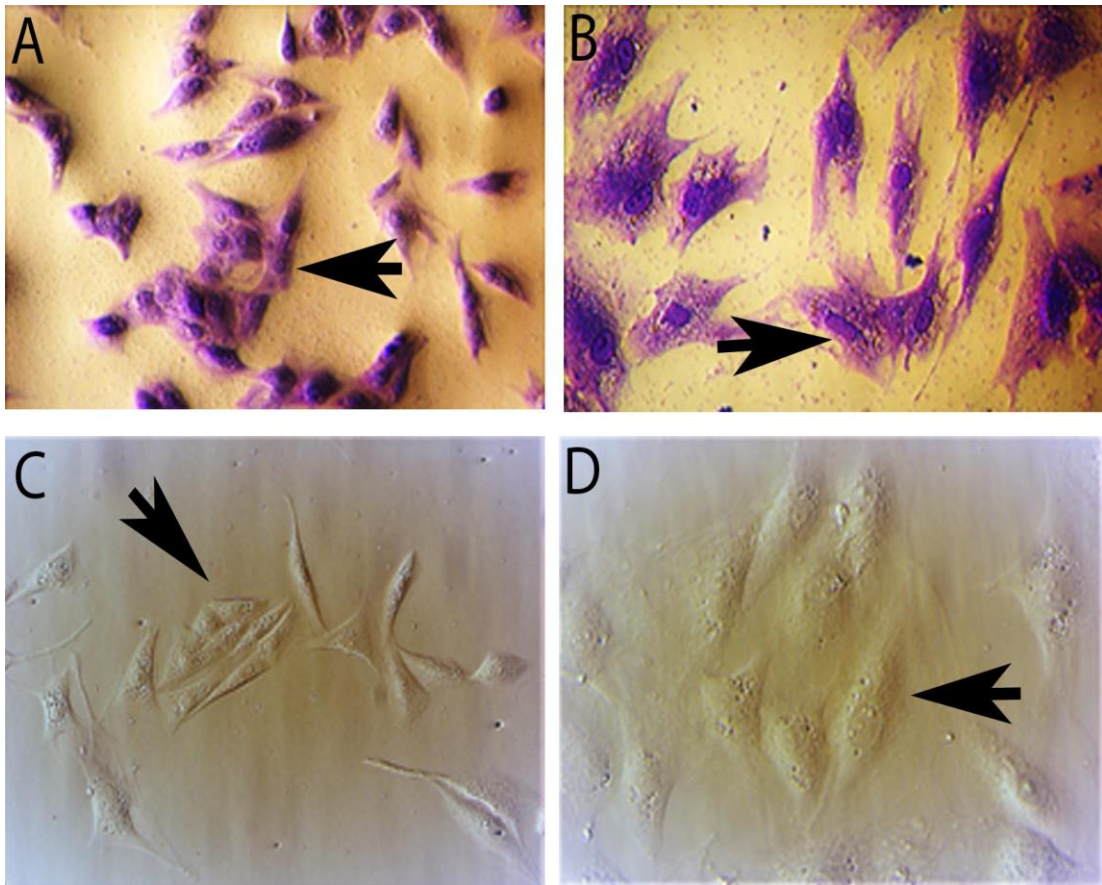
Figure (4-8): Morphological images of HCAM with passages (6, 9, 14) at incubation for 24 h inRPMI-1640 media 10% FBS with and without crystal violet staining which shows adhered cells to the culture flask and formed polygonal cells with clear borders (Black arrows) (A) staining cells 20X (B) staining cells 40X (C) unstaining cells 20X (D) unstaining cells 40X.
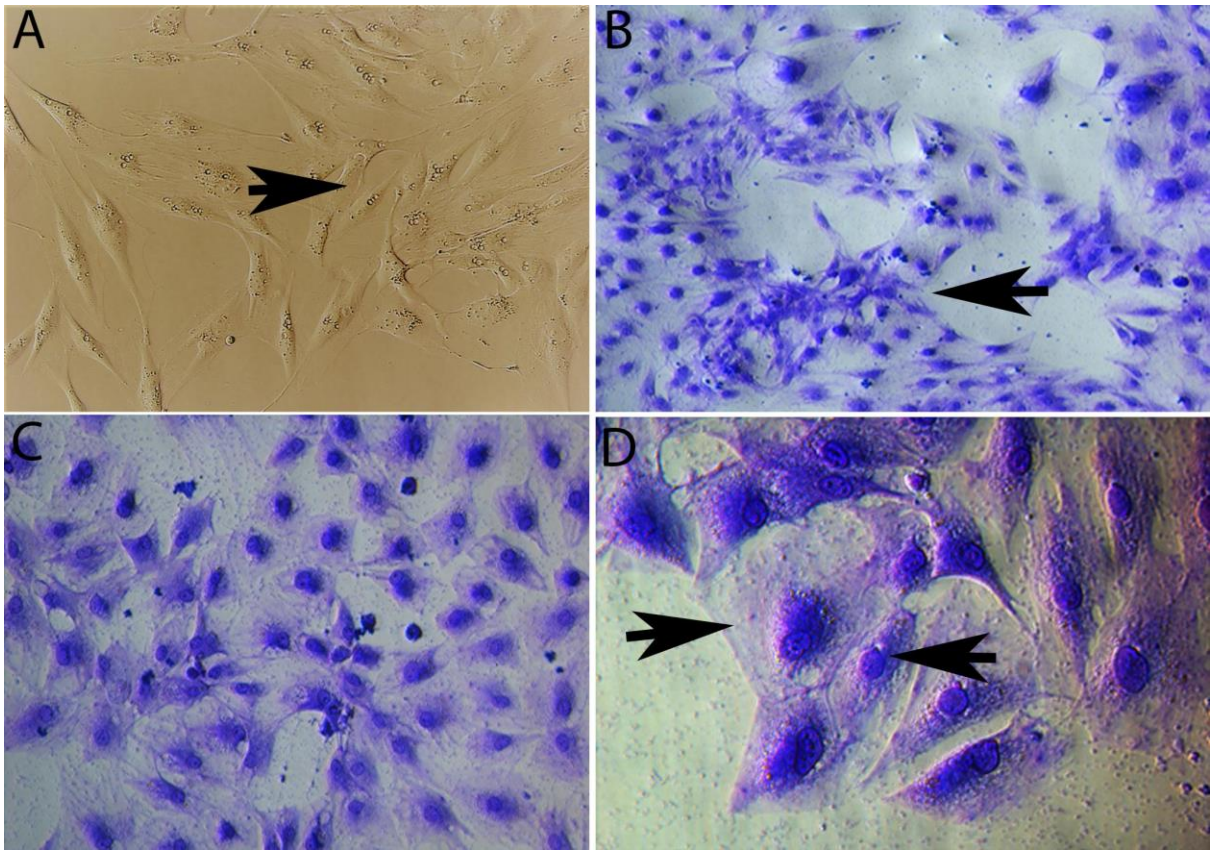
Figure (4-9): The morphological characteristics of HCAM cells in cultured stain with crystal violet at 48h. (A) Cells colonies formed to initiate monolayer of cultures cells (unstaining cells, 20X. (B) Staining cells, 10X. (C) Cells appeared elongated, some are multipolar and epithelial like cells, 20X. (D) Cells appeared in spherical to oval body containing central nucleus, cytoplasmic vacuolated and some of extension from cells surfaces, 40X. (Black arrows)
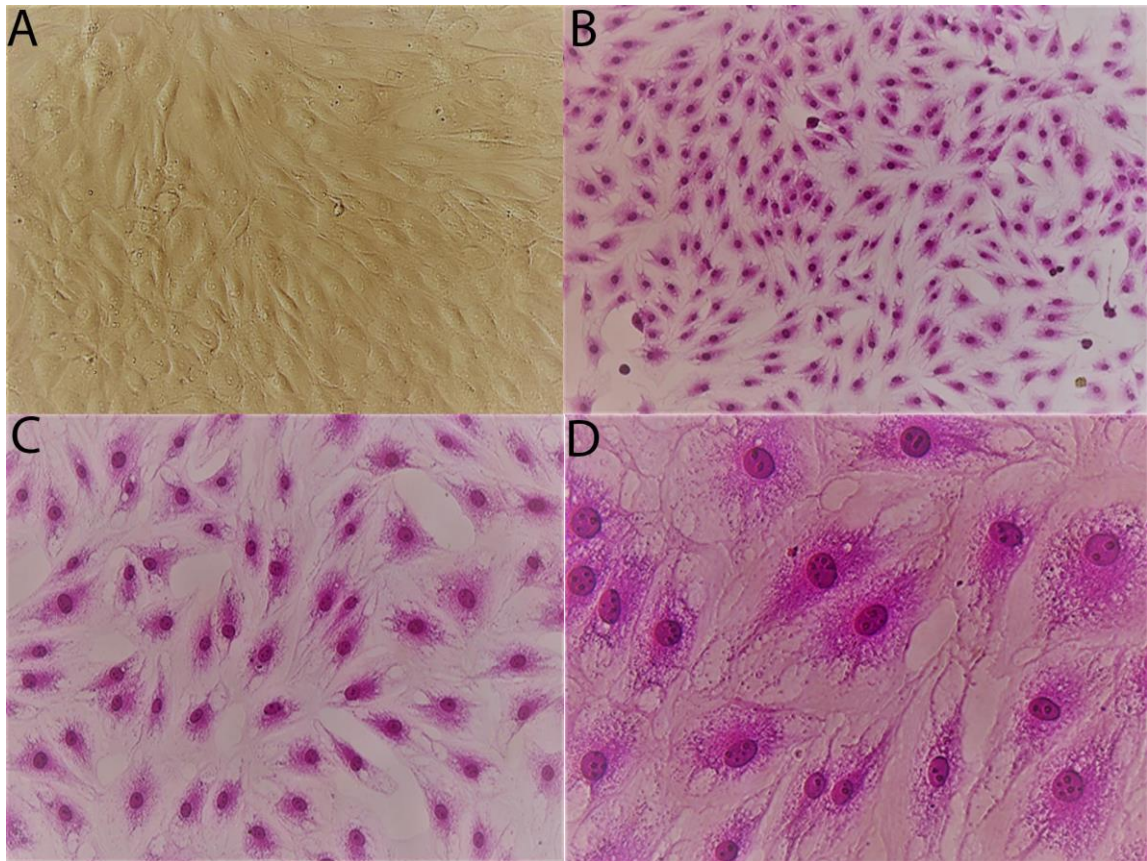
Figure (4-10): **The morphological characteristics of HCAM cells cultured in RPMI-1640 media with 10% FBS with or without H&E for 72 h. The cells continued to multiply, expansion of clusters in size until reaching to monolayer and show high nuclear to cytoplasm ratio. The cells staining with H&E dyes, (A) unstaining cells, 10X, (B) staining cells, 10X, (C) staining cells, 20X, (D) staining cells, 40X.**

4.5 Immunocytochemistry Study of HCAM

Immune enzymes in hepatic cells are affected when they are exposed to cancer. Our study tested HCAM by immunocytochemistry staining kit (santa cruz biotechnology, USA). Under inverted microscope, some enzymes were altered at several passages ranged between (13, 15 and 17) in more than 3 trails within the hepatic carcinoma cell line (HCAM). The findings shows positive expression of the immune enzyme HER2-neu, p53 and EGFR for the HCAM cancer cell line compared with the negative expression for control sample of the same cell line (Figure 4-11).

The (HCAM) cell line showed low positive expression of the HER2-neu in cytoplasm, EGFR and P53 proteins expression in the nucleus of hepatic cancer cell line, compared with the negative expression of control.

The results found that there is a weak level of positive expression of HER2-neu, compared with negative control (Figure 4-12). It is agree with the results which showed that no significant associations between HER2-neu over expression and the pathological parameters of HCC (Xian *et al.*, 2005). Whereas, Shi *et al.* (2018) were showed that HER2-neu protein is overexpressed in hepatoma cell lines of H4IIE, HepG2, JM and the resected HCC tissues. Moreover, overexpression of human epidermal growth factor receptor 2(HER-2-neu) gene and it is protein are associated with cell division increasing and a high rate of tumor growth and have been noted in several malignancies (Cui *et al*., 2014). These results are consistent with increased expression of HER2- neu in HCC and there is correlation between Hepatitis B x antigen (HBxAg) and HER2-neu and the antigen (HBxAg) contributes to the pathogenesis of hepatocellular carcinoma (Liu *et al.*, 2009).

Although HER2-neu is generally indicative of a poor prognosis, its overexpression is associated with a better outcome when inflammatory infiltrates are present in the tumor (Ménard *et al.*, 2004). HER2-neu could be a new goal as a treatment option of hepatocellular carcinoma.

It is consistent with findings that HER2-neu inhibition at early stage might inhibit tumor progression through suppression of growth and metastasis (Shi *et al.,* 2018).
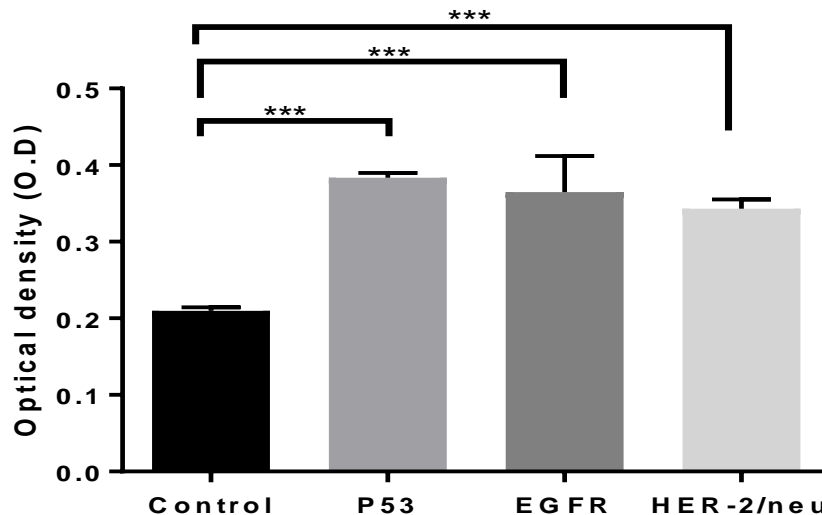
Figure (4-11): **Immunocytochemistry analysis of P53, EGFR, HER2 /neu expression in HCAM cell line derived from primary mice tumor cells. Digital Image Scoring showing significant proteins expression when stained with relative mAbs against the markers that analyzed using ImageJ program. *** Significant differences at P<0.001.**

The results noted that the positive expression of p53 in nuclear of HCAM cells compared with negative control (Figure 4-13). The most significant characteristic of hepatocellular carcinoma is metabolic disorder and significant change in bioenergy, p53 is responsible for metabolic changes that develop in hepatocellular cells because it has the ability to regulate metabolism and participates in DNA repair therefore it is increases during liver cancer (Kim *et al.*, 2016). The regulation of p53 in mice originated from MDM2 (murine double minute 2), MDM2 act as inhibitor of p53 by preventing its transcription activity and promoting to nucleus (Khoury and Dömling, 2012). The balance between p53 and MDM2 is present in normal liver cells. However, when hepatocellular carcinoma is exposed, this balance will be disrupted and lead to the blocking of MDM2-P53 binding and lead to increased expression of P53 protein expression and its increase in hepatic cells (Meng *et al.,* 2014).
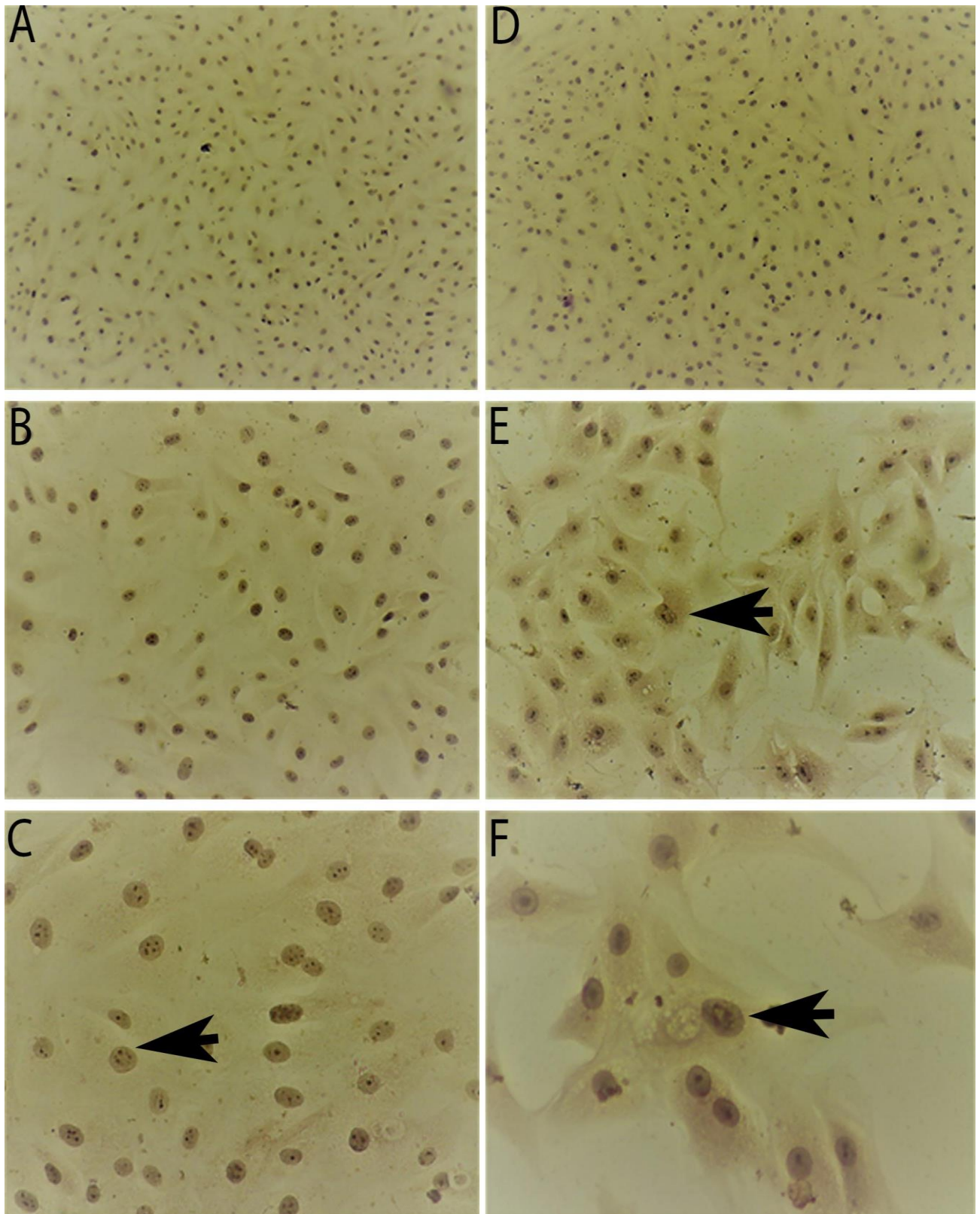
Figure (4-12): Immunocytochemistry analysis in HCAM cell line using antibody marker (HER2-neu gene) showing: low positive - nuclear expression of this gene. (A, B, C): showed negative control expression 10X, 20X, 40 respectively. (D, E, F): showed low positive nuclear expression (black arrows) 10X, 20X, 40X respectively.
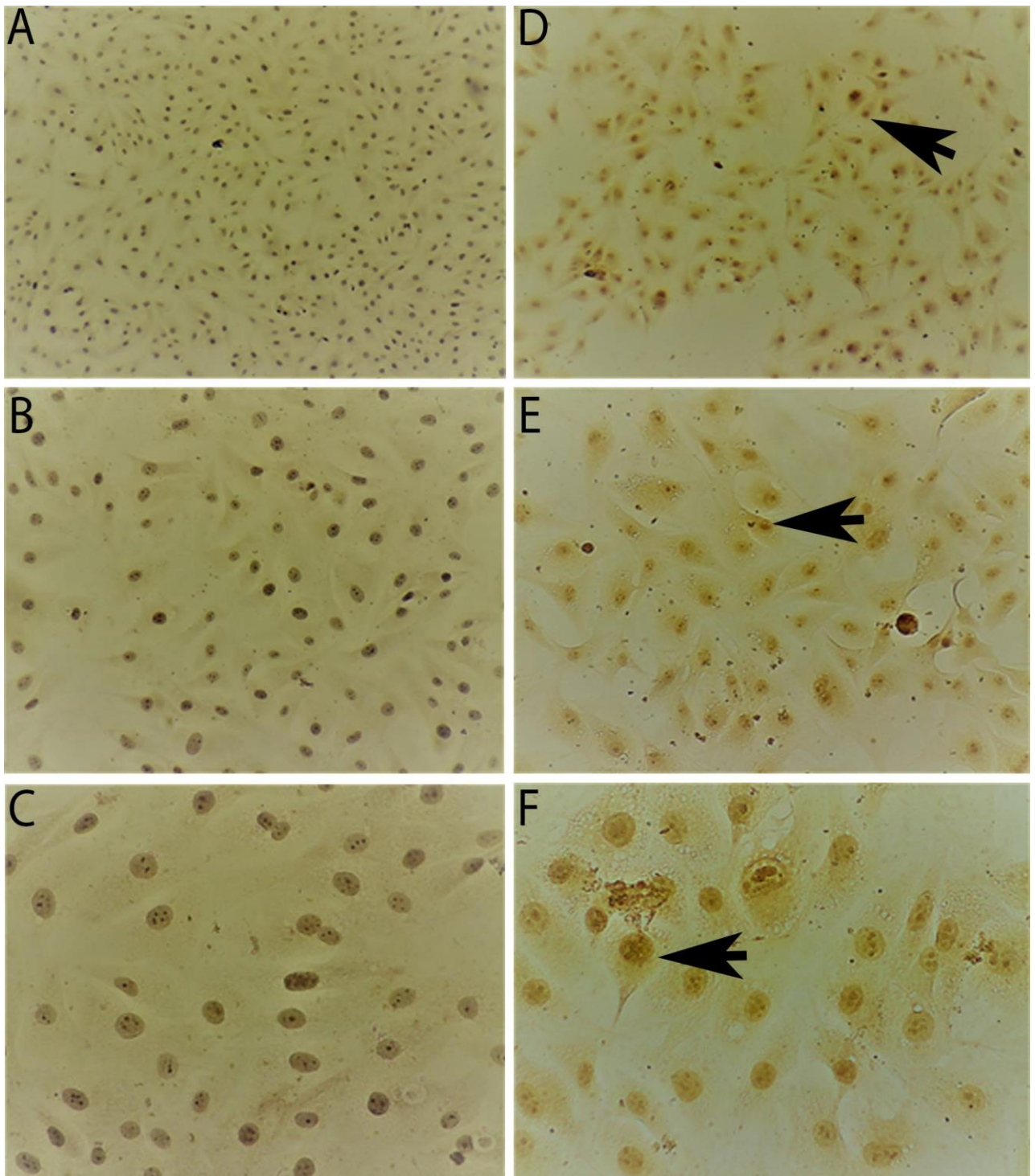
Figure (4-13): Immunocytochemistry analysis in HCAM cell line using antibody marker (P53 gene) with showing: positive nuclear expression of this gene. (Black arrows)
(A, B, C): showed negative control expression 10X, 20X, 40 respectively.
(D, E, F): showed low positive nuclear expression 10X, 20X, 40X respectively.

In addition, we found a positive expression of EGFR protein in nuclear and cytoplasm of HCAM compared with negative control (Figure 4-14). EGFR signaling pathway play a key role in response of the liver to the injury and that takes in extensive crosstalk with other signaling pathways, modulating inflammation and cell proliferation. Komuves *et al.* (2000) were reported that EGF expression increases during cirrhosis and it is overexpressed in cirrhotic liver of human. Therefore, hepatocytes show high expression of EGFR (Nikolova *et al.,* 2018). Also, inflammatory cytokines and their receptors play pivotal roles in the progression and development of hepatocellular carcinoma. Huang *et al.* (2014) were found that EGFR induced the overexpression of seven inflammatory receptors such as CXCL5, XCR1 and CXCL8 in HCC and evidence that EGF could regulate HCC cells to overexpress and produce mRNA and proteins of CXCL8 and CXCL5.
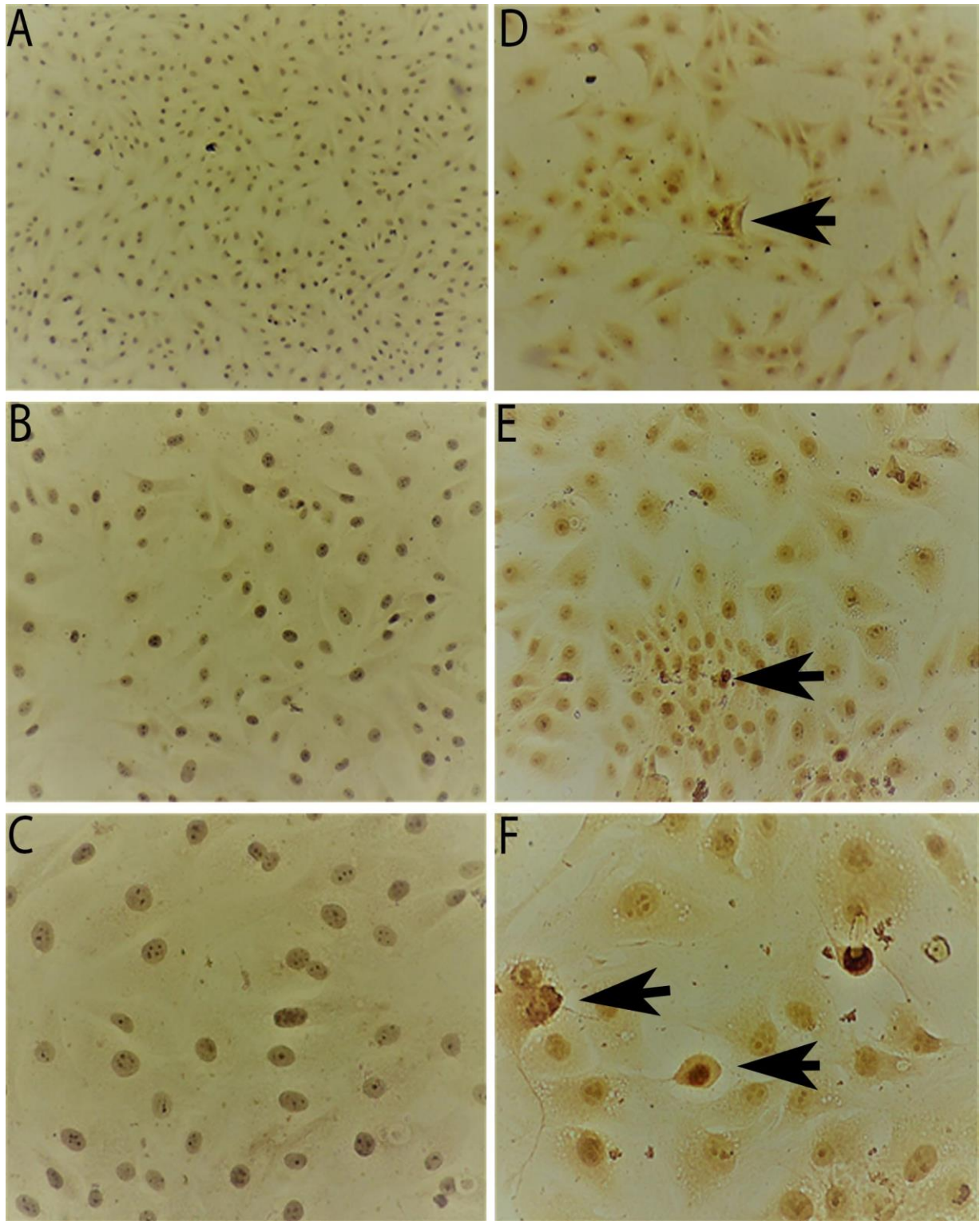
Figure (4-14): Immunocytochemistry analysis in HCAM cell line using antibody marker (EGFR gene) with showing: positive - nuclear and cytoplasm expression of this gene. (Black arrows)

(A, B, C): showed negative control expression 10X, 20X, 40 respectively.

(D, E, F): showed low positive nuclear expression 10X, 20X, 40X respectively.

4.6 Cytotoxicity Assay of (HCAM and MEF) Cell Lines

4.6.1 HCAM and MEF Exposure to Chemotherapy Agents

To examine the anticancer properties of chemotherapy (Cisplatin) and (Docetaxel) on hepatic cancer cell line by time and concentration dependent inhibition, treated HCAM cell line at numerous passages 16, 19 and 21 with decreasing concentration of Cisplatin 50, 25, 12.5, 6.25 and 3.125 µg ml and Docetaxel 100, 50, 25, 12.5 and 6.25 µg/ml through 72h, HCAM cells growth was inhibited by cisplatin and Docetaxel in a dose and time- dependent manner compared with mouse embryonic fibroblast (MEF) cell line treatment.

Methyl thiazolyl tetrazolium (MTT) was used to evaluate the number of viable cells, and the optical density was measured under a wave length 584 nm. The stain was referred to the number of viable cells and the cytotoxic effect was manifested from the inhibition of the growth rate of the treated cells in comparison to the non-treated cells. MTT assay is the best method for determining mitochondrial dehydrogenase activities in the living cells. This method represents an easy, sensitive, safe and quantitative colorimetric assay. It involves the reduction of the tetrazolium salt (MTT) to a purple formazan by NADH. The MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, before measuring the absorbance, an organic solvent like DMSO is required to solubilize the crystals (Bopp and Lettieri, 2008).

Cisplatin is a platinum-based chemotherapy drug that is widely used to treat various types of malignancies. The results revealed that treatment with a high concentration 50 and 25 µg/ml of Cisplatin significantly inhibited cell growth of HCAM cells ($p < 0.05$) for 3 days treatment compared to the low concentration 12.5 and 6.25 µg/ml which showed only a slight decrease in cell proliferation (Table 4-2) (Figure 4-15). Also, the IC50 value of Cisplatin after 72 hours was 10.74 (figure 4-16). These experiments were repeated at least three times.

Through the statistical analysis, which was subjected to the results with the absence of significant differences among the concentrations of the chemotherapy when treated with cancer cell line.

These results are consistent with study of Odii and Coussons (2012) were showed that cytotoxicity degree of Cisplatin (20 µm at 24 hours) in hepatocellular carcinoma cell line is more susceptible than 5-fluorouracil (5-FU) chemotherapy. Cisplatin chemotherapy is an anti-cancer drug used in clinical, can inhibit the replication of DNA and induced the apoptosis of cancer cells.

Whereas, Yang *et al.* (2009) were reported that HCC after 3 month of the establishment , this cell line revealed stable resistance to Cisplatin and exhibited cross- resistance to many other therapeutics. Also, Brito *et al.* (2012) were demonstrated that doxorubicin (5-FU) has no activity on these cell lines HepG2and HuH7 (hepatocellular carcinoma cell lines).

In addition, the gene overexpression responsible for damage cancer cells such as the overexpression of tumor suppressor gene. The increased cell death of HCC due to overexpression of p53 that induced by chemotherapy (Goldstein *et al.,* 2011). Likewise, Cao *et al.* (2018) appeared that p21 gene significantly increased in liver after Cisplatin treatment. Also, it is agree with our results which showed overexpression of p53 and other genes.

Table (4-2): Effects of Cisplatin on the growth inhibition rate, After HCAM cells were treated with descending doses of Cisplatin for 72h, MTT assays were performed. These experiments were repeated at least three times and data are expressed as the mean ± SD.

Concentration 50 µg/ml	Inhibition Rate	
	Mean	±SD
50 µg/ ml	65.4	5.841
25 µg/ ml	60.1	2.491
12.5 µg/ ml	56.4	4.757
6.25 µg/ ml	52.6	7.518
3.125 µg/ ml	47.0	5.838

Figure (4-15): Effect of Cisplatin on the viability of HCAM cells. Cells were treated with Cisplatin at concentrations of (50, 25, 12.5, 6.25 and 3.125) μg/ml and measured after 72 hours. The viability of cells was determined with the MTT assay and data are presented as the percentage of growth inhibition rate (IR). (ns) Non-significant, Data represent mean ± SD.

| IC50 | 10.74 |
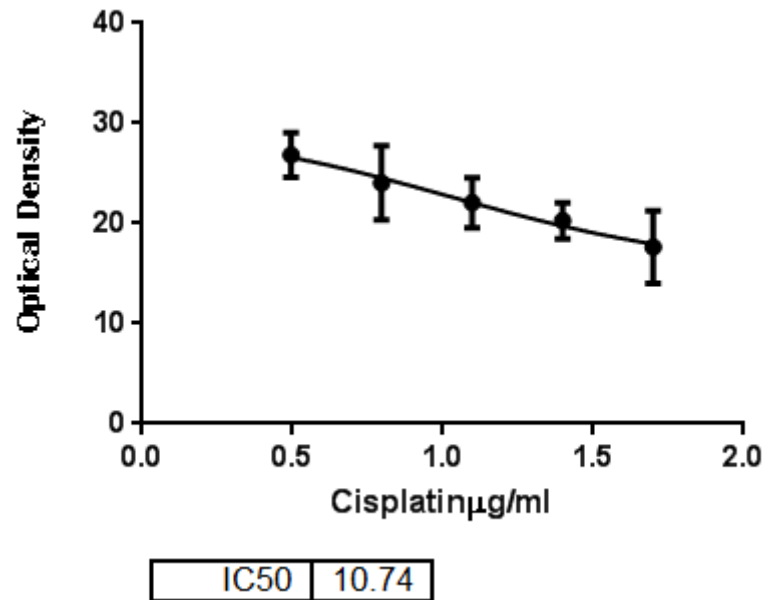
Figure (4-16): shows the IC$_{50}$ of Cisplatin agent for HCAM cell line after exposure of 72 h. the half maximal concentration (ic$_{50}$) is a measure of the effectiveness of a substrate in inhibiting a specific biological or biochemical functions.

Also, the results of this study showed that the toxic effect of Docetaxel was evident in the growth rate of HCAM *in vitro*. These experiments were repeated at least three times. Through the statistical analysis, which was subjected to the results with the presence of significant differences in the concentrations of the chemotherapy when treated with cancer cell line. HCAM cell line was incubated with decreasing concentrations of Docetaxol 100, 50, 25, 12.5 and 6.25 µg/ ml for 72 hours and the cell growth was measured using the MTT assay.

The results showed that the cell growth was significantly inhibited ($p < 0.05$) by high dose of Docetaxol 100, 50 and 25 µg/ml at 3 days compared to the low dose 12.5 and 6.25 µg/ml which showed high decrease in cell proliferation, as shown in Table (4-3), figure (4-17). The IC 50 value of Docetaxel after 72 hours was 12.82 µg/ml , Figure (4-18).

These finding are consistent with research, Geng *et al.* (2003) investigated the activity of Docetaxel of hepatocellular carcinoma cell line and found that chemotherapy inhibited the cell growth at several mechanisms.

Microtubules are playing a critical role in the mitosis. Since, cancer cells divide rapidly compared to the normal cells, therefore this made microtubule a noticeable target in anticancer researches. Li *et al.* (2004) were noted that the effect of chemotherapy on targeting microtubules was confirmed which found the expression of microtubule-associated proteins was increased and tubulin expression decreased in Taxotere-treated cancer cells. Docetaxel induces mitotic arrest and apoptosis in a concentration-dependent manner and at low concentration can induce apoptosis without mitotic arrest (Hernández-Vargas *et al.,* 2007). Chen *et al.* (2018) demonstrated that cabazitaxel (taxane chemotherapy) is highly toxic to hepatocellular carcinoma cell lines in a time- and concentration-dependent manner by inducing apoptosis and metaphase arrest *in vitro*. All these researches were agreed with the present results, which illustrated the effect of docetaxel for HCAM cells at time and concentrations dependent manner.

Also, Docetaxel targeting proteins and induce growth inhibition, apoptosis and cell cycle arrest, which in turn has targeted the proteins indicated by present study (EGFR, P53 and HER2/neu). Burris *et al.* (2005) viewed that chemical agent inhibits EGFR and HER2/neu via docking into the ATP binding site of the two receptors, showed no or little efficiency in advanced HCC.

Table (4-3): Effects of Docetaxel on the growth inhibition rate, HCAM cells were treated with descending doses of Docetaxel for 72h, MTT assays were performed. These experiments were repeated at least three times and data are expressed as the mean ± SD.

Concentration 100 µg/ml	Inhibition Rate	
	M	±SD
100 µg/ ml	59.6	3.089
50 µg/ ml	57.2	2.193
25 µg/ ml	51.4	2.234
12.5 µg/ ml	42.9	1.677
6.25 µg/ ml	37.5	1.701
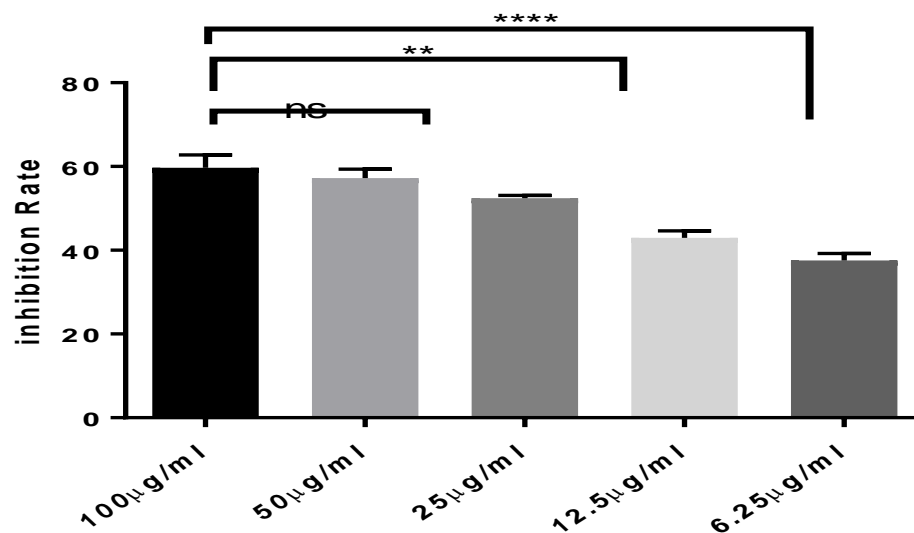
Figure (4-17): Effect of Docetaxel on the viability of HCAM cells. Cells were treated with Docetaxel at concentrations of (100, 50, 25, 12.5 and 6.25) µg/ml and measured after 72 hours. The viability of cells was determined with the MTT assay and data are presented as the percentage of growth inhibition rate (IR). ** Significant differences at P<0.0001, ** significant differences at P<0.001, (ns) non- significant data represent mean ± SD.**
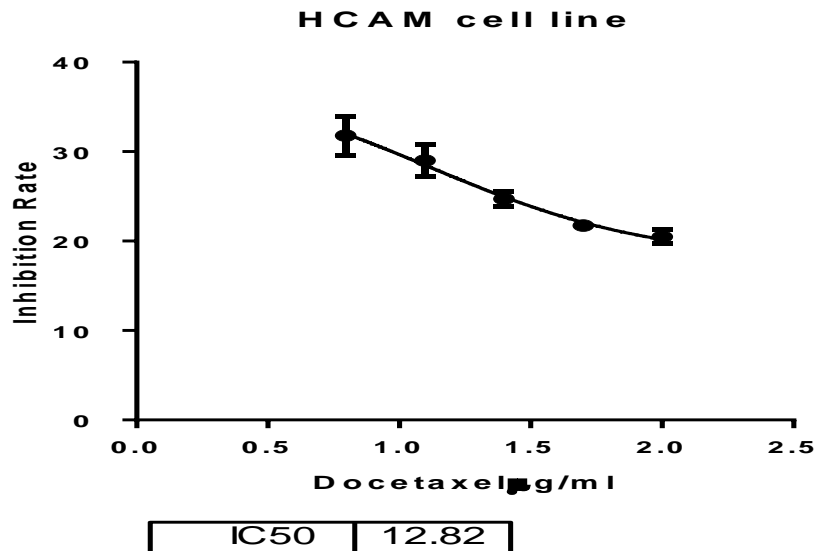
Figure (4-18): shows the IC$_{50}$ of Docetaxel agent for HCAM cell line after exposure of 72 h. the half maximal concentration (ic$_{50}$) is a measure of the effectiveness of a substrate in inhibiting a specific biological or biochemical functions.

Murine embryonic fibroblasts (MEF) have long been considered a relatively homogeneous cell type, and many studies have been conducted on fibroblasts of varying provenance. The cytotoxic effects of Cisplatin and Docetaxel agents on MEF cell line were done by the same period of time and the same concentrations dependent inhibition of HCAM cell line. MEF were shown to be more resistant to Cisplatin and Docetaxel agents, compared with the sensitivity of HCAM cell line (Table 4-4, 4-5), (Figure 4-19, 4-20). Also, the IC50 value of Cisplatin and Docetxel after 72 hours was 14.67 and 16.52 μg/ml respectively (Figure 4-21, 4-22), compared with HCAM cells. There were statistically significant differences in chemotherapy concentrations when treated with MEF cell line.

The current results are consistent with the effect of Cispatin for MEF cell line, which shown to be less sensitive to chemotherapy (Germain *et al.,* 2010). Tumor suppressor genes are critical chemotherapeutic targets for the successful treatment such as p53 family, BRCA1 and Rb (retinoblastoma) genes which are involved in chemotherapeutic drug response (Lai *et al.,* 2012). Loss of p53 in

MEFs leads to resistance to doxorubicin (Stanchina *et al.,* 2015). In addition, studies on various cancer cell lines demonstrate that cells with mutant p53 are more resistant to chemotherapy or drugs (O'Connor *et al.,* 1997). Whereas, Fedier *et al.* (2003) were demonstrated the loss of BRCA1 in MEF leads to increased sensitivity to a number of DNA-damaging agents, including the doxorubicin.

 The main different mechanisms of drug resistant are apoptosis suppression, enhancing DNA repair and altering in the drug metabolism (Mansoori *et al.,* 2017). Depletion of Exonuclease 1 enzyme (Exo1) in mouse embryonic fibroblasts led to a delay in DNA damage-induced apoptosis because Exo1 act as upstream of caspase-3, DNA fragmentation and cytochrome C release (Bolderson *et al.,* 2009).

Table (4-4): Effects of Cisplatin on the growth inhibition rate, After MEF cells were treated with descending doses of Cisplatin for 72h, MTT assays were performed. These experiments were repeated at least three times and data are expressed as the mean ± SD.

Concentration 50 µg/ml	Inhibition Rate	
	Mean	±SD
50 µg/ ml	35.70	0.70
25 µg/ ml	29.70	0.88
12.5 µg/ ml	22.70	1.57
6.25 µg/ ml	18.73	2.34
3.125 µg/ ml	12.66	2.43

Table (4-5): **The Effects of Docetaxel for the growth inhibition rate, After MEF cells were treated with descending doses of Docetaxel for 72h, MTT assays were performed. These experiments were repeated at least three times and data are expressed as the mean ± SD.**

Concentration 100 µg/ml	Inhibition Rate	
	Mean	±SD
100 µg/ ml	39.96	2.73
50 µg/ ml	29.46	2.17
25 µg/ ml	23.96	2.56
12.5 µg/ ml	8.4	3.83
6.25 µg/ ml	6.33	0.86
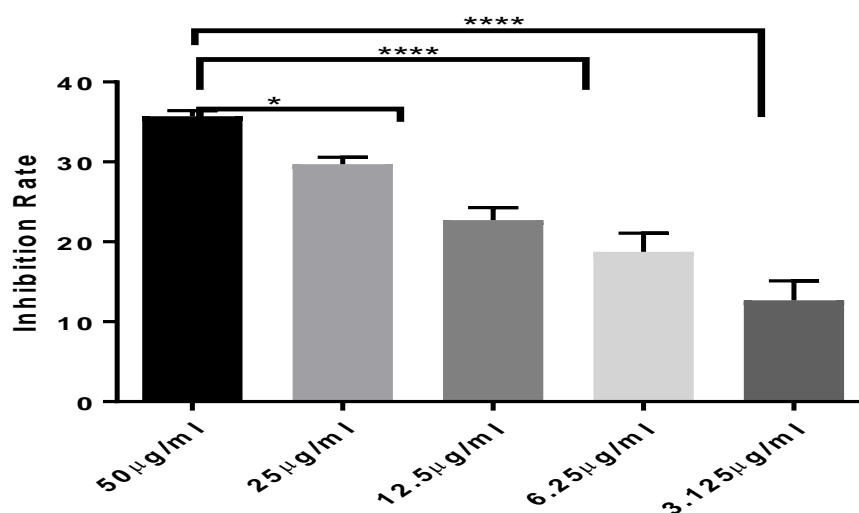
Figure (4-19): **Effect of Cisplatin on the viability of MEF cells. Cells were treated with Cisplatin at concentrations of (50, 25, 12.5, 6.25 and 3.125) µg/ml and measured after 72 hours or they were left untreated. The viability of cells was determined with the MTT assay and data are presented as the percentage of growth inhibition rate (IR). **** Significant differences at P<0.001, * significant differences at P<0.001, (ns) non- significant data represent mean ± SD.**
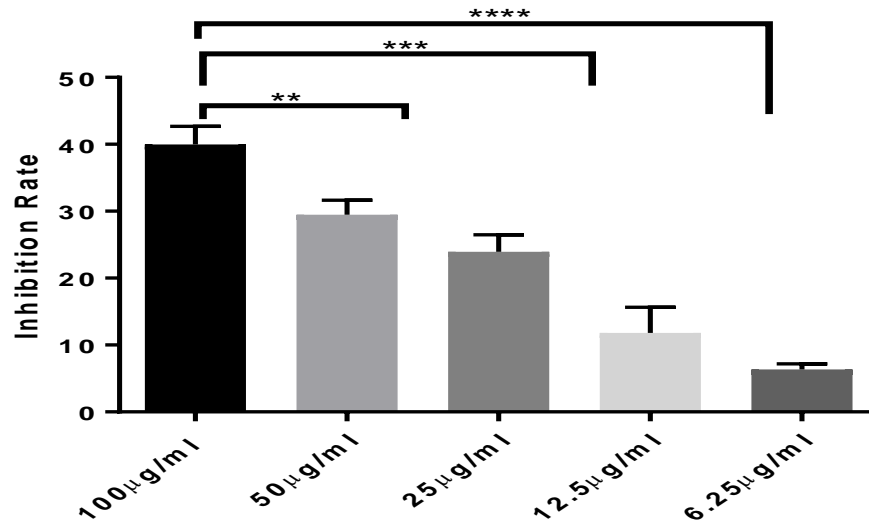
Figure (4-20): Effect of Docetaxel on the viability of MEF cells. Cells were treated with Docetaxel at concentrations of (100, 50, 25, 12.5 and 6.25) μg/ml and measured after 72 hours or they were left untreated. The viability of cells was determined with the MTT assay and data are presented as the percentage of growth inhibition rate (IR). ** Significant differences at $P<0.0001$, ** significant differences at $P<0.001$, (ns) non- significant data represent mean ± SD.**

| IC50 | 14.67 |
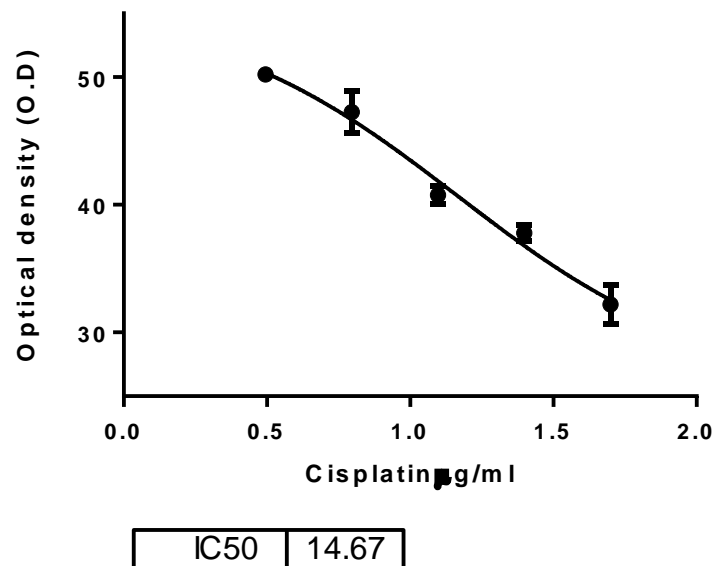
Figure (4-21): shows the IC_{50} of Cisplatin agent for MEF cell line after exposure of 72 h. the half maximal concentration (ic_{50}) is a measure of the effectiveness of a substrate in inhibiting a specific biological or biochemical functions.

IC50	16.52
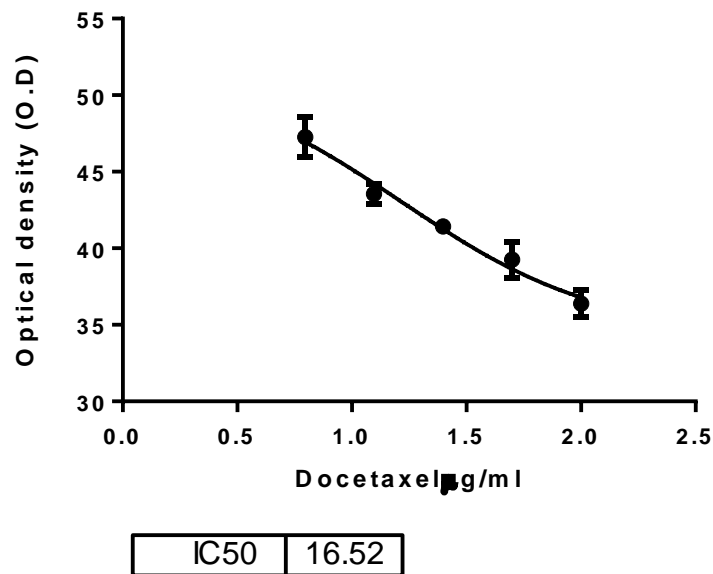
Figure (4-22): shows the IC$_{50}$ of Docetaxel agent for MEF cell line after exposure of 72 h. the half maximal concentration (ic50) is a measure of the effectiveness of a substrate in inhibiting a specific biological or biochemical functions

4.6.2 Morphological Changes of HCAM Cells after Exposure to Chemotherapy

The morphological changes in HCAM cell line, after treated with chemotherapeutic agent (Cisplatin , Docetaxel) appeared cytopathic effect which were observed in exposed cells with IC$_{50}$ concentration (Cisplatin 10.74 and Docetaxel 12.82) at 72 hours. The treated and non- treated cells (HCAM) were stained with H&E dyes to observe the morphological changes. The microscopic analyses of untreated cells showed a large number of confined cells, which result in opaque foci of cells that leads eventually to the separation and floating of the infected cells in the culture media, also tissue culture showed a large empty plaque spaces between cells compared with control cells, (Figure 4-23).

After the exposure of chemotherapy, the HCAM cells stained with H&E dyes, the microscopic analyses observed changes of treated HCAM cells such as degeneration of cells, cell membrane decomposition, enlarged and shrink of nucleus and cytoplasm vacuolation, decreasing of cells proliferation, loss of

adhesiveness, rounded and aggregated of HCAM cell compared with untreated HCAM cell which it is nucleus remained in a uniform rounded compact form and size, unbroken plasma membrane and natural cells proliferation and adhesion in tissue culture flask, (Figure 4-24, 4-25). In addition cells treated with Cisplatin and Docetaxel showed many cytopathic changes included vacuole degeneration, multinucleated cells, mixture of necrotic and apoptotic cells and change in shape of some cells to rod shape. Present results consistent with this study of Zhang *et al.,* (2015) which observed the morphology changes of hepatic cells after exposure to Cisplatin for 72 h, many of the cells exhibited nuclear and cytoplasmic shrinkage and detached from each other or floated in the medium. Also, Geng *et al.* (2003) were indicated that after hepatocellular carcinoma cell line (SMMC-7721) exposed to Docetaxel agent the cell membrane lost integrity and exhibited nuclei condensation.
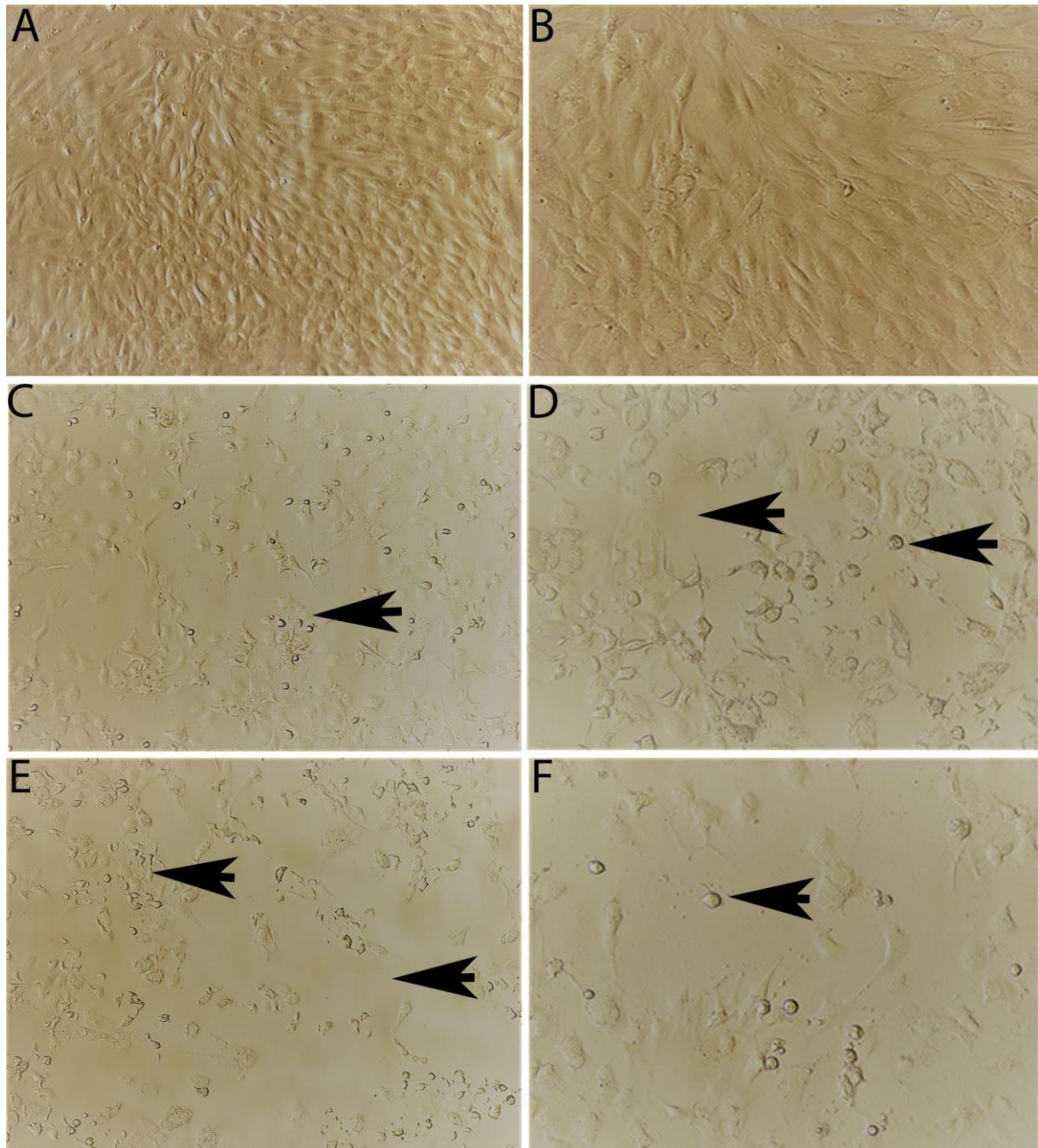
Figure (4-23): HCAM cells were treated with IC_{50} (10.74μg/ml) of Cisplatin and (12.82μg/ml) of Docetxel for 72 hours, untreated cells showed a large number of confined cells (A 10X, B 20X). While treated cells showed separation and floating in culture media and showed large space between cells. (C, D): treated cells with Cisplatin, 10X, 20X respectively. (E, F): treated cells with Docetaxel, 10X, 20X respectively. (Black arrows)
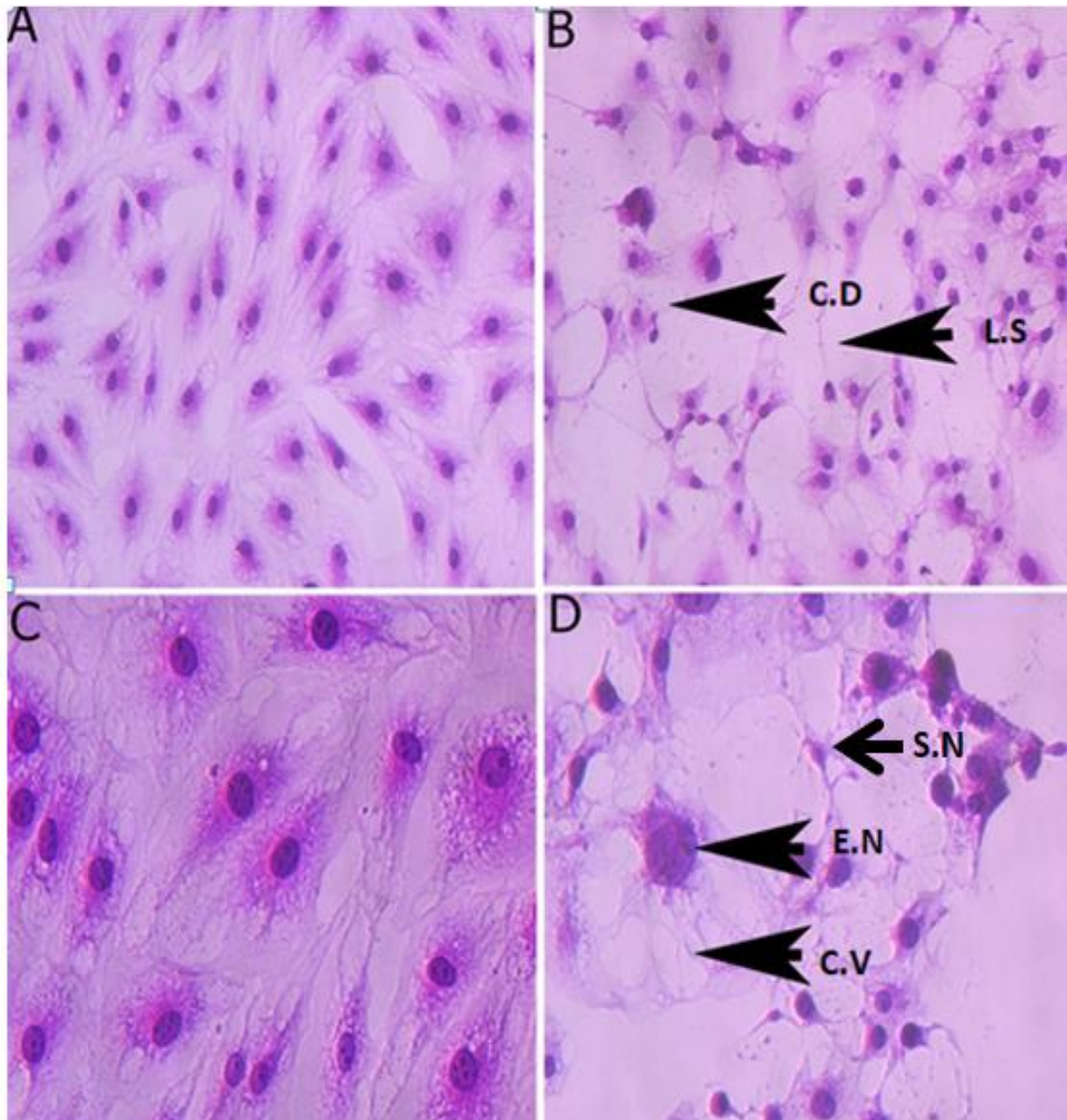
Figure (4-24): HCAM cells were treated with IC_{50} (10.74 µg/ml) of Cisplatin for 72 hours and stained with H&E dyes, under inverted microscope, showed large space [L.S] between cells, decreasing of cell proliferation and morphological changes such as cytoplasm vacuolation [C.V], enlarged of nucleus [E.N] shrink of nucleus [S.N] and cell membrane decomposition [C.D]. (A) Untreated cells, 200X, (B, C, D) treated cells, 20X, 40X, 40X respectively.
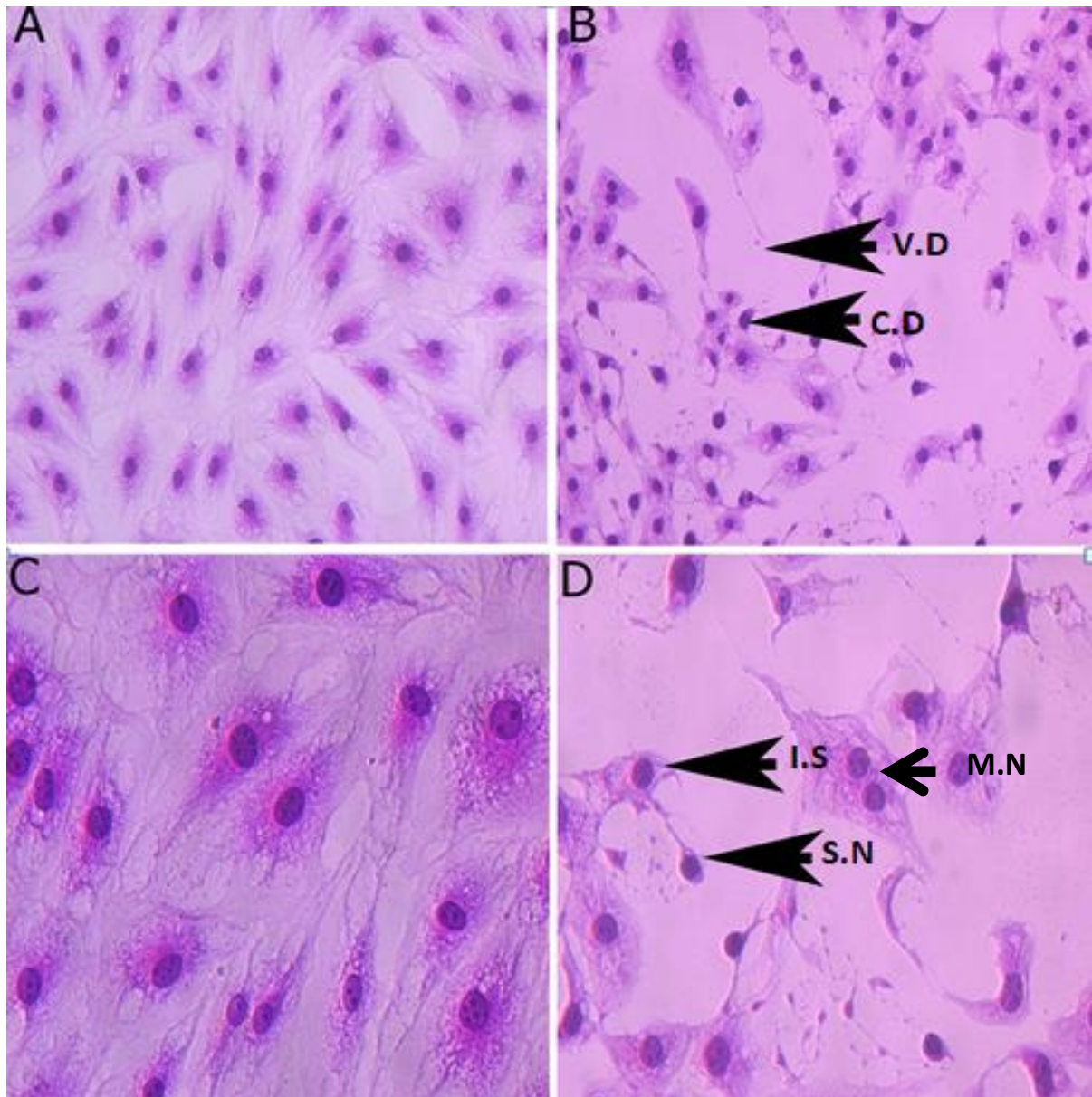
Figure (4-25): HCAM cells were treated with IC50 (12.82 µg/ml) of Docetaxel for 72 hours and stained with H&E dyes, under inverted microscope, cells showed viability decreased [V.D], non-adhesive and morphological changes including (cells decomposition [C.D], cell membrane decay, irregular shape of cells [I.S] Multinucleated cells [M.N] and shrink of nucleus [S.N]. (A) untreated cells 20X, (B, C, D) treated cells 20X, 40X, 40X Respectively.

4.7 Molecular Study

4.7. 1 Conventional PCR

In the present study DNA was extracted from mouse hepatic carcinoma cell line (HCAM). Specific primers were used, the amplification in PCR products was accomplished. Amplified DNA products from HCAM cell with passage (7) were visualized on agarose gel electrophoresis for part of the P53 gene (the size of product 400 bp) Figures (4-26) and β- actin gene (the size of product 900 bp) figure (4-27). The results showed that theses DNA products were gave single sharp bands when running by gel electrophoresis, which mean that these DNA products were ready to use in the next experiment (Gene sequencing).

The optimization process of globin gene was running by gel electrophoresis method to determine the DNA concentration and annealing temperature for primer, and tested the isolated DNA before studying the sequencing method. After optimization process the results showed that the DNA isolated from HCAM cells was negative with globin gene, as shown in figure (4-28).
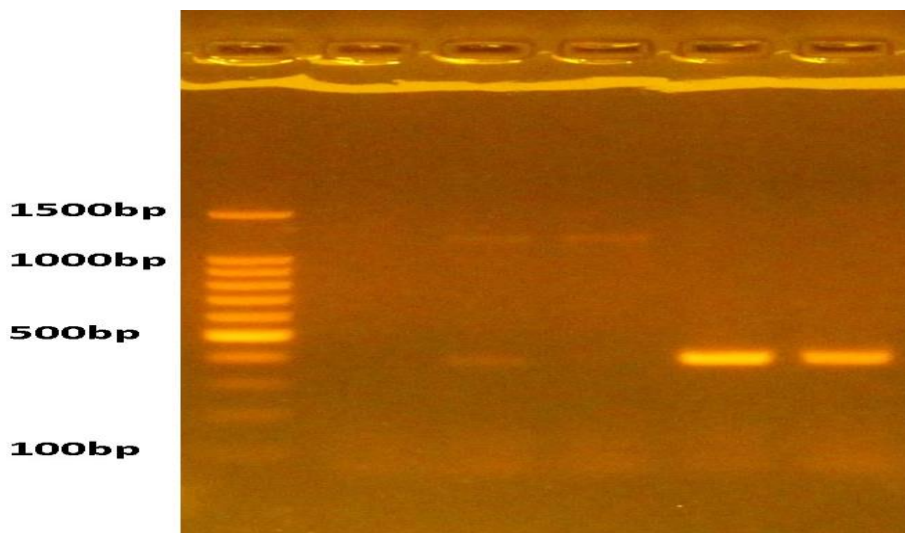
Figure (4-26): Gel electrophoresis for P53 PCR product visualized under UV light. M: 1500 bp marker: negative control (HCAM DNA) Lane 4-5: HCAM Cell line, the product size is 400 bp.
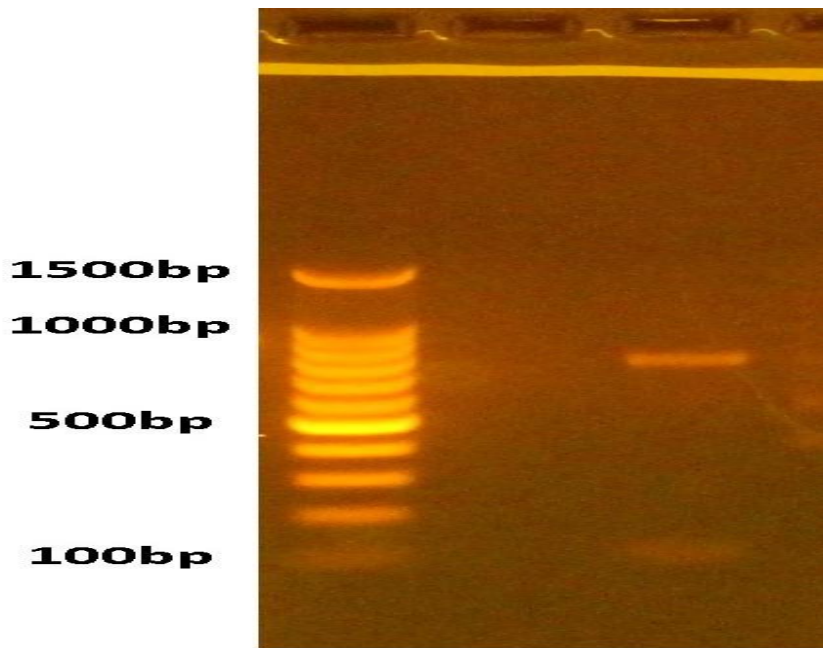
Figure (4-27): Gel electrophoresis for *β*-actin PCR product visualized under UV light. M: 1500 bp marker, Lane 1: negative control (HCAM, DNA) Lane2: HCAM Cell line, the product size is 900 bp.

The PCR method appears to be a useful technique to detect fragment of DNA, and may be a useful marker for identifying patients with HCC (Ikeguchi *et al.,* 2012). The P53 gene is a transcription factor related to repair of DNA damage, apoptosis and growth arrest leading to uncontrolled of cells proliferation, associated with more than 50% of human cancers (Lee and Park, 2015). The present results agreed with the study of Noordin *et al.* (2008) that detected p53 gene in HCC cell line by using gel electrophoresis and showed different types of p53 gene mutations in HCC.

The *β*-actin plays different roles in cell functioning including cell, maintenance of cell shape, contractile ring formation during cytokinesis, signal transduction, cell adhesion and muscle contraction. Simiczyjew *et al.* (2014) concluded that increased level of *β*- actin leads to actin cytoskeletal remodeling followed by an increase in migration and invasion capacities of human cells. Therefore, Liu *et al.* (2017) were recommended that *β*-actin gene act as a best references gene in HCC cancer cell line.

The *β*-globin is selectively deregulated in cancer cells, mediating the effect of cytoprotective during blood-borne metastasis (Zheng *et al.,* 2017). It is consistent with our results that showed no expression of *β*-globin in HCAM cell line. Whereas, mouse hepatocellular carcinoma cell line (BNL 1ME A.7R.1) showed specific expression of a specific segment DNA of the mouse *β*-globin gene by gel electrophoresis (Ogunwobi *et al.,* 2013).

4.7.2 Sequencing Analysis

The DNA sequencing involved part of P53 gene, (Figure 4-29). Sequenced sample represents HCAM cell line. According to National Center of Biotechnology Information (NCBI) this stretch of the gene (p53) shows their allelic variants, and their alleles in the study subjects. From these single nucleotides polymorphism (SNPs) there was similarity with two partial P53 gene sequenced of *Rattus norvegicus*, partial cds AH010014.2 (94% identities), and *Rattus norvegicus* p53 tumor suppressor gene, partial cds; AF190270.1 for 99% identities, except of some nucleotides were inserted and alternated in the study gene, as shown in Figure (4-29), (4-30) respectively. The p53 gene is the most commonly mutated cancer gene and has therefore been the topic of extensive research for nearly 30 years (Boyd and Vlatkovic, 2008). Atypical expression and structure of p53 gene is frequent occurrence associated with hepatocellular carcinoma. These changes of p53 are accompanied of the presence or absence of p53 transcripts. For example, Bressac *et al.* (1990) were revealed that at the genomic level, the p53 gene in two HCC cell lines was discovered to be abnormal and the major portion of p53 is deleted due to the absence of p53transcripts.

BASE COUNT   55 A   42 C   41 G   43 T
Origin

1 AAAAGACAAT AACTAACTCC ACCAGATGTC CATCGTCCGC CAGACGATGT
GGTTATGGTT
61 ACTCAGCCCT GGCAAATGCA AGGGTCCTAT GAAGTCTTAC TACTCAACCA
TTTCAGGCTC
121 TGGAAAATGG GATGAGGGAC AAGGTATGGT ATCATGCTCC TATAATCTCA
CCAGTAGGGA
181   A

Figure (4-28): The nucleotide sequencing of partial p53 gene obtained from HCAM cell line with a number (1-181 bp).

Figure (4-29): The identification percentage of p53 gene sequencing according to (NCBI), which was (94%) between Rattus norvegicus p53 tumor suppressor (p53) gene ID: AH010014.2 and partial p53 gene of HCAM *mus musculus* (house mouse) ID:2235321, Some nucleotides were inserted in p53 gene of HCAM (617-C), (621-A), (627-T), (636- T), (644-C), (660-G), (694-A), and alternated (740 G----C)

Figure (4-30): **The identification percentage of p53 gene sequencing according to (NCBI), which was 99% between Rattus norvegicus p53 tumor suppressor (p53) gene ID: AF190270.1 and p53 gene of HCAM *mus musculus* ID: 2235321, Except of one nucleotide was alternated in Rattus norvegicus p53 (305 ,G----C).**

CONCLUSIONS

1- The study was successful to characterized Hepatic Cancer Ahmed Majeed (HCAM) cell line, the cultured cells continue to grow as cluster of cells at 24 -48 h and become overgrowth at confluent 90% monolayer at 72 h. for HCAM propagation the cells detached from the flask and redistributed to another tissue culture flask by trypsinization.

2- Population doubling time of Hepatic Cancer Ahmed Majeed (HCAM cell line was 17 hours. HCAM cells receding lag phase at 24-48 h, log phase at 72-144h and decline phase at 168-240h

3- HCAM cell line exhibited many numerical and structural changes of chromosomes, such as telocentric, increasing of chromosomal length and breakage in the chromosomal arm.

4- HCAM cells were positive expressed for EGFR, P53 and weak positive expressed for Her-neu2 proteins with immunocytochemistry assay.

5- HCAM cell were shown to be more sensitive to chemotherapy (Cisplatin, Docetaxel).While, MEF cells were resistant to the same chemotherapy agents.

6- The morphological characteristics of HCAM were shown that cells appeared in a polygonal shape, spherical to oval body containing multi-nuclei (central nucleus), cytoplasmic vaculation and some of extension and dendritic from cells surfaces.

7- The treatment of chemotherapy could effect for the morphology of hepatic cancer cells HCAM. The morphological changes include: shrink and enlarged of nucleus, cells decomposition, viability decreased and multinucleated in cells.

8- PCR technique and gel electrophoresis were carried out to detect many genes, and the result showed positive expression of P53 and β- actin and negative expression of β- globin in HCAM cell line.

9- Sequence analyzing was carried out for the identification of HCAM cancer cell line that established from mice infected with tumor by using partial p53 gene and submitted in NCBI.

RECOMMENDATIONS

1- Establishment of human hepatocellular carcinoma cell line or other digestive tumor cell lines from Iraqi patients.

2- Characterization study for new digestive cancer cell lines such as esophagus, stomach and colon cell lines by the detection of ICC, cytogenetic, cytotoxicity and molecular studies.

3- Using other techniques to observe the chromosomal alterations such as Spectral Karyotyping (SKY), Fluorescence *in situ* hybridization (FISH) and Comparative Genomic hybridization (GCH).

4- Measure the expression of some genes in cancer cell after treatment with chemotherapy agents by RT-PCR.

5- Conducting further cytotoxic *in-vitro* studies on the other digestive tumor cell lines.

6- Studying the angiogenesis process *in-vivo* by initiating tumors by implantation of (HCAM) cells in the laboratory animals, then examine the effects of angiogenesis inhibiter by tested many anti-angiogenesis therapies according to tumor size and distance metastasis in laboratory animals.

7- Studying of hepatic cancer cells before and after exposure to chemotherapy by using transmission electron microscope.

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الخلاصة

تعد دراسة خصائص الخط الخلوي لسرطان الكبد في الفئران Hepatic Cancer Ahmed Majeed (HCAM) وسيلة مهمة في أبحاث سرطان الكبد. لقد أنشئ هذا الخط الخلوي من ورم اصاب كبد الفئران البيض السويسرية *Mus Musculus*. لقد هدفت التجارب المختلفة للدراسة الحالية الى وصف خط الخلايا السرطانية الكبدية HCAM ، والتي تشمل الاتي:

تنمية الخط الخلوي لسرطان الكبد في احدى الاوساط الزرعية RPMI-1640 وتمت عملية الزرع الثانوي واجراء التمريرات اللازمة كل 48- 72 ساعة. تمت دراسة منحنى النمو ولتمريرات مختلفة (5 - 9 - 12) ولاعداد الخلايا (10000خلية / مل) اذ كان طور السكون lag phase في 24 ساعة بينما الطور اللوغارتمي Log phase بين 72- 144 ساعة ووصلت اعداد الخلايا الى طور الانحدار تدريجياً من 168 - 240 ساعة فضلاً عن حساب زمن تضاعف الخلايا Population Doubling Time (PDT) اذ كان 17 ساعة.

اظهرت الدراسة الوراثية الخلوية للخلايا السرطانية وللتمريرات الاتية (7- 8- 12) تشوهات كروموسومية وتغيرات عددية فيما بينها، ومن هذه التغيرات التركيبية هي :الطول الغير طبيعي ، تكسر اذرع الكروموسومات وغيرها.

اظهرت الدراسة المظهرية للخلايا السرطانية وباستعمال المجهر مقلوب العدسة Inverted microscope وباستعمال ملون الهيماتوكسلين – ايوسين و الصبغة البنفسجية البلورية crystal violet وللتمريرات (6 , 9 , 14) ان الخلايا تكون بشكل متعدد الاضلاع مشابهة للخلايا الظهارية في النمو داخل قارورة الزرع النسجي، كذلك اظهر الفحص المجهري ان الخلايا تمتلك نواة مركزية ونويات متعددة فضلا عن نسبة السايتوبلازم الى النواة.

من ناحية اخرى اظهرت دراسة الكيمياء الخلوية المناعية للخلايا السرطانية Immunocytochemistry (ICC) وللتمريرات الاتية (13 , 15 , 17) في شرائح الزرع النسجي الكشف عن بعض الانزيمات المناعية. منها : الانزيم P53 والذي اعطى نتيجة ايجابية واضحة ،الانزيم Epidermal growth factor receptor (EGFR) اعطى نتيجة ايجابية ، اما الانزيم (Human epidermal growth factor receptor (HER2/neu اعطى نتيجة ايجابية ضعيفة.

تم تعريض الخلايا السرطانية للكبد وللتمريرات الاتية (16 , 19 , 21) الى نوعين من العلاجات الكيميائية (السيسبلاتين 50 ميكروغرام/ مل ، الدوسيتاكسل 100 مايكروغرام / مل) ولمدة 72 ساعة وقد اظهرت نتائج الدراسة ان السمية الخلوية لخلايا سرطان الكبد كانت اكثر حساسية للمادتين الكيميائية بعد ذلك تم تعريض الخلايا الجنينية الليفية للفئران للمادتين الكيميائية ولنفس التركيز والمدة وقد بينت نتائج

الخلاصة

الدراسة كون الخط الخلوي لل (MEF) Mouse embryonic fibroblast كان اكثر مقاومة للمادتين الكيميائية.

اظهرت الدراسة بعض التغيرات المظهرية عن طريق تصبيغها بملون الهيماتوكسلين ايوسين فقد لوحظت بعض التغيرات المظهرية للخلايا السرطانية الكبدية بعد تعرضها للمادة الكيميائية وبتركيز IC_{50} (السيسبلاتين 10.74 µg/ ml والدوسيتاكسل 12.82 µg/ ml) ولمدة 72 ساعة. ومن هذه التغيرات هو تحلل الغشاء الخلوي للخلايا ،انكماش النواة وضمور بعض الخلايا والشكل غير المنتظم لبعض الخلايا وتفجي السايتوبلازم فضلا عن وجود فسح كبيرة بين الخلايا.

وبينت الدراسة الوراثية الجزيئية للخلايا السرطانية المزروعة باستعمال تفاعل البلمرة المتسلسل التقليدي نتيجة ايجابية للمورث p53 و β-actin وسلبية للمورث الكلوبين β-globin.

كذلك بينت نتائج التسلسل الجيني للمورث p53 في خلايا سرطان الكبد للفئران البيض ومن خلال المركز الوطني لمعلومات التقانة الاحيائية NCBI ان هنالك تطابق بنسبة ٩٤-٩٩ % بين اثنين من التسلسل الجيني للمورث p53.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بغداد

كلية التربية للعلوم الصرفة ان الهيثم

قسم علوم الحياة

توصيف الخط الخلوي للخلايا السرطانية الكبدية (HCAM) المعزولة محلياً من أورام الجهاز الهضمي للفئران

رسالة مقدمة الى

مجلس كلية التربية للعلوم الصرفة (ابن الهيثم)/ جامعة بغداد

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة علوم في

علوم الحياة / علم الحيوان / زراعة نسجية

من قبل

أيمن راضي حبيب

(بكالوريوس علوم حياة/ جامعة بغداد 2005)

(ماجستير علوم حياة/ جامعة بغداد 2015)

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تشرين الثاني	ربيع الاول

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