2. Review of Literature

Liver cancer is predicted to affect more than one million people worldwide by 2025, making it a persistent global health concern (50). For liver cancer, hepatocellular carcinoma (HCC) is the most prevalent types which are derived from the hepatocyte. The stage at which HCC is diagnosed determines how long people with HCC can expect to live. Some months are expected in an advanced stage; however, a five-year survival rate can be achieved with early diagnosis and adequate treatment. Early diagnosis allows for more focused and successful treatment; later on, when conventional chemotherapy is no longer effective, a poor outcome is anticipated (51).

Curative therapies like liver transplants, surgical resection, and local ablation can increase a patient's chances of survival when their HCC is still in its early stages. Consequently, early detection and adequate therapy are crucial to improving the quality of life and increasing survival rates for individuals with HCC.

The etiology of HCC is multifactorial and extremely complex. The risk factors for HCC include diabetes, obesity, alcoholic fatty liver disease (AFLD), and nonalcoholic fatty liver disease (NAFLD). Additional risk factors that are also known to increase the occurrence of HCC include smoking, certain environmental chemicals that act as carcinogens, family or genetic factors, food pollutants such as aflatoxins, and other factors that are illustrated in Figure 2.1 (52). The primary cause of liver cancer, other than fatty infiltration, obesity, diabetes, and alcohol, is viral liver cirrhosis (53). HCC is brought on by hepatic injury, which includes hepatocyte necrosis, inflammation, and regeneration. This chronic liver disease progresses through the phases of fibrosis, cirrhosis, and finally hepatocellular carcinoma (54).

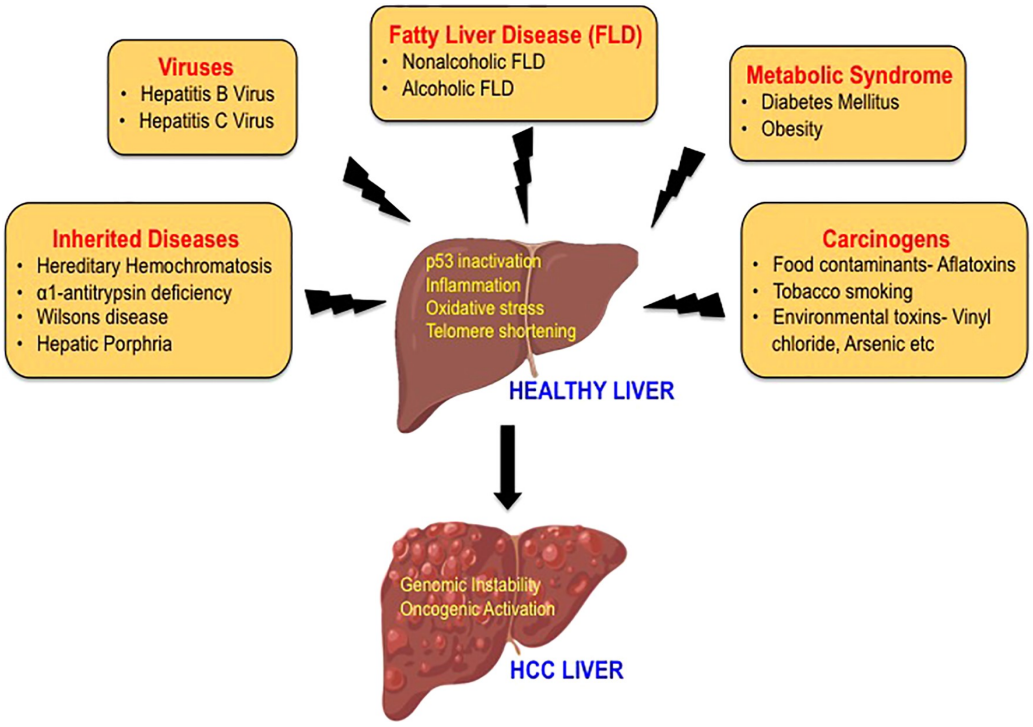

Figure 2.1: Causative agents of HCC

(Suresh et.al., 2020)(52)

2.1 Causative agent of HCC and their molecular mechanisms

Hepatocarcinogenesis is a highly intricate process in the body. When normal hepatocytes turn malignant and become malignancies, a multistep biological process takes place. Different agents, disease status, and viruses are responsible for HCC, which are listed and described as below:

2.1.1 HBV

The most typical reason for HCC is the four HBV genes (C, S, X, and P). DNA polymerase is encoded by the P gene, sizes (large, medium, and small) by the S gene, hepatocarcinogenesis-related HBx protein (X) by the X gene, and the core protein (C) by the C gene. The hepatitis B X protein (HBx) is one of them and is recognized to play a crucial part in the development of HCC (55).

HBx plays important roles in hepatocarcinogenesis by different mechanisms, such as

(1) HBx interrupts the apoptosis in hepatocytes (56).

(2) Alterations to the p53 tumor suppressor's DNA binding specificity, which lead to changes in the expression of its target genes (57) and

(3) Regulation of cellular signaling pathways, such as activation of the Ras-Raf-MAPK pathway, Src-dependent pathway, PI3K-Akt pathway, inflammation-associated NF-kB/STAT-3 pathways, and wnt/β-catenin pathway (58-62).

2.1.2 HCV

The main risk factor for HCC is persistent HCV infection, which first results in liver fibrosis and cirrhosis and finally develops HCC. The HCV core protein can induce oncogenic transformational changes in hepatocytes by up- regulating telomerase activity and stimulating STAT3 via an IL-6 autocrine pathway (63).

In addition, a protracted HCV infection induces neoplastic transformation by multiple pathways, such as DNA mutagenesis brought on by oxidative stress, severe and long-lasting inflammation via NF-kB, and mutations in tumor suppressor genes (64).

2.1.3 Alcohol

Alcohol abuse leads to cirrhosis, fatty liver, alcoholic steatohepatitis (ASH), and eventually, hepatocellular carcinoma (HCC). Reports state that ASH progresses to HCC at a rate of 3% to 10% annually (65).

Chronic alcohol consumption can induce cytochrome p450 2E1 (CYP2E1), a member of the cytochrome p450 mixed-function oxidase system, which can lead to a number of biologic effects, including increased hepatotoxicity, enhanced oxidative stress, increased alcohol metabolism, and interactions with different medications, xenobiotics, and carcinogens. In instance, the metabolism of alcohol produces acetaldehyde, which potently stimulates oxidative stress and aggravates liver disorders. After a liver insult, chronic alcohol use triggers Wnt/β-catenin signaling, which increases hepatocyte proliferation and promotes carcinogenesis (56).

2.1.4 Non-alcoholic steatohepatitis (NASH)

NASH comprises a spectrum of liver disorders, from simple fatty liver to hepatic fibrosis/cirrhosis and HCC. NASH patients advance to HCC at a pace of 0.5% per year (66).

Additional factors accelerating the transition from simple fatty liver (SFL) towards NASH and HCC include the gut microbiota, adipose-related inflammation, and excessive intake of lipids. NASH is associated with end-stage liver disease (ESLD). Generally, SFL is reversible through weight control by exercise and calorie restriction. However, once SFL has progressed to NASH, medical attention is required because of its progression to ESLD or HCC (67).

Oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, autophagy, and intrahepatic NKT and CD8+ T lymphocytes are all involved in the development of liver injury (68).

Through the hepatocyte regenerating process a number of cytokines, adipokines, and lymphokines contribute to hepatic fibrogenesis during the inflammatory process in NASH (69).

Furthermore, it has been shown in recent research that obesity and hyperinsulinemia both promote the production of IL-6 and TNF and upregulate the insulin-like growth factor 1 (IGF1)/insulin substrate 1 pathway, which in turn contributes to hepatocarcinogenesis. In conclusion, the formation and progression of HCC are significantly influenced by the hepatic microenvironment of NASH (70, 71).

2.2 HCC in India

As reported in GLOBOCAN 2020 data, HCC is the 8th most common cause of cancer-related death in India. In India, the male-to-female ratio for HCC is 4:1 (72). In our nation, HBV accounts for 70%–80% of all HCCs; about 15 percent are linked to HCV, and 5% are linked to both HBV and HCV. About 8% of HCCs are caused by alcohol alone. About 10% of patients have no apparent cause at all. In certain regions of India, aflatoxin and iron excess may be related to HCC. The underreporting of HCC is possibly because of no surveillance of patients with chronic hepatitis and cirrhosis. Due to the excellent investigation methods, a majority of HCC cases in Western nations are identified early. However, the majority of patients in India are found at advanced stages, which results in high death rates (73). Although the hepatitis B virus is prevalent in India, no thorough analysis of HCC data has been conducted. Also, the cancer registries most likely do not accurately reflect the incidence.

2.3 Pathophysiology of hepatocellular carcinoma

Chronic necrosis and inflammation of the liver are important driving forces in the multistep process of hepatocarcinogenesis in the context of underlying risk factors such as HBV and HCV infections, iron overload, aflaotoxin exposure, and the presence of fatty liver diseases. It is believed that HCC emerges from normal hepatocytes through sequential acquisition of essential molecular alterations that empower them with cancer hallmark capabilities. Under persistent clonal selection pressure, some subclones of cells with growth and survival advantages will dynamically undergo clonal expansion. This is a gradual and cumulative

process. Consequently, the altered hepatocytes' clonal development gradually gives rise to HCC (74).

Recurrent inflammation and fibrinogenesis in cirrhotic livers predispose the liver to dysplasia and malignant transformation, which is the primary cause of HCC. In response to shocks and injury, the structure and function of the liver change pathologically, leading to fibrosis, necrosis, inflammation, and cirrhosis. Hepatic fibrosis, abnormal hepatocyte regeneration, and inflammation have all been observed in patients with chronic liver illness. These defects support a sequence of genetic and epigenetic events that lead to the production of dysplastic nodules, which are legitimate preneoplastic lesions, and can induce cirrhosis. A final set of molecular changes that give dysplastic cells the ability to proliferate, invade, and survive completes the transformation to hepatocellular carcinoma (28, 75).

Patients with persistent liver disease may also develop hepatocellular cancer. (e.g., HBV infection) who do not yet have developed cirrhosis or severe inflammation. HBV and HCV viral infections cause an increase in hepatocyte turnover as the liver attempts to repair immune-attacked infected cells. Hepatic stem cells that multiply in response to viral injury-induced persistent regeneration may be the source of hepatic cancer (76). The cells in small dysplastic nodules appear to carry markers consistent with stem cells. HBV can also cause HCC in the absence of cirrhosis. HBV integrates its deoxyribonucleic acid into the host genome, leading to genomic instability and chromosomal rearrangements. HCV does not integrate into the host genome because it stores genetic information in ribonucleic acid. HCV-related HCC is found almost exclusively in patients with cirrhosis (75, 77).

Alterations in numerous signaling pathways occur in cancer, and several specific pathways have been observed to be dysregulated in HCC. HCC pathways and their role in disease progression and therapy are summarized in Table 2.1. Changes in liver tissues induced either by chronic viral infection or by exposure to hepatotoxic agents cause up regulation of components of a number of cellular signaling pathways. The major signaling pathways of HCC are shown in Figure 2.2 (78).

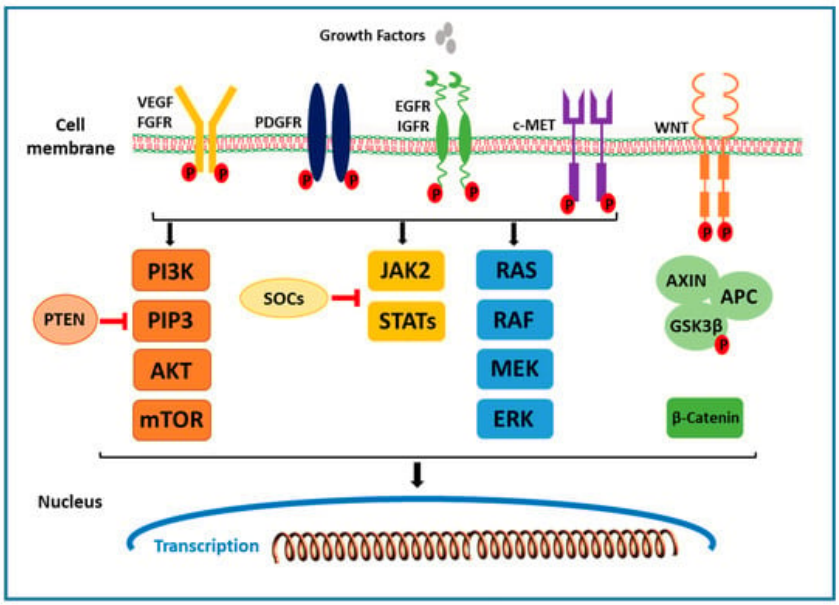

Figure 2.2: Major signaling pathways involved in HCC

(Dimri et. al. 2020) (78)

Pathways regulating growth factor, cell differentiation, and angiogenesis are predominant in HCC. Insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF/MET) regulate the growth factor signaling in HCC. The WNT, Hedgehog, and Notch pathways are involved in cell differentiation. While the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) pathways regulate the angiogenesis.

The RAS/RAF/MEK/ERK and P13K/AKT/mTOR are the major downstream receptor tyrosine kinases pathway. Upregulation of these pathways is correlated with the poor prognosis of HCC patients (28). The RAS/RAF/MEK/ERK pathway is one of the most critical signaling cascades for liver tumorigenesis. Raf kinase is activated in a high percentage of HCC tumors and it can be activated by HBV, HCV infection, or mitogenic growth factors. Its activation is linked to the aggressive nature of tumors (27).

The PI3K/AKT/mTOR pathway can promote cell proliferation and tumor metastasis. It is also one of the most frequently activated signaling pathways in HCC and is the main mechanism of HCC therapy resistance (79).

Table 2.1: HCC pathways, their role in disease progression and therapy

Pathway	Role in HCC progression	Role in HCC therapy
RAS/RAF/MEK/ERK	Phosphorylation of Ras allows delivery of signals to the nucleus	Related to the poor prognosis of HCC patients
PI3K/AKT/mTOR	Promote cell proliferation and tumor metastasis	Most frequently activated signaling pathways in HCC and is the main mechanism of HCC therapy resistance
Wnt/β-catenin	Involves in the regulation of tumor migration, invasion, stem cells maintenance	Usually found in advanced HCC
JAK/STAT	Modulating cell proliferation, angiogenesis, and cellular metabolism.	Involves in the regulation of immune microenvironment, which is expected to provide a new strategy for targeted and immunotherapy combination in HCC
Hedgehog (Hh)	Controls a number of cellular functions, such as migration, differentiation, apoptosis, and proliferation.	Associated with tumor metastasis and chemotherapy resistance
The Hippo	Regulates cell proliferation, apoptosis and stem cell self-renewal	-
Vascular endothelial growth factor	Increase vascular permeability and promote angiogenesis, thereby promoting the growth, invasion, and metastasis of HCC	Play a role in chemoresistance by acting on autophagy through NRP2 and mTOR pathway

In HCC, over expression of epidermal growth factor (EGF) stimulates tyrosine kinase receptors that, in return, stimulate cell surface signal transmission to the nucleus. There is also the role of MAPK pathway activation acting through the Ras protein in HCC. The Ras protein undergoes activation via phosphorylation, which allows the delivery of signals to the nucleus via downstream components of the pathway, such as ERK1, RAF, and MEK. HCC is a highly vascularized tumor with strong angiogenesis activity that is enriched with VEGF on

the surface of tumor cells and is one of the targeted treatments for HCC. When patients with HBV infection have chronic liver illness, VEGF expression is elevated (79).

The VEGF family mainly includes VEGF-A, B, C, D, and E. VEGF-A is directly referred to as a VEGF, which plays a leading role in regulating angiogenesis and promoting tumor growth. VEGF-B works in non-neovascularized tumors, while VEGF-C and VEGF-D are mainly involved in tumor lymphangiogenesis. VEGF-E has a proangiogenic function. VEGF receptors are classified into three primary subtypes as VEGFR1, VEGFR2, and VEGFR3. VEGFR1 has the ability to bind with VEGF-A, VEGF-B, and placental growth factor (PIGF). VEGFR2 can bind with VEGF-A, VEGF-C, VEGF-D, and VEGF-E. VEGFR3 is the receptor for VEGF-C and VEGF-D (80).

In HCC, cells accumulate somatic DNA alterations, including mutations and chromosomal aberrations. Mutations in the TERT promoter are the most frequent genetic alterations, accounting for approximately 60% of cases (81). They can be detected in dysplastic nodules, and the TERT promoter is a recurrent insertion site for the genome of HBV.

Other mutated genes affect the cell cycle (e.g., TP53, accounting for approximately 30% of cases), WNT signaling (CTNNB1 and AXIN1, accounting for approximately 30% and 10% of cases, respectively), or chromatin remodeling (ARID1A and ARID2, accounting for approximately 10% and 5% of cases, respectively) which are shown in Figure 2.3 (25, 82).

HCC is well associated with various metabolic changes including biochemical alterations. Alfa-fetoprotein (AFP) is a glycoprotein in serum that was first recognized as a major marker for HCC. Moreover, HCC development has also been associated with plasma lipid and lipoprotein alterations. These alterations result in cellular dysfunction, reduction in the membrane integrity, fluidity and regulation of cellular processes related to growth and cell survival causing cancer development.

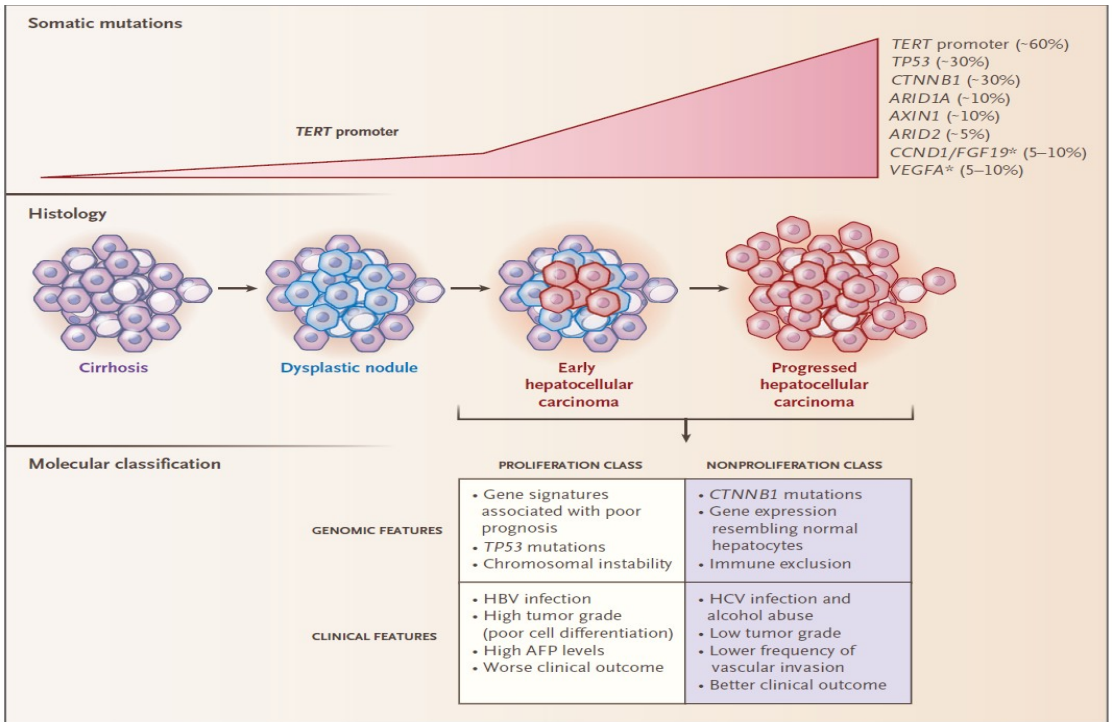

Figure 2.3: Genetic alteration in hepatocellular carcinoma
(Villanueva et. al. 2019) (54)

Furthermore, the development and progression of HCC are well associated with the oxidative stress produced by increasing level of reactive oxygen species (ROS) resulting alteration and decrease the antioxidant activity in the tissues. Lipid peroxidation (LPO) is responsible for formation of many toxic products, such as 4-hydroxynonenal and malondialdehyde (MDA) which attack cellular targets, thereby inducing carcinogenicity. Many biochemical and molecular changes leads to free radical metabolites causing the chemical carcinogens induce oxidative stress leading to tumor promotion. The production of cytokines, ROS, and inflammation-mediated events leads to tumor formation. HCC has a high anti-apoptotic genes expression and rapid cell proliferation. (54).

Hypoxia, oxygen (O_2) deprivation, is frequently found in regions of HCC that are devoid of functional blood vessels. Hypoxia can also be induced during palliative HCC treatment such as transcatheter arterial (chemo) embolization (TAE/TACE) with the initial principle to restrict tumor growth through blockade of blood supply. In addition, rapidly proliferating HCC cells quickly consume O_2 therefore deplete O_2 in the tumor microenvironment of HCC. The key molecular mechanism by which cells adapt to hypoxia is through transcription factor, hypoxia inducible factors (HIF). Through hypoxia-inducible factor 1 (HIF-1) which

elicits various molecular events, cells are able to overcome low O_2. HIF-1 allows hypoxic cells to survive oxidative stress by generating ATP through glycolysis (25).

2.4 Classification of HCC

A critical stage between diagnosis and treatment for HCC is staging of HCC. Staging is essential to: 1) divide patients into discrete prognosis groups; and 2) assist in selecting the most suitable treatment method (83).

The most accepted clinical classification of HCC has been proposed by the Barcelona Clinic Liver Cancer (BCLC). The BCLC staging system, as shown in Figure 2.4, has come to be widely accepted in clinical practice and is also being used for many clinical trials of new drugs to treat HCC (84).

The 5-stage clinical classification stratifies patients into very early HCC (stage 0), early HCC (stage A), intermediate HCC (stage B), advanced HCC (stage C), and end-stage HCC (stage D).

Stage 0 - In this "very early" stage, there is a single nodule with size < 2 cm without vascular invasion/satellites; portal pressure and bilirubin may be normal or increased.

Patients should undergo curative treatments, such as liver transplantation, local ablation with percutaneous ethanol injection (PEI), or radiofrequency ablation (RFA).

Stage A – In this early-stage single HCC nodule > 2 cm but < 5 cm, or three nodules < 3 cm; ECOG 0; Child Pugh Class A or B; and (85) absence/presence of associated extra-hepatic diseases.

In the absence of associated diseases, the patients might be candidates for liver transplantation; otherwise, local ablation with PEI or RFA should be considered.

Stage B - Patients in the intermediate stage B show multinodular asymptomatic HCC without an invasive pattern. Liver function may be preserved (Child A), or early decompensation might be seen (Child B). Performance Status is = 0. These patients might receive a survival benefit from transarterial chemoembolization, while other treatments such as PEI or RFA should be avoided.

Stage C - These subjects suffer from advanced HCC that consists of macroscopic vascular invasion (portal vein invasion), extra hepatic spread (lymph nodes and metastasis) or cancer-related symptoms (performance status 1-2). They cannot receive treatments other than first line therapy with sorafenib.

Stage D - Patients with terminal stage (stage D) have cirrhosis (Child C) and PST > 2. Only supportive, symptomatic treatment can be offered.

Patients presenting with very early (stage 0) and early-stage diseases (stage A) represent 20%-30% of patients with HCC. This group, suitable for curative treatments such as resection, liver transplantation, or local ablation with PEI or RFA, has a 5-year survival of 50%-70%. By contrast, patients in intermediate stage B and more advanced stage C, who account for 50%-60% of patients, have a poor prognosis, presenting a 3-year overall survival of 10%-40%. Finally, symptomatic subjects with end-stage disease (stage D; 10%-20%) have a survival < 3 months.

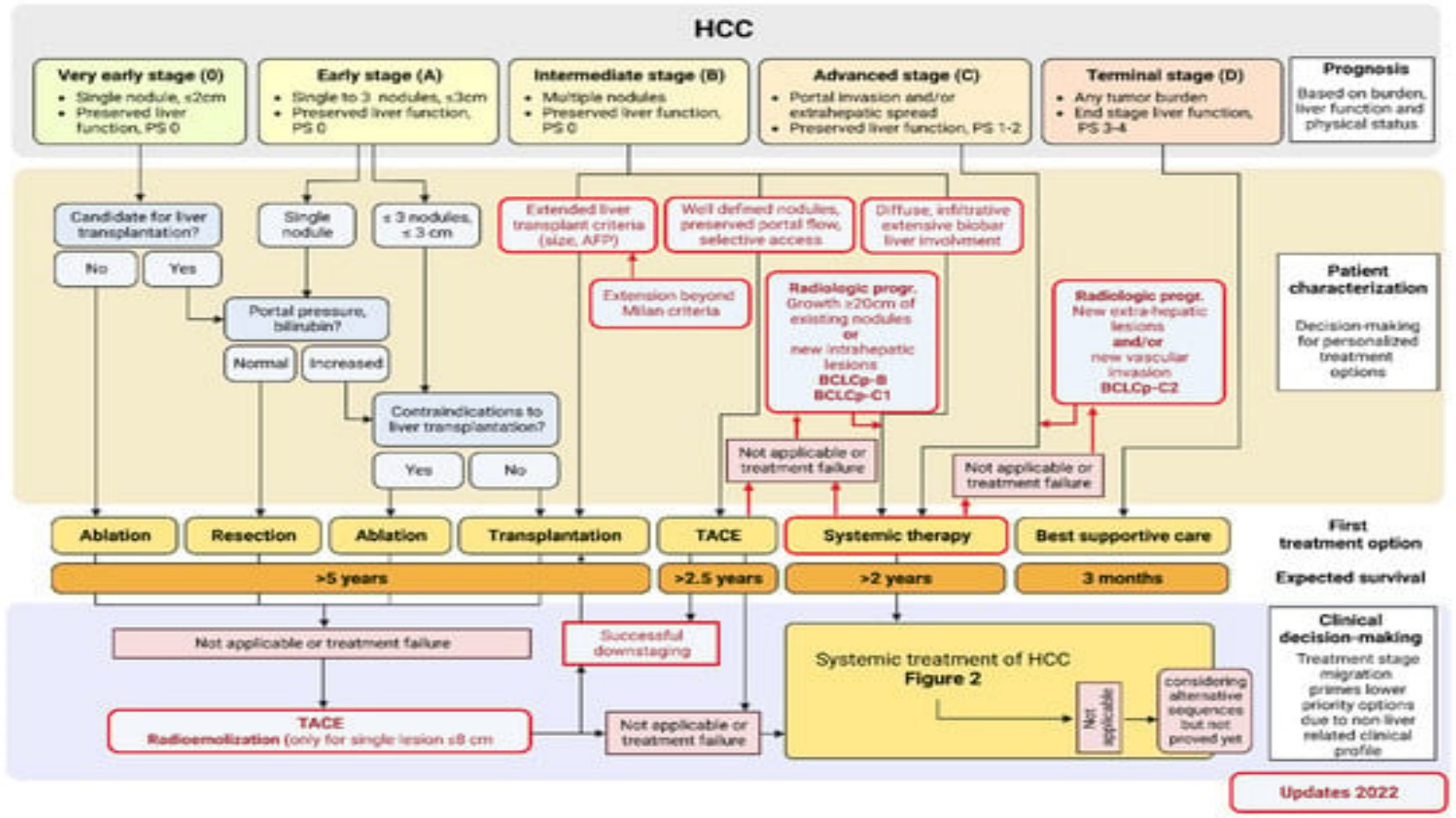

Figure 2.4: BCLC staging system and treatment strategy for HCC
(Tumen et. al. 2022) (84)

2.5 Diagnosis and disease marker of HCC

Clinically, HCC can be detected by screening and radiological evaluation in patients who are otherwise asymptomatic, or by the appearance of a right-upper quadrant mass, significant weight loss, and deterioration of the overall health of a patient diagnosed with cirrhosis (86).

The serum tumor marker alfa-fetoprotein (AFP), radiographic imaging, and liver biopsy are standard diagnostic methods for HCC. For patients with liver cirrhosis, the "gold standard" for HCC screening is the level of AFP in conjunction with abdominal ultrasonography. (87). Role of certain markers in different stages of HCC is summarized in Table 2.2.

The diagnosis of HCC poses many challenges, which can vary among different regions and centers. AFP and US imaging are most often used every 6 months for surveillance purposes in high-risk individuals.

Table 2.2: HCC disease marker

Protein Name	Purpose	Sample	Range		Sensitivity & Specificity (%)	Reference
			Normal Individual	Cancer Patient		
Alpha-feto Protein	Early diagnosis	Serum	0-40 ng/mL	>400 ng/mL	43/78	88
Vascular endothelial growth factor (VEGF)	Diagnosis	Serum/plasma	27-30 pg/mL	37-310 pg/m	-	93
Glypican-3	Diagnosis	Serum and Tissue	4.14-31.65 ng/Ml	99.94-267.2 ng/mL	77/96	89
Des-γ- carboxy prothrombin (DCP)	Early diagnosis	Serum	<7.5 ng/mL	> or =7.5 ng/mL	44/60	90
Transforming growth factor β1 (TGF β1)	Metastasis	Serum	-	-	70/77	91
Golgi protein 73	Early diagnosis	Serum	-	-	84/77	92

2.5.1 Alfa-fetoprotein (AFP)

The human AFP is a 591 amino acid, 70 KD glycoprotein. HCC is regarded as diagnosable by an AFP level of 400–500 ng/ml. When monitoring people who are more susceptible to developing HCC, ultrasonography and the serum marker AFP are often used in tandem (87). It has been shown that AFP correlates with tumor size and volume at the time of diagnosis. A Thai study found that patients with HCC who had an AFP level of 400 ng/ml or above usually had larger tumors, portal vein thrombosis, bilobar involvement, and shorter survival periods (94, 95).

AFP has three major isoforms depending on their affinity for the lectin Lens culinaris agglutinin (AFP-L1, AFP-L2 and AFP-L3). Serum level of AFP, AFP-L3 and DCP are used as parameters to refine the staging system of HCC (96). If serum AFP levels are noticeably increased at the time of diagnosis—which happens in less than half of cases—then using serum AFP alone may be beneficial. Under certain conditions, a liver biopsy might be used for confirmation when the diagnosis of HCC is still ambiguous. To assist in identifying tiny hepatic tumors larger than 3 cm, ultrasound imaging is frequently used either in addition to or instead of AFP.

2.5.2 Vascular endothelial growth factor (VEGF)

The most well-known regulators of angiogenesis are VEGF and VEGF receptors, which are necessary for the expansion and development of HCC. It has been demonstrated that patients with HCC who have higher circulating VEGF levels have a poorer prognosis and the disease progresses more quickly. Furthermore, VEGF appears to be involved in chemoresistance. As prognostic and predictive biomarkers for patients, key angiogenesis factor (VEGF) expression levels appear to be associated with overall survival and response to treatment (93).

2.5.3 Des-gamma-carboxy prothrombin (DCP)

In Japan, DCP, also known as PIVKA II (protein induced by vitamin K absence or antagonist-II), is a commonly utilized tumor marker that is a highly specific aberrant form of prothrombin for hepatocellular carcinoma.

2.5.4 Dickkopf-1 (DKK1)

It is a member of the secreted proteins family that plays an important role in HCC progression by promoting cytoplasmic/nuclear accumulation of β-catenin in HCC cells via the Wnt/beta-catenin signaling pathway (96).

2.5.5 Golgi protein 73 (GP73)

GP73 is a transmembrane glycoprotein with a molecular weight of 73 kDa. The expression of this protein is significantly increased in liver diseases such as HCC. GP73 is more sensitive and specific for HCC than AFP (97).

2.5.6 Glypican-3 (GPC3)

Significant upregulation of GPC3 mRNA was observed in HCC. It can be detected in 40 to 53% of HCC patients.

2.5.7 Angiography

Angiography has been used as a diagnostic tool for HCC because of its highly vascular nature; nevertheless, the detection of tumors by angiography has been disappointing, especially when the tumors are smaller than 2 cm in diameter. These days, angiography is more frequently employed to outline the structure of the liver prior to resection or to provide direction for transarterial chemoembolization therapy (97).

2.5.8 Liver biopsy

A safe and efficient way to confirm suspected lesions for HCC is with a liver biopsy. Under US or CT guidance, percutaneous fine-needle aspiration (FNA) and needle core biopsy, respectively, can be used to collect cytologic and histological samples. When both FNA and core biopsy procedures are utilized at the same time, instead of one alone, the diagnostic accuracy of liver biopsy is increased. Liver biopsy has the highest sensitivity and specificity of any diagnostic test (98).

2.6 HepG2 cell line

HepG2 is a human liver carcinoma cell derived from the liver tissue of a 15-year-old Caucasian male who had a well-differentiated hepatocellular carcinoma. HepG2 is the most widely used human hepatoma cell line in pharmaco-toxicological research. The HepG2 cell line is commonly used in drug metabolism and hepatoxicity studies. HepG2 cells exhibit an epithelial-like morphology with a modal chromosome number of 55 (99).

2.7 Treatment of HCC

Treatment options for HCC are as follows-

1. Multi-Kinase Inhibitors (MKI) like sorafenib, regorafenib, lenvatinib, cabozantinib.

2. Vascular endothelial growth factor (VEGF) receptor antibody- bevacizumab, ramucirumab

3. Programmed death receptor-1 (PD-1) inhibitors- nivolumab, pembrolizumab, atezolizumab.

The American Gastroenterological Association (AGA) lists the following as the first course of treatment for HCC. For the treatment of advanced HCC, oral systemic medications include sorafenib, (the first drug approved by the USFDA for HCC) and lenvatinib. Furthermore, there is a slight to moderate increase in survival over sorafenib when the anti-angiogenic drug bevacizumab and the checkpoint inhibitor atezolizumab are combined.

HCC patients have been prescribed IV monoclonal antibody ramucirumab (anti-vascular endothelial growth factor) and checkpoint inhibitor pembrolizumab (anti-PD1) as second line treatments, in addition to oral multi-kinase inhibitors cabozantinib and regorafenib (78). First-line treatment options for each stage of HCC and treatment option with their mechanisms of action are summarized in Figure 2.5 and 2.6.

The primary therapeutic approach for advanced HCC is targeted therapy, such as oral tyrosine kinase inhibitors (TKIs). At present, there are six approved tyrosine kinase inhibitors for the treatment of HCC. Approved TKI and other drugs used in treatment of HCC are summarized in Table 2.3 (100).

Table 2.3: Summary of approved TKI and other drugs in HCC treatment

Sr. No.	Drug	Targets	Approved by regulatory agency	Treatment 1st or 2nd line	Approval year	References
1	Donafenib	Multitargeted TKI (PDGFR, Raf, VEGFR)	NMPA	2	2021	78
2	Apatinib	Multitargeted TKI (VEGFR2, RET, KIT)	NMPA	2	2020	100
3	Cabozantinib	Multitargeted TKI (VEGFR1-3, KIT, MET)	FDA	2	2019	100
4	Ramucirumab	VEGFR inhibitor	FDA	2	2019	101
5	Lenvatinib	Multitargeted TKI (VEGFR1-3, PDGFR, RET, KIT)	FDA	1	2018	100
6	Pembrolizumab	PD1 immune checkpoint inhibitor	FDA	2	2018	102
7	Nivolumab	PD1 immune checkpoint inhibitor	FDA	2	2017	102
8	Regorafenib	Multitargeted TKI (VEGFR1-3, KIT, PDGFR, FGFR, BRAF)	FDA	2	2017	78
9	Sorafenib	Multitargeted TKI (VEGFR1-3 FGFR, TIE2, PDGFR, BRAF, KIT, FLT-3)	FDA	1	2007	100

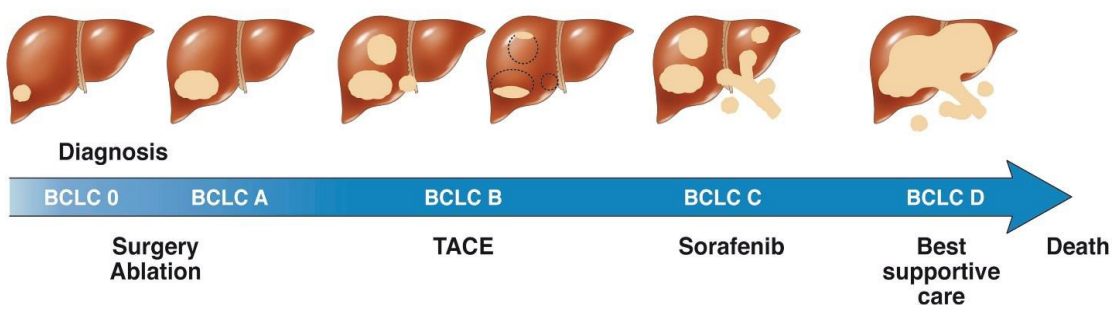

Figure 2.5: Development of HCC and first-line treatment options for each stage

(Ahmad et. al., 2019) (103)

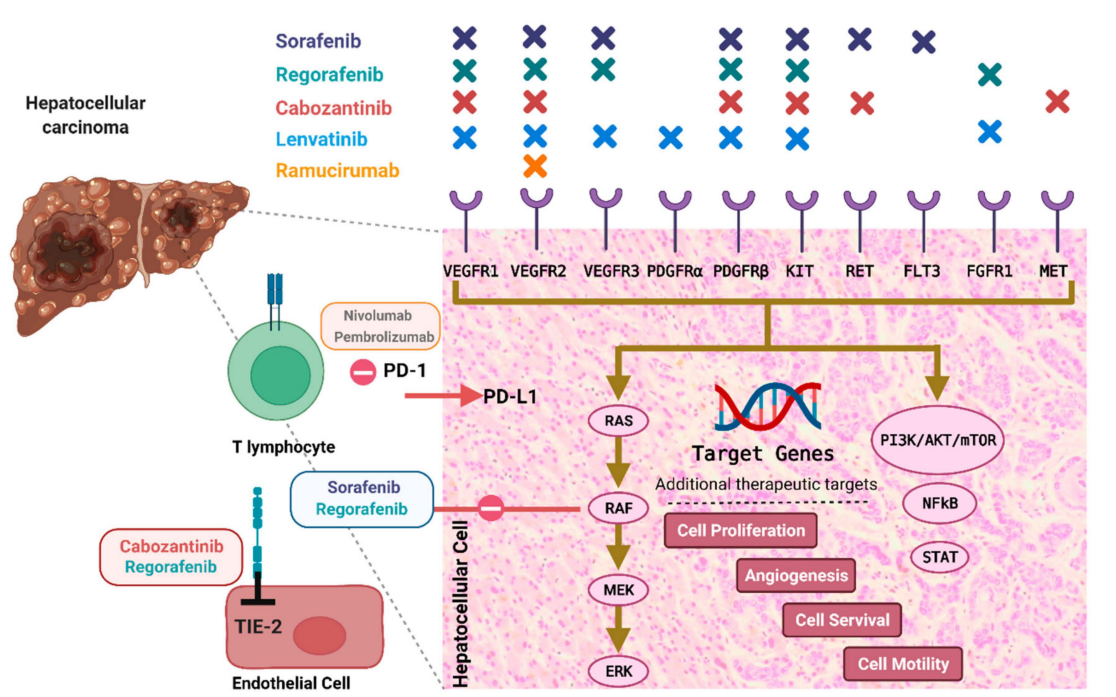

Figure 2.6: HCC treatment option with their mechanism of action

(Farasati et. al., 2023) (104)

2.8 Prevention of HCC by vaccination

In HBV-endemic areas, immunization against HBV has proven to be the most effective method of preventing HCC. In Taiwan, a universal vaccination program was launched in 1986 for all neonates. This vaccination had resulted in a decrease of HCC incidence in 6 to 9 year children from 5.2 per million to 1.3 per million (105). HBV-relevant HCC is the first cancer

that has been shown to be preventable with immunization. Unlike to HBV, HCV cannot be primary prevented and there is no vaccination (106).

2.9 Animal models for screening of anti-cancer drugs

Animal models play an important role in drug development and studies of molecular mechanisms. The researcher faces a hurdle in trying to understand the disease mechanism because there are no animal models of liver cancer that are comparable to liver tumors in humans. The "ideal" animal model should replicate the biopathology, biochemistry, and natural history of human HCC. It should also enable the assessment of novel therapeutic medicines in preclinical trials for the treatment of this illness and aid in the development of molecularly targeted therapy. However, it should be noted that no model is "ideal" for HCC research objectives or for the investigation of other diseases (107). There are currently ample rodent models accessible for the research of hepatocarcinogenesis, and they are as follows: There are four types of models: chemically induced, cell derived xenograft (CDX) (syngeneic and xenograft models), viral, and genetically engineered mouse (GEM) models (1).

2.9.1 Chemically induced models

The human society trusts in the use of chemical compounds to fulfill a large number of needs for food production, processes of conservation/storage, agriculture purposes, and novel applications in industrial processes.

Compounds that cause cancer may be classified as genotoxic or nongenotoxic. Although the distinction between these is not always precise. The mechanism of action of nongenotoxic carcinogens is diverse and not clearly understood. It is believed that the initiation of cancer by these compounds does not involve their direct interaction with DNA but rather the changes that they induce in the control of cellular proliferation. In contrast, the mechanism of action of genotoxic carcinogens, which comprise at least half of reported human chemical carcinogens, is much more clearly understood. These compounds have the general property of being able to react with DNA to form covalently bound products, known as adducts (108). Details of carcinogen inducing different types of cancers are summarized in Table 2.4.

Chemical substances used to promote tumors must have a high tumorigenicity, specificity for a target tissue, a long half-life, be readily available, and be bioprocessable (109). Chemically induced carcinogenesis is often regarded as a sophisticated and sequential process including at least three distinct stages (tumor initiation, promotion, and progression) (110, 111).

Tumor initiation stage

The initial step in the genesis of cancer is initiation. Initiators are chemicals that aren't usually reactive with DNA, but are altered by drug-metabolizing enzymes in the body and can then cause changes in DNA (mutations) following a covalent bond. Many initiators are species- or tissue-specific. Initiation is irreversible, which means that once a cell is touched by an initiator, they are prone for next stage of promoting. Because initiation results in irreversible genetic change, all daughter cells resulting from the mutant cell's division will likewise carry the mutation. The quantity of generated tumour cells is proportional to the initiator dose, implying that the greater the exposure, the greater the risk of carcinogenesis.

Tumor promotion stage

Promotion is the next stage for cells that have already been modified by an initiator. The promoters are the substances that encourage a cell's multiplication into a large number of daughter cells that carry the initiator's mutation. Promoters only work if the organism has already been exposed to an initiator. Its do not covalently link to macromolecules or DNA within the cell, but many bind to the cell surface receptors to impact intracellular pathways that drive cell proliferation.

Promoters are not able to cause cancer by themselves, but they do increase the clonal growth of started cells, which eventually leads to malignant cells (112).

Tumor progression stage

The progression step is the third stage, which describes the progression of a benign tumour into a neoplasm and then into malignancy. The advancement is irreversible once this step is triggered.

A two-stage carcinogenesis technique is often used in practice for animal models of malignancy to abbreviate the cancer development period by treating animals with an organ-specific cancer initiator followed by a promoter (113). DEN is the most widely used carcinogen as an initiator and phenobarbital, CCl_4, 2-AAF as a progression of cancer.

Among commonly adopted cancer models, chemically induced primary malignancies in mammals have multiple advantages, including eased procedures, abundant tumor generation, the ability to study sequential stages of carcinogenesis, and a high analogy to human primary cancers seen in the clinic with high heterogeneity.

These models do have certain drawbacks, though. Animal models created with chemicals do not accurately depict the normal progression of the disease, and there will be significant inter-

animal heterogeneity. In addition to the time-consuming progress of carcinogenesis, the major drawback is the difficulty in noninvasive tumor burden assessment (113).

Table 2.4: Details of carcinogen inducing different type of cancers

Carcinogen	Dose	Route of administration	Duration of induction	Animal species	Type of cancer	References
DENA	200 mg/kg	Intraperitoneal	20-24 week	Rat	Liver cancer	114, 115
2-AAF	30 mg/kg	p.o.	18-20 week	Rat	Liver cancer	116, 117
MNU	1 mg in 20 µL	Intraductal	10-15 week	Rat	Breast cancer	118
DMBA	20 mg/mL	p.o.	120	Rat	Breast cancer	119, 120
N-nitroso-tris-chloroethylurea (NTCU)	4, 8, or 40 mmol/L	Topical	32 week	Mice	Squamous cell carcinoma	121, 122
Vinyl carbamate	60 mg/kg	Intraperitoneal	24 week	Mice	Lung cancer	123
MNU	20-40 mg/kg	Intravenous	12-15 month	Rat	Prostate	124
DMBA	60 µg	topical for 2 week	20 week	Mice	Skin	125, 126
BBN	0.05% in drinking water	p.o.	20 week	Mice	Bladder	113, 127

MNU-N-methyl-N-nitrosourea, DMBA- 7,12-dimethylbenz(a) anthracene, DENA-diethyl nitrosamine, NTCU-N-nitroso-tris-chloroethylurea, BBN-N-butyl-N-(4-hydroxybutyl)-nitrosamine

2.9.2. Cell-derived xenograft (CDX) model

The simplest kind of tumor model is the subcutaneous model, wherein mouse tumors (syngeneic model) or human tumors (xenograft model) are originally created from cell lines that are injected subcutaneously into the mouse's flank, and the progress of the tumor is tracked using calipers. By ensuring that the compounds have the proper pharmacological action in a biological system, these models are essential to the discovery stage of the process. Since its introduction in 1972, this method has been extensively utilized in preclinical drug development as a time- and money-efficient experimental design.

The models used to assess treatment efficacy are produced from human tumor cell lines and categorized based on the transplant site, such as orthotropic xenograft and ectopic xenograft. Because they are less expensive, easier to handle, and have known genetic information, mouse models are more appealing than large animal models. These models have the advantages of immunocompetence, ease of inducing different tumor forms, and repeatability (128).

2.9.2.1 Ectopic tumor xenograft model

Human cancer cells are subcutaneously injected into the hind leg or back of mice. In an ectopic tumor xenograft model, the transplanted site is different from the origin of the cultured cells. The ectopic model is the standard model of cancer used for validation and assessment in oncology studies.

This model is utilized for the evaluation of lead compounds obtained from an in-vitro screening test. In this model, the same cancer cells can be useful and predictive, which is helpful for the selection of an applicable cancer compound for translation into a clinical trial. Several parameters, such as the ratio of the treated group (T) to the control group (C) (% T/C), tumor growth delay, and tumor regression, were utilized based on this data, and anticancer activity can be evaluated.

The ectopic models are very reproducible, homogenous, and amenable to use. However, not all tumors can be used as an assessment tool because some tumors show necrosis during tumorigenicity and some tumors are not solid. The immunosuppressed mice used for making animal models represent a different microenvironment than that of human cancer (129).

2.9.2.2 Orthopic tumor model

Orthotopic mouse models are invaluable for studying HCC as they closely mimic the tumor's natural hepatic microenvironment. Through intrahepatic injection, tumor cells are directly transplanted into the mice's livers in the orthotopic model. Luciferase-labeled cell lines can be utilized in orthotopic models to mimic the metastatic activity of cells, offering a realistic environment for researching the development of tumors and the effects of treatment. To ensure proper tumor implantation and reduce animal discomfort, skilled surgeons are needed for this model development (130, 131).

2.9.3. Viral models for HCC

Virus models are essential for studying hepatocellular carcinoma (HCC), particularly in understanding the role of viral infections in liver cancer development. Chronic HBV and HCV infection is a significant risk factor for HCC. Animal models, such as the woodchuck model, mimic HBV-induced hepatocarcinogenesis, providing insights into the disease's progression and potential treatments (132).

Hydrodynamic injections are used to introduce viral DNA into mouse liver cells, creating models that closely replicate human HCC. These models are used to study the molecular mechanisms of HCC and the interaction between viral infections and liver cells (133).

2.9.4. Genetically engineered mouse (GEM) model

Researchers can better understand tumor genesis, progression, tumor microenvironment, and disease pathways by using genetically modified mouse models. Modifying certain genes, such as p53 and PTEN, as well as oncogenes linked to HCC, such as c-Met and β-catenin, results in GEM models. The sleeping beauty transposon system and hydrodynamic injection (HDI) are utilized to alter the mice's DNA. These animals are a useful platform for preclinical research since they closely resemble human HCC. They aid in the comprehension of the relationship between the immune system and cancer cells. Despite their advantages, GEM models have drawbacks, such as the fact that human and mouse tumor biology differs, which may make it more difficult to apply research in clinical settings (134).

2.10 Plant profile

2.10.1 *Achyranthus aspera*

A. aspera plant is distributed throughout the tropical world. The plant is an erect, annual herb, distributed throughout India, Baluchistan, Ceylon, and Australia (135). *A. aspera* was reported to contain many phytochemicals like alkaloids, flavonoids, tannins, terpenoids, saponins, glycosides, and steroids. The plant can be found growing in many places as an introduced species and a common weed. References are found for the use of plants in Ayurveda and Chinese medicines (136).

Figure 2.7: *A. aspera* plant and root

Scientific/taxonomical classification:	**Other names:**
Kingdom: Plantae	Sanskrit: Apāmārga
Order: Caryophyllales	Gujarati: Agharo
Family: Amaranthaceae	Hindi: Aghara, Madhukar
Genus: Achyranthes	Telugu: Apamargam
Species: *A. aspera*	English: Prickly chaff flower
Part used: Whole plant, leaves, seeds, roots, flowers and fruits.	

Macroscopic characters

The plant has thick, long, cylindrical primary roots in addition to secondary and tertiary roots. The roots have a yellowish brown color, a faint smell, and a sweet flavor. The stem is hairy, cylindrical, branching, and yellowish brown in color. Petiolate, alternating, elipatic-obovate or

sub-orbicular leaves are seen. Greenish-white flowers are in tiny, thick spikes or axillary heads. The seeds are black and shiny, subcylindric, truncate at the apex, and rounded at the base (137).

Microscopic characters

The lower epidermal cells of a leaf have wavy walls; the transverse section of the top epidermal cells is mostly straight. The anomocytic and few anisocytic stomata that cross both the top and lower epidermal cells. Trichomes emerge from the lower epidermis and are simple, covering, uniseriate, multicellular, and many. Calcium oxalate rosette crystals with sizes ranging from 20 to 45 μ are implanted in the mesophyll parenchymatous cells and in the midrib ground tissue. The stem has six to ten conspicuous ridges, and beneath each ridge is collenchyma. There are rosettes, prismatic or granular types of calcium oxalate crystals and a lack of phloem fibers. The immature root's transverse section reveals an epiblema layer with long, unicellular hairs. The cortex has six layers, which are thin, and parenchymatous. Anamolous growth is seen in the stellar area (30).

Chemical constituents

Achyranthes plants have been proven to be a rich source of natural compounds with varying structural patterns. The plant contains oleanolic acid as a major triterpenoid saponin. Various parts of the plant, viz., seeds, stem, leaves and root are reported to contain ecdysterone. Other constituents of the plant are long chain alcohol, viz. 17-penta triacontanol, 27-cyclohexyl heptaeosan-7-ol, 16-hydroxyl 26-methyl heptacosan-2-one and 36, 47-dihydroxy hen-pentacontan-4-one. It also contains a water-soluble base, betaine (136).

Therapeutic uses mentioned in Ayurvedic pharmacopoeia

All of the Indian traditional medicine systems, including Ayurveda, Unani, Sidha, and homeopathy, have used *A. aspera*. The plant has a significant role in Indian culture and folk medicine (138). Around the world, *A. aspera* has also been added as active components to a wide range of products, such as sanitary napkins, health care products and wine, sex pills, cream, and toothpaste (139).

A. aspera was utilized by the tribes people in the Chittoor area of Andhra Pradesh to treat epilepsy, and its seeds were boiled in milk and used to make Payasam or Kheer, a delicious therapy for brain disorders (140). In western Uttar Pradesh, India, and central Nepal, it is one of the potent Ayurvedic herbs used in the management of gynecological diseases (35). The dried plant is employed in arsa (haemorrhoids), medroga (obesity), sula (colic), udararoga

(abdominal disorders), and apaci (lymphadenitis-cervical). In cases of chardi (vomiting), adhmana (tympanitis), kandu (itching), sula (colic), apaci (lymphadenitis), granthi (tumor), bhagandara (fistula-in-ano), hrdaroga (heart disease), jwara (pyrexia), switra (leucoderma), vadhirya (deafness), udararoga (abdominal diseases), yakrtroga (liver disorders), dantaroga (tooth disease), and raktavikara (blood disorders) are treated with the dried roots of the plant (30). Different traditional products of *A. aspera* are available in the market such as Apamarga powder, Apamarga churna, Mutrakrichanta churna, Livol, and Stonhills.

Pharmacological activity

Therapeutic category

Anti arthritis activity

A. aspera extract's saponin-rich fraction was given orally at 50–100 mg/kg, and it was shown to reduce the index of the spleen and thymus, alter antioxidant parameters, reverse tissue marker enzyme levels, and inhibit inflammatory cytokines in rats. These effects decreased the threshold for pain, which minimized paw swelling and arthritis (141). Achyranthes saponins 75-300 mg/kg, p.o., have been given to rats with collagen-induced arthritis to effectively inhibit the advancement of joint swelling, soft tissue inflammation, and bone erosion (142).

Anti-microbial activity

The whole plant exhibited antibacterial efficacy against Bacillus typhosus, Streptococus heamolyticus, and Staphylococcus aureus (143). S. aureus and E. coli were susceptible to the antibacterial properties of both the alcoholic and aqueous leaf extracts of *A. aspera* (144). In another study, *B. subtilis* and *S. aureus* bacterial strains were suppressed by a leaf and stem ethanolic extract of the plant (145).

Anti-hyperlipidemic activity

In rats treated with triton-induced hyperlipidemia, an alcoholic extract of *A. aspera* at a dose of 100 mg/kg reduced total serum cholesterol, phospholipid, triglyceride, and total lipids. (146).

Anti-diabetic activity

The ethanolic, aqueous, and methanolic extracts of A. aspera lowered blood sugar levels in diabetic rats and rabbits that had been given alloxan (147, 148).

Diuretic activity

As rats were given 10–20 mg/kg i.m. dosages of the saponin extracted from *A. aspera* seeds, their urine output increased significantly after 2, 6, and 24 hours as compared to rats that were not given any treatment. The diuretic impact was similar to that seen with an oral dose of acetazolamide (10 mg/kg) and a dose of mersalyl (3 mg/kg). Similar to acetazolamide, saponin's diuretic action was linked to increased potassium and sodium excretion in the urine (149).

Anti-asthmatic activity

At the Central Research Institute for Siddha in Madras, a pilot study on 15 cases of bronchial asthma was conducted. These patients received three daily doses of the oil extracted from the *A. aspera* root that had been soaked in cow urine and placed on betel leaves. The majority of the time, symptoms including coughing, wheezing, gasping, dyspnea, and sneezing were gone away. There was a decrease in erythrocyte sedimentation rate (ESR), eosinophil levels, and total WBC count (150).

Anti-inflammatory activity

In albino male rats with carrageenan-induced hind paw edema and cotton pellet granuloma models, an alcoholic extract of *A. aspera* showed anti-inflammatory action (151). Additionally, it has been reported that *A. aspera's* ethanolic extract has anti-inflammatory and anti-arthritic properties in rats at doses of 100–200 mg/kg (152).

Antitumor activity

A large number of *in vivo* and *in vitro* experiments have clearly demonstrated that *A. aspera* can inhibit tumor growth through the following common mechanisms:

1. Induction of tumor cell apoptosis (153);
2. Activation of immune system
3. Inhibition of Akt phosphorylation

A methanol extract of *A. aspera* leaves exhibited cytotoxicity on pancreatic cancer cells. The methanol extract (34 μg/mL) selectively suppressed the transcription of MMP-1, MMP-2,

TIMP-2, VEGF-A, and VEGF-B (33). Further study by the same group in human pancreatic tumor-bearing athymic mice reduced tumor volume and weight, increased caspase-3 level (apoptosis induction) and inhibited the Akt phosphorylation (32).

Another recent study demonstrated that *A. aspera* polyphenols (100 mg/kg, *p.o.*) have played a role in cytokine based immunomodulatory activity, resist DNA conformational changes in mice, and produce anticancer activity in urethane-induced lung cancer (154). The ethanolic extract of the entire *A. aspera* plant significantly reduces oxidative stress and liver diagnostic markers, suggesting that it is protective against the two stages of hepatocarcinogenesis caused by NDEA and CCl_4. Reduced levels of increased SGPT, SGOT, SALP, GGT, bilirubin, and LPO indicated that extract administration inhibited hepatic diagnostic and oxidative stress indicators. After extract treatment, the liver and relative liver weight reduced, and the liver architecture returned to normal compared to the positive control group (36).

Gastroprotective activity

Ethanolic extract of *A. aspera* leaves at 600 mg/kg, p.o. protected the rats from ulcers in pylorus ligation and chronic ethanol induced ulcers in rats (155).

Toxicity study

Based on the extant literature, *A. aspera* plants have a low level of toxicity, making them suitable for use as an herb. According to an acute toxicity investigation done on rabbits at 8 gm/kg, oral administration of *A. aspera* did not result in any indications of poisoning (148). Similar research revealed that neither the hematological variables nor the biochemical parameters changed significantly when a methanol extract of *A. aspera* leaves was given orally to an adult male albino mice up to a dosage of 4 g/kg (156). In another study, there was no observed mortality and behavioral change in response to a leaf extract at a dose of 2000 mg/kg (35).

2.10.2 *Boerhaavia diffusa*

B. diffusa is a medicinal herb, commonly known as a punarnava in the Indian medicine system. It is a perennial weed belonging to the family of Nyctacinaceae. The plant is widely distributed throughout tropic and subtropical areas, including India, Brazil, Africa, Australia, China, Egypt, Pakistan, USA, and Sri Lanka. *B. diffusa* is utilized as a green vegetable in India and has a high nutritional content (157).

The roots and leaves of *B. diffusa* are frequently used in folk medicine to alleviate a variety of ailments, even though the entire plant is utilized to treat various disorders (158).

Figure 2.8: *B. diffusa* plant and root

Scientific/taxonomical classification:	**Other names:**
Kingdom: Plantae	Sanskrit: Punarnava, rakta punarnava
Order: Caryophyllales	Gujarati: Dholisaturdi
Family: Nyctaginaceae	Hindi: Gadapurna, sant
Genus: Boerhavia	Marathi: Raktavasu, tambadivasu
Species: *B. diffusa*	Bengali: Rakta punarnava
Part used: Roots	Telgu: Atikamamidi

Macroscopic characters

The elongated, fusiform, tapering, cream or light brownish yellow roots of *B. diffusa* have a slightly bitter, sweet, and pungent taste without a pronounced odor. The stiff, slender, cylindrical, greenish-purple stems swell at nodes and have a length of around one meter. The

leaves are opposite in uneven pairs, with bigger leaves being 25–37 mm in length and smaller ones measuring 12–18 mm. They are also green and pale on the underside and glabrous on top. The leaves can be ovate-oblong or suborbicular, with a rounded or slightly pointed tip. Petioles almost as long as the blade, thick texture, dorsal side pinkish in certain cases, complete or sub-undulating margin. The tiny flowers have an ovoid, greenish base section and a pink, funnel-shaped top part. Fruits are spherical, glandular, 0.5 cm in size, with one seed, 6 mm in length, clavate, widely spaced, and clearly five ribbed. The entire plant tastes bitter and has no scent.

Microscopic characters

The transverse section of the leaf has anomocytic stomata and multicellular glandular trichomes on both sides, with the stomata being more prevalent on the top surface. It also shows a few short 3-4 celled hairs present on the border and on veins. A single-layered palisade and 2-4 layers of spongy parenchyma interspersed with small air holes. Idioblasts with raphides occasionally group calcium oxalate crystals and the orange-red resinous material found in mesophyll.

The stem epidermal layer is composed of 1-2 layers of parenchyma and contains uniseriate glandular trichomes with an ellipsoidal head and 8–12 stalked cells. The cortex spans 150–220 µm. A ring of six to twelve loosely organized vascular bundles in the ground tissue with an intrafascicular cambium present, makes up the stele, which is composed of two big vascular bundles in the center.

A cork of one or two layers of thin-walled cells is visible in the transverse section of the root. The cortex is made up of two to three layers of parenchymatous cells, which are followed by five to twelve layers of thin-walled, oval-to-polygonal cells and several concentric bands of xylem tissue. Below the cortical regions is a wide zone of parenchymatous tissue, the number of which varies depending on the thickness of the root and is made up of vessels, tracheids, and fibers. Small, thick-walled, spindle-shaped, elongated, and pointed-end vessels with reticulate thickening typically begins as clusters of two to eight in radial rows. Sections of parenchyma include bundles of starch grains and acicular calcium oxalate (159, 137).

Chemical constituents

Numerous chemical elements, including alkaloids, steroids, triterpenoids, lipids, flavonoids, lignins, carbohydrates, proteins, and glycoproteins, are present in the plant. Punarnavoside, Boeravinone A-F, hypoxanthine 9-Larabinofuranoside, ursolic acid, lirodendrin, α-sitosterol,

palmitic acid, arachidic acid, β-sitosterol, ester of β-sitosterol, hexacosonoic, stearic, tetracosanoic, urosilic acid, β-Ecdysone, triacontanol, Hentriacontane, and other substances are mostly present. Punarnava's herb and roots are high in lipids and proteins. The root has fourteen amino acids and seven essential amino acids, whereas the herb has fifteen amino acids and six necessary amino acids.

Pharmacological activity

Traditional use

B. diffusa is categorized as a "rasayana" herb in Ayurveda, which strengthens life and brain power, prevents disease, is anti-aging, and rebuilds youth. This plant helps in increase the body's resistance against any attack by offering hepatoprotection and immunomodulation. Further, abdominal tumors are treated with the *B. diffusa* plant in traditional medicine.

It is a significant natural ingredient in many ayurvedic formulas. It has been used in a number of formulations intended to treat a variety of conditions, including gynecological diseases, anemia, asthma, rheumatism, inflammation, jaundice, and ascites (160). Different traditional formulations having *B. diffusa* as a main ingredient are Punarnavasava, Punarnavadi Mandura, Punarnavasataka, Punarnavambu, Punarnava Guggula, Punarnavasak Kwath/ Churna, Sukumar Ghrit, and Sothaghna Lepa.

Anti-diabetic activity

Pari et. al. reported that leaf extract in alloxan induced diabetic rats decreased the blood glucose levels, the amount of glycosylated haemoglobin, and a major rise in plasma insulin. The extract increased pancreatic insulin secretion, blood glucose transportation to peripheral tissues, or a decrease in gastrointestinal glucose absorption (161).

Antibacterial activity

Umamaheswari and coworkers studied the ethanolic extract of *Boerhaavia diffusa* leaves, which showed good antibacterial activity against both Gram-negative and Gram-positive bacteria (162). The Sahu and colleagues studied the anti-microbial activity of the extract against bacterial pathogens that cause urinary tract infections (163).

Kant et al. conducted clinical trials on 50 newly diagnosed patients with pulmonary tuberculosis, and found that the extract was more beneficial as an adjuvant to chemotherapy than for the control group of patients. When compared to the control group, the extract-treated

patient experienced superior relief from fever and cough at the conclusion of the 4-6 week follow-up period (164).

Hepatoprotective activity

B. diffusa possesses marked hepatoprotective activity against thioacetamide-induced hepatotoxicity and supports various theories proposed for the mechanism by which thioacetamide damages the liver (165). The plant extract resulted in a substantial decrease in serum bilirubin levels, and reduced the prothrombin time (which was increased by Ccl_4) which indicates that the drug is effective in the maintenance of the normal functional status of the liver and capable of protecting the prothrombin synthetic activity of the liver in Ccl_4 induced hepatotoxicity in rats (166).

Immunomodulatory activity

Using an E. coli-induced abdominal sepsis stress model, Mungantiwar and colleagues examined the immunomodulation by oral administration of aqueous extract at 50–200 mg/kg/day and demonstrated substantial leucocytosis and a 50% reduction in mortality in pretreated mice. The alkaloidal component of the extract, at 25–100 mg/kg p.o., metabolized to its active form and significantly reduced and delayed hypersensitivity reactions in rats. It has been revealed that the plant contains quercetin, punarnavine, and syringaresinol mono-D-glucoside, which all have immunomodulatory properties (167, 168).

Anticancer activity

Two rotenoids, boeravinones G and H, were identified from *B. diffusa* roots by Ahmed-Belkacem and colleagues, who discovered that they may act as efflux inhibitors for the protein known to cause breast cancer resistance (ABCG2) (169). Sreeja and colleagues examined the antiproliferative and antiestrogenic properties of the whole plant's methanolic extract in the MCF-7 cell line, demonstrating a 46.8% decrease in cell viability at 320 μg/mL in 48 hours (43). Leyon et al. investigated the impact of a whole plant aqueous methanolic (3:7) extract on metastasis using a B16F10 melanoma model in C57BL/6 mice (44). Additionally, the author extracted punarnavine from the extract, which demonstrated complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity in addition to an increase in NK cell activation.

IFN-γ and IL-2 production was elevated by punarnavine. Punarnavine administration resulted in a significant decrease in GM-CSF levels as well as proinflammatory cytokines such as IL-1α, IL-6, and TNF-α. It also downregulated the production of MMP-2, MMP-9, VEGF,

ERK-1, and ERK-2 (170). In cancer therapy, *B. diffusa* provides a multiple target regimen. It possesses immunomodulatory, anticancer, and radioprotective properties. In a DEN-induced carcinogenesis model, an aqueous extract of *B. diffusa* has demonstrated hepatoprotective and anti-cancer activity. By significantly decreasing the effects of DEN, *B. diffusa* may result in markedly improvment in lipid profile, renal and liver functions. Additionally, a histological and gross examination showed that the extract might decrease DEN's carcinogenic effects (171).

2.10.3 *Enicostemma littorale*

E. littorale blume a perennial herb of the family Gentianaceae often termed Nagajihva in Ayurveda. The leaves, root, and whole plant part are used in traditional practice in Ayurveda, Unani, Siddha, homeopathy naturopathy, and the tribal population of Gujarat for treating various disorders like fever, diabetes, stomach ache, and malaria (172, 173).

Figure 2.9: *E. littorale* plant

Scientific/taxonomical classification:	**Other names:**
Kingdom: Plantae	Sanskrit: Mamajjakah
Order: Gentianales	Gujarati: Mamejavo
Family: Gentianacae	Hindi: Chota chirayata
Genus: Enicostemma	Marathi: Kadvi-nayi, Mamijwa
Species: *Littorale*	Tamil:Vellarugu
Part used: Leaves, root and whole plant	Bengal: Nagajivha

Macroscopic characters

The dark green, sessile, 4 to 6.5 cm-long leaves have a lanceolate form, a sharp tip, and reticulate veins. Stems are 4-5 cm long, slightly greenish, and upright. Blossoms measure 4-5 mm in diameter, with an oval calyx measuring 1 mm, a white corolla measuring 7 mm, a tube of corolla measuring 5 mm, and lobes measuring 2 mm. Epipetallus filament white, anther yellow, ovary greenish, bicarpellary, style small, stigma lobed are the characteristics of stamens. Fruits have a 2 mm diameter, subglabase capsule-like shape, with bright yellow seeds. The entire plant has a bitter flavor.

Microscopic characters

In the transverse section, the leaf has prominent hemispherical midrib lamina. The whole midrib is dorsiventral in nature. A distinct epidermal layer composed of small squarish cells with a prominent cuticle. In mesophyll, calcium oxalate crystals are observed. Stomata are seen in epidermis. The stem shows single-layered epidermis, polygonal cells, with thick and smooth cuticles. Below the epidermis, two to three layers of loosely arranged cells contain chloroplasts and vascular bundles (174).

Chemical constituents

E. littorale contains many chemical components. There have been reports of the presence of swertiamarin, betulin, a triterpene sapogenin, phenolic acids like vanillic acid, syringic acid, p-hydroxy benzoic acid, protocatechuic acid, p-coumaric acid, and ferulic acid, as well as alkaloids like enicoflavin and gentiocrucine. Aspartic acid, L-proline, L-tyrosine, threonine, phenyl alanine, L-histidine monohydrochloride, methionine, isoleucine, Larginine monohydrochloride, DOPA, L-glutamic acid, tryptophan, alanine, serine, and 2-amino butyric acid are among the several amino acids found to be present in the plant.

Pharmacological activity

Traditional medicinal use

E. littorale has been traditionally used to treat a variety of diseases, including leprosy, diabetes, arthritis, stomachic, bitter tonic, laxative, back pain, fever reduction, and appetite loss as a "tonic." Its traditional uses include laxative, anti-inflammatory, antidiabetic, anthelmintic, and urinary astringent properties. *E. littorale* is administered in combination with other herbs in Ayurvedic treatment, particularly for diabetes. Indian traditional healers utilized it to treat liver disorders (175).

Therapeutic category

Antidiabetic activity

In clinical research, 84 individuals with type 2 diabetes received daily doses of 2000 mg of *E. littorale* in the form of ghavantis, an Ayurvedic tablet, for a duration of three months. In addition to considerably enhancing renal function, lipid profile, systolic and diastolic blood pressure, and pulse rate, *E. littorale* also decreased blood glucose and serum insulin levels [176]. *E. littorale* has been shown in several studies to have potential antidiabetic effect and to enhance lipid profile at a low dose of 0.5 g/kg. (177, 178). According to research by Mokashi et al., the flavonoid-rich fraction of *E. littorale* exhibits the potential as a treatment for type 2 diabetes mellitus as it can increase insulin sensitivity and glucose uptake through the IRS/PI3K/Akt pathway (179).

Hepatoprotective activity

An ethanolic extract of *E. littorale* significantly reduced the fat metabolism and demonstrated antioxidant ability and free radical scavenging capabilities to protect rats' livers from oxidative stress-induced liver damage caused by CCl_4 (180). In another study, aqueous extract showed hepatoprotective activity by preserving the structural integrity of the cell membrane of a hepatocyte and maintaining normal function of the liver damaged by CCl_4-induced hepatotoxicity in rats (181).

Significant antioxidant and hepatoprotective activities have been identified in the swertiamarin that was extracted from *E. littorale* in response to D-GalN-induced hepatotoxicity (182).

Anti-inflammatory activity

In a chronic model of rat hind paw edema caused by formalin as well as carrageen, the plant's alcohol extract at concentrations of 300 and 600 mg/kg and its ethyl acetate fractions at 25 and 50 mg/kg showed a strong dose-dependent anti-inflammatory effect (183).

Antimicrobial activity

E. littorale chloroform, ethyl acetate, aqueous methanolic, and hydro alcoholic extracts showed prominent antimicrobial activity against Bacillus subtilis, Staphyloccous aureus, Escheichia coli, Shigella sonni, Pseudomaonas aeruginosa, Proteus vulgaris, Aspergillus niger, and Candida albicans (184).

Anticancer activity

In Dalton's ascetic lymphoma (DAL), a methanolic extract of *E. littorale* exhibited anticancer activity. This action was either mediated by the extract's stimulation and activation of macrophages or by some cytokine product created inside the peritoneal cavity (48).

Because of its antioxidant capacity, *E. littorale* extract postponed and suppressed the oral cancer in hamsters with DMBA-induced buccal cancer (47). In the DEN-induced HCC rat model, the methanolic extract of *E. littorale* demonstrated anticancer action together with its antioxidant potential (185).