Chemically-Induced Hepatocarcinogenesis

4

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Contents

4.1 Introduction

The biology of the liver, the biological processes involved in cancer development, and the etiological factors involved in liver cancer development provide a focus on the early processes and signaling pathways important in primary liver cancer development. Perhaps, the most important point to consider is the cell population at risk for initiation of the cancer process in the liver. Since most hepatocytes are in G0 phase, first proliferation must be stimulated. Under normal conditions, single cell death is followed by replacement of that hepatocyte. One hypothesis is that cancer stem cells are bipotential and can be stimulated to proliferate [4]. Their (oval cells) outgrowth can occur under situations where a large percentage of the liver is damaged. The stem cells then differentiate into hepatocytes or cholangiocytes depending on the degree and duration of damage. Agents that cause extensive damage to the liver can result in neoplastic changes that are fetal in nature. A second hypothesis is that mature hepatocytes are the cell population at risk for early preneoplastic changes [5]. Mature hepatocytes can develop into focal areas of proliferation that in turn can become nodular areas of hyperplasia. In this case, both poorly differentiated, small cell lesions (that are primarily diploid) and large cell, more highly differentiated (tetraploid or higher ploidy) lesions develop [6]. Understanding the etiology, proliferative and differentiation cues for the liver, and the mechanisms of the carcinogenesis process in the liver is key to understanding the role of chemicals in the development of HCC.

Chemical, biologic, and physical agents can contribute to cancer development. Perturbations in single cells lead to the focal outgrowth of putatively preneoplastic lesions. The altered areas can evolve into nodular hyperplasia, focus in nodule pathology, and areas of frank malignancy [6]. To determine the contributions of chemicals to the carcinogenic process in the liver, a variety of animal models have been developed. Since the liver is the primary site for cancer induction in the bioassays used for carcinogen testing, there is a need for extrapolation of animal of neoplasms that arise

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at this site to man. The utility of defining common biomarkers for the conversion of benign to malignant transition will assist in developing appropriate inter-species extrapolation. The analysis of early lesions will permit assessment of the early changes that occur prior to the onset of clinically detectable disease to our understanding of HCC.

4.2 Liver Cancer Is an Important Biological Problem

Liver cancer is an important form of cancer worldwide ranking in the top ten in both incidence and mortality [7, 8]. Hepatocellular carcinoma (HCC) is the primary form of liver cancer. Primary liver cancer is the sixth most common form of cancer (750,000 cases/year) in terms of incidence [9]. In addition, it is the third most common cause of death (725,000 deaths/year) from cancer [10], with eighty percent of cases (and deaths) resulting from hepatitis B and/or C infection and occurring in developing countries. Surveillance Epidemiology and End Results [11], the National Cancer Institute's statistical unit, estimate that 35,000 new cases of liver and intrahepatic bile duct cancer were diagnosed and nearly 24,000 people will die from this disease in the US in 2015 [11]. Understanding the processes that contribute to the cancer development process is an important component of determining how and where certain compounds contribute to liver cancer development and progression. Environmental influences, including carcinogen exposure, are believed to contribute to the distinct geographical distribution pattern of primary liver cancer [12]. Another important cause of primary liver cancer in humans is viral with both HCV and HBV infection contributing to its incidence. According to NHANES 3, the number of individuals with chronic HCV infection is greater than 2 million in the part of the US population sampled [13, 14]. Chronic infection with hepatitis C virus (HCV) is known to be a major risk factor for development of HCC. In general, HCC develops only after 2 or more decades of HCV infection and in those with advanced fibrosis [14, 15]. Cirrhosis is also an important factor associated with the development of primary liver cancer and hence is an important control for liver cancer biomarker development, most liver cancer arises in the context of cirrhosis. In the US, less than 30 % of HCC is viral in etiology. Excess alcohol use and diabetes mellitus are independent risk factors for liver cirrhosis and are associated with liver cancer development in the US [16]. In addition, smoking may contribute to the risk of liver cancer development. The residual 10 % of attributable risk of HCC may be due to or influenced by hereditary metabolic disease factors (such as hemochromatosis). Although rare genetic disorders can contribute to liver cancer development, ethanol and dietary factors are known to contribute to its incidence and progression [2, 3]. The prevalence of liver cancer and its high mortality rate indicate the need for appropriate animal models of this disease in order to develop treatment and intervention strategies. In addition, the pathogenesis of primary liver cancer development for different etiologies needs to be better delineated. The influence of genetic background and environmental factors on neoplastic development is readily studied in rodent models of this disease.

4.3 Chemical Carcinogens

Carcinogenesis can be induced by physical, biological or chemical means. Agents that act to increase the incidence of cancer in appropriate organisms compared with concurrent and/or historic controls are considered carcinogens. The identification of a carcinogenic potential for an agent delineates the conditions of exposure (dose, time and duration) under which the agent may induce cancer. Animals are surrogate models of humans since they possess similar physiology and biochemistry. This similarity is not absolute; hence any hazard detected must be examined in the context of human relevance in order to understand the conditions of exposure that may pose a plausible risk to humans. Each human HCC is detected at different points along the pathogenesis continuum and is the result of distinct etiologies and pathogenesis. Several factors are important for cancer development including a loss of normal growth control with contributions from inhibition of apoptosis and enhanced but altered proliferation control [17]. In addition, an altered differentiation status can contribute to cancer development and progression. The morphology and certain aspects of the natural history of rodent and human cancer are coincident although the etiology and the exact molecular pathogenesis may diverge between rodents and man. Although several parallel pathways may be induced, the pathway for cytogenetic alterations observed in a specific cancer type is similar in rats, mice, and men. The latency period between initiation of early precancer changes in a single cell and their selection for malignant growth comprises the reversible stage of tumor promotion. In the human, exposure to dietary contaminants such as aflatoxins, as well as calorie overload, ethanol over use, and methyl deficiency can contribute to the risk of primary liver cancer. Certain metals (iron and copper) have been associated with an increased risk of primary liver cancer. Thus, a number of classes of chemical agents can increase the incidence of hepatic neoplasms depending on their dose and duration of exposure.

4.3.1 Genotoxic Carcinogens

Chemically induced carcinogenesis has been examined experimentally for nearly 100 years [18, 19]. Initial studies provided the compounds typically in the diet for extended periods of time. For example, the studies of Sasaki and Yoshida [20] demonstrated that chemicals could cause hepatic neoplasms in animals. Provision of o-aminoazotoluene in the diet led to liver neoplasms in rats. Similarly, Kinoshita [21] demonstrated that feeding 4-dimethylaminoazobenzene to rats resulted in liver neoplasms. These findings suggest that agents can be carcinogenic at sites distant from their initial application. Importantly, analogues of these agents have also been examined allowing some structural information to be gathered about the properties of agents that have a carcinogenic potential [22]. There is some tissue specificity for carcinogenic action as polycyclic aromatic hydrocarbons are not typically carcinogenic to the liver (except in some circumstances during the neonatal period), while they are to the skin [23]. Similarly, certain azo dyes, while carcinogenic to the liver, do not have this activity in the skin [24]. The agent 2-acetylaminofluorene but not its related regioisomer, 4-acetylaminofluorene, is carcinogenic in the rodent liver [25]. However, dialkyl nitrosamines and several analogs are cytotoxic to the liver and are carcinogenic in rodents and many other mammals [26]. These activities are dose dependent and high doses induce acute toxicity, while lower doses are tolerated but can result in neoplasms if the dose and duration of exposure is sufficient. Similarly, aflatoxin produced by the fungus Aspergillis flavus is acutely cytotoxic. This agent is also carcinogenic in all species examined, although the mouse is relatively resistant to its carcinogenic action [27]. A variety of other agents in food can also be carcinogenic to the liver including certain mycotoxins [28] in addition to aflatoxin (fumonisin in rodents) and pyrrolizidine [29] alkaloids (found in comfrey and riddelline). In addition, a dearth of antioxidants and a lack of lipotropes [30, 31] can lead to cancer development in the rodent.

4.3.1.1 DNA Adducts

This initial class of agents is capable of altering the genetic material either directly, through one of its metabolites, or through perturbation of the processes controlling its actions. Agents that modify the DNA can initiate the carcinogenic process [32]. Many of these agents can be metabolized to form DNA adducts or may directly form them. Alternatively, such agents can alter the methylation status of the DNA. In each case, the DNA is modified in a manner that results in heritable changes. In the case of DNA adducts when they are coupled with cell proliferation mutations can result [33]. Such mutations can alter the function of selected genes, in some cases inactivating them and in other cases enhancing their activity [33]. The dose and duration of exposure of an agent is an important contributing factor to understanding the carcinogenic risk of an agent at doses to which humans are exposed. Many agents with a carcinogenic potential can be metabolized to an electrophilic form. These reactive metabolites can bind to cellular nucleophiles including DNA, RNA, proteins, and lipids [24]. The biological consequences of these actions differ. Early studies by Miller and Miller [34] demonstrated that certain carcinogenic agents did not directly bind to proteins, but that following incubation of the compound with tissue extract, the compound or some derivative could be found bound to protein in normal liver but not in the resulting neoplasm. This metabolic activation or reactive metabolite formation would lead to the determination that the cell could metabolize some compounds to a reactive form. For example, AAF is metabolized by ring hydroxylation [35] and by *N*-hydroxylation [36]. The *N*-hydroxy metabolite is more carcinogenic than the parent AAF [24]. The *N*-hydroxy AAF is further metabolized by esterification with glucuronyl, acetyl, and sulfate groups. Although conjugation can lead to inactivation of reactive metabolites, in certain cases it can result in more reactive agents with facile leaving groups. This is the case for some esters of *N*-hydroxy AAF [24]. In addition to the formation of reactive metabolites, certain agents can form free radicals [37]. Free radicals have no charge, but have an unpaired electron that makes them reactive. This process can be facilitated by the presence of free iron or copper. Endogenous processes can form free radicals and metabolism of certain carcinogenic agents can also lead to their generation [38]. Many agents with a carcinogenic potential can be metabolized to reactive forms providing a mechanism to understand species differences and individual risks. Understanding the structural basis for metabolic activation permits the prediction of agents that are likely to be directly genotoxic or that can be metabolized to a genotoxic form. In addition, it generates a physicochemical basis for understanding mutagenesis at specific sites in the DNA and in specific tissues. Careful analyses of structures of agents that are positive in rodent bioassays have yielded reactive groups that yield structural alerts for carcinogenic risk [39, 40].

4.3.1.2 Mutations and Their Consequences

The reaction of electrophilic substances with the DNA results in physicochemical changes in the DNA. The high prevalence of cancer in individuals with an inability to remove DNA adducts in DNA repair deficiencies indicate the important role of DNA damage in cancer induction [41]. Similarly, the high incidences of mutations in selected genes in animal models of cancer further demonstrate that DNA damage is the basis of early cancer development [42]. Alkylation of DNA can occur by carcinogenic agents that can be metabolized to reactive

forms. In this case, the reactive metabolite can covalently adduct to the DNA [43]. For example, aflatoxin B1 can be metabolized to 8,9 epoxide of AFB1, which then binds to N7 guanosine leading to mutations [44]. Mutation of G to T can occur at multiple sites, most notably at 249Ser of P53 [45]. Methylation, ethylation, and other alkylations can occur with each of the bases as well as the sugar and phosphate backbone [46, 47]. Direct acting electrophiles can bind to the N7 of guanine, while softer electrophiles can bind to the ring oxygens of the bases. Formation of bulky adducts can occur on the purine ring, while small alkylations can occur more ubiquitously. At lower exposures, selective alkylation can occur, which may or may not be repaired. The presence of DNA adducts and the repair of these lesions can result in mutation. As the adduct burden increases with increased dose/duration of exposure, the repair can be more extensive and over a greater span of the DNA. In addition, as dose/duration increases more cell types may become involved as metabolism shifts and conjugation reserves are depleted. Repair can outpace adduct conversion to mutations under some circumstances. When the lesion is repaired, either the base is removed or a larger segment of DNA is removed. Each of these processes can have different rates and consequences and each is dose dependent.

Point mutations, frameshift mutations, chromosome aberrations, and aneuploidy can occur following chemical administration. Because the degree of adduct formation, the site of adduct formation, the ability of adducts to be repaired, and the degree of metabolism to reactive forms, differential activity can be seen in individual cells, tissues, organisms and species. One consequence of the presence of DNA adducts is cell death. Apoptosis is observed at lower concentrations followed at higher exposures and degrees of damage by necrosis. Direct-acting carcinogens are reactive without requiring metabolic activation and are often carcinogenic at the sites of exposure in multiple species [48]. Methylation or ethylation of DNA can lead to base mispairing [46, 49]. Because these simple alkylations are similar to or can result from endogenous processes, they are not as actively repaired. In part, the more persistent DNA adducts/lesions are the ones that have an important mutagenic consequence. For example, ethylating agents can adduct at O6 alkylguanine and O4 alkylthymidine. The O6 adduct is readily repaired, while the O4 adduct is more persistent leading to base mispairing with different consequences for both lesions [50, 51].

The consequence of bulky adduct presence is to block DNA synthesis resulting in noncoding [47]. However, the DNA synthetic machinery can bypass such lesions placing in its stead the most abundant nucleotide, generally an adenine [52]. Since bulky adducts typically occur at guanines, this is a useful endogenous strategy that can however result in more marked consequences when more than one base is affected or the adduct was not at guanine. Using 2-AAF as an example, the parent is not mutagenic, but it can be metabolized to the sulfate ester that is highly reactive; binding to the N7 of guanine as well as the N3 of guanine [24]. In contrast to the formation of a covalent bulky adduct by 2-AAF that distorts the DNA structure, 2-aminofluorene, which also forms bulky adducts at the same sites, sits outside of the helix and does not distort it. As a consequence, 2AF can induce point mutations, while 2AAF can lead to frameshift mutations [53]. Biological consequence of the presence of DNA adducts is a function of their persistence in the DNA [54] and impacts their tissue and species specificity. The persistence of DNA adducts in viable cells has consequences when cell proliferation occurs to fix the mutation before repair can occur [33]. Once the mutation is fixed, its location in the genome, the expression of that DNA and the importance of the affected gene in that stage of the differentiation of the cell, both impact its consequent mutation and the ultimate consequence of a given adduct. Although susceptibility to cancer induction can be modified by polymorphisms in DNA repair genes [41], carcinogen metabolism [55], and immune system [56] differences, genes that regulate cell growth and proliferation are more frequently the targets of carcinogens. Both protooncogene and tumor suppressor gene function can be altered by carcinogen exposure [57–59]. For example, oncogenes such as Ha-ras can be activated by a single point mutation [60]. Activation of Ha-ras is an important mechanism of HCC induction and development in the mouse [42, 61], but not in rats or humans [19]. In the liver, activation and mutation of β-catenin (and possibly axin) is an important aspect of some types of liver cancer [62, 63]. Similarly, mutations in HNF1 can result in loss of differentiation status as evidenced by loss of expression of a number of drug metabolizing genes in the neoplasm. Although mutations have been observed in a number of genes in HCC development and progression, only a few genes have been described with non-random mutations. Etiologic agents have been examined with respect to the resulting mutations observed in specific genes including p53, β-catenin and HNF1. There appear to be multiple pathways that can lead to HCC initiation and progression [63].

Endogenous DNA modifications can be perturbed and this perturbation can contribute to chemical carcinogenesis. Hydroxylation of DNA bases can also occur both through endogenous processes and by certain DNA damaging agents [64]. Repair processes for oxidative damage are pervasive in most cell types nonetheless oxidized bases can persist [65]. Although all of the DNA bases can be oxidized, the most common are 8-hydroxy deoxyguanosine [66] and 5-hydroxymethylthymine [67]. These oxidative bases likely arise through endogenous processes [68] and they are readily repaired. The most prevalent endogenous modification of DNA is methylation of deoxycytidine [69, 70]. Chemical carcinogens can perturb this process by adduct formation, altered one-carbon pools, single strand break formation, or

inactivation of the enzymes involved in the methylation process [71]. Diets deficient in lipotropes can result in marked steatosis followed in time by HCC formation in rodents [31]. Methyl deficient diets can result in DNA hypomethylation. Global hypomethylation results in re-expression of genes in general, while hypermethylation results in their silencing [72]. Perturbation of nucleosomes, of minor and major groove protein binding, and the DNA repair process can likewise lead to DNA perturbations. The presence of a DNA adduct does not mean that a mutation will occur, but it does increase the probability. Both endogenous and exogenous derived DNA alterations can result in cancer initiation [64].

4.3.1.3 The Role of Cell Proliferation in Cancer Initiation

The presence of DNA adducts coupled with cell proliferation can lead to mutation. This process is called fixation wherein the mutation is fixed when an adduct or other DNA alteration persists through a cycle of DNA synthesis [33]. Thus, the rate of cell proliferation and DNA synthesis can impact DNA damage [73]. In situations where repair processes are normal, high rates of cell proliferation can still lead to mutations. Inherited defects in DNA repair lead to an increased risk of neoplasia [47] in many cell types especially in the GI tract with its high rate of exposure to potentially mutagenic agents and its high rate of proliferation. Hepatocytes turn over slowly by comparison except in circumstances of persistent inflammation induced by hepatitis (viral, alcohol, or drug induced). DNA polymerases are not completely faithful in their replication of the DNA [74, 75]. Since a variety of types of DNA damage can occur, many processes exist to remedy their activity. Excision repair can remove either a modified base or nucleotide. The presence of an adduct will result in excision and repair with more bases removed and potentially misrepaired for nucleotide excision compared with base excision repair. Single strand breaks are readily repaired. The repair of double strand breaks is more problematic [76] and a nonhomologous end joining process is used that is error prone [77]. Mismatch repair can occur when bases are mispaired or when it appears that they are mispaired due to the presence of a DNA modification [78]. Perturbation of the mismatch repair process can result in mutations. Larger DNA damage including amplifications, deletions, and aneuploidy can occur. Agents that lead to these lesions contribute to the carcinogenesis process by altering gene dosage of critical genes and/or perturbing their expression. Although mutations alone do not lead directly to neoplasia, they can contribute to the process when they occur in genes critical for cell survival, proliferation, apoptosis, and differentiation status.

4.3.2 Non-genotoxic Mechanisms of Chemical Carcinogenesis

A variety of compounds other than mutagenic agents can contribute to liver cancer development. These agents have in common the ability to alter cell survival either by increasing cell proliferation or decreasing apoptosis. Agents that have this activity include those that cause cytotoxicity and those that perturb signaling pathways associated with growth factors, some of which act through nuclear receptors [19, 79]. Certain agents are cytotoxic at either high doses or with chronic administration [80]. These agents such as chloroform do not pose a risk when exposure occurs below the threshold for cytotoxicity [81]. For example, chronic high dose ethanol consumption results in high levels of acetaldehyde generation [82]. Aldehydes can covalent adduct to proteins through Schiff base reactions and with other cellular components. In addition, CYP2E1 that generates acetaldehyde is loosely coupled to oxidoreductase resulting in the generation of reactive oxygen species. Acetaldehyde can result in exocyclic etheno DNA adducts [83]. The resulting oxidant damage and lipid peroxidation can lead to chronic hepatitis. In addition, the marked steatosis that can occur in conjunction with excess alcohol consumption may perturb the insulin/IGF1 signaling pathway of cell survival in the liver [83]. Similarly, the one carbon cycle with eventual folate/choline depletion can contribute to cancer development [84]. Ethanol over consumption in conjunction with HCV increases the risk of cancer development [85]. In addition, alcohol abuse in the context of hemochromatosis increases both cirrhosis and HCC risk [86]. In part this may be due to increased oxidant stress in the presence of both increased lipid deposition and increased iron. Low alcohol intake does not appear associated with an increased risk of HCC, while higher levels are associated with an increase in risk of both cirrhosis and HCC [87]. In some parts of the world, alcohol is made with moldy food staples containing other liver toxins that can compound the problem. Similarly, intake of high levels of iron in conjunction with alcohol can similarly exacerbate the oxidant stress in the liver leading to cirrhosis. Since cirrhosis is associated with more than 60 % of HCC in the human [8], this is an important pathway through which ethanol contributes to primary liver cancer development.

Studies in animal models indicate that agents that act through selected nuclear receptors are associated with the ability to regulate cell proliferation/survival, apoptosis, and differentiation can promote tumor development [18, 19, 79]. Such agents can promote the outgrowth of cells with genetic damage into preneoplastic lesions and hence can under certain circumstances of exposure increase the incidence of

hepatic neoplasia in rodents and humans. Tumor promoting agents are believed to alter the balance between proliferation and apoptosis in initiated cells relative to the normal surrounding cells [88, 89]. Studies with prototypical hepatic tumor promoting agents including phenobarbital, PPARα agonists, and ethinyl estradiol indicate that a generalized mitosuppression of non-focal hepatocytes is an early and sustained activity of such agents. In addition, reversible alteration of gene expression is associated with tumor promotion. Furthermore, tumor promotion is reversible and exhibits a threshold for the selction of initiated cells [27].

4.3.2.1 Phenobarbital

Phenobarbital and related agents are not genotoxic, yet they can result in the development of cancer in susceptible organisms [90]. While selected mouse strains can develop neoplastic lesions following chronic exposure to Phenobarbital or related agents, certain rat strains can develop adenomas and rarely adenocarcinomas after chronic exposure. At therapeutic doses, man does not appear susceptible to liver tumor development with chronic Phenobarbital administration (c.f. [91]). Initiation-promotion studies indicate that Phenobarbital has a promoting action [92]. Importantly, a dose dependent promoting activity is observed that exhibits a threshold [92, 93]. Interestingly, phenobarbital and related agents can increase the background proliferation rate transiently in the liver [94]. Specifically, Phenobarbital increases the focal relative to the non-focal hepatic labeling index [95]. Importantly, Phenobarbital promotes eosinophilic, but not basophilic lesions [96]. In addition, a mitosuppression can be observed in the non-focal hepatocytes [97], while the discrete focal hepatocytes have an increased rate of proliferation compared with control hepatocytes or the surrounding normal appearing ones [98, 99]. Phenobarbital increased DNA synthesis and decreased apoptosis in hepatocytes in vitro [99, 100]. Studies with Phenobarbital showed that only the promoting dose resulted in changes in gene expression associated with apoptosis suppression and cell proliferation, while dose dependent changes in selected drug metabolizing agents was observed [100]. It has been suggested that the increased growth rate of the eosinophilic lesions compared with the surround is due to the decreased responsiveness of the altered focal cells to TGFβ family members that are responsible for apoptosis [101, 102]. IGF2R modulates cell proliferation in response to insulin and IGF family members and apoptosis in response to TGFβ. The expression pattern is altered in focal compared with non-focal areas of the liver for IGF2R and TGFβR [102, 103]. Phenobarbital can promote those initiated cells with a low level of TGFβR, while increasing ligand expression in surrounding hepatocytes [102–104]. TGFβ is a potent mitoinhibitor of hepatocytes and phenobarbital increases this ligand in non-focal hepatocytes and TGFβ is increased at the protein level during mitosuppression induced by Phenobarbital exposure [103, 104].

Previous work has demonstrated that Phenobarbital-like compounds cause the increase in gene expression of a number of genes including CYP2B1/2 [105] and is transcriptionally regulated [106]. The tumor promoting action of this type of agent is correlated with the induction of CYP2B1 [107]; therefore, the mechanism underlying tumor promotion by phenobarbital and related compounds has been associated with the mechanism of CYP2B1 induction. Since a structurally diverse group of compounds act in a similar manner, it has been under consideration as to whether a receptor was responsible for this action. The constitutive androstane receptor (CAR) plays a role in the induction of CYP2B family members [108]. Agents that act to alter the metabolism of testosterone derivatives, specifically androstenedione, can alter endogenous activation of the CAR receptor [109]. There are two forms of CAR and Phenobarbital can displace the ligand from CARβ [109]. Agents such as phenobarbital activate the CAR receptor to perturb gene expression [110–113]. Studies in knock-out mice indicate that certain genes are expressed or repressed when the CAR receptor is present while a separate set is affected when it is not present [113, 114]. It is clear that CAR is associated with the gene expression acutely associated with phenobarbital exposure, but how this is associated with tumor promotion is unclear. CAR knock-out mice have been used to confirm that CYP2B expression is dependent on CAR [112]. Nonetheless, CAR knock-out mice are resistant to Phenobarbital induced hepatic tumor promotion [114]. Interestingly, chronic Phenobarbital administration results in DNA hypomethylation that is CAR-dependent [115]. The mouse strain susceptible to spontaneous and chemical carcinogenesis is sensitive to promotion by Phenobarbital, while the resistant strain C57B6l6 is resistant. The tumors arising spontaneously in C3H mice are Ha-ras-mutation positive [116], lack CAR, and are not promoted by phenobarbital [117]. These tumors lack CAR, but express β-catenin and are promoted by phenobarbital [117, 118].

Nuclear receptors are frequent targets of drugs and of environmental chemicals. The function of these ligand activated transcription factor receptors is to regulate endogenous metabolism; hence, homeostasis can be perturbed when their function is modulated. Drugs and environmental chemicals can alter the effects of multiple nuclear receptors due to their broad and overlapping substrate specificity. The interaction of nuclear receptors with coactivators and corepressors provides another level of control of their function within cells. The CAR is a nuclear receptor that regulates the expression of drug metabolizing enzymes [110–113]. CAR is an important regulator of many genes involved in drug metabolism including a number of P450s,

phase 2 enzymes, and transporters. Species specificity in response to CAR agonists have been detected although that of Phenobarbital (PB) is only 1.5 fold (the human is less sensitive) and human CAR is not sensitive to the same bile acids as mice [119]. The mode of action of phenobarbital for hepatic tumor promotion has been reviewed [120].

4.3.2.2 Estrogenic Agents

In the human, certain estrogenic formulations can result in adenoma development and rarely in carcinomas. Estrogenic agents can be carcinogenic to rat liver, but tend to inhibit cancer development in the mouse liver. Estrogenic agents are clearly promoting toward the rat liver, but the basis for this action is unknown [121–126]. Estrogenic agents can increase cell proliferation in the rat liver and can induce focal proliferation with mitosuppression in the surrounding hepatocytes [127, 128]. Examination of altered gene expression during the mitosuppression observed with chronic ethinyl estradiol treatment demonstrated an increase in TGFβ and IGF2R/M6PR without a change in myc or CEBPα levels [129, 130]. The increase in TGFβ leads to CKI induction that may lead more directly to the mitoinhibition [131]. Similarly, EE exposure induces TGFβ1 expression. Hepatocytes with decreased levels of TGFβR are at a selective growth advantage compared to cells without this characteristic [102]. Hepatocytes that survive TGFβ exposure have decreased HNF4α activity, but increased fos, jun, myc, and ras levels [132]. Oncogene expression can confer tumor characteristics that TGFβ responsiveness can limit [133]; thus, loss of TGFβ responsiveness is permissive to acquisition of the tumor phenotype. In certain, hepatocarcinogenesis protocols administration of tamoxifen results in the regression of a component of the lesions suggesting an estrogen- (and estrogen receptor-) dependence for those lesions [134–136].

Sustained estrogen receptor activation is known to increase the incidence of liver neoplasms in animals and humans [137–140]. An increase in adenomas was observed in young women taking an early form of oral contraceptives (with a higher dose and different formulation to the current available forms). Rarely, HCC were observed in women taking early formulations of estrogens for oral contraceptive purposes [90, 137]. Estrogenic agents are effective tumor promoting agents in the rat liver and their action to initiate cells through catechol estrogen formation [138] or induction of aneuploidy [139] needs to be assessed at physiological concentrations. For example, certain estrogenic agents can cause a burst of increased proliferation in the rodent liver [140]. This transient increase in cell proliferation is associated with stimulation of the estrogen receptor [124, 128]. There is a mitosuppression in the normal appearing hepatocytes, while the focal, putatively, preneoplastic hepatocytes have a sustained increase in proliferation [128, 129, 141]. Although the incidence of HCC in humans following chronic (greater than 5 years) estrogen exposure is low, the incidence is definable and permits one to anchor the incidence in rats where a clear carcinogenic response to high dose, potent carcinogens is observed under defined exposure conditions. This observation permits more accurate risk assessment from animal hazard identification studies. Extrapolation of potential for risk across species could be performed using the low incidence human tumor data as an anchor for the calculations.

Estrogenic agents have a carcinogenic potential at several sites including the mammalian liver [90]. Estrogenic agents are known liver tumor promoting agents in the rat [122, 123, 135] and in the human [142]. There is an apparent threshold for promoting action [142–144]. The mechanism of tumor promotion is not known although an increase in focal proliferation and a decrease in focal apoptosis have contributing roles. Although tamoxifen has an estrogenic action in the liver that may contribute to its promoting action, the phenotypes of the liver lesions that arise with mestranol and tamoxifen treatment differ [145]. In addition, tamoxifen can inhibit the development of mestranol promoted lesions indicating a divergent mechanism of action [124, 135]. The mechanism of estrogenic/antiestrogenic action for tamoxifen is only incompletely understood. While this action may in part be due to an interaction with the estrogen receptor, other factors may also be involved. For example, antiestrogens bind to sites other than the estrogen receptor including covalent binding to P450s [146], tubulin [147], and other interactions with "antiestrogenic binding sites" [148]. In addition, antiestrogens inhibit protein kinase C and calmodulin activity [149]. In addition, antiestrogens alter the production of several peptide growth factors including TGFα [150], TGFβ [151], and IGF1 [152], and affect some calcium dependent processes [153]. Estrogenic and antiestrogenic agents additionally alter cholesterol metabolism [148]. Tamoxifen appears to promote the diploid hepatocyte population [154], similar to ethinyl estradiol [155]. The triphenylethylene antiestrogens have differential effects on the hepatic proliferative rate in the rat [156, 157]. In the liver itself, triphenylethylene antiestrogens have an estrogenic action; however these drugs are mixed agonist/antagonists in a species, strain, tissue, gene, and hormone status basis.

Mestranol is a synthetic steroidal estrogen that is metabolized [158] to the potent rat liver tumor-promoting agent, ethinyl estradiol [150]. Mestranol use in oral contraceptives was associated with an increased incidence of hepatic adenomas and a few HCCs in young women [90, 159–161]. Studies in rats indicate that mestranol and its active metabolite ethinyl estradiol promotes the development of previously initiated liver cells through induction of elevated cell proliferation levels. Mestranol does not have a marked effect on P450 profiles in the liver [162], but it can cause cholestasis [163] and clearly enhances liver growth

[162]. Chronic administration of ethinyl estradiol results in mitosuppression of liver cells with selection of resistant hepatocytes for outgrowth [127, 128] and this in combination with its ability to increase cell proliferation [124, 164]; is believed responsible for its tumor promoting properties [121–124, 127, 128, 144, 165, 166]. Tumor promotion by ethinyl estradiol is effected through the estrogen receptor, since it can be inhibited by tamoxifen [135, 136]. At low doses and for short durations of administration, ethinyl estradiol can increase hepatic hypertrophy and a transient increase in cell proliferation [124, 164], while with chronic administration a mito-inhibition is observed [124, 127].

4.3.2.3 PPAR Agonists

The peroxisome proliferators activated receptors (PPARs) are members of the steroid/retinoid receptor superfamily. Three mammalian nuclear receptors of the PPAR class have been isolated including PPAR alpha, delta, and gamma [167]. The PPAR alpha receptor is a ligand activated nuclear transcription factor that is responsible for the regulation of lipid catabolism [168]. The PPARα receptor and the retinoid X receptor nuclear receptor (RXR) can heterodimerize and bind to peroxisome proliferator response elements (PPRE) to alter the transcription of genes including those that are involved in lipid metabolism [169–171]. Peroxisome proliferators include structurally diverse chemicals that can activate the PPARα receptor including industrial chemicals, plasticizers, herbicdes, and some lipid lowering drugs [171–173]. Agonists of PPARα induce peroxisome proliferation [173, 174], hepatomegly [173, 175], cell proliferation [173, 176, 177], and liver neoplasms in rodents [171, 177, 178]. Although numerous theories exist regarding the mechanism of hepatocarcinogenesis in the rodent following chronic exposure to PPARα agonists, the mechanism is not fully understood. In general, PPARα agonists are not genotoxic and demonstrate a promoting activity [179]. Similar to other receptor-mediated, non-genotoxic rodent carcinogens, PPARα agonists, including WY14, 643, methylclofenapate, Nafenopin and clofibric acid increase the TGFβ1 ligand, while these agents excluding clofibric acid increase expression of the IGFII/Man6P receptor [180]. Sustained PPARα receptor activation is required for induction of liver tumors, since PPARα knock-out mice do not develop hepatic neoplasms even after a one year exposure to a PPARα agonists [181]. Similarly, peroxisome proliferation and gene expression regulated by PPARα are not altered by exposure to PPARα agonists in the knock-out mice [181]. The lack of carcinogenic action in the human relative to the rodent has been explored with human PPARα receptor knock-in mice [182]. Although the precise mechanism of the hepatocarcinogenesis of PPARα agonists in rodents is not fully understood, it appears dependent upon PPARα receptor activation [183–185]. Thus, PPARα agonists are non-genotoxic carcinogens that function through receptor activation [186] and appear to be carcinogenic in the rodent, but not in primates.

4.3.2.4 AhR Agonists

The aryl hydrocarbon receptor (AhR) is structurally distinct from the nuclear receptors, and contains a bHLH-PAS domain [187–189]. The ligand bound receptor interacts with arnt and this dimerization partner regulates the expression of specific genes. The ligand-binding domain of AhR is within the PAS domain. The PAS domain of AhR binds ligand, binds to a repressor (probably hsp90) and has some of the interaction function with arnt. The function of excess AhR ligand may be to block the function at the other sites of arnt binding. The low affinity allele of AhR found in some mouse strains is similar to that observed in humans [190–192]. In addition, the transactivation domain part of AhR is highly divergent with only a 60 % identity between rat and human [192]. This suggests that human gene expression in response to an AhR ligand will differ qualitatively as well as in magnitude from that in rats and mice containing the high affinity AhR allele.

TCDD and related agents can induce a range of toxicities that may be mediated by AhR [187]. Dioxin lacks any genotoxic activity, yet increases the incidence of hepatic neoplasms in rats [193]. Dioxin can cause marked cytotoxicity at higher doses and this may contribute to its tumor promoting activity. Activation of arylhydrocarbon receptor (AhR) by 2,3,7,8 tetrachlorodibenzoparadioxin (TCDD) and related compounds of the furan and PCB classes results in alterations in gene expression including an induction of CYP1A1 [194]. Although the role of CYP1A1, if any, in tumor promotion is unclear, CYP1A1 expression is a useful marker for ascertaining exposure to this class of compounds. Over 100 genes may be regulated by AhR activation [195]. Genetic differences between mouse strains have been used to demonstrate that TCDD-mediated liver tumor promotion is AhR dependent [196]. Transgenic mice overexpressing a constitutively active AhR are more sensitive to diethylnitrosamine-initiation resulting in a higher yield of preneoplastic lesions than the genetically matched control animals [197]. Knock-out animals have been generated [198–200]. The gene expression patterns [201] and toxicity [202] have been examined after acute but not chronic administration of TCDD to the knock-out animals. The genetic background of the animal is important for its potential to develop neoplasms in response to TCDD administration. Since a selection for neoplastic clones resistant to the toxic insult that permits their outgrowth occurs, Ha-ras mutated hepatocytes might be resistant to AhR dependent toxicity. Liver tumors from TCDD treated mice have a high incidence of Ha-ras mutations [203] suggesting that the C3H background would be exquisitely

sensitive to TCDD induced tumor promotion [119]. When IL1-like knock out mice are generated on an AhR knock-out background, hepatic tumor induction by TCDD is decreased [203] similar to the dual receptor dependence on the IL1R and AhR receptor for TCDD-induced hepatotoxicity.

Initiation-promotion studies in the rat [204, 205] indicate that there is a threshold for the promoting action of TCDD and related compounds. A variety of studies indicate that TCDD causes a generalized mitosuppression in the liver [206, 207]. However, an increased cell turnover in focal lesions was noted relative to the surrounding liver [208, 209]. The initiated cell population is resistant to apoptosis [209, 210]. Interestingly, the AhR null hepatocytes both secrete TGFβ ligands and are quite sensitive to the apoptosis induced by TGFβ [210], indicating that AhR deficiency leads to increased TGFβ ligand production wherein selection for resistance to its apoptotic effects would permit promotion. Perhaps, TGFβR or processing of TGFβ through IGF2R would confer selective growth advantage to AhR−/− mouse hepatocytes that secrete TGFβ ligands. The AhR null mice have been used to demonstrate that the gene induction profile associated with AhR activation are altered [201] and the acute toxicities associated with AhR activation are diminished [202]. For example CAR is increased by AhR activation [211], while growth hormone receptor and janus kinase 2 are decreased [212]. Future studies should address the question of carcinogenicity in mice with AhR overexpressing and null alleles on different mouse strain backgrounds. In the human, exposure to TCDD has been associated, but not causally linked to an increased cancer risk [213, 214]. In part, the human AhR receptor is less sensitive to activation by AhR ligands [192] and in part, the exposure level in humans has been below that required to cause sustained tumor promotion [214]. Other agents in the class including certain of the polychlorinated biphenyls and the tetrachlorofurans may act in part through an AhR-dependent mechanism. Each agent has a unique contribution of AhR, CAR, and ER-dependent activity, as well as other actions including cytotoxicity that may contribute to its carinogenicity in rodents and provide a potential risk to the human. Certain exposures to mixtures of PCBs and furans have been associated with an increased risk of human liver disease and cirrhosis [215], but a causal link has not been made to cancer. Even in worker populations, the low incidence and lack of consistent dose trend prohibits the conclusion of causality [216]. The risks at high dose exposure differ from the risks posed by ambient exposures, sine multiple modes of action occur at the higher exposures.

4.3.2.5 Ethionine

Ethionine is an antimetabolite of the amino acid methionine when administered in the diet for extended periods can result in the development of liver cancer in rats [30]. This was the first example of direct interference with the metabolism of a normal metabolic constituent, resulting in the development of cancer. Ethionine induces marked steatosis that progresses to NASH, cirrhosis and HCC [31, 217]. Its ability to disturb one-carbon pools (rats are ten times more sensitive than humans to choline deficiency), folate metabolism, and to induce steatosis is similar to alcohol-induced changes that progress to cirrhosis and ultimately to HCC. This compound interferes with methylation causing hypomethylation upon chronic administration [217]. This agent is not used in the human.

4.4 Pathogenesis of HCC

The pathogenesis of human HCC has been examined extensively [6–8, 218]. Generally, the neoplasms are detected at late stage when many concurrent genetic changes are apparent. Tracing the earliest genetic changes in clinical samples has been limited. Studies using CGH arrays and gene expression analysis indicate that multiples pathways and multiple mechanisms lead to HCC development and progression due in part to different etiologies and time during pathogenesis of clinical detection. Primary liver cancer associated with cirrhosis evolves from precancerous lesions. Dysplastic nodules have variable degrees of atypia and can exhibit a focus or nodule in nodule appearance that can range from normal appearing to neoplastic in appearance. The formation of dysplastic nodules is not required for HCC development. Large cell dysplasia appears to be a response to injury and is not strictly a preneoplastic lesion although it is associated with an increased risk of HCC in a cirrhosis background of more than 3 fold [6]. On the other hand, small cell dysplasia seems more characteristic of preneoplastic change with greater than a 6 fold risk [6]. These small cell dysplastic cells are more diploid and less differentiated in character than the large cell dysplasias.

4.4.1 Rodent Models of Hepatocarcinogenesis

Examination of the epidemiology of liver cancer in humans indicates that both genetic and environmental factors are involved in the etiology and evolution of this disease. Studies in rodents can provide insight into the various factors involved in liver carcinogenesis. Early studies on rodents exposed to carcinogens indicated that male rodents are more likely to develop liver tumors [219, 220]. Rats, although relatively resistant to the spontaneous induction of liver neoplasms, will develop hepatic tumors later in life with a sex-bias in incidence that differs between strain and study [221]. This compilation of strain background effects on spontaneous liver tumors in rats suggests that females

have a slightly higher rate in Charles River CD, Osborne-Mendel, and Fischer rats and the incidence in males being marginally greater in the Wistar strain. Hepatic tumors can be readily induced in the rat by a variety of carcinogenic agents, with the male generally more sensitive than the female. The cancer bioassay is performed in 2 species of rodent, the rat and mouse. The sex specificity of liver tumor induction is, however, carcinogen specific due in large part to the sex dependence of the metabolic pathways.

4.4.2 Rat Models

The rat liver has been used extensively as a model of the carcinogenic process [5, 17]. Three basic protocols with numerous variations have been described including resistant hepatocyte model, neonatal rat model, and the partial hepatectomy model. These models couple carcinogen administration with a period of rapid cell proliferation due to the intrinsic growth of the tissue in the neonate, the wave of proliferation that occurs following surgical resection, or the extensive necrosis induced by excessive carcinogen administration. These studies can be used to examine very early changes in the pathogenesis of preneoplasia in the rat liver. The initiation-promotion-progression (IPI) model [222], the Solt-Farber model [223], and transgenic [224] rat models can be used to analysis later focal hepatic lesions, adenomas and carcinomas. The utility of the rodent as a model lies in the ability to assess the changes associated with early premalignant changes that would not be detected in clinical samples that present late in the progression process. In addition, rodents can be used to model gene-environment interactions in a controlled manner. Thus, the early premalignant changes, as well as the initial stages and pathways in progression of primary liver cancer are tractable in rodent models, while human cases are more amenable to analysis of later progression.

The rat has been used extensively as a model in which to examine the process of liver cancer development and to ascertain which compounds can influence cancer development in the liver. Studies by Bannasch [225] indicate that two pathways that evolve toward HCC in the rat are thyroidmimetic and insulinmimetic (insulin signaling pathway) with resulting glycogen accumulation phenotype). With progression, a shift from anabolic to catabolic glucose utilization occurs in the insulin dependent signaling pathway. Similarly in humans, diabetes mellitus predisposes to HCC development as an independent risk factor [16]. This effect is observed in livers of rats treated with Phenobarbital and related types of agents that promote eosinophilic lesions, while a thyroid like effect is observed for the basophilic lesions that arise with PPARα agonist administration [225]. Although PGST has been used as a marker of putatively preneoplastic lesions in the rat and is increased in expression in single cells following carcinogen exposure, in focal lesions with promotion, and in some neoplastic nodules and neoplasms, a deficiency of glucose 6-phosphatase expression may be more representative of hepatic lesions that will progress to neoplasia [225, 226].

Analysis of the gene expression changes across the carcinogenesis process and especially in preneoplastic lesions or following carcinogen exposure can illuminate the processes impacted by carcinogens. Recently, gene expression analysis has been applied to gain a clearer understanding of the changes that accompany liver cancer development in the rat. Many of these studies have been performed using variations on the Solt-Farber selection model for rat liver cancer induction [223]. Preneoplastic lesions have a higher level of expression of genes that are anti-apoptotic (p53, NK-kB and Bcl-2 pathways) and pro-proliferation [226]. Proliferation gene changes are also common in liver tumors, while apoptosis was decreased [227, 228]. Early nodules demonstrate a decrease in both growth hormone receptor and growth hormone binding proteins [229]. Specifically, IGF2 is expressed during liver cancer development, while IGF1 is decreased during liver cancer development [230]. These more fetal-like gene expression patterns are observed during early tumor development [231]. The increased expression of TGFα and HGF and their respective receptors, EGFR and met, observed in early nodules is lost with neoplastic progression [232]. Gene expression analysis demonstrates many genes in common between neoplastic nodules and HCC with only a few genes uniquely observed in HCC [226, 232].

4.4.2.1 Multistage Nature of Cancer Development

Molecular analysis of the pathogenesis of the natural history of liver cancer induction and progression has been extensively examined in the rodent. In the rat, single hepatocytes aberrantly expressing glutathione S transferase P (GSTP) can be observed within two days of carcinogen exposure [233–238]. Under many conditions, GST expression has been suggested to represent a population of initiated hepatocytes in the rat liver [235, 236, 238]. This is true for several types of genotoxic carcinogens including diethylnitrosamine [233, 238], an alkylating agent, aflatoxin B1 [233] that results in the formation of bulky DNA adducts, and choline deficient diet that result in depletion of methyl pools [237]. Single GSTP expressing hepatocytes are found in a dose-dependent manner following carcinogen administration [233]. Some subset of these cells will grow into colonies of hepatocytes also expressing GSTP. These findings suggest that the single GSTP expressing cells are precursors of those that form colonies and by definition of some of those that will progress into hepatic neoplastic nodules and HCC. Single hepatocytes expressing GST have the characteristics

associated with initiated liver cells; namely, dose dependent induction with carcinogen administration, rapid appearance after carcinogen treatment, enhanced intrinsic proliferation compared with surrounding apparently normal hepatocytes, and response to the selective growth pressure exerted by a promoting agent [233]. Expression of genes at the single cell level has been inadequately characterized, but GSTP and GGT are increased in certain hepatocytes following carcinogen administration.

4.4.2.2 Promotion

The promotion stage of cancer development has been operationally defined as the clonal expansion of the initiated cell population. The growth kinetics of GST expressing hepatocytes can be followed over time through the analysis of the size and volume fraction of the liver occupied by GST expressing hepatocytes [233]. The hepatocytes within AHF during promotion are primarily diploid [239, 240] and additionally lack demonstrable karyotypic changes [240]. Promoting agents stimulate the growth of the focal hepatocytes in a reversible manner and this can be determined by assessment of the size of the observed (GST expressing) hepatic lesions and by determination of focal increase in the expression of cell proliferation markers [234]. The net growth rate of GST expressing hepatocyte colonies can be determined from the volume fraction occupied by such lesions as a function of time. The net growth rate thus reflects the balance between the birth and death rate within this population in relation to that observed in the surrounding apparently normal cells. While many of the GSTP expressing lesions will regress, the nodules that concurrently express GSTP and gamma glutamyltranspeptidase (GGT) appear to be the ones that progress. The loss of expression of glucose 6-phosphatase has also been associated with progression, but it is unclear whether this is through a different mechanism than for GSTP expressing lesions. Gene expression has been examined in these early putatively preneoplastic lesions that precede nodule-in nodule of HCC.

4.4.2.3 Progression

The stage of progression encompasses the spectrum of changes that occur in the conversion of preneoplastic cells into malignant neoplasia [32]. There is not as yet a validated method for the quantitation of hepatocytes in the stage of progression. This stage is characterized by an evolving karyotypic instability and aneuploidy indicating the necessity of understanding alternative pathways in progression of liver neoplasia. Morphologically, the focus in nodule configuration is the earliest endpoint for detection of progression in the liver [32, 222, 241, 242]. Interestingly, gene expression differences between resistance and sensitivity of rat strains to liver cancer progression have been described [243].

4.4.3 Mouse Models

Certain mouse strains are more susceptible to spontaneous [244] and chemically induced [245] hepatic tumors than other strains. An upregulation of c-jun may mark single altered cells in the mouse liver [246] analogous to the increased GSTP expression in the rat. The focal areas of change can be detected in frozen sections by the loss of expression of glucose 6Phosphatase. Alternatively, H&E stained sections demonstrate the presence of two distinct lesion types (A and B). Discussions by Schwartz indicate that one class contains Ha-ras mutations, while the other class contains β-catenin mutations. The C57Bl/6 (resistant) and the C3H (sensitive) strains differ in their susceptibility to spontaneous and chemically induced liver cancer development [247]. The hepatocarcinogenesis susceptibility allele (Hcs) is autosomal and is inherited in a semi-dominant manner with the F1 between the sensitive and resistant strain demonstrating an intermediate phenotype. This phenotype is believed to be cell autonomous factor [248]. In a study performed by Drinkwater et al. [249], BXH (RI strains developed from a cross between C57Bl/6 (B) and C3H (H) mice were subjected to neonatal ENU administration. BXH strains 6, 14, and 10 were resistant, while BXH strains 8, 9, 7, and 3 were sensitive to ENU induced increases in liver tumor multiplicity. A number of susceptibility gene loci have been described genetically for mouse liver cancer development. These cancer modifier loci have been mapped to specific chromosomal locations based on the Mendelian inheritance patterns in inbred mouse strains that are sensitive and resistant to cancer development [250]. Strain differences in sensitivity to liver cancer development were described by Andervont [244] indicating a genetic component to the spontaneous development of liver cancer in mice. A few of these genes have been identified by positional cloning approaches. In addition, human homologues of cancer sensitivity and resistance alleles have been proposed. The C3H strain is susceptible to spontaneous and carcinogen induced liver cancer development, while the C57/Bl6 mouse is by comparison resistant. The hepatocarcinogenesis sensitivity (HCS) and resistance (HRS) alleles have been defined for the mouse. A hepatic susceptibility locus on mouse chromosome 1 accounts for 85 % of the variance between these two mouse strains [247, 251]. Studies with other mouse strains and other carcinogens have also been performed [252].

The National Toxicology Program assesses cancer risk in the B6C3 F1 mouse that carries the dominant susceptibility allele for liver cancer development. The most common experimental cancer assessment tool is the neonatal mouse model [253] as first described by Vessilinovitch [254]. Numerous models of human liver diseases exist. Many of these are developed as a complicated toxin or carcinogen

regimen [18]. In addition, genetically modified mice have been made against signaling pathway members believed important in liver cancer development [224]. These rarely are a complete recapitulation of the human disease, but are nonetheless useful for modeling one component of the disease [224]. The challenge is to couple etiologic agents, with pathway perturbations and disease models to unravel components of the pathogenesis of human primary liver cancer [18, 224, 255]. Analysis of early and progressive lesions that arise in the mouse, rat, and human will provide a mechanism by which to develop models of human liver cancer development, pathogenesis, and progression.

4.5 Etiology in the Human

Patients at risk for HCC include those with chronic hepatitis B virus (HBV) and/or HCV infection [14, 256], certain metabolic liver diseases, such as hereditary hemochromatosis [257], Wilson's disease, α-anti-trypsin deficiency, and porphyria cutanea tarda [7, 8]. Individuals with cirrhosis are at risk of HCC [7, 258]. Heavy alcohol consumption is also a common major risk factor for developing HCC [7, 8, 83, 85, 258]. Other predisposing factors include gender (males are times more likely to develop HCC than females), smoking, and diabetes [258]. Environmental influences, including carcinogen exposure and viral hepatitis prevalence, are believed to contribute to its distinct geographical distribution pattern [8]. Specifically, chronic infection with HBV and exposure to aflatoxin in the diet contribute to high-risk levels of HCC [259]. Thus, primary liver cancer is a product of environmental exposures with genetic consequences. In the US, the largest cross-sectional study of HCC identified infection with HCV and/or HBV as the most common risk factor for HCC (47 % HCV, 15 % HBV, 5 % both) Approximately, 33 % of primary liver cancer in the US are not associated with HBV or HCV) [8]. The incidence of HCC is increasing in the US primarily due to an increase in HCV infection [8]. It has also been proposed that the rising incidence of obesity, type 2 diabetes, and non-alcoholic liver disease contributes to this increased incidence of HCC [120].

4.5.1 Cirrhosis

Individuals with cirrhosis, regardless of its etiology are at risk for HCC [7, 258]. Fibrosis of the liver can result as a response to liver injury or as a component of selected genetic diseases [260, 261]. Cirrhosis is the endstage of fibrotic disease. Cirrhosis of the liver can occur during the progression of alcoholic hepatitis, non-alcoholic steatohepatitis (NASH), viral hepatitis, and cholestatic liver diseases [262]. Viral hepatitis (HBV and HCV) and alcohol are the primary causal factors in liver cirrhosis, while NASH, certain genetic diseases (e.g. hemochromatosis), and immune-mediated damage provides other contributing factors [7, 8]. There is an increased risk of primary liver cancer in individuals with hepatitis C associated cirrhosis and diabetes mellitus [263]. In some conditions, cirrhosis can progress to HCC.

4.5.2 Non-alcoholic Steatohepatitis (NASH)

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of elevated serum enzymes indicative of liver injury and may be due to many etiologies [264–269]. An independent diagnostic test or disease marker is not available for NAFLD. The NAFLD disease continuum, which has a worldwide prevalence of 20 %, is defined to exclude viral hepatitis, autoimmune diseases, metabolic changes due to hemochromatosis, alpha 1 antitrypsin, and ceruloplasmin changes, and alcoholic liver disease despite the similarities of disease presentation. Steatosis appears to be a benign condition, but steatohepatitis is progressive [264, 265, 267]. Essentially all morbidly obese individuals have NAFLD and approximately 25–50 % exhibit steatohepatitis. For NASH patients (prevalence of 1–5 % in the general population) approximately 20 % will progress to cirrhosis, with a small percentage of these progressing to HCC. Approximately 10 % of individuals with NASH will die of liver related diseases [265, 266]. NASH is common in type two diabetes and has a prevalence of 60 % [265–267, 269, 270]. Morbid obesity is another risk factor for NASH. Approximately, 2–3 % of lean individuals exhibit NASH, while 15–20 % of obese individuals have steatohepatitis at non-liver initiated autopsies. Individuals that have insulin resistance are susceptible to the development of steatosis (fatty liver) and its progression to NASH. In some individuals, steatohepatitis can progress to cirrhosis and in a limited number of cases can progress to primary liver cancer [270]. Recently animal models of NAFLD and NASH have been developed, but these do not completely recapitulate the pathogenesis of the related diseases and do not progress to cirrhosis or HCC without additional provocation [271, 272]. Current trends suggest that the NAFLD continuum is not as benign as once thought and that progression to NASH, cirrhosis, and potentially HCC can occur depending on the interaction of genetic, environmental factors and underlying disease including diabetes, HFE, among others [273–276].

4.5.3 Viral Hepatitis

Chronic infection with HBV or HCV is the predominant risk factor for development of HCC, accounting for up to 80 % of liver cancer cases in geographic regions of high incidence

of the disease [7, 8, 277]. Although much of the HCC incidence is attributable to chronic HBV infection, only a low percentage of individuals that are infected with HBV go on to develop progressive liver disease even though 80 % or more develop chronic infection. Approximately one third of individuals with chronic infection will develop cirrhosis and HCC develops in less than 5 % of those that develop cirrhosis [278]. Carriers of HBV have100 fold risk of developing HBV [14] that has been suggested to be closer to 5–15 fold in case control studies with a lifetime risk of 10–25 %. The annual incidence in HBV carriers is less than 1 % [14]. It increases to greater than 1 % in those with hepatitis and to 2–3 % in those with cirrhosis. Although rates of infection with the viruses are similar in men and women, there is some evidence that progression of the disease is more likely to occur in men [7]. Among chronic carriers of hepatitis B surface antigen (HBsAg) in Taiwan, the ratio of men to women was 1.2 for asymptomatic individuals, but there were six times as many men as women among patients with chronic liver disease [279] in concert with the greater prevalence of chronic hepatitis and cirrhosis in men [279]. A prospective study of liver cancer development among men in Taiwan has indicated a relationship between serum testosterone levels and risk for HCC [279, 280]. Men, whose testosterone levels was in the highest tertile (>5.7 ng/ml), had a relative risk of 2 for development of HCC when compared with men having lower testosterone levels. When other risk factors, including HBsAg carrier status, anti-HCV positivity, and alcohol consumption, were take into account, the relative risk for men with high testosterone levels was 4 [14, 278]. However, this difference may have been due to a higher proportion of HBsAg carriers among the liver cancer cases. In developed countries, HCV infection is a more prevalent risk factor for HCC. HCV infection results in a 15–fold increase in risk of HCC compared with uninfected individuals. Approximately, 90 % of HCV carriers develop hepatitis, while 20 % of HCV carriers develop cirrhosis. Cirrhotic HCV patients develop HCC at a rate of 1–4 % per year [7, 8, 5 286]. The high rate of cirrhosis development results in a risk of HCC over the lifetime of 1–3 %. The risk of HCC is further increased in HCV carriers for alcohol excess and HFE carriers [14, 278].

4.5.4 Aflatoxin and Other Dietary Carcinogens

A number of dietary factors have been associated with HCC risk including exposure to aflatoxin (a fungal product of *Aspergillus flavus* and related species. The risk of HCC is exposure (dose and duration) dependent [27, 281]. The risk is heightened in those with HBV [282]. This toxic substance is produced by certain strains of the mold *Aspergillus flavus*. Aflatoxin B_1 is one of the most potent hepatocarcinogenic agent known and has produced neoplasms in rodents and primates [27]. This agent is a potential contaminant of many farm products (the common food staples, grain and peanuts) that are stored under warm and humid conditions for some time. Aflatoxin B_1 and related compounds may cause some of the toxic hepatitis and hepatic neoplasia seen in various parts of Africa and the Far East [283]. Thus, an important environmental and experimental hepatocarcinogenic agent is aflatoxin B_1. Other products of molds and fungi are potentially carcinogenic in humans and animals including fumonosins [284]. Other fungal [285, 286] and microbial products [287] may similarly be associated with HCC risk. Certain alkaloids are cytotoxic to the liver and may be associated with an increased risk of liver cancer. A number of plants, some of which are edible, also contain chemical carcinogenic agents whose structures have been elucidated [288]. These include the pyrrolizidine alkaloids are found in comfrey, and riddeline [289]. The use of Senecio, Crotalaria, Heliotropium, and Synphytum species can result in veno-occlusive disorder. Acute toxicity can occur with high dose exposure, but lower doses and longer durations of treatment can result in chronic disease. While these agents are used as teas and herbal remedies, they have been associated with acute toxicity and when there is a genotoxic metabolite in addition to cytotoxicity the combination of DNA adduct formation and cell proliferation permits mutation induction and fixation. Similarly, a low intake of retinoids, selenium, Vitamin E and other antioxidants may also be associated with an increased risk when combined with other risk factors [290–294].

4.5.5 Alcohol and Tobacco

Alcohol abuse has been associated with HCC development that occurs in a background of hepatitis and cirrhosis [258, 295]. Alcohol abuse can potentiate HCV and HBV to increase the incidence of HCC [87]. This incidence is markedly increased in individuals with high AFP levels, high cell proliferation index, and in uncompensated patients with atypical macroregenerative nodules. In those with compensated liver fibrosis, the risk of HCC is 3 % [87, 296, 297]. Both case-control and prospective studies have indicated that excessive alcohol consumption increases the risk of liver cancer development by up to 3-fold, a result likely due to the induction of liver cirrhosis [296, 298, 299]. Liver cirrhosis due to excessive alcohol intake is an important risk factor in countries with a low incidence of HCC. Since chronic alcohol abuse is more prevalent among men than women, this risk factor may also contribute to the higher incidence of HCC in men than women [300]. Alcohol abuse may be an independent risk factor for HCC in areas of

endemic HBV or HCV infection with an attributable risk of approximately 20 % in one study [299]. Alternatively, associations between gender and lifestyle-associated risk factors, including smoking and alcohol consumption, have been suggested as potential determinants of the sex difference in HCC risk resulting in a male bias in the prevalence of this disease. There is a positive impact of cigarette smoking on HCC risk [301–307] and a higher rate of HCC are observed in heavier smokers when all other risk factors were taken into account [307]. Thus, the lifestyle factors of smoking and alcohol intake contribute to the induction and progression of HCC in a dose dependent and synergistic manner in both high and low risk geographical areas [304, 305]. Alcohol abuse can increase the risk of HCC in hepatitis virus carriers at least 2 fold [87].

4.5.6 Steroids

The factors underlying the sex difference in human risk of developing liver cancer have not been determined. However, the geographical and ethnic diversity in the populations at risk indicate that sex hormone-related factors may underlie the higher incidence of liver cancer development in men. Similarly elevated levels of testosterone result in an increased incidence of hepatic adenomas [308]. In men taking anabolic steroids, an increased incidence of liver adenomas has also been observed [309–311] and these lesions may or may not regress upon cessation of androgen therapy [312, 313]. Oxymetholone, methyltestosterone, and danazol administration were associated with hepatic neoplasms in certain cases. HCC were associated with oxymetholone and methyltestosterone in some patients, while adenomas were associated with danazol exposure [311]. These studies support the potential for elevated testosterone levels to contribute to the development of HCC development [259, 279]. Significant associations have been observed between polymorphisms in three hormone related genes and HCC. These include androgen receptor, 5 alpha reductase, and cytochrome P450 17 alpha [259].

Exposure to either anabolic steroids or certain oral contraceptive formulations has been associated with the increased incidence of hepatic adenomas and in rare instances with HCC development in humans. The earliest report of an association between liver cancer induction and exposure to exogenous sex hormones described seven cases of benign hepatomas in young women with a history of oral contraceptive use [314]. Women of child-bearing age appear to be sensitive to the induction of benign hepatic adenomas and the induction of these liver tumors is enhanced by exposure to oral contraceptives. These tumors respond to hormonal manipulations such that they regress upon cessation of hormonal administration [142] and grow or progress upon continued administration of these agents. While a dose (estrogenic potency) and duration effect is seen for oral contraceptive use and adenoma development, the association with carcinoma induction is very low and only detectable with greater than 8 years of exposure [315]. Several investigators reported that the relative risk for adenoma development increased sharply beyond 5 years of oral contraceptive use [142, 316]. While formulations containing mestranol and ethinyl estradiol have led to equivalent risks, the incidence of liver cancer among women using high potency oral contraceptives was significantly greater than that for users of low potency formulations. Oral contraceptive use has also resulted in an increased risk for malignant liver cancer [317]. Case-control studies in the United States, Britain, and Italy demonstrated a 5-fold increased risk for HCC among women with more than 5 years use of oral contraceptives relative to women with exposures of shorter duration [315, 317–319]. In contrast, estrogen replacement therapy does not increase the risk for HCCs [315]. Thus, excess exposure to hormonally active agents can increase the risk of HCC.

4.5.7 Genetic Disorders

A number of metabolic diseases have been associated with an increased risk of HCC [7, 8]. These include hemochromatosis, tyrosinemia, citrullinemia, porphyrias, and α1 antitrypsin. Individuals with cirrhosis and genetic hemachromatosis have a markedly increased rate and shortened time until HCC development that is exacerbated by viral infection and alcohol abuse [273, 279]. Other metabolic diseases can increase the risk of HCC but to lesser degree. These include Wilson's disease, fructose intolerance, and type I and III glycogen storage disease. Thus, the variety of the underlying disease base that contributes to HCC demonstrates the multifactorial risk profile for primary liver cancer development.

4.5.7.1 Metal Overload Disorders

Iron overload [257, 320, 321] has been associated with hepatic fibrosis, cirrhosis, and HCC. Hereditary disturbances in iron uptake [322–324] and metabolism results in one form of iron overload and dietary ingestion excess [325] a second. A variety of iron overload conditions have been associated with HCC even in the absence of cirrhosis including sideroblastic anemia and thalassemia [320, 326]. In certain areas of sub-Saharan Africa, the natives ingest drinks with concentrated iron. These individuals have an increased incidence of both cirrhosis and HCC [325]. Porphyrias occur due to defects in the heme biosynthetic pathway. Both acute intermittent porphyria and porphyria cutanea tarda have been associated with an increased risk of HCC [324]. The

mechanism is unknown, but the presence of free iron in the tissue may be a contributory factor. In combination with HBV infection, HCV infection, alcohol cirrhosis, iron overload induced an increase in lipid peroxidation and the rate of progression to steatohepatitis, cirrhosis and HCC [86, 258]. Underlying liver disease including cholestasis, steatosis, and cirrhosis can impact the degree and latency to disease onset and progression with iron overload syndromes.

Hereditary hemochromatosis was first described as a hereditary disease associated with HLA linkage and a form of pigment associated cirrhosis typically associated with diabetes. A prevalent gene mutation [323] was found to underlie hereditary hemochromatosis (HFE) and a knock-out mouse [327]. Although several genetic factors can be involved in iron overload, the most common is in HFE (85–90 %). Although several polymorphisms exist, the most prevalent is C282Y (85–100 % attribution to HFE). The prevalence is 1 in 250 with an allelic frequency of 5 %. The second polymorphism allele that is common in HFE is H63D. Carriers of this allele comprise 15–20 % of the American population, but the consequence of this allele is not known [323]. The HFE is an MHC class 1 molecule that is associated with β2 microglobulin (B2M) and the major polymorphism C282Y prohibits this interaction. Studies in a B2M knock-out mouse demonstrate an iron overload syndrome [328]. In the HFE knockout mouse, periportal iron deposition in conjunction and elevated transferrin saturation [327]. Interestingly, HFE and B2M are in a complex with transferrin receptor HFE results in an increase in intestinal iron absorption. HFE mutation carriers cannot facilitate iron update by transferrin receptor resulting in an upregulation of the iron responsive gene dimetal transporter 1 that enhancing iron uptake [329, 330]. Transferrin receptor Ser142 alleles are increased in liver cancer cases and in addition, TfR expression is increased in hepatic preneoplasia and in HCC [330]. The odds ratio for C282Y allele carriers with TFR142Ser alleles for HCC is 17.2, while it is 62.8 in those with cirrhosis for HCC development demonstrating the contribution of TfR to risk of HCC [321].

The long term consequences of iron overload on the liver include fibrosis and cirrhosis that can be exacerbated by the presence of underlying liver disease [257, 320]. The incidence of HCC in HH is increased over 100X relative to a comparative control population [257, 320]. Outcomes in heterozygotes for HFE seem similar to wildtype, except for those 1–2 % individuals who are compound heterozygotes with C263Y/H63D [331, 332]. The odds ratio of HCC in HFE C282Y carriers or homozygotes is 3.5, while it is 7 in those with cirrhosis indicating that HFE is a risk factor for HCC [332]. The HCC population is enriched for C282Y carriers than is found in the general population indicating a possible risk factor for its development and progression [331–333]. The increased risk from HFE alleles is found in alcoholic cirrhosis and some cases of HCV viral hepatitis, but not HBV viral hepatitis patients [331, 333]. Animal models of liver disease in combination with iron overload also demonstrate an increase in disease progression [334]. For example, transgenic mice overexpressing the HCV polyprotein fed a diet enriched in iron develop microvesicular steatosis indicative of mitochondrial damage and impaired energy use with fatty acid retention and earlier onset of HCC than their littermates similar to those humans that develop fatty liver with HCV infection [334]. A wide range of hepatic tumor phenotypes is observed in human HFE [335]. Interestingly, a high incidence of p53 mutations has been observed in one series of HCC from HFE patients [336]. Importantly, epigenetic defects are observed in liver tissue from 75 % of the HFE patients examined prior to the onset of cirrhosis with hypermethylation and hence gene expression decreases [337].

Wilson's disease or inherited copper-overload disease can result in cirrhosis, hepatitis, and HCC. Wilson's disease is found in 1:30000 with a carrier rate of 1:250 [338]. Ceruloplasmin is decreased in the serum of Wilson's disease patients. This autosomal recessive disorder is due to a mutation in the P-type ATPase responsible for biliary copper excretion (ATP7B) located in the trans golgi network [339]. The most prevalent mutation, H1069Q, is observed in 30 % of Wilson's patients of European decent. Other mutations of the ATP7B gene exist and can also result in Wilson's disease [338]. In addition, modifier genes that impact the severity of the disease also exist. Copper is normally ingested and absorbed through the GI tract and excreted through the bile. Copper is transported in the serum bound to histidine. Copper binds to glutathione or metallothionein, and ceruloplasmin. It is excreted into the bile in part through a secretory pathway involving ATP7B. The Long Evans Cinnamon rat is susceptible to non-viral hepatitis with subsequent formation of liver neoplasms, the male is more susceptible to the development of liver tumors [340, 341]. The LEC rat is a model of Wilson's disease that develops a non-viral hepatitis due to copper overload. These rats also have disturbances in iron metabolism. Those animals that survive the hepatitis will develop HCC. The toxic milk mouse has a mutation in M1356 V and G712D have defects in copper transport [342] and a knock out mouse (ATP7B) has also been generated [343]. If intracellular copper accumulates beyond the ability of the hepatocyte to buffer it, then hepatic damage will ensue with copper release into the circulation and its accumulation in other tissues.

4.5.7.2 Alpha-1 Anti-trypsin

Alpha-1 Anti-trypsin (AAT) is a prevalent protease inhibitor (Pi) found in the plasma [344]. The most prevalent mutation is a Glu342Lys caused by a G to A transition called the Z mutation [345, 346]. Adult males that are homozygous for

the Z mutation (PiZZ) may have an increased risk of cirrhosis and HCC [345–347]. Alpha 1 antitrypsin results in an increased risk of HCC in the absence of cirrhosis in homozygotes [347]. Carriers (PiZ) are also believed to be at an increased risk for HCC [348] especially in combination with other risk factors [349, 350]. While the mechanism of α1AT alleles on disease etiology is unclear, the altered protein structure may induce the unfolded protein response. Alternatively, this acute phase serum protein, which acts as an inhibitor of elastase and is synthesized by the liver and macrophage is retained in the liver resulting in a plasma insufficiency. Retention in the liver and consequent polymerization can result in cirrhosis and to HCC [345, 346].

4.5.7.3 Hereditary Tyrosinemia

Tyrosinemia is an autosomal recessive disorder that can lead to HCC. This inborn error of metabolism results [351] from inactivation of fumaryl acetoacetate hydrolase (FAH) resulting in the buildup of its substrate fumarylacetoacetate (FAA) and malylacetoacetate (MAA). As a consequence, these individuals excrete high levels of succinylacetone into the urine [352]. MAA and more specifically FAA have multiple effects on liver cells including apoptosis, ER stress response, redox balance including GSH depletion, and cell cycle arrest. Since the last step in the catabolism of tyrosine is blocked, tyrosine is elevated in the serum. These patients have a rapid conversion from micro to macronodular cirrhosis and later conversion to dysplasia and HCC. Without pharmacological (nitisinone) treatment or now surgical intervention, the prognosis was poor with acute liver failure predominant, followed by HCC [352, 353]. A mouse model has been developed in which FAH is knocked out [354]. This mutant recapitulates the pathogenesis of human hereditary tyrosinemia type 1 and can be protected by nitisinone [355]. Intervention with nitisinone does not reverse gene expression changes associated with tyrosinemia [356]. Thus, pharmacological treatment can delay, but may not prevent HCC development. Genetic manipulation reversal of double mutant FAH mice formed through ENU mutagenesis do not develop preneoplastic lesions or HCC, suggesting that the lack of complete reversal of the phenotype by pharmacological intervention is due to incomplete blockage of the formation of toxic intermediates [357].

4.5.7.4 Citrullinemia

The inborn errors of disease associated with the urea cycle [358, 359]; namely, mutation of arginosuccinate results in acute liver toxicity [360]. Citrullinemia type I is an autosomal recessive disorder that is caused by a deficiency in the rate limiting enzyme in the urea cycle, argininosuccinate synthetase (ASS1). In severe cases, a hyerammonia can occur that is fatal neonatally. An argininosuccinic aciduria with an increase in citrulline and ammonia in the serum is observed. Since citrulline is essential in nitrogen homeostasis, disruption of ammonia removal results in toxicity to the liver. There is a broad mutational pattern and each genotype has different phenotypes [360]. A knock out mouse has been generated that has high citrilline blood levels and a severe hyperammonemic phenotype [361, 362]. The aspartate-glutamate carrier (AGC), SLC25A13, gene mutations result in citrin deficiency [363] and may develop hepatic steatosis and steatohepatitis [364]. These type 2 citrullinemia patients have an increased level of pancreas derived trypsin inhibitor and are associated with pancreatistis [363]. A decrease in this mitochondrial ACG, citrin, results in hepatic apoptosis through a caspase pathway in which the bax to bcl2 ratio is inverted [357]. A knock-out model has been described, but does not recapitulate all of the pathologies associated with adult onset type 2 citrullinemia [363]. The citrin/mitochondrial glycerol-3-phosphate dehydrogenase double knock-out mutant is a better model for type 2 citrullinemia [365]. Urea cycle disruption and perturbations of nitrogen removal can have adverse effects on the liver as exemplified by citrullinemia.

4.5.8 Genomic Landscape of HCC

The genomic landscape of cancer has evolved as a concept in cancer to account for the many genetic changes observed in neoplasms [366–368]. It has been suggested that primary hepatocarcinoma has an average of 6 mutations per megabase of DNA [369]. This high number may in part due to the late stage of life in which the cancer is detected as well as the late stage of its lifecycle when it is detected. The genetic changes observed in cancer especially liver cancer are considered to have an environmental and lifestyle component reflected in the genetic and epigenetic changes observed [370]. The recent ability to deeply sequence whole exomes or entire sequences as compared with single genes has emphasized this point. While many genetic signatures have been detected in neoplasms [366–368], six have been demonstrated in liver cancer using COSMIC [369; http://cancer.sanger.ac.uk/cosmic]. Specifically, the genetic landscape of hepatocellular adenocarcinoma has been associated with the etiology of the disease, while the stage of disease has been more correlated with the expression and pathway alterations although these two factors and sets of changes are interdependent. One of the primary genetic signatures present in HCC (COSMIC signature 1B) is that of C > T that has been associated with aging. This may in fact reflect oxidative stress that is prevalent in cirrhosis and in viral and alcohol induced liver cancer and which can be found in aflatoxin excess. In this situation, a helix-distorting adenine adducts at GpCpN on the transcribed strand are contributory. Similarly, diseases such as NAFLD/NASH and hemochromatosis also have ongoing

oxidative stress and damage that would contribute to this type of genetic signature and to HCC development. A second signature (COSMIC signature 5) has a similar, albeit less prominent pattern of C > T changes that in this case are associated with dinucleotide mutation and strand synthesis bias. In the third signature (COSMIC signature 6), interstitial deletions at nucleotide repeats are common. This microsatellite instability is associated with mismatched repair deficiency resulting in high C > T, lower C > A, and even lower levels of T > C. The fourth identified gene mutation pattern for HCC (this is COSMO signature 4) is associated with the transcribed strand and has not been associated with a single predominant mutation, but rather may be associated with the infidelity of the polymerase and of transcription-coupled repair. In the fifth signature associated with HCC (COSMIC signature 16), a high level of T > C is observed and has been associated with transcription-coupled repair. In addition, a high level of T to C transversions is associated with the presence of G adducts as are frequently observed following polyaromatic hydrocarbon exposure as observed, although not exclusively, with tobacco smoke and exposure to other combustible products. A final predominant signature associated with HCC has a high level of T > G and a medium amount of T > C changes (COSMIC signature 17). The genetic landscape of a cancer reflects the cumulative environmental exposure, the impact of underlying liver disease, the etiology of the neoplasm, and its pathogenesis. This has been examined extensively in liver cancer for p53 and ras loci, but has now been extended across the genome. This whole genome examination has been instrumental in deciphering the complexity and heterogeneity of HCC. Genome wide analysis is now possible with the combined development of deep sequencing and big data based bioinformatics approaches. Besides mutations, insertions, deletions and amplifications, copy number variants and other factors that alter gene expression. In addition, mechanisms that impact gene dosage are important in liver cancer development and progression.

With respect to gene expression, a number of kinases and potentially phosphatases are of importance in altered gene expression in the liver and with liver cancer development [370, 371]. Specifically, Met, EGFR, and IGFR families have been implicated in liver cancer development and progression. Other receptors including VEGF2, PDGF, and FGF have roles in HCC pathogenesis. In addition, downstream signaling pathways (MAPK and AKT) and transcription factors (ras, mTOR, and have been implicated in HCC development and progression. One of the most important signaling pathways associated with HCC is the WNT pathway [370, 371]. An inflammatory mechanism is associated with some HCC and may be associated with estrogen-dependent regulation of IL6, NFkB and other mechanisms including those that signal through JAK/STS and TGFb. Recent, studies of mutations in HCC have confirmed the incidence of mutations in p53 and beta-catenin. Furthermore, the many mutations have been mapped against pathways and network to reveal the importance of proliferation, apoptosis, tumor microenvironment, neural signaling, metabolic pathways, and circadian pathways [371]. These pathways include cell cycle, p53 signaling, Wnt, MAPK, PI3 K/AKT and apoptosis, but also calcium signaling and Hippo pathways based on TGAC analysis. While these pathways are associated in general with HCC, their association with etiology, pathogenesis, and prognosis requires additional analysis. Additionally, chromatin- remodeling genes are altered in HCC. These include ARIAD1a/d, ARID2, MLL, MLL3, TERT among others [372]. The advent of deep sequencing as applied to the whole genome or all exons in conjunction with improved bioinformatics tools and well characterized sample banks of well defined pathology samples and their accompanying metadata have enabled important insights into the genomic landscape of liver cancer as demonstrated with the TGAC and COSMIC databases [373, 374].

4.5.9 Summary

Chemicals from a variety of chemical classes can initiate, promote, and lead to the development or progression of HCC. The effects of chemical agents occur on the background of a variety of genetic alterations and disease backgrounds. Animal models have proven invaluable in the assessment of the early pathogenesis of primary liver cancer by chemicals. The late stage neoplasms analyzed from the human demonstrate that multiple etiologies, molecular pathways, and genetic changes accompany neoplastic development in the liver. Combinations of genetic factors, environmental exposures, and background liver disease will be modeled in increasing complex ways in the future to better recapitulate the role of chemicals in HCC development and progression. Systems biology tools as applied to the pathogenesis of HCC will be informative about the pathways that chemicals disregulate in different genetic and disease backgrounds to lead to HCC development and progression.

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